The inhibitory receptor PD-1 differentially regulates effector and anergic autoreactive CD4 T cells during Type 1 Diabetes

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ABSTRACT

A variety of mechanisms act to prevent the inappropriate targeting of host tissues by the immune system, but these mechanisms can fail, leading to development of autoimmunity. Type 1 Diabetes is an autoimmune disease caused by T cell-mediated destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans. This work focused on understanding islet-reactive CD4 T cells during Type 1 Diabetes progression by comparing T cell responses in diabetes-susceptible non-obese diabetic (NOD) mice to diabetes-resistant (B6.g7) mice. Our knowledge of how these cells are normally regulated and how that regulation breaks down leading to diabetes is limited due to a lack of reagents to track these cells in mice with normal T cell repertoires. The goal of this work was to develop models to study physiological numbers of islet-reactive CD4 T cells in mice in order to gain a better understanding of how these cells are regulated with an emphasis on one critical inhibitory pathway involving Programmed-Death 1 (PD-1). Using these models, we showed that in diabetes-susceptible NOD mice, the majority of islet-reactive CD4 T cells become activated, but the pathogenic subset capable of contributing to disease was relatively small. Rather, the majority of the population was adequately controlled by the host through induction of T cell anergy. In diabetes-resistant mice, islet-reactive CD4 T cells were present, but failed to become activated and subsequently did not infiltrate the pancreas. The inhibitory receptor PD-1 was important for suppressing CD4 T cell effector functions in NOD mice, including proliferation, trafficking to the pancreas, and localization within the pancreas. However,

blockade of PD-1 did not promote CD4 T cell trafficking to the pancreas in diabetes-resistant mice. Unexpectedly, in NOD mice, PD-1 was predominantly involved in suppressing the functions of an activated effector population, not maintaining the anergic population. These findings provide new insight into the regulation of islet-reactive CD4 T cells, and show that PD-1 differentially contributes to the suppression of these cells.

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INTRODUCTION

The immune system has evolved to protect the host from a variety of pathogens including viruses, bacteria, and parasites, as well as tumor cells. Once activated, the immune system has the capacity to potently eliminate these harmful threats. A long standing problem in the immune system has been distinguishing what is harmful from what is not, and sparing the innocuous tissue while eliminating the threat from the body. There are a variety of mechanisms that the immune system has developed to distinguish between harmful and innocuous; however, these mechanisms are not always effective, resulting in the immune system targeting and destroying self tissues. Depending on the tissue that is targeted, a variety of different clinical outcomes occur, ranging from loss of function of an organ, deregulated production of different small molecules such as hormones, and, in the most severe cases, death. This inappropriate targeting of self tissues by the immune system is referred to as autoimmunity, and the range of diseases that fall into this category are diverse both in terms of severity and the tissues that are involved. The focus of the work presented herein is Type 1 Diabetes, an autoimmune disease characterized by loss of the insulin-producing beta cells in the pancreatic islets of Langerhans. The immune system selectively targets and destroys this population,

requiring Type 1 Diabetic patients to inject insulin daily for the remainder of their lives to regulate glucose metabolism. In this thesis, issues regarding the immune components responsible for the disease and the mechanisms that normally regulate these cells will be discussed, with a particular emphasis on one inhibitory pathway involving Programmed Death-1 (PD-1). Additionally, clinical perspectives will be addressed including potential cures for this devastating autoimmune disease. With a better understanding of how the self-specific immune response is controlled and why these regulatory mechanisms fail, we will enhance the possibility of developing a cure for Type 1 Diabetes.

TYPE 1 DIABETES PATHOGENESIS

Type 1 Diabetes in human patients

Type 1 Diabetes is an autoimmune disease that manifests when the adaptive immune system selectively targets and destroys the insulin-producing beta cells in the pancreatic islets of Langerhans. This aberrant autoimmune response leads to the permanent destruction of the insulin-producing cells, resulting in the requirement for Type 1 Diabetic patients to take exogenous insulin for the remainder of their lives to regulate glucose metabolism. While insulin therapy can be an effective way to regulate glucose metabolism, stringent control of blood glucose levels is difficult to maintain, which can lead to severe end organ damage in the kidney, eyes, peripheral nerves, and blood vessels. This organ damage can lead to kidney failure, blindness, peripheral neuropathy, coma and/or death (1, 2). Therefore, there is intense interest in the field to develop a cure for Type 1 Diabetes. A cure for Type 1 Diabetes will require both

restoration of beta cell mass so patients can produce their own insulin, as well as limitation of the anti-islet autoimmunity response. Issues involved in achieving this goal will be discussed in Chapter 5.

Type 1 Diabetes is one of the most common chronic diseases in children in the United States. This form of the disease accounts for approximately 5-10% of diabetes cases, and is the most prevalent diabetes subtype observed under the age of 20 (2). Type 1 Diabetes has also been referred to as juvenile onset diabetes due to the tendency of this disease to develop during childhood; however, it has become appreciated that Type 1 Diabetes can develop at any age including adulthood, so the term juvenile onset is no longer widely used. Diabetes in children typically occurs either between 5-7 years of age or at or near puberty (2). Symptoms typically include polydipsia (chronic thirst), polyuria (increased urination), and polyphagia (increased hunger) and hyperglycemia (2). By the time patients are diagnosed, typically 80-90% of the insulin-producing beta cells have been destroyed (1). While these patients can retain some insulin production for the first year or two after diagnosis, they will ultimately require exogenous insulin for the remainder of their lives to maintain glucose homeostasis. Murine models of Type 1 Diabetes show strong gender bias towards females developing the disease; however, this gender bias is not observed in human Type 1 Diabetes (2). The lack of gender bias in human diabetes is particularly interesting since most autoimmune diseases are more prevalent in women, and may be partially due to the early onset of the disease before puberty. Despite decades of research investigating this autoimmune response, the triggers that cause Type 1 Diabetes are still largely unknown. There is evidence for both genetic

and environmental components contributing to the disease, both of which will be discussed below. However, there are still several unresolved questions that remain in order to prevent or cure Type 1 Diabetes in humans.

Genetics are the highest risk factor predisposing people to developing Type 1 Diabetes. There is a high rate of familial transmission, which is particularly high for identical twins (35-40% concordance) (3-6). Additionally, there is a 7% increase in the risk of developing Type 1 Diabetes if a sibling has the disease, and a 6% increase for a child of a diabetic person (6). Furthermore, several genes have been associated with increased susceptibility to Type 1 Diabetes development. The highest associated genetic risk factor is MHC class II. For humans, combinations of the DRB1, DQA1, and DQB1 genes heavily influence susceptibility to diabetes, with haplotypes HLA-DR4-DQ8 (DRB1*0401-DQA1 *0301-DQB1 *0302) and HLA-DRB1*0301-DQA1*0501-DQB1*0201 most commonly associated with diabetes (7-11). The HLA-DQ8 molecule is of particular interest because it contains an atypical non-aspartic acid mutation at position 57 of the MHC class II beta chain which disrupts a salt bridge used for proper folding of the molecule. This mutation is also observed in the non-obese diabetic (NOD) mouse model of Type 1 Diabetes, where homozygous expression of this atypical MHC molecule is required for diabetes (12-14). Due to its role in antigen presentation to $CD4^+$ T cells, MHC class II heavily impacts T cell selection in the thymus, which may bias towards selection of an autoreactive T cell repertoire. Additionally, MHC class II controls $CD4^+$ T cell activation in the periphery, which may lead to aberrant activation of autoreactive T cells in diabetes-prone hosts (15). These two features likely both contribute to MHC

haplotype leading to enhanced susceptibility to developing Type 1 Diabetes. A variety of other genes have now been identified which increase the susceptibility to Type 1 Diabetes. At least fifteen more loci in addition to MHC class II have been identified, though the functions of all of these polymorphisms are not fully characterized (11). Polymorphisms in the insulin gene (contains a variable nucleotide tandem repeat at the 5' end of the gene) (16, 17), PTPN22 (11), IL-2RA (11), and Cytotoxic T-Lymphocytes-Associated Antigen-4 (CTLA-4) (presence of an alanine at codon 17 increases susceptibility to disease) (18, 19) have been identified to increase the risk of developing diabetes. However, the contribution of these genes is minor compared to MHC class II (11).

While the genetic component of Type 1 Diabetes is widely appreciated, genetics cannot entirely account for disease development. For example, even though the genetic composition between identical twins is the same, the concordance is only 35-40%, leaving 60-65% discordance (3-5). Additionally, the disease frequency varies substantially between countries in a manner that cannot be explained by genetics, including immigrants from a country with a low frequency of diabetes acquiring a higher risk for diabetes development in the new country (20, 21). Hence, other factors must contribute to the development of Type 1 Diabetes, including the environment as well as stochastic factors that remain to be defined. Patients often have severe viral infections shortly before clinical symptoms of Type 1 Diabetes manifest, including rubella virus (22) and coxsackie B virus (23, 24). Other environmental factors that have been proposed to contribute to Type 1 Diabetes include diet and stress (25). Hormones have also been an

area of interest in Type 1 Diabetes due to the dramatically higher incidence of the disease in female NOD mice compared to male; however, since Type 1 Diabetes in humans often develops before puberty, it is less likely that sex hormones play a critical role in diabetes development in humans (2).

The identification of Type 1 Diabetes as an autoimmune disease was critical for beginning to understand disease pathogenesis and continues to shape therapeutic strategies to manage the disease. Evidence to suggest that Type 1 Diabetes is autoimmune in nature comes from the presence of autoreactive T cells and autoantibodies specific for pancreatic islet antigens. Autoantibodies during Type 1 Diabetes in humans are better characterized than the islet-reactive T cell response, though production of peptide/Major Histocompatibility Complex (MHC) tetramer reagents to track antigen-specific T cells in humans is allowing us to gain a better understanding of this population as well. Autoantibodies against islet constituents including insulin, preproinsulin, and GAD are detectable in patients prior to diabetes onset (26-28). Importantly, levels of anti-insulin antibodies correlate with age of onset and rate of progression towards diabetes (29). In humans, $CD4^+$ T cell responses to beta cell antigens including insulin, glutamic acid decarboxylase 65 kDa (GAD65), heat shock protein (HSP)-60 and HSP-70, insulinoma antigen (IA)-2, islet cell antigen (ICA)-69, and others, and $CD8^+$ T cell responses to insulin, GAD65, IA-2, and islet amyloid polypeptide (IAPP) have been identified (30). There is a substantial effort from several groups including our own to further investigate the dynamics of these islet-antigen specific T cell populations in patients during pre-

clinical stages as well as following diabetes onset in an effort to develop better therapies to inhibit these pathogenic T cell responses.

In summary, Type 1 Diabetes is an autoimmune disease that typically develops early in life in humans and is caused by T cell-mediated destruction of the insulin-producing beta cells in the pancreas. While the triggers leading to diabetes development remain unclear, genetics appear to play the most substantial role in diabetes risk. However, other factors including the environment, stress, and infections contribute to diabetes development as well. In order to cure diabetes, the insulin-producing cells must be replaced and the autoimmune response must be inhibited. Later sections will focus in detail on the regulation of the autoreactive T cell response using preclinical mouse models as well as potential immunotherapies to selectively inhibit autoreactive T cells in Type 1 Diabetic patients.

The non-obese diabetic (NOD) mouse model for studying Type 1 Diabetes pathogenesis

The non-obese diabetic (NOD) mouse is the best characterized and most commonly used mouse model for studying Type 1 Diabetes (31, 32). The NOD mouse was developed over 30 years ago by Makino and colleagues in an attempt to isolate strains susceptible to developing cataracts (32, 33). The observation that this strain developed diabetes spontaneously, and subsequently the establishment that this mouse shared many similarities with human Type 1 Diabetes, has led to a wealth of information on how diabetes progresses and importantly how it can potentially be treated. Compared to the human disease, the NOD mouse has similar genetic linkages, pancreas-specific

autoantibodies, and aberrant activation of autoreactive $CD4^+$ and $CD8^+$ T cells. Additionally, many of the antigens involved in disease progression are shared between Type 1 Diabetic patients and the NOD mouse, including insulin and GAD65 (30). A critical advantage of the NOD mouse model is that the disease develops spontaneously, allowing investigation of the natural breakdown of self tolerance mechanisms. Furthermore, the similarities between the NOD mouse and human diabetes have allowed development of potential therapies to treat Type 1 Diabetes in the NOD mouse that have subsequently been translated into humans (31, 34). The characterization and use of different therapies targeted at immune regulation and prospective therapies will be further detailed in Chapter 5.

Diabetes incidence in NOD mice varies between mouse colonies, but it typically averages 60-80% for female mice and 20-30% for male mice by 30 weeks of age (32). However, there is evidence of immune cell infiltration of the pancreas by 3-4 weeks of age. The infiltration of the pancreas begins as peri-insulits, which is defined as mononuclear cell infiltrates surrounding the islet but not deeply penetrating within the islet, and eventually progresses to destructive insulitis as the protective barrier between the infiltrate and insulin-producing cells is lost and the T cells induce damage that leads to disease onset. All female NOD mice develop peri-insulitis; however, 100% of these mice do not develop the disease (31, 32). The inability to discern which mice would progress to clinical diabetes and which would not has made studying the events leading to Type 1 Diabetes difficult, particularly the imitating events that occur very early in life. However, recent imaging advances by Mathis and colleagues have made it possible to

determine which mice will eventually develop clinical disease months prior to symptom onset (35). This technology, termed MRI-MNP, employs non-invasive MRI imaging of magnetic nanoparticles (MNP). During diabetes development, insulitis results in inflammation that leads to vascular damage and leakage, which can be detected by the MRI. Using this technique, it can be determined whether a NOD is going to develop diabetes later in life by 6 weeks of age, demonstrating the importance of events early in life during Type 1 Diabetes pathogenesis (35). This technology will likely aid in further delineating the mechanisms that contribute to the loss of self tolerance in NOD mice, particularly the pathogenic events that occur early in life.

Autoimmune diseases develop due to the aberrant activation of a small portion of the adaptive immune system that is specific for self antigens. In Type 1 Diabetes, $CD4^+$ and $CD8^+$ T cells as well as B cells all play indispensable roles during disease progression, which is most evident by NOD mice deficient in any one of these adaptive immune components being protected from developing Type 1 Diabetes ($CD4^+$ T cell deficiency using CIITA knockout (KO) NOD (36), $CD8^+$ T cell deficiency using β2m KO NOD (37, 38), and B cell deficiency using μMT mice (39)). However, diabetes is considered a T cell-mediated autoimmune disease because disease can be transferred from one mouse to another using diabetogenic T cells but not serum autoantibodies (31). The primary role of B cells during diabetes development is thought to be antigen presentation to $CD4^+$ T cells rather than autoantibody production, since B cells must express MHC class II for diabetes to develop and serum antibody transfer into B cell-deficient NOD mice does not restore disease (40, 41). Additionally, NOD mice

engineered to contain B cells that express MHC class II but cannot secrete antibody developed both insulitis and diabetes, suggesting that antibody is not required for diabetes pathogenesis (42). For $CD4^+$ T cells, there are a variety of different effector subsets that arise following antigen encounter, and these subsets are defined based on the cytokines they are capable of producing (Th1 cells produce IFNγ, Th2 cells produce IL-4, and Th17 cells produce IL-17) (43). During Type 1 Diabetes, IFNγ-producing Th1 cells are critical for disease progression, since IFNγ KO and IFNγR KO mice on the NOD background are largely protected from developing diabetes (44, 45), and NOD.T-bet KO mice (the master transcription factor required for Th1 differentiation) are completely protected from developing Type 1 Diabetes (46). Th2 cytokines are thought to be protective during Type 1 Diabetes (47, 48), and the role of Th17 cells during disease progression remains controversial (49-51). However, the indisputable role for T cells in the pathogenesis of Type 1 Diabetes has led to a substantial interest in understanding how these cells escape deletion during development (central tolerance), how these cells are normally regulated (peripheral tolerance), why these cells become activated and destroy the pancreas, and how these self-destructive cells can be stopped.

THE ROLE OF ISLET-ANTIGEN SPECIFIC $CD4^+$ T CELLS DURING TYPE 1 DIABETES DEVELOPMENT

$CD4^+$ T cells are required for Type 1 Diabetes development in NOD mice

The previous section outlined the indispensable roles of $CD4^+$ and $CD8^+$ T cells as well as B cells in Type 1 Diabetes development based on studies using NOD mice

deficient in any one of these subsets (36-39). While $CD8^+$ T cells and B cells are important for the disease, our lab specifically focuses on understanding the pathogenic $CD4^+$ T cell subset in this autoimmune response. Several lines of evidence support the importance of $CD4^+$ T cells in Type 1 Diabetes. The MHC class II locus is the highest genetic susceptibility allele for Type 1 Diabetes in NOD mice and human patients, implicating $CD4^+$ T cells as an important component in disease progression (31). The H-2^{g7} haplotype in NOD and HLA-DR3/4 and DQ8 haplotypes in humans are thought to enhance susceptibility to diabetes through mediating selection of a T cell repertoire that selects for diabetogenic clones and promoting aberrant activation of these pathogenic cells in the periphery (15, 52). The NOD MHC class II molecule H-2^{g7} differs from other mouse MHC class II molecules by having histidine and serine residues at positions 56 and 57 of the beta chain, respectively, rather than the proline and aspartic acid residues that are typically found at these positions (53). Additionally, the H-2^{g7} haplotype encodes H-2A (g7), but does not encode H2-E due to a deletion in part of the gene encoding the alpha chain (54). In NOD mice, Type 1 Diabetes development requires homozygous expression of H-2^{g7} and the absence of the H2-E locus, highlighting the importance of this atypical MHC molecule in disease progression (12-14). A similar amino acid substitution is also observed in the human HLA-DQ8 haplotype which is highly associated with Type 1 Diabetes susceptibility (52). The histidine and serine residues disrupt a salt bridge in the MHC class II molecule, altering the nature of the peptides that preferentially bind as well as impacting the on/off rate of peptides once bound. The latter results in peptides bound to H-2^{g7} and HLA-DQ8 being abnormally unstable, causing loss

of peptides from the groove on the surface of antigen presenting cells (52, 55, 56). Some speculate that the abundance of empty MHC class II molecules on the surface of dendritic cells in the pancreas leads to loading of peptides derived from beta cell granules onto the surface of these cells, leading to the aberrant activation of diabetogenic T cells (56, 57). While the exact mechanism of diabetogenic T cell activation is still debated and will be discussed in detail in a later section, the critical role for the MHC class II haplotype in this process remains well established.

In NOD mice, $CD4^+$ T cells are required for Type 1 Diabetes pathogenesis. NOD mice deficient in MHC class II (CIITA KO mice) and subsequently $CD4^+$ T cells are protected from diabetes development (36). Additionally, antibody-mediated depletion of $CD4^+$ T cells prevents diabetes onset in NOD mice (58). Furthermore, in vitro activated diabetogenic $CD4^+$ T cells are sufficient to induce Type 1 Diabetes in lymphoreplete mice (59), demonstrating that $CD4^+$ T cells can be directly pathogenic during diabetes. The mechanism of diabetes induction in this case is likely cytokine mediated, since in vitro activated $CD4^+$ T cells produce high levels of IFNγ. This critical cytokine enhances the antigen presenting capacity of antigen presenting cells within the islet (60), and in cooperation with other cytokines including TNFα and IL-1β can directly induce apoptosis in the insulin-producing beta cells (61-63). During spontaneous diabetes progression, $CD4^+$ T cells likely play a more cooperative role in disease development rather than a directly pathogenic role. $CD4^+$ T cells are known for providing help to activate both B cells and $CD8^+$ T cells, two other immune constituents that are required for Type 1 Diabetes pathogenesis (37-39). Diabetogenic $CD4^+$ T cells comprise the

majority of the early infiltrate in the pancreas of young NOD mice, and may be critical for the initiating events that subsequently lead to Type 1 Diabetes later in life (64). There is currently a substantial interest in the field in understanding the contribution of $CD4^+$ T cells to diabetes with the hope of targeting this T cell subset for therapeutic benefit.

A variety of pancreatic antigens are recognized by $CD4^+$ T cells during Type 1 Diabetes

Diabetogenic $CD4^+$ T cells are activated early in life in NOD mice and persist in the pancreas throughout the course of the disease. These cells recognize a variety of different pancreatic antigens, though the epitopes responsible for initiating as well as sustaining Type 1 Diabetes have been highly debated. There is interest in identifying which epitopes are important at which stages of disease so that these $CD4^+$ T cells can be targeted using antigen-specific therapies, including antigen-coupled ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI)-fixed cells, which will be discussed later. Identifying which antigens are critical at different stages of disease is particularly problematic in the context of developing antigen specific therapy because antigens can vary between patients, requiring each therapy to be personalized, and there is evidence of epitope spreading, suggesting that the antigens targeted by the anti-islet T cell response change as disease progresses. Two of the major antigens recognized by $CD4^+$ T cells early during the initiation of Type 1 Diabetes in NOD mice are GAD65 and insulin (30). Epitopes from GAD65 in NOD mice include amino acid residues 206-220, 247-266, 509-528 and 524-543. Tolerizing three week old NOD mice against GAD65 by either immunizing intravenously or by intrathymic injections practically eliminated insulitis and

diabetes (65, 66), demonstrating the importance of GAD65 as an autoantigen during Type 1 Diabetes. Importantly, the 247-266 and 206-220 epitopes have also been identified in human patients with Type 1 Diabetes. However, several reports strongly argue against GAD as an immunodominant antigen during Type 1 Diabetes. First, GAD65 KO NOD mice develop diabetes normally (67). Second, insulitis and diabetes developed normally when tolerance to GAD was induced by expressing it under the control of the invariant chain promoter (68). Third, there are substantial differences in GAD65 expression between humans and mice, with expression levels in NOD mice being substantially lower than humans (69). These studies have led to substantial resistance against GAD being an immunodominant epitope during Type 1 Diabetes. The majority of the field now acknowledges insulin as the major immunodominant antigen. While the evidence for this consensus will be discussed below in detail, the most substantial finding suggesting the importance of insulin came from mice where insulin was engineered to be a foreign antigen (70). Insulitis and diabetes were completely prevented in these mice, highlighting the importance of insulin in the initiation of diabetes (70). It should be noted, however, that whether other epitopes are essential for sustaining diabetes progression is still debated. Other autoantigens that serve as $CD4^+$ T cell epitopes during Type 1 Diabetes include IA-2, HSP-60, ICA-69, S100β (30) and chromogranin A (71). However, these antigens appear to play less significant roles in diabetes progression than insulin or GAD65 (72).

Insulin is the major immunodominant antigen for spontaneous Type 1 Diabetes development in NOD mice and human patients

While the autoantigens responsible for the development of Type 1 Diabetes are still debated, several lines of evidence highlight the importance of the immune response against insulin during disease development. $CD4^+$ and $CD8^+$ T cell epitopes have been identified within the insulin protein, and anti-insulin autoantibodies are detectable in both NOD mice and diabetic patients (26, 64, 73-79). Importantly, insulin is the only diabetes-associated autoantigen where autoantibodies correlate with age of onset and rate of disease progression (29) Work conducted in NOD mice strengthens the argument that insulin is a major immunodominant epitope both in terms of initiating the disease as well as sustaining the response through diabetes onset. T cells that react to insulin are detectable in the pancreas as early as 4 weeks of age (64), and persist in the pancreas long term as evidenced by their presence at weeks 5, 6, 7, 8, 9, and 12 weeks of age (80). Early work investigating insulin-specific T cells in the pancreas showed that in 12 week old mice the majority of T cells that responded to insulin were of the $CD4^+$ T cell lineage (64), though a $CD8^+$ T cell epitope within insulin (B chain 15-23) has been identified that can comprise up to 10% of $CD8^+$ T cells infiltrating the pancreas (73, 81). Greater than 90% of the insulin-specific $CD4^+$ T cells in the pancreas respond specifically to the 9-23 peptide from the insulin B chain ($insB_{9-23}$) (74). Consequently, a substantial amount of work has been conducted to understand the role of diabetogenic $CD4^+$ T cells specific for $insB_{9-23}$ in NOD mice.

The importance of the T cell response directed against insulin, and more specifically the insB_{9-23} peptide, during Type 1 Diabetes progression has come from two key observations. The first observations came from genetic approaches to study the impact of insB_{9-23}-specific $CD4^+$ T cells during diabetes. First, Maki Nakayama and George Eisenbarth created a NOD mouse line that had the two genes that encode mouse insulin (mouse *ins1* and *ins2*) knocked out, and transgenic expression of insulin containing a critical point mutation in the gene encoding the insulin B chain (alanine for the tyrosine at position 16) (70). This mutation does not impact the metabolic activity of insulin, allowing these mice to regulate glucose metabolism normally. However, previous work from this group had shown that the tyrosine at position 16 was required for insB_{9-23}-specific $CD4^+$ T cell responses (82). Hence, the native mouse insulin peptide sequence is considered a foreign antigen in these mice. Using this mouse model, Nakayama made the seminal observation that when mouse insulin was a foreign antigen, NOD mice did not develop insulitis or Type 1 Diabetes, presumably due to the lack of induction of an efficient insB_{9-23}-specific $CD4^+$ T cell response (70). Introduction of one copy of either mouse insulin 1 or 2 into this transgenic line restores insulitis and diabetes, highlighting the importance of the immune response against mouse insulin in diabetes progression. If the other diabetes-associated epitopes were critical for disease initiation, insulitis and/or diabetes would have been observed in those mice (70). Further evidence that insB_{9-23}-specific T cells are required for diabetes progression comes from generation of a T cell receptor transgenic mouse specific for the 9-23 peptide. These transgenic mice, termed BDC12.4.1, develop Type 1 Diabetes with approximately 40% penetrance on a Rag-

deficient background, demonstrating that when insulin-specific $CD4^+$ T cells are the only T cell specificity in the mouse, diabetes can still develop (83). In this study transfer of $insB_{9-23}$-specific T cells from the BDC12.4.1 mouse into lymphopenic NOD mice (NOD.*scid*) induced diabetes, further supporting the direct pathogenic role of this specificity during Type 1 Diabetes progression (83). These studies firmly established the importance of the insulin-specific $CD4^+$ T cell response during Type 1 Diabetes development in NOD mice.

The second key observation that has led to intense interest in targeting the anti-insulin immune response in patients is that tolerogenic immunization against the insulin protein, the $insB_{9-23}$ peptide, or variants of $insB_{9-23}$ that have been modified to bind with high affinity to $I\text{-}A^{g7}$ can prevent and/or reverse Type 1 Diabetes in NOD mice (59, 84-86). Work using antigen coupled to apoptotic splenocytes using the chemical ECDI revealed a role for $insB_{9-23}$ in the initiation of disease, since treating 4-6 week old mice with splenocytes coupled to $insB_{9-23}$ or whole insulin protein could prevent disease later in life (85). Importantly, treating with cells coupled to $GAD65_{509-528}$, $GAD65_{524-543}$, or $IGRP_{206-214}$ did not confer protection, highlighting the importance of the anti-insulin response early during the initiation of the disease (85). Interestingly, treating older pre-diabetic NOD mice (19-21 weeks) with whole insulin coupled to apoptotic cells, but not $insB_{9-23}$, prevented diabetes, suggesting that the $insB_{9-23}$ epitope may be less important for sustaining disease progression in older mice (85). These results have been confirmed and extended using tolerogenic immunization (84, 86) with an $insB_{10-23}$ peptide variant that had been modified to bind with higher affinity to $I\text{-}A^{g7}$ (87). Zhang and Daniel

demonstrated that immunizing 4-6 week old NOD mice with this modified peptide could prevent diabetes, supporting the conclusion by Prasad and colleagues that the insulin B peptide is important for initiating disease (84, 86). However, Daniel and colleagues also showed that immunizing 12-14 week old NOD with the modified peptide could prevent diabetes, contrary to the conclusions made by Prasad using the natural 9-23 peptide coupled to apoptotic cells (84, 85). It is possible that modifying the insulin B peptide to increase stable binding to I-A^{g7} increased the potency of the tolerogenic stimulus in Daniel's work compared to the natural peptide sequence in Prasad's work, and this issue should be further pursued to determine the efficacy of using peptide variants for therapeutic benefit in patients with Type 1 Diabetes. Use of the whole protein as a tolerogenic stimulus should also be considered for therapeutic benefit, since work from our lab using whole insulin coupled to cells using ECDI reversed diabetes in approximately 50% of new onset diabetic NOD mice (59). Whether tolerogenic immunization with $insB_{9\text{-}23}$ or variants of this peptide will be able to reverse diabetes in NOD mice remains to be investigated. Collectively, these data show that the immune response against insulin is important both early during the initiation of disease and late near and/or at disease onset.

Antigen recognition by $insB_{9\text{-}23}$-specific $CD4^+$ T cells in vivo

Activation of diabetogenic T cells is requisite for Type 1 Diabetes development; therefore, there is interest in understanding how these pathogenic cells get activated by autoantigen in vivo. The early events that lead to the activation of autoreactive T cells in

NOD mice and patients with Type 1 Diabetes are poorly understood; however, the most favored model is that T cells get activated in the pancreatic lymph node (LN) since naïve diabetogenic T cells proliferate in the pancreatic LN prior to observable insulitis (88, 89) and removal of the pancreatic LN (but not the spleen) prevents diabetes onset (90). Antigen can enter the pancreatic LN in two ways: (1) intra-islet dendritic cells can take up antigen from dying beta cells and migrate to the pancreatic LN (91), or (2) small particulate antigen may drain directly from the pancreas to the pancreatic LN through lymphatics (92). Evidence supporting migration of dendritic cells from the pancreas to the pancreatic LN comes from work using NOD.MIP-GFP mice (91, 93), where the gene encoding green fluorescent protein is under the control of the mouse insulin promoter (94). Intra-islet dendritic cells containing GFP could be detected in both the pancreas and pancreatic LN, and T cell-mediated inflammation promoted dendritic cell trafficking to the pancreatic LN (93). Importantly, dendritic cells isolated from islets were uniquely capable of activating diabetogenic T cells (57). Collectively, these data support a model where naïve diabetogentic T cells get activated in the pancreatic LN either by a migrating population of intra-islet dendritic cells or presentation of pancreatic antigen that had drained to the pancreatic LN and was presented by LN-resident dendritic cells.

The source of the pancreatic antigen responsible for activating ins$B_{9\text{-}23}$-specific $CD4^+$ T cells is atypical compared to other immune responses. Canonical $CD4^+$ T cell activation occurs when whole proteins are broken down by antigen presenting cells such as dendritic cells, processed in the MIIC endocytic compartment, loaded onto MHC class II molecules, and presented on the cell surface. $CD4^+$ T cells capable of responding to

antigens processed through this pathway are considered Type A cells. However, insB_{9-23}-specific $CD4^+$ T cells in NOD mice do not potently respond to immunization with whole insulin protein, suggesting that the natural cleavage products of insulin presented on MHC class II do not efficiently activate this population in vivo (57). One hypothesis to explain this result is that cells which respond with high affinity to the natural cleavage products of insulin get deleted in the thymus. Rather, the pathogenic insB_{9-23}-specific $CD4^+$ T cells that contribute to diabetes pathogenesis escape deletion because they specifically respond only to the B_{9-23} peptide that is present in the pancreas which is not a natural cleavage product of insulin that arises in the thymus. The source of the insB_{9-23} peptide that activates these cells comes from granules in beta cells that contain a high concentration of pre-processed peptides that are a by-product of insulin production (57). Cells that respond to specific peptide fragments but not natural cleavage products from the endogenous protein are referred to as Type B cells, and evidence suggests that insB_{9-23}-specific $CD4^+$ T cells that are Type B in nature are pathogenic during Type 1 Diabetes (57). While it is still unclear whether the insulin peptides from beta cell granules are taken up by intra-islet dendritic cells and processed through the canonical MHC class II-processing pathway or whether these peptides are directly loaded onto empty MHC class II molecules on the cell surface, it is generally agreed that these peptides serve as the main source of antigen responsible for activating insulin-specific $CD4^+$ T cells in NOD mice.

The importance of insulin-specific $CD4^+$ T cells during the initiation and development of Type 1 Diabetes has generated substantial interest in investigating the

dynamics of insB_{9-23}-specific $CD4^+$ T cells during diabetes development in vivo. However, the field has been limited by the inability to track endogenous insulin-specific $CD4^+$ T cells in unmanipulated NOD mice. Recent advances in the generation and use of peptide/MHC class II tetramer reagents have greatly improved our ability to track endogenous $CD4^+$ T cell populations (95, 96). Of particular importance has been the development of enrichment techniques to improve the sensitivity of detecting incredibly rare antigen-specific populations in intact immune repertoires (95-97). Despite advances in detecting rare populations of cells, peptide binding registers remain an important issue when constructing peptide/MHC class II tetramer reagents. Due to the way MHC class II molecules form, the ends of the peptide-binding groove are open and therefore can accommodate peptides that are longer than the nine amino acid core that fits into the groove. Because a portion of the peptide can be found outside of the groove, the entire peptide can be frame-shifted along the MHC molecule. Each time the peptide frame shifts by one amino acid, the amino acid residues that stick out of the peptide binding groove and contact the T cell receptor change. Therefore, for one peptide, there are several different binding registers, and for each register the T cell receptor is encountering a completely different set of amino acid residues. When tracking antigen-specific $CD4^+$ T cells, this issue of register is particularly problematic, since each frame shift will provide a unique peptide that is binding a unique population of T cells, and potentially not all populations of these T cells are involved in the immune response of interest.

Groups led by John Kappler and Emil Unanue have extensively investigated the impact of different insB_{9-23} peptide binding registers on the insulin-specific $CD4^+$ T cell

response. Three primary registers have been postulated for the insulin B_{9-23} peptide. Registers 1 (core segment 12-20 (VEALYLVCG) and 2 (core segment 13-21 (EALYLVCGE) can stably bind I-A^{g7} (98). Register 3 (core segment 14-22 ALYLVCGER) poorly binds I-A^{g7} due to a charge clash between the p9 anchor residue (arginine) and the positively charged p9 binding pocket unique to the NOD I-A^{g7} molecule (87). Kappler and colleagues performed a series of modifications to the insB_{9-23} peptide to stabilize binding to I-A^{g7}. Modifying the amino acids at p1 and p9 of the nonomer core from the insB_{10-23} peptide to enhance binding to I-A^{g7} did not alter the ability of register 1 or 2 to bind I-A^{g7}, but substantially enhanced binding of register 3 (87). Only the mutation at p9 was required to enhance binding, likely due to the alleviation of the charge clash with the p9 binding pocket. Importantly, mutating register 3 to allow strong binding I-A^{g7} caused the register 3 peptide to stimulate IL-2 production from more insulin-specific hydridomas than either register 1 or 2 (87). Further work by Kappler's group highlighted the importance of register 3 in stimulating insulin-specific $CD4^+$ T cells (99). In this study a series of tetramers were constructed with the insulin B peptide bound in register 3, but with a critical point mutation to mimic registers 1 and 2. Using these reagents, this group determined that the glutamic acid at B:21 (position 8 of the nonomer core in the peptide-binding groove for register 3) could either promote or inhibit binding to different insulin-specific hybridomas. Hence, the authors concluded that all insulin-specific $CD4^+$ T cell hybridomas could recognize the register 3 peptide, though some favored the glutamic acid at B:21 (mimicking registers 2 and 3) while other were inhibited by it (mimicking register 1) (99). These data have caused a substantial

shift in the field to study insulin-specific $CD4^+$ T cells capable of binding this modified register 3 peptide ($insB_{10\text{-}23r3}$) (87, 99), though there is still some interest in register 1 and 2 in stimulating these cells as well (100). Studies incorporating tetramers containing multiple binding registers are needed to understand this pathogenic population.

$CD4^+$ T cells from the BDC2.5 T cell receptor transgenic mouse are used to study Type 1 Diabetes pathogenesis

The last specificity that will be discussed is the BDC2.5 T cell population which recognizes Chromogranin A. This specificity is discussed because it is used throughout the thesis as a model antigen-specific population. Like many of the other known diabetogenic T cell clones, the $CD4^+$ T cell encoding the BDC2.5 T cell receptor was originally isolated from the islet infiltrate of a diabetic NOD mouse (101). Since T cells are thought to traffic to and get retained in the pancreas based on their specificity for pancreatic antigens and pathogenic potential during diabetes (102), it is often predicted that cells cloned out of the pancreas are important for disease progression. Creation of a $CD4^+$ transgenic mouse encoding this diabetogenic T cell receptor confirmed the hypothesis that BDC2.5 T cells were pathogenic and could cause Type 1 Diabetes (103). Transfer of BDC2.5 T cells derived from either the original clones (104) or the transgenic mouse (103) accelerated diabetes onset in vivo. Due to its ability to transfer disease into pre-diabetic NOD mice, the BDC2.5 transgenic mouse has provided a model system used by several investigators for over twenty years to study $CD4^+$ T cells during Type 1 Diabetes (105). However, studying this population has been complicated by a lack of

knowledge of the natural antigen that this specificity recognizes. It was appreciated early after isolating this clone that unlike many of the other clones isolated from the pancreas within that time frame (64, 74), BDC2.5 T cells did not respond to insulin. These cells did, however, respond to pancreatic islet cells, beta cell extracts, and beta cell adenomas (106, 107). In 2001 Darcy Wilson's group substantially enhanced the ability to study BDC2.5 T cells by constructing a library of "mimics of epitopes" or "mimetopes", which are synthetic peptides that could be recognized by the BDC2.5 T cell receptor (108). This mimetope screen identified a series of peptide ligands that bound to the BDC2.5 T cell receptor with varying affinities, ranging from incredibly low to high (108). For the ten amino acid core that binds MHC class II, all of these peptides share the consensus sequence XXXPLWX(R/K)M(D/E). The presence of the amino acids P, L, W, R/K, and M at positions 4, 5, 6, 8, and 9, respectively, are required for activity; however, varying the amino acids as positions 1, 2, 3, and 7 provided a wide range of affinities for the T cell receptor that correlated with a range of stimulatory capacities (108). Many studies have relied on in vitro activation of BDC2.5 T cells with the high affinity mimetopes p31, p63, or PS3 (46, 59, 109, 110). However, the availability of low affinity mimetopes provides the opportunity to interrogate issues of affinity in diabetogenic T cell functions.

Use of the mimetope library defined by Wilson and colleagues has generated substantial data on the biology of islet-reactive $CD4^+$ T cells during Type 1 Diabetes; however, there was a need to identify the natural antigen recognized by this T cell population in order to gain a deeper understanding of how these cells became activated in vivo. While the specific peptide epitope that binds MHC class II is still debated (71, 109),

it is generally accepted that the natural antigen is contained within the protein Chromogranin A (71). This critical finding was evidenced by the inability of beta cell membranes isolated from Chromogranin A-deficient mice to stimulate IFNγ by BDC2.5 T cells in vitro, while Chromogranin A-sufficient beta cell membranes stimulated substantial IFNγ production (71). The issue that remains debated is which exact peptide sequence from Chromogranin A is the natural epitope. Two candidate epitopes are WE14 (Chg358-371) or Chg29-42. Haskins and colleagues identified WE14 as a potential candidate first based largely on searching for the consensus sequence identified by the mimetope library screen (71, 108). While multiple candidates were identified in Chromogranin A containing this consensus sequence, none of the candidates stimulated BDC2.5 T cells except WE14. WE14 is a natural cleavage product of Chromogranin A (Chg358-371), but the N terminal end of the peptide starts at position 5 of the consensus sequence from the mimetope screen, not position 1 (71). The authors speculated that the four N terminal amino acids that would normally occupy the peptide binding groove are simply missing in this case, with WE14 binding I-A^{g7} in an atypical manner by occupying only half of the binding groove (71). Other groups have not successfully repeated these results, failing to show that WE14 has any stimulatory capacity (109)(KEP unpublished observation). Considering the lack of reproducibility with WE14, Singh and colleagues proposed Chg29-42 as the natural epitope for BDC2.5 instead of WE14 (109). This peptide is derived from Vasostatin-1, another natural cleavage product of Chromogranin A (ChgA1-76). This peptide contains only three of the amino acids from the consensus sequence predicted from the mimetope library, but is better at stimulating

proliferation of BDC2.5 T cells in vitro than WE14 (109). Which peptide (if either) is the natural epitope remains debated. In Chapter 2 we developed a model that allowed us to study activation of BDC2.5 T cells without knowledge of the natural antigen, circumventing issues associated with identifying the natural ligand for this diabetogenic T cell receptor.

MECHANISMS OF IMMUNOLOGICAL SELF TOLERANCE SUPPRESS AUTOIMMUNE ACTIVATION AND TYPE 1 DIABETES

Overview of central and peripheral tolerance mechanisms

The lymphocyte receptors of T cells and B cells develop randomly to increase the likelihood that these cells will have a receptor capable of recognizing a diverse repertoire of foreign antigens. This process, which involves the rearrangement of small gene segments to provide incredible diversity, is independent of foreign antigen exposure. This random process of receptor development, coupled with the fact that lymphocyte receptors recognize ligands that are physically small (only four or five amino acids within a peptide bound to MHC contact the T cell receptor), creates a major problem for the adaptive immune system. The problem is that there is a high likelihood that lymphocyte receptors will recognize self antigens in the body rather than being specific for foreign antigens. In an attempt to protect self tissues from the potentially devastating effects of an anti-self or autoimmune response, mechanisms of central and peripheral tolerance have evolved to eliminate or restrain autoreactive cells. Central tolerance refers to events that occur during lymphocyte development in the central or primary lymphoid organs, including the

bone marrow for B cells and the thymus for T cells. The main mechanism of central tolerance involves deletion of autoreactive lymphocytes that bind with high affinity to antigens present in these primary lymphoid organs. Deletion of these cells is an effective mechanism of tolerance because it completely eliminates the risk of aberrant activation in the periphery. One unique mechanism in the thymus that promotes deletion of self-reactive T cells is Aire, or autoimmune regulator (111-113). Aire is expressed in the thymic medulla by medullary thymic epithelial cells, and allows expression of self proteins in the thymus that are normally restricted to peripheral tissue sites, including insulin, thyroglobulin, and zona pellucida glycoprotein (111). The importance of Aire in promoting deletion of self-reactive T cells comes from *aire* knockout mice, which develop mutli-organ autoimmunity (111) and human patients with autoimmune polyendocrinopathy, candidiasis and ectodermal dysplasia (APECED), which manifests as the spontaneous development of autoimmunity in multiple endocrine organs (112, 113).

An additional mechanism of central tolerance is lineage deviation to a regulatory phenotype. $CD4^+$ T cells with a high affinity for self antigens expressed in the thymus can induce expression of the master transcription factor Foxp3 and subsequently acquire a regulatory phenotype rather than being deleted (114-116). Therefore, regulatory T cells (also referred to as Treg cells) that develop in the thymus not only reduce the number of autoreactive T cells, but these cells also substantially contribute to the regulation of conventional T cells in the periphery (117). The importance of Treg cells in regulating autoreactive T cells comes from mice that lack functional Foxp3, including the *scurfy* mutation (118) or Foxp3 KO mice (114, 115), which develop fatal multi-organ

autoimmunity early in life. A similar phenomenon is observed in human patients with loss of function mutations in Foxp3 (119, 120). These patients die early in life from immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which manifests as multi-organ autoimmunity due to a lack of Treg cells (119, 120). Consequently, central tolerance acts as a front line defense against autoimmunity, and defects in these tolerance mechanisms (e.g. Aire and Foxp3) result in devastating multi-organ autoimmune disorders.

Despite the well-established role for central tolerance in reducing the frequency of potentially autoreactive T cells in the periphery, autoreactive T cells are present in the periphery of all healthy individuals (121-123). Therefore, mechanisms of peripheral tolerance are required to control these cells to prevent autoimmunity. Peripheral tolerance mechanisms include: antigen sequestration, ignorance, extrinsic regulation by Treg cells, intrinsic regulation by anergy, and clonal deletion. This section will provide a brief introduction to the different peripheral tolerance mechanisms, and a more detailed discussion of how these mechanisms contribute to regulation during Type 1 Diabetes will subsequently follow.

Antigen sequestration results when tissue antigens are being isolated from the potentially autoreactive cells of the immune system. Naïve T cells and B cells circulate through the secondary lymphoid organs (lymph nodes and spleen), and are restricted from entering non-lymphoid tissues (124). Therefore, the likelihood that these cells will encounter tissue antigens is limited. Antigen sequestration is particularly important for sites of immune privilege, which either lack or have reduced lymphatic drainage to limit

the probability of antigen entering the secondary lymphoid organs (125). These sites include the brain, eye, testis, and the uterus during pregnancy. This sequestration leads to ignorance, or a lack of activation of autoreactive T cells in the periphery (125). However, since activated effector T cells are capable of infiltrating the majority of non-lymphoid tissues (124, 126, 127), the premise of antigen sequestration as a tolerance mechanism pertains mostly (though not exclusively) to naïve T cells.

Treg cells mediate cell extrinsic regulation of other conventional T cells. There have been several mechanisms proposed for how Treg cells suppress, including: direct cytolysis, cytokine deprivation, inhibitory receptors (e.g. CTLA-4), suppression through cytokine release (e.g. IL-10, TGFβ, IL-35), and alteration of antigen presenting cells including promoting up-regulation of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) by antigen presenting cells (117). Through these mechanisms, Treg cells contribute to homeostatic control of the adaptive immune system, regulate cells at sites of inflammation to mitigate tissue damage, aid in the resolution of immune responses, and promote tolerance (117). The importance of the Treg cells in regulating the adaptive immune system is evidenced by the severe phenotype of mice lacking these cells (e.g. *scurfy* mice (118) and Foxp3-deficient mice (114, 115)) as described above, which develop severe multi-organ autoimmunity that results in death early in life. The role Treg cells during Type 1 Diabetes will be discussed in more detail in subsequent sections.

The remaining three mechanisms of peripheral tolerance described herein manifest when autoreactive T cells encounter autoantigen in the periphery. First, these

cells can simply be deleted. This outcome typically occurs when T cell have a high affinity receptor for autoantigen, though this mechanism is likely uncommon during physiological conditions in vivo since most of these cells are deleted in the thymus. Second, $CD4^+$ T cells can induce Foxp3 expression in the periphery, becoming adaptive or peripheral Treg cells. Treg cells that are induced in the periphery (rather than the thymus) have some redundant functions with thymic-derived Treg cells, though an important area of research is currently focused on how these cells differ (128). One notable difference is likely T cell receptor specificity: Since adaptive Treg cells develop post-selection in the thymus, these cells should have a unique set of T cell receptors compared to thymic Treg cells (129, 130). While evidence suggests that there is minimal overlap between the T cell receptor specificities between natural Treg cells and adaptive Treg cells (131), whether there is some overlap and the extent of that overlap is debated (132). The role of T cell receptor specificity in Treg suppression remains controversial, though some favor a model where antigen-specific Treg cells are better at suppressing conventional T cells of the same specificity than non-specific Treg cells (133). The third major fate is anergy induction, which leads to a state of functional hyporesponsiveness. Following anergy induction, cells persist long term rather than getting deleted, but upon reencounter with their cognate antigen fail to produce effector cytokines or proliferate (134). Since inducing T cell anergy is a goal of our lab to inhibit autoimmunity in patients, a more detailed discussion will follow in the next section. In summary, both central and peripheral tolerance mechanisms act to restrain potentially autoreactive T cells. Since central tolerance in incomplete, several mechanisms of peripheral tolerance

are needed to control these cells. Strategies to enhance peripheral tolerance therapeutically to inhibit autoreactive T cells in patients will be discussed in Chapter 5.

Anergy is an important mechanism of peripheral tolerance used to restrain autoreactive T cells following autoantigen encounter

Anergy is cell intrinsic mechanism of peripheral tolerance broadly defined as functional inactivation following exposure to antigen. These cells are impaired in cell division, differentiation, and cytokine production, particularly following secondary exposure to antigen. Rather than undergoing cell death, anergic cells live for an extended period of time in this hypofunctional state (134). The nomenclature involved in studying T cell anergy has become convoluted over the years, and now "anergy" can be subdivided into at least two different types defined by Schwartz as "clonal anergy" and "adaptive tolerance" or "in vivo anergy" (134). The requirement for antigen in the maintenance of anergy, the ability of IL-2 to reverse anergy, and the cellular events leading to anergy induction vary depending on the subtype of anergy being considered. Classical "clonal anergy" is caused when pre-activated T cells undergo incomplete secondary T cell activation, whereas "in vivo anergy" is typically caused when naïve T cells encounter antigen either in the absence of positive costimulation or in the presence of high levels of inhibitory signals such as PD-1 or CTLA-4. Clonal anergy can be maintained without antigen, while in vivo anergy requires the persistence of antigen to be maintained. Additionally, clonal anergy can typically be reversed by high doses of IL-2, while in vivo anergy cannot (134). Additional work is needed to identify the molecular

requirements involved in the induction and maintenance of anergy, with a focus on the safety and efficacy of exploiting anergy in patients to inhibit the function of autoreactive T cells. Since anergy is induced following stimulation through the T cell receptor, T cells can be shut off in an antigen-specific manner. This approach to inducing tolerance leaves patients far less immunocompromised than broad-spectrum non-antigen specific therapies targeted at wide spread immunosuppression such as corticosteroids. Therefore, investigators are actively pursuing ways to induce anergy clinically. As will be discussed in a later section, work from our lab has shown that treatment with antigen-coupled ECDI-fixed cells can induce anergy in islet-reactive $CD4^+$ T cells to prevent and reverse Type 1 Diabetes (59), which could provide an exciting therapeutic platform to inhibit the autoimmune response in diabetic patients.

Role of central versus peripheral tolerance mechanisms in controlling islet-reactive T cells during Type 1 Diabetes

The relative contribution of central and peripheral tolerance mechanisms to the control of diabetogenic T cells in NOD mice is controversial; however, it is clear that for the mice that develop Type 1 Diabetes both mechanisms ultimately fail. Defects in central tolerance in NOD mice lead to increased numbers of autoreactive T cells in the periphery, which periphery tolerance mechanisms fail to control in cases when diabetes develops. It had been speculated that defects in selection would be correlated with the I-A^{g7} molecule because it directly impacts the peptides that select $CD4^+$ T cells in the thymus. However, the MHC class I allele is not a diabetes susceptiblity locus, and $CD8^+$

T cells are required for Type 1 Diabetes pathogenesis. Sprent and colleagues provided the first evidence that there were defects in negative selection in NOD mice, and this defect was independent of the I-A^{g7} allele (135). Rather, this defect was thought to be caused by enhanced expression of anti-apoptotic molecules following T cell receptor engagement such as caspase-8-homologous Fas-associated death-domain-like interleukin 1β-converting enzyme inhibitory protein (cFLIP), which antagonizes apoptosis induced by Fas-dependent negative selection (135). Work by Zucchelli and colleagues further supported the conclusion that there were defects in central tolerance in NOD, showing that deletion of self-reactive thymocytes was impaired in fetal thymic organ cultures derived from NOD compared to B6 mice expressing the NOD MHC class II allele (B6.g7 mice) (136). Collectively, these data showed that clonal deletion during thymocyte development is impaired in NOD mice, which leads to increased numbers of autoreactive T cells in the periphery.

Despite the growing evidence that defects in central tolerance lead to Type 1 Diabetes development in NOD, the majority of the field continues to focus on defects in peripheral tolerance in these mice because it is more feasible to enhance peripheral tolerance to inhibit autoreactive T cell functions clinically than to alter central tolerance through thymic selection. Multiple pathways involved in peripheral tolerance are implemented in Type 1 Diabetes pathogenesis (31). Genes involved in costimulatory and inhibitory pathways are involved in diabetes development. Polymorphisms in CTLA-4 have been associated with increased susceptibility to Type 1 Diabetes (18, 19), and blocking CTLA-4 in young NOD mice accelerates diabetes (137). Additionally, NOD

mice deficient in the inhibitory receptor PD-1, which is involved in the induction and maintenance of T cell tolerance (59), develop accelerated diabetes (138). NOD mice deficient in the positive costimulatory molecule CD28 develop accelerated diabetes, though this effect is due to defects in Treg cell development in the absence of CD28 (139). Treg cells are important during Type 1 Diabetes development, since loss of this population exacerbates disease in NOD (139) and transfer of large numbers of Treg cells or therapies targeted at enhancing their function slows diabetes (133, 139-141). These findings have led to preclinical work and early clinical trials involving the adoptive transfer of Treg cells to induce tolerance in Type 1 Diabetes patients (133, 140, 142), which will be discussed in Chapter 5. In NOD mice, however, there are contradictory reports as to whether loss of suppressive capacity by Treg cells contributes to disease pathogenesis. Some studies have shown decreases in Treg cell numbers or function with age and subsequently disease progression in NOD mice (139, 143), and the *idd3* diabetes susceptibility locus has polymorphisms in the genes encoding IL-2 and IL-21, which are involved in Treg homeostasis (144). However, work from the Mathis and Benoist group has suggested that Treg cells in NOD are not functionally impaired, but rather diabetes develops due to over-activity of conventional T cells, leading to their inability to be suppressed (145). These findings have important therapeutic implications for Treg cell-based therapy for Type 1 Diabetes, which will be discussed further in Chapter 5.

THE ROLE OF THE INHIBITORY RECEPTOR PROGRAMMED DEATH-1 (PD-1) AND ITS LIGANDS IN REGULATING T CELL FUNCTIONS

Members of the CD28 superfamily regulate T cell activation and function

T cell activation is a highly coordinated process that involves at least three distinct signals: (1) the T cell receptor binding peptide/MHC, (2) positive costimulatory signals such as CD28 on the T cell binding B7, and (3) signals from inflammatory cytokines such as IL-12 and Type I IFN that aid in effector differentiation (146, 147). This activation process is subject to a high degree of regulation to provide specificity to the adaptive immune response (through peptide/MHC) and limit immunopathology. Inhibitory receptors are critically involved in regulating initial T cell activation as well suppressing on-going effector responses. Two of the most well-characterized inhibitory receptors are CTLA-4 and PD-1, both of which belong to the CD28 superfamily of immune receptors (147). Unlike CD28, which is expressed on naïve T cells, CTLA-4 and PD-1 expression are specifically induced following T cell activation by peptide/MHC complexes, increasing the susceptibility of activated T cells to immune regulation. An interesting feature of PD-1 and its ligands are their broad expression patterns: CD28 and CTLA-4 are largely restricted to T cells, while PD-1 is expressed by T cells as well as B cells and some myeloid cells. CD28 and CTLA-4 share the ligands CD80 (or B7-1) and CD86 (or B7-2), which are largely restricted to antigen presenting cells (147). On the contrary, PD-1 has distinct ligands from CD28 and CTLA-4, including Programmed Death Ligand (PD-L) 1 and PD-L2. PD-L1 has an incredibly broad expression pattern, including antigen presenting cells, T cells, B cells, and several non-hematopoietic cell lineages including pancreatic islet cells, vascular endothelial cells, liver non-parenchymal cells including sinusoidal endothelial cells and Kupffer cells, keratinocytes, and placental

synctiotrophoblasts. PD-L2 is more restricted in its expression, and is largely limited to antigen presenting cells (148). Additionally, PD-L1 can interact with CD80, providing further complexity in terms of ligand interactions (149, 150). The broad distribution of PD-L1 suggests that this pathway plays a critical role in regulating PD-1-expressing T cells in both lymphoid and non-lymphoid tissues, and has made targeting this pathway an attractive therapeutic option to manipulate T cell responses at effector sites.

While PD-1 and CTLA-4 are both inhibitory receptors expressed following activation, these pathways are not redundant in terms of regulating T cell functions. Differences between PD-1- and CTLA-4-mediated suppression were revealed by analysis of mice genetically deficient for either molecule. CTLA-4 KO mice die early in life from lymphoproliferative disease regardless of genetic background (151, 152). CTLA-4 expressed by Treg cells is required to suppress lymphoproliferative disease (153), and supplementing CTLA-4-deficient mice with wild type regulatory T cells using bone marrow chimeras prevented lymphoproliferative disease, highlighting the importance of CTLA-4 on Treg cells (154). PD-1 KO mice do not develop lymphoproliferative disease; rather, these mice develop accelerated autoimmunity that is dependent on genetic background. C57Bl/6 mice develop a lupus-like nephritis (155), BALB/c mice develop dilated cardiomyopathy (156), and NOD mice develop accelerated Type 1 Diabetes (138). These data reveal important differences between PD-1 and CTLA-4, and suggest that CTLA-4 is more important in regulating the threshold for T cell activation, while PD-1 is more important in fine tuning effector responses, particularly in non-lymphoid tissues. This difference between these two inhibitory receptors has engendered the hypothesis

that blocking PD-1 therapeutically to enhance immunity will have less adverse side effects than CTLA-4. An additional discussion involving PD-1 blockade in clinical trials will follow in Chapter 5.

Regulation of PD-1 and its ligands

The regulation of PD-1 and its ligands is an area of on-going investigation. However, it is clear that PD-1 on T cells is regulated at least in part by antigen engagement through the T cell receptor. PD-1 is not expressed on naïve T cells (157). Activation through the T cell receptor causes PD-1 to become expressed, and it is considered a marker of recent activation during acute antigen settings (157). Chronic antigen engagement causes PD-1 levels to remain sustained, and continual signaling through PD-1 can result in progressive loss of function leading to exhaustion (158, 159). Whether there are signals independent of antigen that can regulate PD-1 expression on T cells is unclear. Additionally, how PD-1 is regulated on other cell types, such as myeloid cells that lack antigen receptors, is also unclear.

Our understanding of how the PD-1 ligands are regulated is also limited, though both PD-L1 and PD-L2 appear to be induced by inflammation. Type I (IFNα and IFNβ) and Type II (IFNγ) interferons induce the up-regulation of PD-L1 (160, 161), and IFN regulatory factor (IRF) binding sites (IRF-1) have been identified in the PD-L1 promoter (162). Additionally, pathways known to be activated during inflammation, including MyD88, TRAF6, MEK, and JAK2 have all been implicated in the induction of PD-L1 (162, 163). Less is known about the regulation of PD-L2, but it can be induced by GM-

CSF, IL-4, and IFNγ (164, 165). A better understanding of how PD-1 and its ligands are regulated will enhance our ability to exploit this pathway therapeutically.

Mechanism of PD-1-mediated suppression

In order to deliver an inhibitory signal, PD-1 must be ligated in cis with the T cell receptor, meaning the PD-L and peptide/MHC must be expressed on the same cell to signal (166). This signaling mechanism is shared with CTLA-4, which must bind B7 on the same cell that is presenting MHC to the T cell in order to deliver an inhibitory signal (167). One hypothesis for why PD-1 must be engaged simultaneously with the T cell receptor is that the inhibitory effects delivered by PD-1 involve dephosphorylation of key signaling intermediates downstream of the T cell receptor that are phosphorylated in the activated state. Alternatively, PD-1 may counteract the positive signals associated with promoting T cell differentiation and survival, including those regulated by CD28 and IL-2 (168-170). These models are not necessarily mutually exclusive, and it is likely that PD-1 acts to oppose several positive signals being delivered to activated T cells. PD-1 is a 55 kDa Type I transmembrane protein that contains two key tyrosine-containing signaling domains, one ITIM motif and one ITSM motif (148). Phosphatases SH2-domain containing tyrosine phosphatase (SHP)-1 and SHP-2 can be recruited to the ITIM and ITSM of PD-1, leading to inhibition of T cell receptor-mediated phosphorylation of CD3ζ, ZAP70, and PKCθ (171, 172). Interestingly, despite the abundance of ITIM motifs in immunoreceptors, only the ITSM motif is required for PD-1 to inhibit T cell functions (169, 171). Ultimately PD-1 signaling inhibits PI3K (173) and Erk activity (171), leading

to decreases in cytokine production, proliferation, and survival. Despite advances in our knowledge of the mechanism of PD-1-mediated inhibition, additional studies are needed to fully elucidate the mechanism by which PD-1 controls T cell functions, particularly during chronic antigen settings where anti-PD-1 antibodies are being tested for use in humans to enhance immunity.

The downstream effect of PD-1 ligation is dampened effector T cell responses. Several in vitro and in vivo studies have described roles for PD-1 in suppressing proliferation and effector cytokine production and impairing survival (59, 169, 174-177). During acute antigen settings, PD-1 is important for dampening T cell effector functions to limit immune pathology. For example, during adenovirus infection, PD-1 KO mice clear the virus more rapidly, but also develop more severe hepatocellular damage than PD-1 sufficient mice (178). During persistent antigen settings such as chronic viral infections and malignancies, continued engagement of PD-1 by $CD8^+$ T cells leads to a progressive loss of function referred to as exhaustion (148, 158, 159). This loss of function occurs in a hierarchical manner, with IL-2, cytotoxicity, and robust proliferation being lost early and TNFα and IFNγ being lost later at more terminal stages of exhaustion (179). PD-1 blockade can restore functional capacity to a subset of exhausted T cells (158, 159, 180), providing a promising therapeutic avenue that will be discussed in more detail in Chapter 5. PD-1 and its ligands also function during autoimmunity, which will be discussed in the next section. However, whether the PD-1 pathway can be targeted therapeutically to inhibit T cell functions in patients with autoimmunity is unclear.

Role for PD-1 and its ligands during Type 1 Diabetes

PD-1 interacting with its ligand PD-L1 is important for slowing Type 1 Diabetes progression (137, 138, 174). NOD mice genetically deficient for either PD-1 (138) or PD-L1 (174) develop accelerated diabetes, and blocking either PD-1 or PD-L1 with neutralizing antibodies accelerates the disease (137). Blocking PD-L2 does not accelerate diabetes, highlighting the importance of PD-1/PD-L1 interactions in disease progression. Interestingly, PD-1/PD-L1 blockade precipitates disease when administered to both young (4 weeks of age) and old (10 weeks of age) NOD mice, but the rate of disease onset is far more rapid in older mice (137). This result differs from CTLA-4, where administering neutralizing antibody only to extremely young mice (1 week of age) causes accelerated diabetes on the NOD background (137). Importantly, polymorphisms in the gene encoding PD-1 have been described in humans with susceptibility to developing Type 1 Diabetes (181), suggesting that perhaps PD-1 is less functional during human diabetes development.

One interesting feature of the PD-1 pathway is the broad distribution of PD-L1 in both lymphoid and non-lymphoid tissue. Several lines of evidence suggest that PD-1 is controlling T cell effector functions in both the pancreas-draining LN and pancreas during Type 1 Diabetes. Removing the pancreatic LN prior to anti-PD-L1 treatment revealed interesting differences in PD-1-mediated regulation of young (3 weeks of age) versus old (10 weeks of age) NOD mice (182). When the pancreatic LN was removed at 3 weeks of age, anti-PD-L1 no longer accelerated disease; however, when the pancreatic

LN was removed at 10 weeks of age, anti-PD-L1 accelerated disease similarly to control-treat mice (182). This result suggested that the effector T cells in the pancreas of a 10 week old NOD mouse were sufficient to induce diabetes, but were being actively regulated by PD-1/PD-L1 interactions in the islets. At 3 weeks of age there were likely few activated autoreactive T cells in the pancreas, rendering PD-1 blockade ineffective at accelerating disease. This work emphasizes the importance of PD-1 in the pancreas, but does not exclude a role for PD-1 in regulating autoreactive T cells in the pancreatic LN. Since PD-1 regulates activated T cells during antigen engagement, it is likely that PD-1 acts in both the pancreatic LN and pancreas to suppress diabetogenic T cell functions. Evidence generated by our lab supports this idea, since anti-PD-L1 treatment induced the T cell stop signal in both the pancreatic LN and islets, an event associated with acquisition of potent effector functions. This stop signal was not induced in the non-antigen containing inguinal LN, showing the importance of antigen in PD-1-mediated suppression (110). Hence, PD-1 acts to suppress T cells in both the pancreatic LN and pancreas. However, the temporal requirements for PD-1 may change during Type 1 Diabetes progression due to the relative functional importance of suppression in the pancreatic LN versus the pancreas as disease progresses.

Work from several groups has addressed the effect of anti-PD-L1 on Type 1 Diabetes progression both in NOD mice as well as B6 mice expressing model antigens under the control of the insulin promoter, a model which provides a reductionist approach to studying antigen-specific T cell responses against islet antigens (182-186). By treating NOD mice deficient for B cells (NOD.μMT), $CD4^+$ T cells (NOD.CIITA KO), or $CD8^+$

T cells (NOD.β2m KO), Guleria and colleagues demonstrated that $CD4^+$ T cells and $CD8^+$ T cells were required for anti-PD-L1 treatment to accelerate Type 1 Diabetes, but B cells were dispensable for this effect (182). Anti-PD-L1 caused increased proliferation of two islet-reactive $CD4^+$ (BDC2.5 and 4.1) and one $CD8^+$ (8.3) T cell receptor transgenic populations in the pancreatic LN but not the non-antigen containing inguinal LN (182). Interestingly, anti-PD-L1 did not influence survival measured by apoptosis (182), which contradicted predictions made based on PD-1 ligation preventing up-regulation of the pro-survival molecule Bcl_{XL} (169). Work using expression of the model antigen ovalbumin (OVA) under the rat insulin promoter (RIP-mOVA) on the B6 genetic background has further elucidated the role of PD-1/PD-L1 interactions in regulating islet-reactive $CD8^+$ T cell responses. Treating mice with anti-PD-L1 antibody, Martin-Orozco and colleagues observed a more substantial impact of PD-1 blockade on enhancing OVA-specific (OT-1) $CD8^+$ T cell granzyme B and IFNγ production than proliferation (185). Keir et al. utilized the same OT-1/RIP-mOVA model, but used OT-1 cells genetically deficient for PD-1 to show that the results obtained from Martin-Orozco and colleagues using anti-PD-L1 were due to the direct effect of PD-1 on the $CD8^+$ T cell regulating functions (184). Interestingly, when PD-1 KO OT-1 cells were transferred into RIP-$mOVA^{low}$ mice, which express less than 1/30 of the OVA as the RIP-$mOVA^{high}$ mouse used in the remainder of the study, PD-1-deficient OT-1 failed to proliferate or induce diabetes. If PD-1 KO OT-1 were activated in vitro and then transferred into RIP-$mOVA^{low}$ mice, diabetes was induced, suggesting that these cells were not intrinsically incapable of executing their effector functions (184). It could therefore be reasoned that if

antigen levels were sufficiently low in the pancreatic LN, then PD-1 would not be required for the induction of tolerance since the absence of PD-1 was not capable of overcoming this defect.

While it is established that PD-1 can regulate islet-reactive T cells during Type 1 Diabetes, an important unanswered question in the field is which cellular interactions are required for this regulation, particularly in the pancreas. As discussed earlier, PD-1 must signal in cis with the T cell receptor to inhibit T cell functions (166), meaning PD-L and peptide/MHC must be expressed by the same cell. $CD8^+$ T cells recognize peptide presented on MHC class I, which is expressed by nearly all nucleated cells; therefore, any nucleated cell could theoretically deliver an inhibitory signal through PD-1 on a $CD8^+$ T cell. On the contrary, $CD4^+$ T cells recognize peptides presented on MHC class II, which is largely restricted to antigen presenting cells. It is therefore likely that only MHC class II-expressing cells (predominantly antigen presenting cells) will be capable of inhibiting $CD4^+$ T cells through PD-1. Due to MHC restriction, we hypothesize that PD-L1 expressed by hematopoietic cells (mostly dendritic cells, a subset of antigen presenting cells) will be essential for regulating islet-reactive $CD4^+$ T cells, while PD-L1 expressed by either hematopoietic or non-hematopoietic cells (such as pancreatic islet cells) will be capable of regulating islet-reactive $CD8^+$ T cells. Work conducted by Keir and colleagues investigated the role of PD-L1 on hematopoietic versus non-hematopoietic cells during Type 1 Diabetes (174). Selective ablation of PD-L1 on innate immune system cells accelerated diabetes compared to wild type controls (100% diabetic at 7 weeks versus 13 weeks post-irradiation, respectively); however, this result was not statistically significant

and both groups eventually developed diabetes, yielding the interpretation by the authors that PD-L1 expressed by cells of the innate immune system (including antigen presenting cells) was not sufficient to suppress diabetes onset. To address the role of PD-L1 on non-hematopoietic cells, PD-L1-deficient or -sufficient islets were transplanted into diabetic NOD recipients. In this setting, islets expressing PD-L1 survived longer than islet lacking PD-L1. The authors concluded from this result that PD-L1 in the islet was responsible for controlling autoreactive T cell functions (174). While this study was informative, it failed to address (1) the role of PD-1/PD-L1 in the pancreatic LN in regulating islet-reactive T cells, (2) which cell type in the islet had to express PD-L1 since both insulin-producing beta cells and intra-islet dendritic cells express this molecule and both were present in the transplant, and (3) the relative importance of PD-L1 on hematopoietic versus non-hematopoietic cells in regulating islet-reactive $CD4^+$ versus $CD8^+$ T cells. A more thorough understanding of these issues is required in order to determine whether the PD-1 pathway can be engaged to provide therapeutic benefit for Type 1 Diabetes patients.

Associations between the PD-1 pathway and autoimmune diseases in humans

In addition to the importance of PD-1 in suppressing autoimmunity in several murine models, there is increasing evidence that PD-1 is linked to autoimmunity in humans. Greater than 30 single-nucleotide polymorphisms (SNPs) have been identified in the human PD-1 gene, and several SNPs have been linked to susceptibility to autoimmunity in various populations. Autoimmune diseases in humans that have had SNPs identified in PD-1 include Type 1 Diabetes (181, 187) as well as systemic lupus

erythematosus (SLE) (188-191), multiple sclerosis (192), rheumatoid arthritis (191, 193, 194), psoriasis (195), and ankylosing spondylititis (196). Additionally, enhanced expression of PD-1 has been described on T cells in the synovial fluid during rheumatoid arthritis (RA) and salivary glands during Sjogren's syndrome (197-199). Interestingly, a splice variant of PD-1 was recently identified in patients with rheumatoid arthritis (200). This splice variant lacks the transmembrane domain, and is therefore secreted. Soluble PD-1 can be found in sera and synovial fluid from RA patients, and it is speculated that the soluble form interferes with the functions of the membrane bound form, leading to exacerbated disease. Understanding how these SNPs impact PD-1 functions in human cells will be important for exploiting this pathway clinically. It is critical to understand how PD-1 operates in humans to suppress autoimmunity, particularly since antibodies against this molecule are entering the clinic to enhance immunity in both chronic viral infections and tumors. Discussions in Chapter 5 will be dedicated to outlining the potential risks of blocking this pathway in patients.

CONCLUSION

Type 1 Diabetes is an autoimmune disease caused by T cell-mediated destruction of the insulin-producing beta cells in the pancreatic islets. The NOD mouse is a well-established model used to study the pathogenesis of Type 1 Diabetes since it shares many similarities with the human disease. Diabetes is caused by the adaptive immune system responding to a variety of pancreatic antigens, including insulin. $CD4^+$ and $CD8^+$ T cells as well as B cells are required for the disease, though this focus of this thesis is on

understanding the dynamics of islet-reactive $CD4^+$ T cells. Diabetes results from defects in both central and peripheral tolerance mechanisms which normally act to restrain autoreactive lymphocytes in vivo. The inhibitory receptor PD-1 interacting with its ligand PD-L1 is an important mechanism of peripheral tolerance that suppresses autoreactive T cells during Type 1 Diabetes development in NOD mice. The work presented in this thesis aims to address how the PD-1 pathway regulates islet-reactive $CD4^+$ T cell effector functions in the secondary lymphoid organs as well as the pancreas, and our ultimate goal is to determine the efficacy of targeting this pathway therapeutically to restore tolerance in Type 1 Diabetic patients.

FOOTNOTES

[1]Portions of this work have been previously published. Reprinted from *Annuals of the New York Academy of Sciences*, Volume 1217, Brian T. Fife and Kristen E. Pauken, **The role of the PD-1 pathway in autoimmunity and peripheral tolerance**. p. 45-59.

CHAPTER 2:

Development of a novel adoptive transfer model to track islet antigen-specific $CD4^+$ T cells following activation by endogenous autoantigen during Type 1 Diabetes[1]

INTRODUCTION

Type 1 Diabetes is an autoimmune disease mediated by T cell destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans (25). The non-obese diabetic (NOD) mouse is a classic model for studying this disease because it shares many similarities with human Type 1 Diabetes, including the requirement of $CD4^+$ T cells (32, 36, 58). However, knowledge of how diabetogenic $CD4^+$ T cells are regulated and how this regulation fails causing diabetes is limited due to a lack of tools to monitor endogenous islet-antigen specific $CD4^+$ T cells in vivo.

Common models used to study islet-reactive $CD4^+$ T cells in NOD mice include adoptive transfer of high numbers of naïve or in vitro activated T cell receptor transgenic cells into wild type (WT) or lymphopenic NOD recipients (46, 59, 109, 110, 174, 182). While informative, these approaches fail to recapitulate the natural inflammatory environment present in NOD mice and the timing associated with diabetes progression. Previous work in other systems showed transferring lower numbers of naïve T cells allowed greater clonal expansion on a per cell basis and more efficient effector cell differentiation (97, 201-203). Since we speculate that endogenous autoantigen in the NOD mouse is low, we predicted that limiting the diabetogenic precursor frequency would be essential for autoantigen encounter and activation. Therefore, in this study we

developed a new model by transferring a small number of islet-specific BDC2.5 transgenic $CD4^+$ T cells (101, 103) into prediabetic NOD mice to mimic an endogenous pre-immune repertoire.

Following transfer of a low number of naïve BDC2.5 T cells into pre-diabetic NOD mice, these cells encountered autoantigen in the pancreatic LN and converted to an activated ($CD44^{high}$) phenotype. In this model, the majority of the population encountered autoantigen within the first week post-transfer. On the contrary, when a high number of naïve BDC2.5 T cells ($1x10^6$) was transferred, a large population never encountered antigen even at later time points, highlighting the importance of precursor frequency in this model. Following activation, a subset of BDC2.5 T cells in the low transfer model differentiated into T-bet expressing, IFNγ-producing Th1 effector cells, up-regulated the inhibitory receptor PD-1, infiltrated the pancreas, and localized to the islet infiltrate. However, a portion of the population acquired an anergic phenotype and was functionally impaired rather than differentiating into effector cells, and these cells were enriched in the pancreatic LN. These data suggest that when precursor frequency is reduced to mimic an endogenous pre-immune repertoire, islet-reactive $CD4^+$ T cells can be restrained by peripheral tolerance in the form of anergy induction in the pancreatic LN. However, this tolerance is incomplete, allowing IFNγ-producing Th1 effector cells to infiltrate the pancreas and contribute to diabetes.

MATERIALS AND METHODS

Mice

Mice were housed and bred in specific-pathogen free conditions in animal barrier facilities at the University of Minnesota. Female NOD mice were purchased from Taconic (Germantown, NY) and NOD BDC2.5 T cell receptor transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and crossed to NOD Thy1.1^{+} mice. BDC2.5 mice were used for donors between 4-6 weeks of age. Pre-diabetic NOD mice (Thy1.2^{+}) were used as recipients for Thy1.1^{+} BDC2.5 T cells between 7-12 weeks of age. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Adoptive transfer of naïve BDC2.5 T cells

Naïve Thy1.1^{+} NOD BDC2.5 T cells were enriched from spleen and inguinal, axillary, brachial, cervical, and periaortic LNs using negative selection over a magnetic column (Miltenyi Biotech, Auburn CA). Cells were resuspended in DMEM containing 10% fetal bovine serum (Hy-clone), 2 mM L-glutamax, 100 U/ml penicillin-streptomycin, 0.1 M non-essential amino acids, 10 mM HEPES and 50 μM 2-mercaptoethanol (Life Technologies, Grand Island, NY) (referred to as complete DMEM) at 1x10^{8} cells/ml. Biotinylated antibodies (eBioscience, San Diego, CA) against GR-1, MHC class II, CD8α, CD117 (c-kit), CD24, and TER119 were added at a final concentration of 0.15 μg/10^{7} cells, CD19 and B220 were added at 0.3 μg/10^{7} cells, and CD44 was added at 0.015 μg/10^{7} cells. Cells were incubated for 30 minutes at 4^{o}C, washed with DMEM containing 1% fetal bovine serum, and resuspended in 1x10^{8} cells/ml in complete DMEM. Anti-biotin microbeads (Miltenyi Biotech) were added and cells were incubated for 30

minutes at 4^{o}C. Cells were washed with DMEM containing 1% fetal bovine serum and subjected to magnetic enrichment per manufacture's protocol. The unbound fraction was collected, washed with HBSS and 7,500 (for low transfer model) or 1x10^{6} (for high transfer model) CD4^{+} BDC2.5 T cells were transferred intravenously into pre-diabetic Thy1.2^{+} NOD recipients. Diabetes was assessed by urine glucose readings prior to transfer. BDC2.5 T cells were distinguished from endogenous CD4^{+} T cells by expression of the congenic marker Thy1.1.

Flow cytometry

BDC2.5 T cells were enriched from single cell suspensions from spleen and/or pooled non-antigen draining LN samples due to the low frequency of BDC2.5 T cells in these organs compared to the endogenous lymphocyte population (96). Briefly, anti-Thy1.1 conjugated to APC (eBioscience) was added to single cell suspensions in 100-200 μl medium containing 2.4G2 (Fc receptor blocking antibody) and incubated for 30 minutes at 4^{o}C. Cells were then washed, resuspended in 200 μl medium containing 2.4G2 and anti-APC microbeads (Miltenyi Biotech), and incubated for an additional 30 minutes at 4^{o}C. Cells were then eluded over a magnetic column (Miltenyi Biotech) and the bound fraction containing the enriched BDC2.5 T cell population was collected and stained for FACS. Single cell suspensions from the pancreas were generated using a combination of collagenase P digestion (Roche, Indianapolis, IN) and discontinuous percoll separation as described (204). Briefly, the pancreas was removed, injected with collagenase P, and digested at 37^{o}C for 15 minutes. The digested tissue was then washed with RPMI

containing 0.25% fetal bovine serum, 10 mM HEPES (Life Technologies), and 40 μg/ml DNase (Sigma, St. Louis, MO), subjected to a 40%/60% discontinuous percoll gradient, and the interface between the 40% and 60% percoll layers was collected and stained for FACS. Antibodies used to surface stain samples that were purchased from eBioscience included: anti-Thy1.1 (H1S51), CD4 (RM4-5), CD3e (145-2C11), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), CD44 (IM7), and PD-1 (J43). CD8a (5H10, Life Technologies) and CD44 (IM7, BD Biosciences, San Jose, CA) were also used. Antibodies used for intracellular staining from eBioscience included anti-IL-2 (JES6-5H4) and IFNγ (XMG1.2). Intracellular stains were conducted following surface staining as described below. Cell numbers were assessed by FACS using AccuCheck Counting Beads (Life Technologies).

Samples were collected using BD LSRII and Fortessa FACS instruments with FACS DIVA software (BD Bioscience). Data were analyzed using FlowJo software (Treestar, Ashland, OR). The gating strategy to define BDC2.5 T cells was singlet^{+}, CD3^{+} lineage^{-} (B220, CD11b, CD11c), CD4^{+}, Thy1.1^{+} cells. Single cells (singlets) were gated using side scatter area by side scatter width to exclude doublets.

Stimulation of cells for cytokine production

Single cell suspensions from the pancreatic LN and pancreas were generated as described above and suspended in complete DMEM with 10 μg/ml brefeldin A, 100 ng/ml phorbol 12-myristate 13-acetate (PMA), and 1000 ng/ml ionomycin (Sigma). Conditions that received no stimulation were supplemented with brefeldin A but not

PMA and ionomycin. Cells were incubated at 37^{o}C in a humidified incubator containing 5% CO_2 for four hours. Cells were then washed and surface stained for flow cytometry in medium containing Fc receptor block (2.4G2) and brefeldin A. For intracellular cytokine staining, cell membranes were permeabilized using the BD cytofix/cytoperm fixation/permeabilization kit (BD Biosciences) according to manufacturer's recommendations. For intracellular IL-2 staining, single cell suspensions were incubated overnight at 4^{o}C in BD cytofix/cytoperm buffer prior to staining with anti-IL-2 antibody. Gates drawn for intracellular cytokines were on BDC2.5 T cells from samples that received brefeldin A but no PMA and ionomycin. In order to assess cytokine production from anergic and effector BDC2.5 T cells, 500 μg of acetylated p31 peptide (YVRPLWVRME) (Genemed Sythesis, San Antonio, TX) was injected intravenously and cells were harvested 4 hours later into medium containing brefeldin A. The following enrichment and staining steps were also conducted in media containing brefeldin A and azide.

Immunofluorescence Microscopy

Pancreata were frozen in O.C.T, cut at seven μm, and fixed using acetone. Antibodies included guinea pig anti-swine inulin (Dako, Denmark) and donkey anti-guinea pig AF488 (Jackson ImmunoResearch), Thy1.1-PE (clone OX7, Biolegend, CA), and CD3e-APC (clone 145-2C11, eBioscience). Slides were mounted using Prolong Gold with DAPI (Life Technologies). Slides were imaged on a Leica epifluorescent DM5500

microscope (Germany). Data was compiled using MetaMorph software (Molecular Devices, CA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software. P values were calculated using an unpaired two-tailed Student's t test using a 95% confidence interval. Values of 0.05 or less were considered statistically significant. Asterisks used in the figure legends to indicate statistical significance were: not significant >0.05 (ns), significant 0.01 to 0.05 (*), very significant 0.001 to 0.01 (**), and extremely significant <0.001 (***) (GraphPad Prism, La Jolla, CA).

RESULTS

Transferring a low number of naïve BDC2.5 T cells reduces competition for antigen and causes a higher frequency to become activated than transferring a non-physiologically high number of BDC2.5 T cells

To investigate the role of PD-1 in regulating islet-reactive $CD4^+$ T cells during Type 1 Diabetes progression, we first established a model to track these cells in vivo. We utilized the BDC2.5 transgenic mouse, which contains $CD4^+$ T cells specific for an islet peptide-MHC class II complex (101, 103). We did not provide exogenous antigen or adjuvant but rather relied on presentation of endogenous levels of autoantigen to activate the cells. We speculated that since levels of endogenous islet-peptide/MHCII complexes are low, precursor frequency would dictate the ability of T cells to become activated

(201). To test this hypothesis, we transferred naïve BDC2.5 cells into NOD mice and compared the frequency of cells that increased CD44 expression after a low (7,500 cells) or high transfer ($1x10^6$) (Figure 2-1). At day 6 post-transfer, a significantly higher frequency of BDC2.5 cells had converted to $CD44^{high}$ in the low transfer compared to the high transfer in the pancreatic LN (low transfer = 90.67±1.71% versus high transfer 71.71±3.64%, p=0.0087) and spleen and non-antigen draining LNs (non-dLNs) (low transfer = 68.34±3.41% versus high transfer = 25.95±2.81% p=0.0002) (Figure 2-1 A and C). Importantly, at a later time point (day 28), many of the BDC2.5 cells remained naïve in the high transfer situation while nearly the entire population in the low transfer setting had encountered autoantigen (Figure 2-1 B and D) (in the pancreatic LN low transfer = 87.83±1.75% versus high transfer = 61.11±2.74%, p=0.0002, and spleen+non-dLNs low transfer = 83.49±7.18% versus high transfer = 34.47±4.17%, p=0.0011). For this reason, the remainder of this thesis utilized the low transfer model when investigating the dynamics of BDC2.5 T cells during Type 1 Diabetes progression.

BDC2.5 T cells encounter autoantigen, undergo a moderate clonal expansion without a traditional contraction, and infiltrate the pancreas in the low transfer model

To further characterize the low transfer BDC2.5 model, we transferred 7,500 naïve BDC2.5 cells into NOD mice and examined conversion to a $CD44^{high}$ antigen-experienced phenotype, clonal expansion, persistence, and infiltration of the pancreas. Approximately 10-15% of the BDC2.5 T cells survived the transfer (data not shown),

providing a naive pre-immune repertoire of approximately 750 cells. Although this is higher than what has recently been reported for endogenous pre-immune $CD4^+$ T cells using peptide/MHCII tetramers (20-400 cells) (95, 205, 206), it is orders of magnitude closer to reality than other transfer models (59, 182). Figure 2-2 A shows representative FACS contour plots comparing CD44 expression on the transferred BDC2.5 population ($Thy1.1^+$) to the endogenous $CD4^+$ T cell population. Following low transfer, the majority of BDC2.5 T cells (73±6%) in the pancreatic LN increased CD44 expression indicating they encountered autoantigen by day 3 post-transfer, while a minority of cells in the spleen and non-dLNs expressed CD44 (34.8±6.2) (Figure 2-2 A and B). At time points 28 days and later there was no significant difference in CD44 levels between these organs (Figure 2-2 A and B). We conclude from these data that the entire population converted to the $CD44^{high}$ phenotype by day 28, though the majority were $CD44^{high}$ by day 7 (Figure 2-2 A and B).

We next enumerated BDC2.5 T cells to determine the magnitude of clonal expansion and the shape of the clonal expansion curve. Endogenous priming led to a 2.5-fold increase in BDC2.5 T cells between day 3 and day 7 (Figure 2-3 A), which was low compared to a classic acute infection response (205). Interestingly, we did not measure a standard contraction of antigen-specific T cells (Figure 2-3 A-C). The lack of a contraction phase had been observed previously during chronic viral infections (207), and we hypothesized that we did not observe a contraction due to the persistence of autoantigen in this model. Importantly, BDC2.5 T cells trafficked to the pancreas and localized to the infiltrate surrounding the islet (peri-insulitis) (Figure 2-4 A and B).

BDC2.5 T cells were rarely found deep within the islet core (insulitis) (Figure 2-4 B). Due to the ability of the BDC2.5 T cells to become activated and infiltrate the pancreas following exposure to endogenous autoantigen, the low transfer system provided a novel tool to investigate islet-reactive T cell biology under physiological conditions in vivo.

BDC2.5 T cells differentiate into T-bet$^+$ IFNγ-producing Th1 cells

We next determined the effector potential of the transferred BDC2.5 T cells. Previous work identified a critical role for IFNγ-producing Th1 cells in the pathogenesis of Type 1 Diabetes (44-46). We hypothesized that since BDC2.5 T cells were capable of infiltrating the pancreas, these cells would differentiate into the pathogenic Th1 subset. We first measured T-bet, a master transcription factor which controls differentiation into Th1 cells (208). Following activation, the majority of BDC2.5 cells in all organs expressed T-bet (inguinal LN 51.08±7.36, spleen 72.54±4.68, pancreatic LN 79.11±7.00, pancreas 76.71±13.49) (Figure 2-5 A-C), supporting our hypothesis that these cells develop a Th1 phenotype.

We further validated that BDC2.5 T cells differentiated into Th1 cells by testing for production of the hallmark Th1 cytokine IFNγ. BDC2.5 T cells from the pancreatic LN and the pancreas were capable of producing IL-2 and IFNγ following ex vivo stimulation with PMA and ionomycin (Figure 2-6 A and B). Interestingly, there was a substantially higher frequency of cells capable of producing IFNγ (either or alone or co-producing IL-2) in the pancreas compared to the pancreatic LN (48.45% in the pancreatic LN versus 68.245% in the pancreas), suggesting the population in the pancreas is more

enriched for pathogenic effector cells than the pancreatic LN. Collectively these data showed that when a low number of naïve BDC2.5 T cells were transferred into pre-diabetic NOD, they became activated, differentiated into IFNγ– and IL-2-producing Th1 cells, infiltrated the pancreas, and persisted for an extended period of time.

BDC2.5 T cells chronically express the inhibitory receptor PD-1 in the secondary lymphoid organs and pancreas, with the highest levels detected in the pancreas

We were interested in using the low transfer BDC2.5 T cell model to evaluate the role for PD-1 during Type 1 Diabetes. Therefore, we next assessed PD-1 expression kinetics on BDC2.5 T cells in the secondary lymphoid organs and pancreas. PD-1 is expressed following T cell activation (157), and remains elevated during chronic antigen stimulation (158). Therefore, we hypothesized that PD-1 expression would be detected at time points consistent with recent activation (conversion to $CD44^{high}$) and would remain elevated due to the persistence of autoantigen. As expected, BDC2.5 cells in the pancreatic LN were $PD\text{-}1^{+}$ at day 3, consistent with recent T cell activation (Figure 2-7 A). PD-1 expression on the BDC2.5 T cells in the spleen and non-dLNs was lower at day 3 than in the pancreatic LN as expected since a higher frequency of BDC2.5 cells were naïve in this location (157). At day 7, there was a decrease in PD-1 expression in the pancreatic LN compared to day 3, though the significance of this decline is unclear. By day 21 post-transfer PD-1 levels were elevated in the pancreatic LN and spleen and non-dLNs, and these levels remained elevated (Figure 2-7 A and B). By day 7, PD-1 levels were highest in the pancreas and remained highest in this organ (Figure 2-7 A and B). We

predict that the elevated levels of PD-1 reflect chronic antigen engagement in the NOD mouse, and further speculate that the elevated mean fluorescence intensity (MFI) of PD-1 in the pancreas compared to the secondary lymphoid organs reflects enhanced antigen presentation and negative regulation occurring in the pancreas to suppress these cells.

A population of BDC2.5 T cells is anergic in the pancreatic LN, while effector cells are enriched in the spleen and pancreas

The development of Type 1 Diabetes is dependent on the loss of peripheral tolerance that would normally act to restrain autoreactive T cells. However, tolerance induction in islet-reactive T cell populations during spontaneous diabetes development has never been directly tested due to the inability to track these antigen specific cells in vivo under physiological conditions. A variety of peripheral tolerance mechanisms have evolved to restrain autoreactive T cells (as discussed in Chapter 1), and it is the loss of these mechanisms that ultimately leads to autoimmunity. Therefore, we speculated that BDC2.5 T cells would be partially restrained by peripheral tolerance, but that regulation would eventually be lost resulting in infiltration of the pancreas. Anergy is a state of functional hyporesponseviness, and is typically induced following suboptimal activation (134). We hypothesized that anergy induction would occur in the BDC2.5 T cell population following priming in pancreatic LN of NOD mice due to low levels of autoantigen and a lack of inflammatory cues necessary for optimal T cell priming. Martinez et al. recently identified the co-expression of folate receptor 4 (FR4) and CD73 on antigen-experienced ($CD44^{high}$) $Foxp3^{-}$ cells as a marker of T cell anergy (209). We

identified a population of anergic phenotype $FR4^{+}$ $CD73^{+}$ $CD44^{high}$ $Foxp3^{-}$ cells three weeks post-transfer (Figure 2-8 A and B). Interestingly, the majority of the antigen-specific $CD4^{+}$ T cells in the pancreatic LN were the anergic phenotype (67.46±4.5%, Figure 2-8 A and B). We speculate that the remaining population of effector BDC2.5 T cells in the pancreatic LN either failed to become anergic or had broken tolerance, resulting in a population of cells capable of migrating to the pancreas to contribute to disease pathology. In support of this notion, the anergic phenotype was largely absent from the spleen (11.91±2.94% anergic) and pancreas (19.19±6.51% anergic) (Figure 2-8 A and B), which were dominated by effector phenotype ($FR4^{-}CD73^{-}CD44^{high}Foxp3^{-}$) cells (spleen 88.08% and pancreas 80.81% effector phenotype). In order to validate that co-expression of FR4 and CD73 correlated with functional anergy in diabetogenic T cells, we assessed IFNγ production in the pancreatic LN by stimulating BDC2.5 T cells in vivo with cognate peptide (acetylated p31) intravenously. Following peptide challenge, the production of IFNγ (as measured by geometric MFI) and the frequency of IFNγ-producing cells were both lower in the anergic phenotype cells compared to the effector phenotype cells (Figure 2-8 C-E), suggesting that co-expression of FR4 and CD73 correlated with functional anergy in NOD mice despite its susceptibility to Type 1 Diabetes. The data support a model where, following antigen encounter, the majority of islet-reactive $CD4^{+}$ T cells becomes anergic, but a small population of effector cells escapes tolerance induction, differentiates into pathogenic Th1 cells, and infiltrates the pancreas.

DISCUSSION

Current knowledge of how $CD4^+$ T cells are regulated during spontaneous diabetes progression is limited due to a lack of tools to track these cells in vivo. Our study utilized the transfer of a low number of naïve autoreactive T cells to mimic rare self-reactive T cells in the pre-immune repertoire (95, 206). Precursor frequency can influence the magnitude of an immune response, and previous reports showed transferring non-physiologically high numbers of T cell receptor transgenic T cells can lead to abnormal T cell activation and function (97, 202, 203). We reasoned that precursor frequency would be critically important in Type 1 Diabetes because of limited endogenous self-peptide/MHCII complexes. Indeed, analysis of $CD44^{high}$ frequencies in BDC2.5 cells following low or high transfer revealed that a significantly higher frequency of cells encountered antigen and became activated in the low transfer system. Importantly, in the high transfer a large fraction of the cells failed to encounter antigen even at later time points despite the fact that antigen persists, presumably due to competition for limiting peptide/MHC class II complexes (201). In addition to antigen being a limiting factor in activation, we postulate that tolerance mechanisms in NOD mice are also limiting. Overwhelming the system with large numbers of either naïve or previously activated cells reduces the likelihood that endogenous regulatory mechanisms (e.g. PD-L1 in the islet) could induce or maintain tolerance. In order to study how peripheral tolerance is maintained and why it breaks down, we contend that models that more closely mimic physiological conditions should be used. Lastly, this low transfer approach allowed us to investigate diabetogenic T cell dynamics in the natural inflammatory environment in

NOD mice during Type 1 Diabetes pathogenesis, which cannot be recapitulated with in vitro systems, priming regimens, and mice that have transgenic expression of model antigens in the pancreas (59, 110, 169, 184, 185, 210). By limiting the precursor frequency, we were able to define the dynamics of autoreactive T cell activation in response to endogenous autoreactive peptide/MHCII complexes.

Following transfer of a low number of BDC2.5 T cells into prediabetic NOD mice, the majority of cells converted to an activated $CD44^{high}$ phenotype, but the clonal expansion of the population was incredibly weak compared to those observed during infections (205, 211). We speculate that the weak clonal expansion that occurred was due to suboptimal priming by low levels of endogenous peptide/MHC complexes as well as insufficient levels of costimulatory molecules needed to drive high levels of proliferation. The weak clonal expansion that these cells go through likely contributes to the induction of peripheral tolerance through T cell anergy, which is often associated with suboptimal T cell priming. Our data with FR4 and CD73 co-expression support the notion that BDC2.5 T cells become anergic in the pancreatic LN following autoantigen encounter in a relatively low inflammatory context, and our data examining IFNγ production following peptide restimulation further supports that these cells are functionally anergic. Despite anergy induction, we observed a small population of effector phenotype BDC2.5 T cells in the pancreatic LN, and we speculate that this population was capable of exiting the pancreatic LN and trafficking to the pancreas. Anergic phenotype cells were absent from the pancreas, suggesting that effector cells were uniquely capable of infiltrating the autoimmune target organ. What factors drive anergy induction in the pancreatic LN and,

importantly, which factors are required to maintain tolerance to prevent the development of pathogenic effector cells are unclear but an area of interest clinically to induce tolerance in patients. In Chapter 4 the role of PD-1 in tolerance in this low transfer system will be tested based on work suggesting that PD-1 plays critical roles in both the induction and maintenance of anergy in other diabetes models (59, 110, 183, 186).

One hypothesis for why the anergic phenotype cells are uniquely enriched in the pancreatic LN is that the persistence of antigen in this organ prevents T cells from egressing from this organ. Memory T cells can become resident in several non-lymphoid tissues, providing a front-line defense against invading pathogens (212-214). What factors contribute to memory T cell residence, particularly following infection with acute pathogens, are not fully understood. However, during chronic infections the presence of antigen can lead to long term maintenance of antigen-specific T cell populations (207, 211, 215). During chronic infections the T cell population does not go through a standard contraction phase (207), and we also observed a similar lack of contraction in our model. We therefore envision a model where islet-reactive $CD4^+$ T cells encounter autoantigen in the pancreatic LN, become suboptimally activated by endogenous autoantigen and are rendered anergic, and become resident to the pancreatic LN where constant antigen encounter not only keeps these cells alive but also keeps them from leaving the pancreatic LN to infiltrate the pancreas. Preventing trafficking to the pancreas protects this tissue from autoimmune destruction, thereby providing a novel tolerance mechanism. However, since tolerance is incomplete in NOD, a small population of BDC2.5 T cells are either able to avoid tolerance induction or are capable of breaking tolerance, and this effector

population exits that pancreatic LN and infiltrates the pancreas. What factors contribute to this loss of tolerance are unclear, but understanding the mechanism that leads to the release of this effector population will be critical for targeting pathogenic autoreactive T cells in patients. In Chapter 4 the role of PD-1 in the induction and maintenance of this anergic population will be described, though other factors involved in regulating this form of tolerance should also be explored to determine the efficacy of inducing anergy in patients.

FOOTNOTES

[1]Portions of this work have been previously published. Reprinted from *Diabetes*. Volume 62. Kristen E. Pauken, Marc K. Jenkins, Miyuki Azuma, and Brian T. Fife. **PD-1, but not PD-L1, expressed by islet-reactive CD4$^+$ T cells suppresses infiltration of the pancreas during Type 1 Diabetes**. p. 2859-2869. Copyright 2013, with permission from the American Diabetes Association (license number 3196070044473).

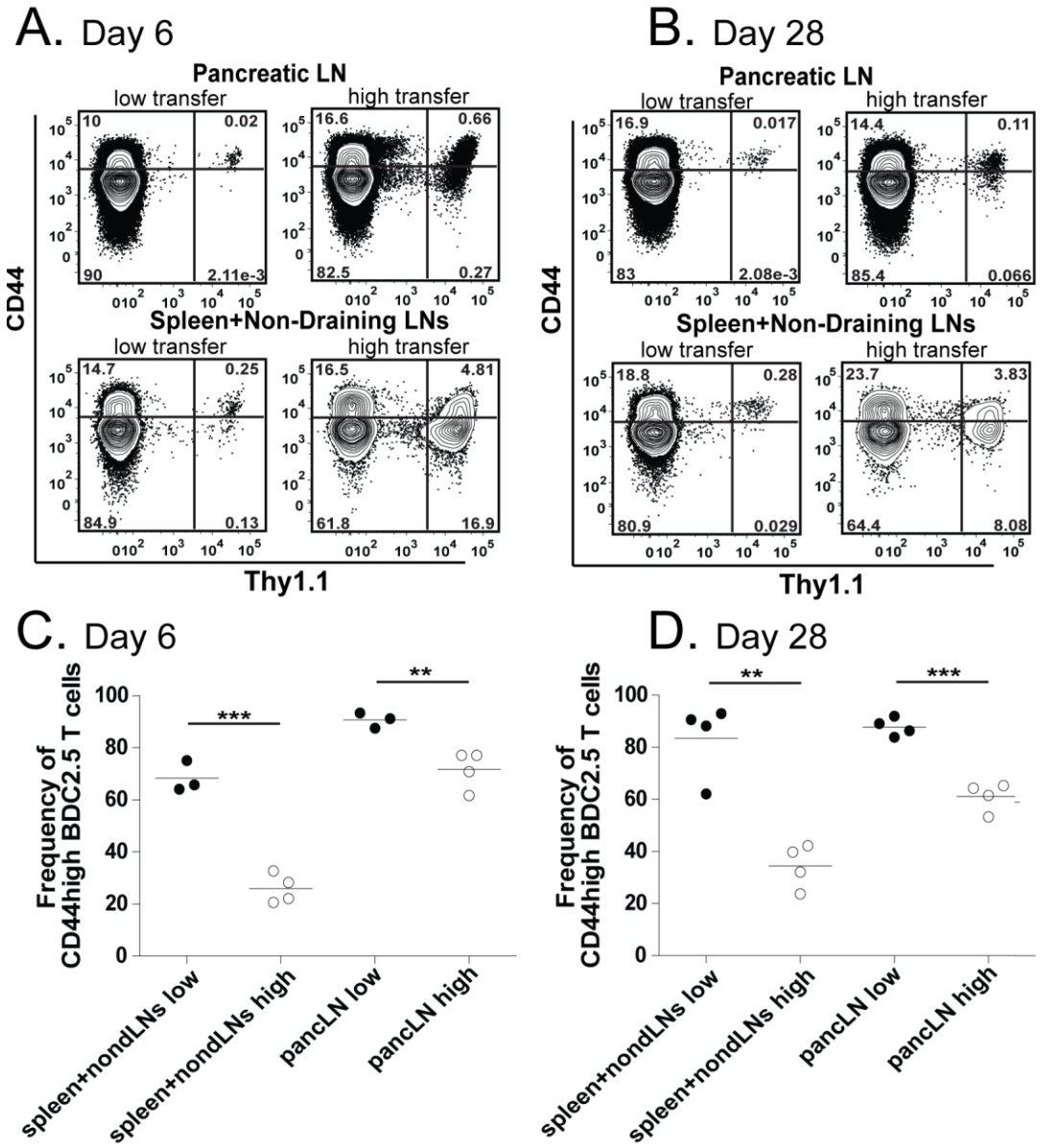

Figure 2-1: Precursor frequency influences the ability of BDC2.5 T cells to encounter autoantigen and become activated in NOD mice.

(A and B) Representative FACS contour plots are shown from the spleen and non-draining LNs comparing CD44 expression on the endogenous $CD4^+$ T cell population ($Thy1.1^-$) with transferred BDC2.5 T cells ($Thy1.1^+$) at day 6 (A) or 28 (B) following transfer of 7500 (low transfer) or $1x10^6$ BDC2.5 T cells (high transfer). (C and D) Quantification of the frequency of BDC2.5 T cells that were $CD44^{high}$ at day 6 (C) and 28 (D) following transfer of 7500 or $1x10^6$ BDC2.5 T cells in the spleen and non-draining LNs and pancreatic LN. Data are representative of two independent experiments with three-five mice per group.

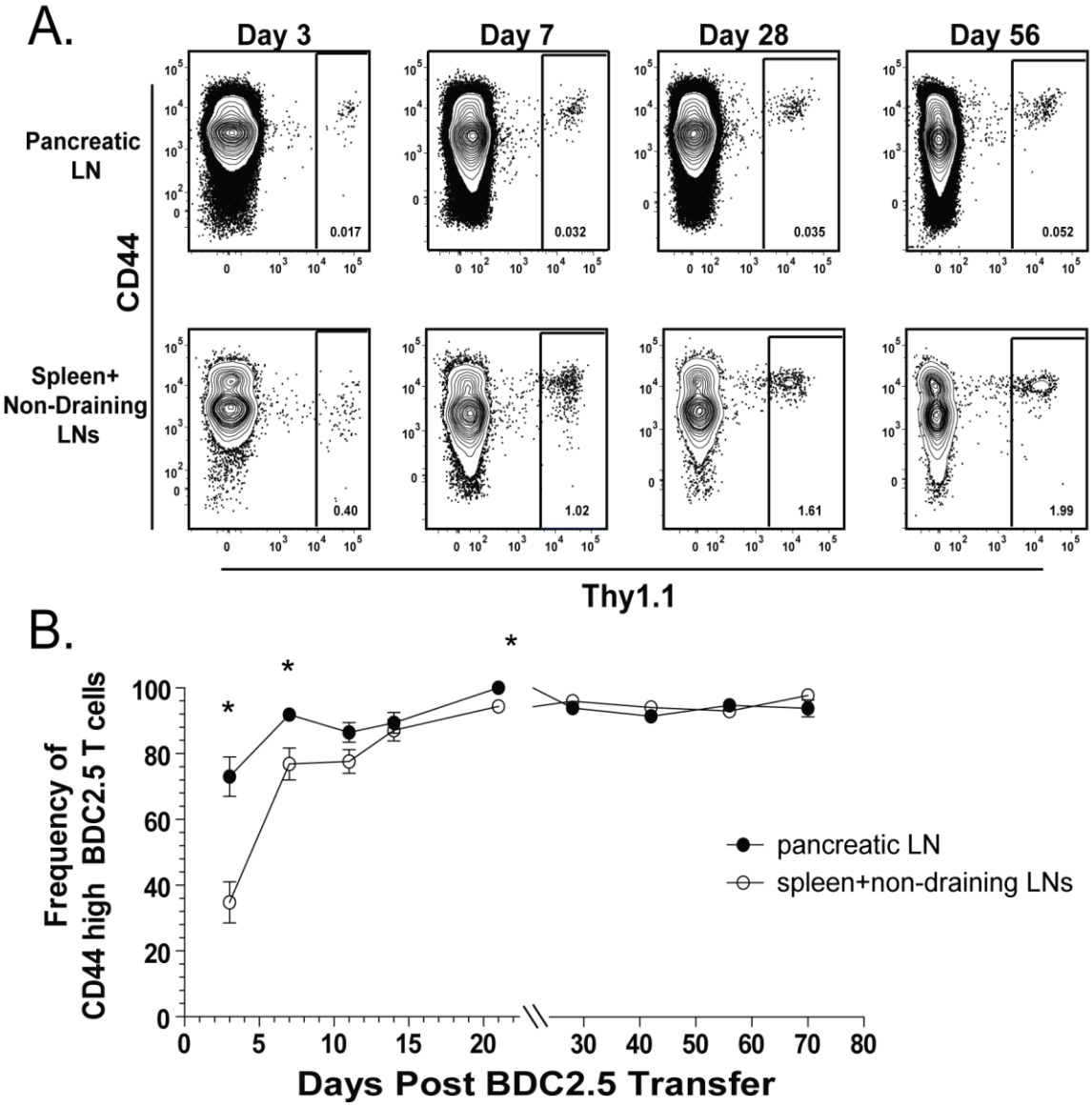

Figure 2-2: BDC2.5 T cells encounter antigen in the pancreatic LN following transfer of a low number of naïve cells into pre-diabetic NOD mice.

(A) Representative FACS counter plots comparing CD44 expression on the endogenous $CD4^+$ T cell population ($Thy1.1^-$) with the transferred BDC2.5 population ($Thy1.1^+$) in the pancreatic LN and spleen and non-draining LNs at days 3, 7, 28, and 56 post-transfer. (B) Quantification of the frequency of $CD44^{high}$ BDC2.5 T cells at various time points (x-axis) between the pancreatic LN and the spleen and non-draining LNs following transfer of 7500 cells. All mice shown were non-diabetic at the time of harvest. Data are representative of two independent experiments with three-four mice per time point.

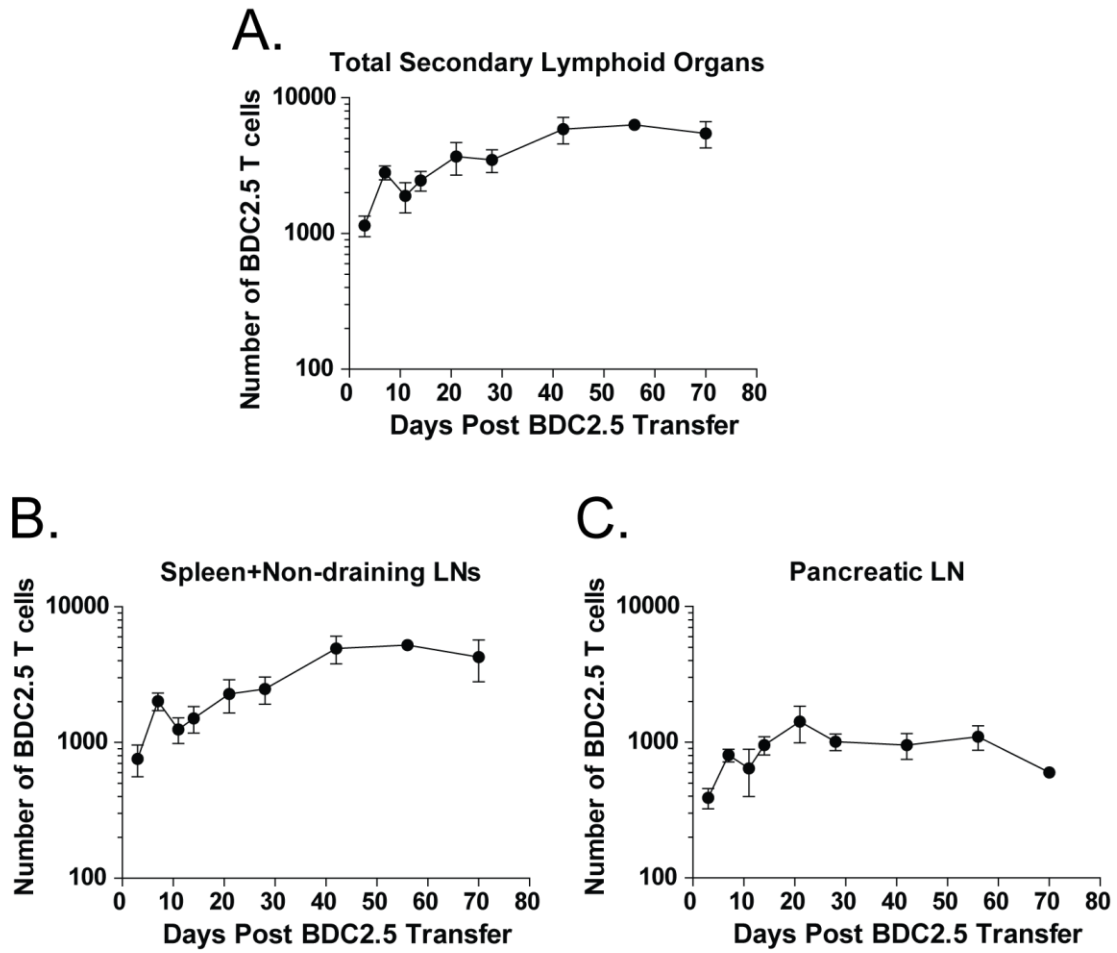

Figure 2-3: Following activation, BDC2.5 T cells undergo a weak clonal expansion and persist without a substantial contraction phase.

(A) The absolute number of BDC2.5 Thy1.1^{+} T cells in the spleen and non-draining LNs and pancreatic LN combined (referred to as "Total Secondary Lymphoid Organs") at various time points over 70 days following low transfer into prediabetic NOD.Thy1.2 mice. All mice shown were non-diabetic at the time of harvest. (B and C) The absolute number of BDC2.5 T cells in the spleen and non-draining LNs (B) or pancreatic LN (C) of mice shown in panel A following low transfer into NOD mice. Data are representative of two independent experiments with three-four mice per time point.

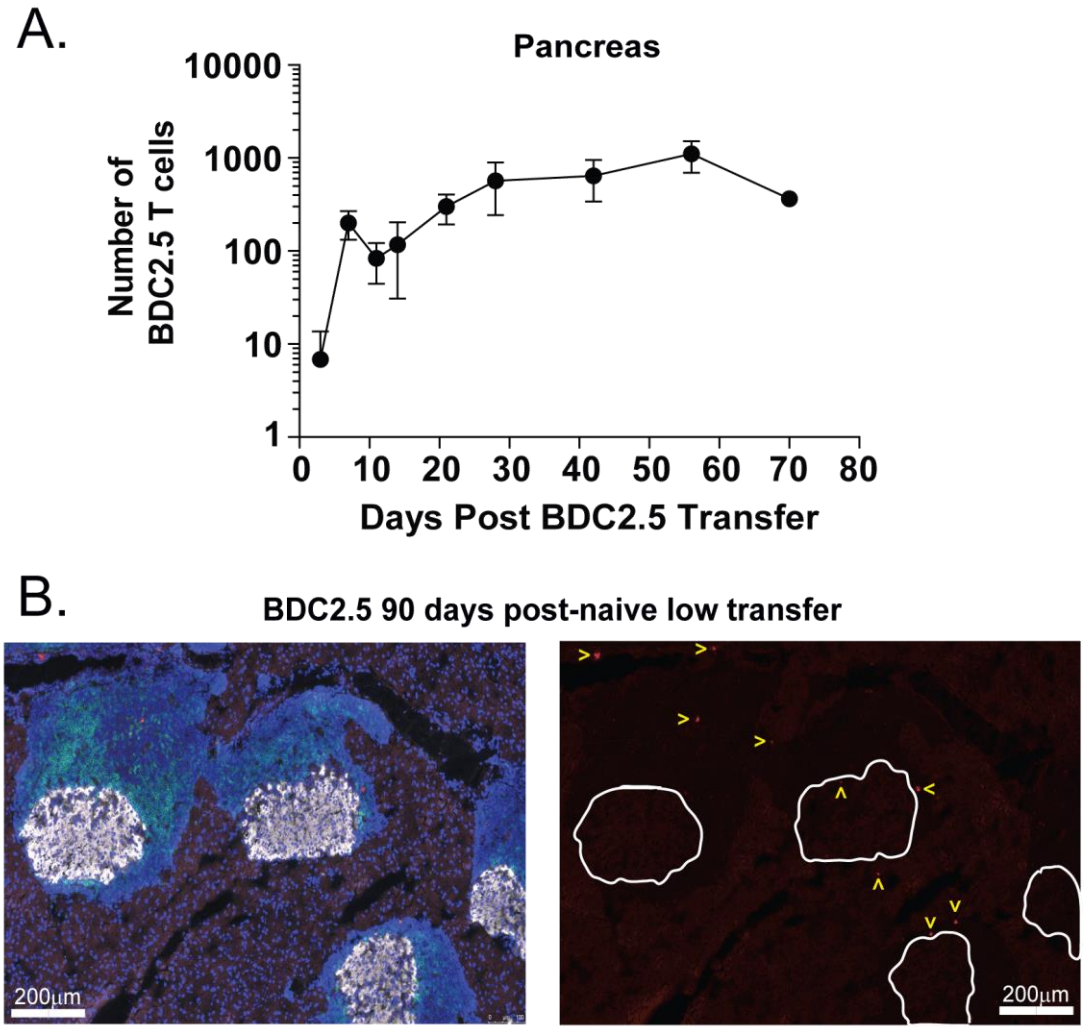

Figure 2-4: BDC2.5 T cells infiltrate the pancreas and localize to the mononuclear infiltrate surrounding the pancreatic islets.

(A) The absolute number of BDC2.5 T cells in the pancreas of mice shown in Figure 2-3 following low transfer into NOD mice. Data are representative of two independent experiments with three-four mice per time point. (B) Histological analysis of the pancreas from non-diabetic mice 90 days post-transfer of BDC2.5 T cells. Images are representative of four mice, 90-114 days post-transfer. Insulin is grey, Thy1.1^{+} BDC2.5 T cells are red, CD3^{+} T cells are green, and DAPI marking nuclei is blue. A single color image showing BDC2.5 T cells in red is to the right of the overlaid image containing all four colors. White lines in the red single color image outline the islet body containing the insulin-producing cells. Select BDC2.5 T cells are marked with a yellow arrow to aid in visualization of the cells. Scale bar =200μm.

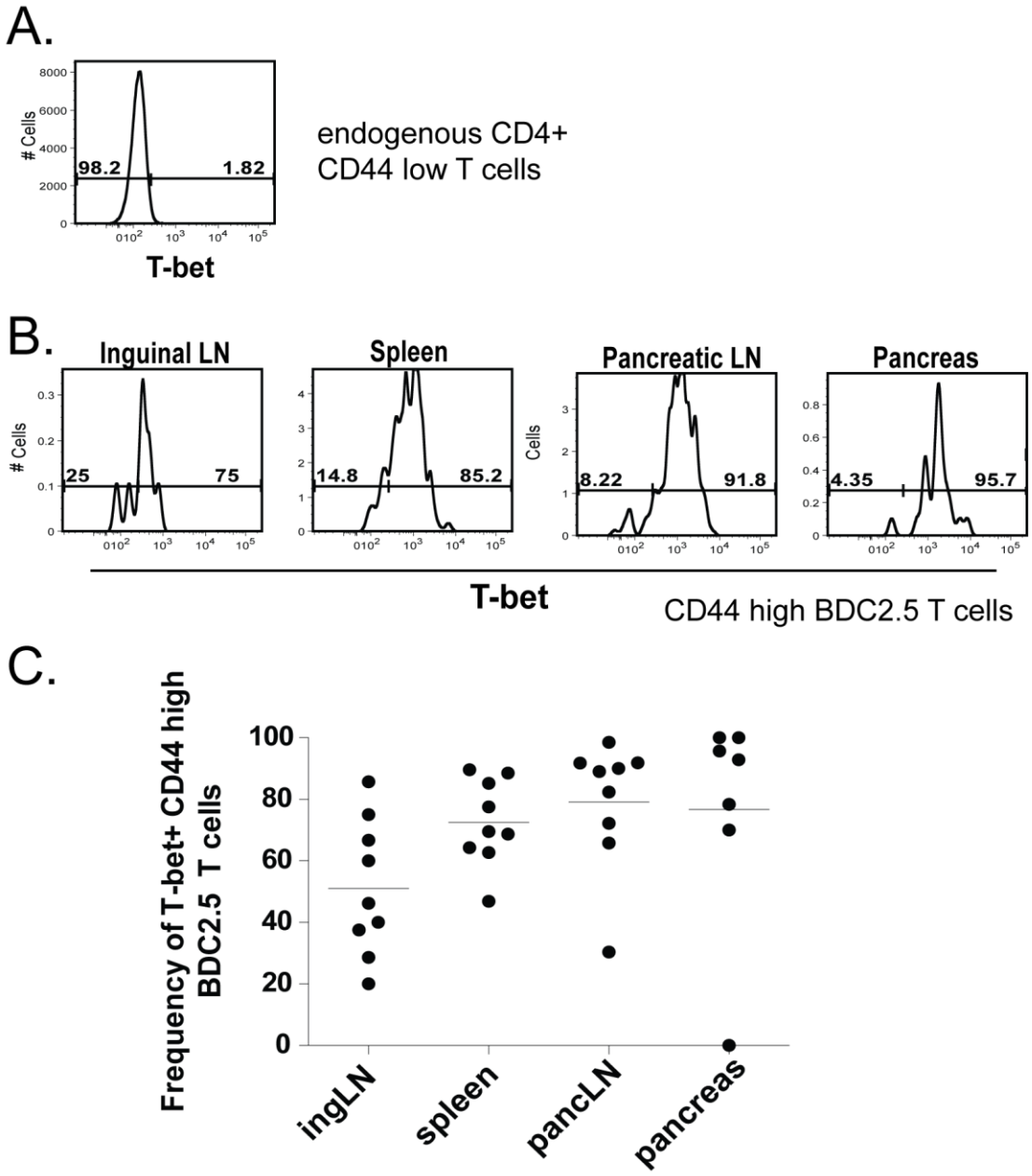

Figure 2-5: BDC2.5 T cells express the transcription factor T-bet^{+}.

(A) Representative FACS histogram showing T-bet expression on CD44 low endogenous CD4^{+} T cells in NOD mice that received BDC2.5 T cells shown in panel B. (B) Representative FACS histograms showing T-bet expression by CD44^{high} BDC2.5 T cells 3 weeks post-transfer into NOD mice. (C) Frequency of T-bet^{+} CD44^{high} BDC2.5 T cells 3 weeks post-transfer. Data are from three combined independent experiments with three mice per experiment.

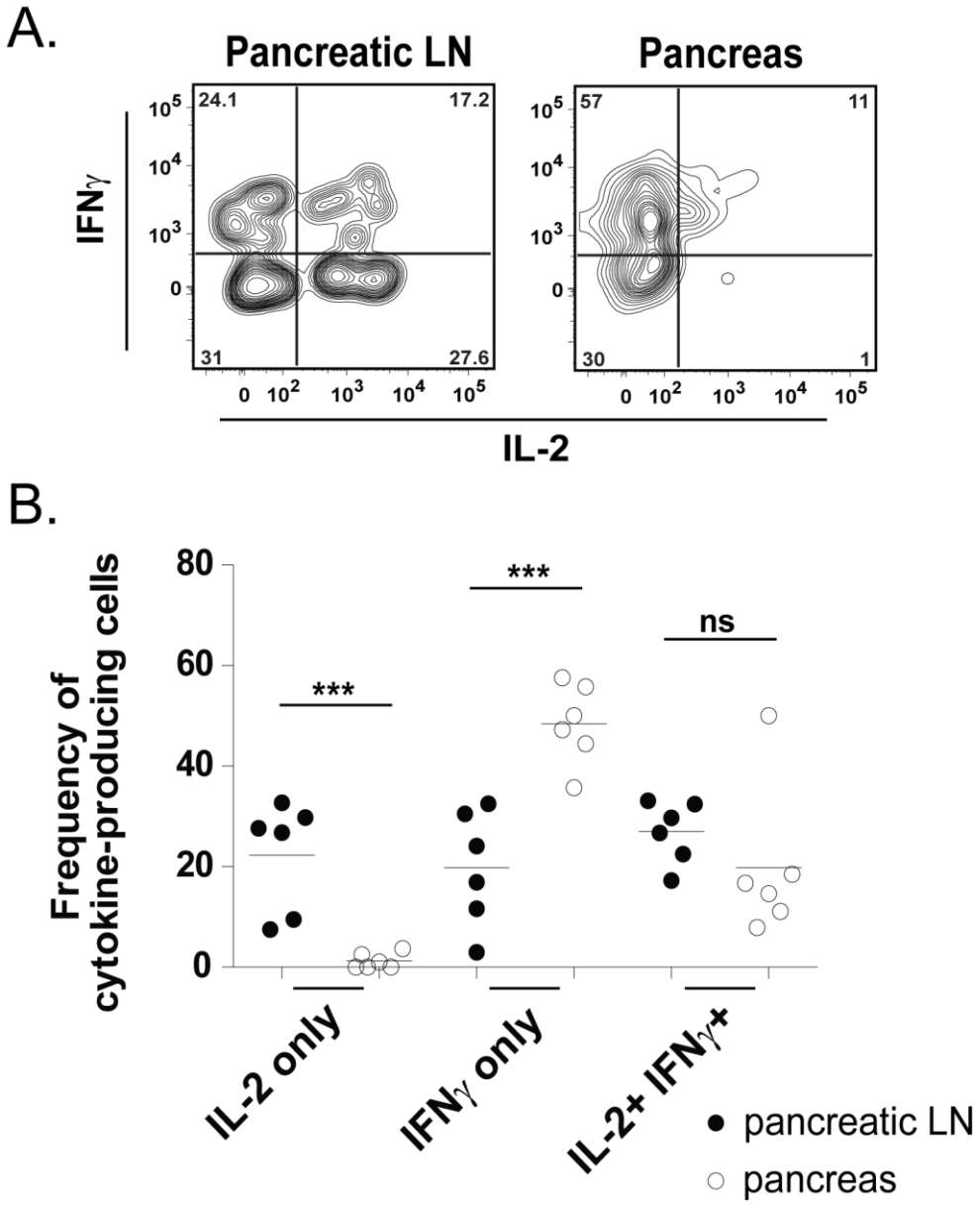

Figure 2-6: BDC2.5 T cells produce IFNγ and IL-2 in the pancreatic LN and pancreas

(A) Representative FACS contour plots gated on BDC2.5 T cells 32 days post-transfer showing IFNγ and IL-2 staining. Gates were set with a control that received BFA but no PMA or ionomycin during the 4 hour stimulation. (B) Frequency of BDC2.5 T cells producing IL-2 and/or IFNγ 32 days post-transfer from pancreatic LN and pancreas. Data shown are representative of three independent experiments with at least three mice per experiment.

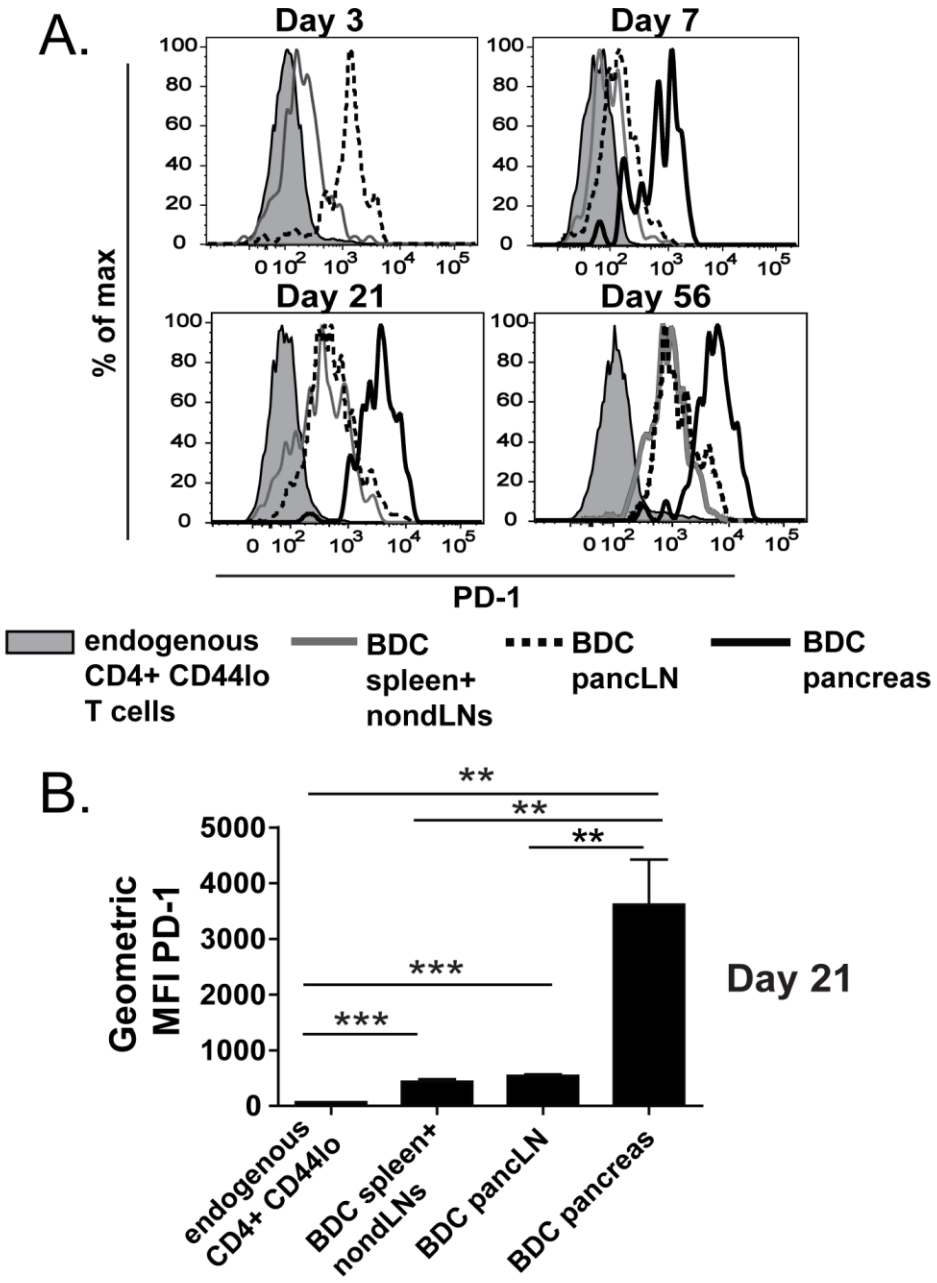

Figure 2-7: Post-activation BDC2.5 T cells express the inhibitory receptor PD-1.

(A) Representative FACS histograms showing PD-1 expression on BDC2.5 T cells from the spleen and non-draining LNs, pancreatic LN, and pancreas at days 3, 7, 21, and 56 post-transfer. PD-1 expression levels are compared to endogenous $CD44^{low}$ $CD4^{+}$ T cells. Data are representative of two independent experiments with three-four mice per time point. (B) Average geometric mean fluorescence intensity (MFI) of PD-1 from four mice analyzed at day 21 post-transfer of BDC2.5 T cells. Data are representative of six independent experiments.

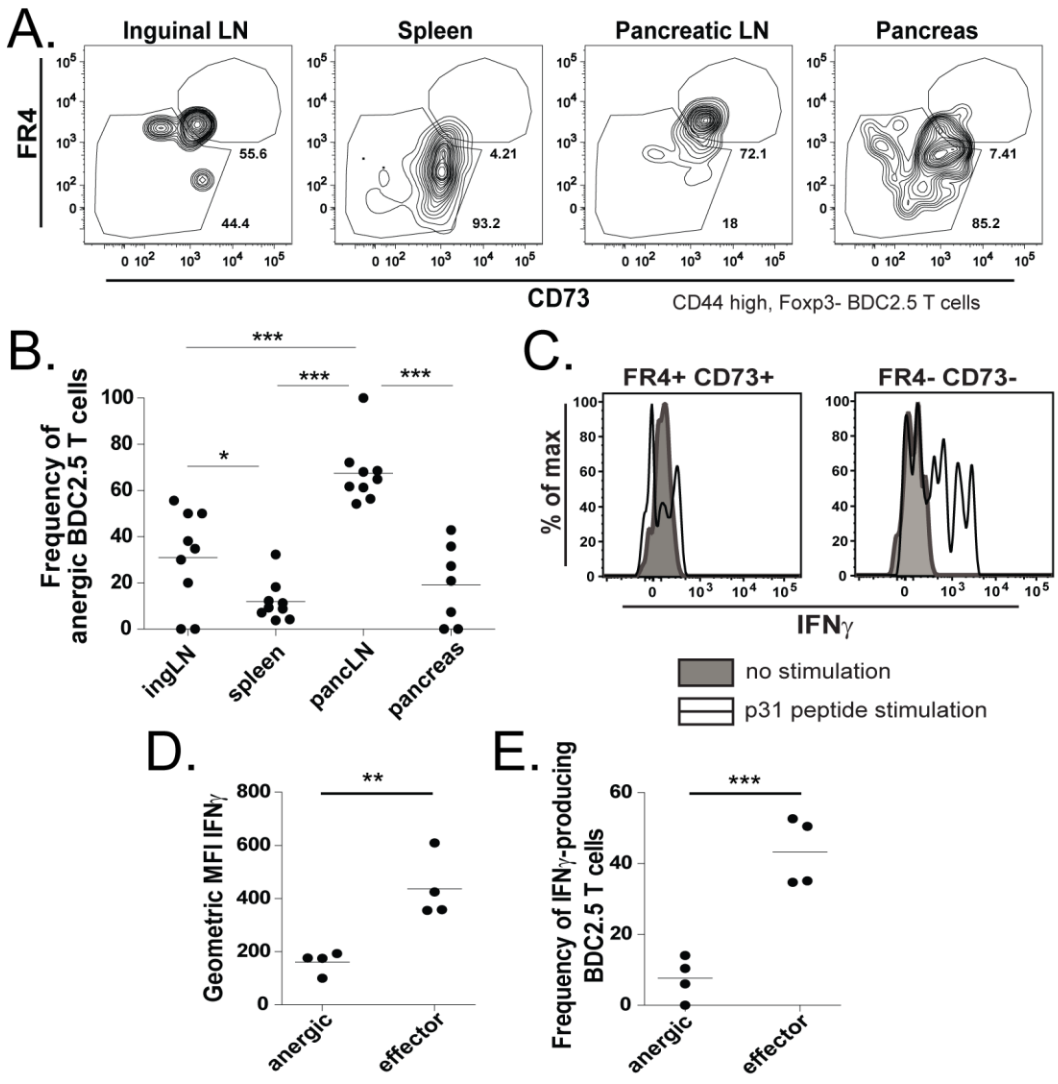

Figure 2-8: Anergic phenotype BDC2.5 T cells are enriched in the pancreatic LN, but largely absent from the spleen and pancreas.

(A) Representative FACS contour plots showing FR4 and CD73 expression on BDC2.5 T cells 17 days post-transfer into non-diabetic NOD. (B) Quantification of anergic phenotype BDC2.5 T cells 17-26 days post-transfer. Panels (A) and (B) are gated on $CD44^{high}$ $Foxp3^{-}$ cells and are representative of three experiments with three to six mice per experiment. (C) FACS histograms showing IFNγ production by effector ($FR4^{-}CD73^{-}$) and anergic phenotype ($FR4^{+}$ $CD73^{+}$) BDC2.5 T cells in the pancreatic LN 56 days post-transfer. Quantification of (D) the geometric mean fluorescence intensity (MFI) of IFNγ produced by and (E) the frequency of IFNγ-producing effector and anergic phenotype BDC2.5 T cells in the pancreatic LN 56 days post-transfer. Data from (C-E) are representative of three experiments with three to four mice per experiment.

CHAPTER 3:

Tracking endogenous insulin-specific $CD4^+$ T cells during Type 1 Diabetes with peptide/MHC class II tetramers revealed differences in T cell priming and effector functions between diabetes-prone and diabetes resistant mice[1]

INTRODUCTION

The importance of the insulin-specific T cell response during Type 1 Diabetes in the non-obese diabetic (NOD) mouse is well established. Insulin-specific $CD4^+$ T cells can be detected in the pancreas infiltrate by 4 weeks of age, and can continually be detected through at least 12 weeks of age (74, 80). The majority of the insulin-specific $CD4^+$ T cells responds specifically to the amino acid peptide residues 9-23 from the insulin B chain (74). Transfer of $insB_{9\text{-}23}$-reactive T cell clones into young NOD mice accelerates diabetes, highlighting the pathogenic nature of these cells during disease progression (74). Furthermore, 40% of transgenic mice expressing a T cell receptor specific for $insB_{9\text{-}23}$ (BDC12.4.1) develop diabetes on a Rag KO background (83). Abolishing the T cell response directed against the 9-23 epitope eliminates diabetes in NOD (70), and administering tolerogenic immunizations against the $insB_{9\text{-}23}$ peptide or variants modified to bind with high affinity to $I\text{-}A^{g7}$ slows or prevents diabetes in NOD mice (84-86). Despite the well-established role of insulin-specific $CD4^+$ T cells during diabetes, little is known about how this pathogenic immune response develops due to the difficulty of tracking these cells in vivo. Important unanswered questions in the field include: what is the frequency and activation status of insulin-specific $CD4^+$ cells, does

this change over time, do these cells exist in diabetes-resistant strains, what frequency participates in clinical diabetes, and how are they regulated? To date there has not been a careful analysis and characterization of this critical antigen-specific $CD4^+$ T cell population during diabetes pathogenesis to understand how peripheral tolerance fails and diabetes ultimately develops. The goal of this chapter was to begin to address these issues, which ultimately will aid in the development of better treatments for diabetic patients.

Peptide/MHC class II tetramers are powerful tools to track antigen-specific $CD4^+$ T cells in vivo. When coupled with magnetic enrichment, incredibly rare populations can be tracked with high precision (95-97, 206). However, one complication of peptide/MHC class II tetramers is determining the binding register for the peptide, since $CD4^+$ T cell epitopes can bind in a variety of different conformations. For the insulin B_{9-23} epitope the field lacks a consensus on the relevant binding register(s) (87, 98-100). Register 1 comprised of the core segment 12-20 (VEALYLVCG) and register 2 containing the core segment 13-21 (EALYLVCGE) can stably bind I-A^{g7} (98). Register 3 containing the core segment 14-22 (ALYLVCGER) poorly binds I-A^{g7} (87). However, modifying the insB_{10-23} peptide to enhance binding to I-A^{g7} caused the register 3 peptide to stimulate IL-2 production from more insulin-specific hydridomas than either register 1 or 2, suggesting that this register can recognize a diverse repertoire of insulin-specific $CD4^+$ T cells (87). Additionally, sub-immunogenic immunization of both young (4-6 weeks) and old (12-14 weeks) NOD mice with this modified register 3 peptide prevented diabetes (84), highlighting the importance of this epitope for spontaneous disease progression.

Based on these data, we constructed a tetramer reagent containing the modified register 3 epitope (HLVERLYLVCGEEG) bound to I-A^{g7} to define the dynamics of the insulin-specific $CD4^+$ T cell response in diabetes-susceptible NOD mice as well as diabetes-resistant B6 mice expressing the I-A^{g7} allele (B6.g7) (136). Using this reagent, we performed a careful time course analysis and detected a population of insulin-specific $CD4^+$ T cells in the secondary lymphoid organs of both strains, but the frequency and number of antigen-experienced cells was substantially greater in NOD. Additionally, these cells produced the inflammatory cytokine IFNγ and infiltrated the pancreas in NOD but not B6.g7 mice. Similarly to the BDC2.5 low transfer model presented in Chapter 2, the majority of insulin-specific cells in the pancreatic LN of NOD mice were anergic with the surface phenotype of $CD73^+FR4^+$ (209). However, this anergic population decreased in frequency as the NOD mice aged. These data suggest that only a small subset of endogenous insulin-specific $CD4^+$ T cells are pathogenic in NOD mice, and that peripheral tolerance acts to restrain those cells but ultimately fails in mice that progress to diabetes. Interestingly, this population was missing from B6.g7 mice. These data suggest that B6.g7 mice are not protected due to deletion of this autoreactive repertoire, nor the development of T cell anergy, but rather because these cells remain naïve. These data provide insight into distinct mechanisms for regulation of insulin-specific $CD4^+$ T cells in diabetes-resistant and -susceptible mouse strains during disease progression.

MATERIALS AND METHODS

Mice

Mice were housed at the University of Minnesota under specific pathogen free conditions. NOD mice were purchased from Taconic (Germantown, NY). B6 mice expressing the NOD I-A^{g7} allele (B6.g7 mice) were generated by Mathis and Benoist (136). Mice were used for tetramer experiments between 3-30 weeks of age. Diabetes was assessed by blood glucose reading with 250 mg glucose/dL blood indicating clinical diabetes (LifeScan, Inc., Milpitas, CA). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Peptide/MHC class II tetramer reagents

The ins$B_{10\text{-}23r3}$:I-A^{g7} tetramer reagent was constructed as described (95). Briefly, the I-A^{g7} monomer containing the peptide sequence HLVERLYLVCGEEG was produced and biotinylated in S2 cells (*Drosophilia melanogaster)*. Biotinylated monomer was further purified by eluting over a monomeric avidin column (Thermo Scientific, Pittsburgh, PA), and the final tetramer product was made by combining with streptavidin-phycocerythrin (PE) and streptavidin-allophycocyanin (APC) from Prozyme (Hayward, CA). The $HEL_{11\text{-}25}$:I-A^{g7} tetramer containing the peptide sequence AMKRHGLDNYRGYSL was obtained from the National Institute of Health Tetramer Core Facility (Atlanta, GA).

Flow cytometry

Single cell suspensions were generated from the pancreatic LN, spleen and non-draining LNs, and pancreas as described previously (216). Tetramer-binding $CD4^+$ T

cells were enriched from the spleen and non-draining LNs (inguinal, axillary, brachial, cervical, mesenteric, and periaortic) pooled using magnetic enrichment (Miltenyi Biotech, Auburn, CA). Briefly, single cell suspensions were incubated with 10 nM of insulin tetramer conjugated to PE and APC for one hour at room temperature followed by anti-PE and anti-APC microbeads for 30 minutes at 4^{o}C and subsequently eluted over magnetic columns. Magnetic enrichment was not conducted on the pancreatic LN and pancreas samples since the low number of total cells allowed the entire sample to be analyzed by flow cytometry. Lymphocytes were enriched from pancreas samples using collagenase P digestion (Roche, Indianapolis, IN) and discontinuous percoll gradient as described (216).

Surface antibodies used included CD3, CD4, B220, CD11b, CD11c, CD44, CD45RB, CD8α, FR4, and CD73. Intracellular stains included Foxp3, IFNγ, TNFα, and IL-17A. Foxp3 staining was conducted using the Foxp3 Fixation/permeabilization kit according to manufacturer's recommendations (eBioscience, San Diego, CA). Intracellular cytokine staining for IFNγ and IL-17A was conducted using the cytofix/cytoperm kit according to manufacturer's instructions (BD Biosciences, San Jose, CA). Samples were collected using BD LSRII and Fortessa instruments. Data was analyzed using Flow Jo software (Tree Star, Ashland, OR). Cell numbers were assessed by FACS using AccuCheck Counting Beads (Life Technologies, Grand Island, NY). Insulin-specific CD4^{+} T cells were defined as CD4^{+} lineage^{-} (B220^{-}, CD11b^{-}, CD11c^{-}), CD8α^{-}, insB10-23:I-A^{g7}-PE^{+} and –APC^{+} cells.

Cytokine stimulation

To assess cytokine production from insulin-specific $CD4^+$ T cells, pancreatic LN, spleen, and non-draining LNs were pooled and stimulated in vitro in complete DMEM (containing 10% fetal bovine serum (Hy-clone), 2 mM L-glutamax, 100 U/ml penicillin-streptomycin, 0.1 M non-essential amino acids, 10 mM HEPES and 50 μM 2-mercaptoethanol (Life Technologies, Grand Island, NY)) with 100 ng/ml phorbol 12-myristate 13-acetate (PMA), 1000 ng/ml ionomycin, and 10 μg/ml brefeldin A (Sigma, St. Louis, MO) for 4 hours (216). Subsequent tetramer staining and enrichment and surface staining was conducted in media containing 2.4G2 (FC receptor block), brefeldin A, and azide.

In vivo priming

The modified ins$B_{10\text{-}23}$ peptide (HLVERLYLVAGEEG) (Genemed Synthesis) (87) or whole insulin protein (Novo Nordisk Pharmaceuticals Inc, Princeton, NJ) was emulsified in complete Freud's adjuvant at a concentration of 100 μg. Mice were immunized subcutaneously in the back and the pancreatic LN, non-draining LNs, and spleen were harvested for FACS analysis 7-14 days post-immunization.

Statistics

Statistical analyses were performed using unpaired two-tailed Student's t tests with a 95% confidence interval using GraphPad Prism 5 software (GraphPad, La Jolla, CA). Asterisks used to indicate statistical significance were: not significant >0.05 (ns),

significant 0.01 to 0.05 (*), very significant 0.001 to 0.01 (**), and extremely significant <0.001 (***).

RESULTS

Development and validation of the insB$_{10\text{-}23r3}$:I-A^{g7} tetramer reagent

In order to study the dynamics of insulin-specific CD4^{+} T cells in vivo, we developed[2] a tetramer reagent containing a portion of the insulin B chain (10-23) bound to I-A^{g7}. Amino acid substitutions at 14 (A to R) and 22 (R to E) were performed to trap the insulin peptide in the register 3 binding conformation based on previous work (Figure 3-1 A) (87). In the previous study, this modified peptide was capable of stimulating IL-2 production from a wider variety of insulin-specific clones than either registers 1 or 2 (87), and further work has shown that sub-immunogenic immunization with this peptide can prevent diabetes in NOD mice (84). These results suggest the modified register 3 epitope provides an ideal detection reagent because it can bind a diverse repertoire of insulin-specific CD4^{+} T cells that are involved for disease pathogenesis.

To validate that our tetramer bound a unique T cell population in unimmunized NOD mice we compared insulin tetramer staining to an irrelevant control tetramer HEL$_{11\text{-}25}$:I-A^{g7} and found distinct staining with no double positive events indicating reagent specificity (Figure 3-1 B). We next confirmed the specificity of the tetramer reagent by immunizing NOD and B6.g7 mice with the modified insB$_{10\text{-}23}$ peptide in CFA and assessing clonal expansion 7-14 days later. The insulin B peptide induced a robust

response in both NOD (17,071±2707 cells) and B6.g7 (36,131±6227 cells) (Figure 3-1 C and D). Interestingly, the clonal expansion was significantly higher in B6.g7 than NOD mice (2.12 fold). These data validate that our reagent is specific for the $insB_{10-23}$ peptide. It should be noted that immunizing with both $insB_{10-23}$ and HEL_{11-25} resulted in the expansion of mutually exclusive populations (data not shown), consistent with these two tetramers binding unique populations in both pre-immune mice as well as immunized mice. We next immunized NOD and B6.g7 with whole insulin protein emulsified with CFA. We failed to detect substantial clonal expansion in either NOD (1,618±562 cells) or B6.g7 (155±64 cells) mice (Figure 3-1 D).[3] This result was expected for this epitope, since previous work by Unanue and colleagues had identified that the pathogenic $insB_{9-23}$-reactive $CD4^+$ T cells are Type B phenotype, or capable of responding to the 9-23 peptide but not whole insulin. Therefore, it is possible that the major cell population we are detecting with our tetramer reagent is Type B rather than Type A cells. However, we cannot completely exclude the possibility that we are also detecting Type A cells using clonal expansion as our only assay. Alternatively, it is possible that Type A cells do not expand as robustly in vivo as Type B cells or the levels of 9-23 peptide/MHC complexes may be far lower because other peptides will be competing for MHC binding in the whole insulin immunization. We therefore conclude that our tetramer is capable of detecting Type B cells, though additional work will be needed to determine if Type A cells are also detected with this tetramer reagent.

Insulin-specific $CD4^+$ T cells were detected in the secondary lymphoid organs of NOD and B6.g7 mice, but only infiltrated the pancreas in NOD mice

We next compared the insulin-specific $CD4^+$ T cell response in unimmunized diabetes-susceptible NOD mice to diabetes-resistant B6.g7 mice by enumerating the insulin-specific $CD4^+$ T cells in the spleen and non-draining LNs, pancreatic LN, and pancreas at a variety of ages that are relevant during diabetes development. However, we found high background staining with the insulin tetramer based on non-specific binding of the tetramer in the $CD8^+$ T cell population (Figure 3-2 A-D), particularly in the pancreatic LN (Figure 3-2 C and D). In order to increase the sensitivity of our reagent we stained with the insulin tetramer conjugated to two different fluorochromes (PE and APC) and gated on cells that bound both tetramers (Figure 3-2 B and D). This approach was utilized for the remainder of this thesis to reduce background and enhance reagent sensitivity.

Using staining with both PE and APC tetramers, we detected insulin-specific $CD4^+$ T cells in the secondary lymphoid organs of both NOD and B6.g7 mice (Figure 3-3 A and B). However, these cells only infiltrated the pancreas of NOD mice (Figure 3-3 A and C). The number of insulin-specific $CD4^+$ T cells increased in the secondary lymphoid organs of NOD mice with age, peaking at 14 weeks (Figure 3-3 B, Table 3-1). There was significantly more insulin-reactive $CD4^+$ T cells in NOD mice at all ages examined compared to B6.g7 mice (Figure 3-3 B and C). The number decreased at 20 weeks in NOD compared to 14 weeks, but the significance of this decline was unclear. It was possible that since disease is not 100% penetrant in NOD mice, the older mice examined

could have individual animals that never would have developed clinical diabetes within the analyzed group (Figure 3-3 B). Infiltration of the pancreas was detected in a few NOD mice at weeks 3 and 5 of life, but substantial and more consistent infiltration of the pancreas did not occur until later in life (at weeks 14-20) (Figure 3-3 C). Importantly, all diabetic NOD mice examined contained insulin-specific $CD4^+$ T cells in the pancreas (Figure 3-3 C). In B6.g7 mice there was a slight, but not significant, increase in cell number between weeks 3 and 5 of life, which was likely due to the increase in the overall number of lymphocytes at this age. The number of insulin-specific $CD4^+$ T cells did not change in B6.g7 mice between weeks 5 and 20, and these numbers remained low compared to age-matched non-diabetic NOD (Figure 3-3 B, Table 3-1 and 3-2). Importantly, no insulin-specific $CD4^+$ T cells were observed in the pancreas of B6.g7 mice at any time point (Figure 3-3 C). We conclude from these data that defects in central tolerance lead to the accumulation of these insulin-specific $CD4^+$ T cells in both NOD and B6.g7, but defects in peripheral tolerance in NOD compared to B6.g7 mice lead to the expansion of these cells in the secondary lymphoid organs and infiltration of the pancreas.

Endogenous antigen results in the up-regulation of CD44 on insulin-specific $CD4^+$ T cells in NOD but not B6.g7 mice.

We next examined the activation phenotype of insulin-specific $CD4^+$ T cells in NOD and B6.g7 mice. We hypothesized that in NOD mice, a conversion to a CD44 high antigen-experienced phenotype would occur early in life on the insulin-specific $CD4^+$ T

cell population, since this is when the critical events are thought to occur that eventually lead to diabetes (31). Conversely, we predicted that majority of insulin-specific $CD4^+$ T cells that escaped central deletion and persisted in the periphery of B6.g7 mice would either be naïve or anergic since the mice are protected from diabetes. To test this hypothesis, we measured CD44 expression. In both the spleen and non-draining LNs and the pancreatic LN, there was a higher frequency (Figure 3-4) and number (Figure 3-5, Table 3-1 and 3-2) of $CD44^{high}$ cells in NOD mice compared to B6.g7 mice. In NOD mice, there was a significant conversion from CD44 low to high expression that occurred in the pancreatic LN between weeks 3 and 5 of life ($p<0.0001$) (Figure 3-4 B), consistent with the notion that the antigen release that led to activation of this pathogenic cell population occurred early in life.

CD44 does not accurately report antigen experience during Type 1 Diabetes

While the frequency of insulin-specific $CD4^+$ T cells that displayed a $CD44^{high}$ antigen-experienced phenotype was higher in NOD mice than B6.g7 mice as expected, we found it striking that the maximal frequency of $CD44^{high}$ cells only reached 40% in the spleen and non-draining LNs and 60% in the pancreatic LN in NOD mice (Figure 3-4) since this population is essential for Type 1 Diabetes pathogenesis. Previous work examining antigen-specific $CD8^+$ T cells during lymphocytic choriomeningitis virus (LCMV) infection showed that during chronic infection (Clone 13), CD44 levels decreased compared to levels during acute infection (Armstrong), causing cells that were chronically encountering antigen to display a CD44 “intermediate” phenotype (207). We

speculated that down-regulation of CD44 due to chronic exposure to insulin was occurring in our tetramer-binding population. In support of this hypothesis, the number of $CD44^{low}$ insulin-specific $CD4^+$ T cells was abnormally high for a truly "naïve" repertoire in older NOD mice (14 weeks, 20 weeks, and diabetic) compared to younger NOD mice (3 weeks and 5 weeks) (Figure 3-5, Table 3-1). The number of naïve $CD4^+$ T cells in pre-immune repertoires for foreign antigens typically ranges from 20-400 (95, 206). In 3 and 5 week old NOD mice, the number of $CD44^{low}$ cells was within this range; however, in older NOD mice, the average number was between 500-800 cells (Figure 3-5, Table 3-1). Interestingly, this increase in the number of $CD44^{low}$ cells was not observed in B6.g7 mice, where we predicted the majority of the insulin-specific population would be naïve (Figure 3-5, Table 3-2). The only significant change in CD44 ^{low} cell frequency occurred in older NOD mice, which was consistent with the hypothesis that CD44 was not accurately reporting antigen experience in NOD mice similar to chronic antigen during viral infections (207).

To test whether insulin-specific $CD4^+$ T cells were chronically stimulated in NOD mice, we conducted further phenotypic analysis comparing CD44 with CD45RB, a marker that is highly expressed on naïve T cells and is down-regulated upon T cell activation (217). In all mice examined, there was a greater frequency of $CD45RB^{dim}$ cells than $CD44^{high}$ cells, suggesting that we underestimated the frequency of antigen-experienced cells using CD44 as our only marker of activation (Figure 3-6 A-C); however, there was only a statistically significant difference between $CD45RB^{dim}$ and $CD44^{high}$ frequencies in the older group of NOD mice (15-20 weeks, $p=0.0122$ in the

spleen+non-dLNs and p=0.0003 in the pancreatic LN). These data suggest that using solely CD44 as an activation marker caused an under-estimation of the frequency of antigen-experienced cells, particularly in older NOD mice. Furthermore, we speculate that the $CD44^{dim}$ $CD45RB^{dim}$ population is chronically stimulated and perhaps functionally exhausted, though additional work is needed to formally test this hypothesis.

Insulin-specific $CD4^+$ T cells express the inhibitory receptor PD-1 following activation in NOD mice, but do not express PD-1 in B6.g7 mice

We next determined the expression of the inhibitory receptor PD-1 on insulin-reactive $CD4^+$ T cells in NOD and B6.g7 mice. PD-1 is expressed following antigen encounter (157), and we showed previously that BDC2.5 T cells in the low adoptive transfer model expressed PD-1 following antigen encounter in NOD mice (Figure 2-7). In the BDC2.5 low transfer model, PD-1 remained elevated in the secondary lymphoid organs compared to the endogenous naïve $CD4^+$ T cell population long after initial antigen encounter; therefore, we hypothesized that a high frequency of insulin-specific $CD4^+$ T cells would express PD-1 in NOD mice following antigen encounter. Furthermore, we hypothesized that the frequency of PD-1^+ cells would be low in B6.g7 mice since the majority of the insulin-specific $CD4^+$ T cells in these mice are not antigen-experienced. The frequency of PD-1^+ cells was similar to what was observed with CD44 in the spleen and non-draining LNs (PD-1 Figure 3-7 A, CD44 Figure 3-4 A) and pancreatic LN (PD-1 Figure 3-7 B, CD44 Figure 3-4 B) for both NOD and B6.g7 mice. Consistent with this notion, PD-1 was typically expressed on $CD44^{high}$ cells (Figure 3-7

C). There were some CD44 low/intermediate cells that expressed PD-1, but it was likely that these cells were also antigen-experienced and perhaps chronically stimulated since we previously showed that CD44 alone does not report all antigen experienced-cells in NOD mice (Figure 3-6). The frequency of PD-1^+ cells was significantly higher in the spleen and non-draining LNs and pancreatic LN of NOD than B6.g7 at 5 (spleen+nondLNs p=0.0042, pancLN p=0.0007) and 20 (spleen+nondLNs p =0.0008, pancLN p=0.0091) weeks of age as expected since there was a significantly greater frequency of CD44 high cells in NOD at these time points (5 weeks spleen+nondLNs p<0.0001 and pancLN p<0.0001 and 20 weeks spleen+nondLNs p=0.0001 and pancLN p=0.0035 (Figure 3-4 A and B)). There was no statistically significant difference in PD-1 in the spleen and non-draining LNs (p=0.0614 ns) or the pancreatic LN (p=0.7578) between 3 week old NOD and B6.g7 as expected since there was less of a difference in CD44 frequencies between these strains at this age (Figure 3-7 A and B). Unexpectedly, there was a higher frequency of PD-1^+ cells in the pancreatic LN of B6.g7 than predicted based on the frequency of $CD44^{high}$ cells at these time points (PD-1 versus CD44: 3 weeks 32.95±9.27% versus 9.83±6.08%; 5 weeks 19.97±8.0% versus 5.02±2.47%; 20 weeks 21.7±7.6 versus 6.79±3.41%), suggesting that some antigen encounter had occurred in this organ in these mice. The frequency of PD-1^+ insulin-specific $CD4^+$ T cells was consistently higher in the pancreatic LN compared to the spleen and non-draining LNs in NOD (spleen+nondLNs versus pancreatic LN, 3 weeks 23.64±4.59% versus 30.26±4.0%, 5 weeks 21.91±2.08% versus 55.34±4.7%, 14 weeks 33.03±6.23% versus 45.00±4.48, 20 weeks 25.96±3.64% versus 37.8±3.4%, and diabetic 28.78±5.28%

versus 40.18±7.9%) (Figure 3-7 A and B), consistent with the notion that antigen is being encountered in the pancreatic LN.

We had previously shown that PD-1 expression levels on BDC2.5 T cells in the low transfer system were higher in the pancreas than the secondary lymphoid organs (Figure 2-7). Therefore, we hypothesized that PD-1 levels on insulin-specific $CD4^+$ T cells would be higher in the pancreas of NOD mice than the pancreatic LN and spleen and non-draining LNs. While PD-1 levels were consistently high in the pancreas, the expression levels compared to the pancreatic LN varied (Figure 3-7 D). In some cases PD-1 levels were higher in the pancreas than the pancreatic LN, and in others the expression was more similar. However, PD-1 was uniformly expressed on all cells in the pancreas, suggesting that the insulin-specific $CD4^+$ T cells were encountering antigen in this location similarly to the BDC2.5 T cell population.

Insulin-specific $CD4^+$ T cells differentiate into IFNγ- producing Th1 cells in NOD mice, but fail to produce this cytokine in B6.g7 mice

IFNγ-producing Th1 cells are the major pathogenic $CD4^+$ T cell subset during diabetes (44-46). We hypothesized that insulin-specific $CD4^+$ T cells would produce IFNγ in NOD mice, but not B6.g7 because the majority of the insulin-specific $CD4^+$ T cells were not activated. We also examined TNFα and IL-17A. IL-17A was examined because its role in diabetes has been controversial, with some sources arguing it is pathogenic and others arguing that it is dispensable (49-51). TNFα was included to test if the cells were healthy and viable, since this cytokine can be produced by naïve and

activated T cells (218). Therefore, if insulin-specific $CD4^+$ T cells in B6.g7 were naïve rather than tolerant we would expect them to produce TNFα. We tested these hypotheses by stimulating cells from pancreatic LN, spleen, and non-draining LNs with PMA and ionomycin ex vivo. IFNγ, TNFα, and IL-17A were all produced in the bulk polyclonal $CD4^+$ $CD44^{high}$ population in both NOD and B6.g7 mice, providing positive controls that all cytokines could be detected (Figure 3-8 A). Following stimulation, tetramer-binding cells in NOD mice produced IFNγ and TNFα but not IL-17A (Figure 3-8 A and B). B6.g7 produced little to no IFNγ or IL-17A as predicted due to the large frequency of $CD44^{low}$ "naïve" insulin-specific $CD4^+$ T cells in these mice (Figure 3-8 A and B). Importantly, insulin-specific $CD4^+$ T cells from B6.g7 produced TNFα, suggesting that these cells were viable (Figure 3-8 A and B). Interestingly, there was not a significant difference in the frequency of IFNγ- ($p=0.5674$) or TNFα-producing cells ($p=0.0551$) in diabetic compared to non-diabetic NOD mice, though there was a trend towards more TNFα produced in diabetic NOD (Figure 3-8 B). These data support a model where insulin-specific $CD4^+$ T cells become activated and differentiate into pathogenic Th1 cells in NOD, but not B6.g7 mice.

We next compared the frequency of cytokine-producing cells in the $CD44^{high}$ to the $CD44^{low}$ compartment, since we speculated that a fraction of $CD44^{low}$ cells were chronically stimulated in NOD (Figure 3-8 C). As expected, the majority of the IFNγ production was from the $CD44^{high}$ compartment in NOD (non-diabetic 30.77±6.1; diabetic 37.76±7.8); however, there was a small frequency of cells that produced IFNγ in the $CD44^{low}$ population (non-diabetic 6.9±1.87; diabetic 8.85±2.1) (Figure 3-8 C). This

result was consistent with the notion that some of the cells in the $CD44^{low}$ compartment were antigen-experienced ($CD45RB^{dim}$). We speculate that a proportion of these cells could be functionally impaired or exhausted compared to their $CD44^{high}$ counterparts due to chronic antigenic stimulation; however, the $CD44^{low}$ compartment could also contain naïve cells.

Insulin-specific regulatory T cells develop in both NOD and B6.g7 mice

Treg cells are important mediators of peripheral tolerance. Lineage deviation into a regulatory phenotype prevents autoreactive T cells from inducing aberrant tissue pathology, and these cells play an important role in controlling Type 1 Diabetes progression (139). We hypothesized that a subset of insulin-specific $CD4^+$ T cells would develop a regulatory phenotype since these cells are specific for self antigen. To test this hypothesis, we determined whether insulin-specific $CD4^+$ T cells express Foxp3, the master transcription factor that controls the Treg cell lineage (114, 115). In both NOD and B6.g7, between 10-20% of the tetramer positive cells expressed Foxp3 in the spleen and non-draining LN and 10-30% expressed Foxp3 in the pancreatic LN (Figure 3-9). There was no statistically significant difference in the frequency of $Foxp3^+$ insulin-specific $CD4^+$ T cells between NOD and B6.g7, suggesting that insulin-specific Treg cells alone were unlikely to contribute to the differences observed in diabetes incidence between the two mouse strains. However, the functional relevance of this population in either mouse strain remains to be tested.

Autoimmune diabetes develops in NOD mice despite robust anergy development in the endogenous insulin-specific CD4$^+$ T cell compartment in the pancreatic LN.

In Chapter 2 we established that a large frequency of BDC2.5 T cells displayed an anergic phenotype (FR4$^+$ CD73$^+$) in the pancreatic LN, but this population was largely absent from the spleen and pancreas (Figure 2-8). We next utilized our tetramer reagent and stained for this anergic phenotype among the insulin-tetramer positive cells in NOD and B6.g7 mice. Since FR4$^+$ CD73$^+$ BDC2.5 T cells were largely absent from the pancreas, we focused on expression of these markers in the secondary lymphoid organs. Similarly to the BDC2.5 model, the majority of ins$B_{10\text{-}23r3}$-specific CD4$^+$ T cells displayed the anergic phenotype within the pancreatic LN (Figure 3-10 A and B). Additionally, CD73$^-$ FR4$^-$ effector phenotype cells were the predominant population in the spleen and non-draining lymph nodes (Figure 3-10 B and data not shown). We speculate it is this population of cells that had escaped peripheral tolerance or broken tolerance and would contribute to diabetes pathogenesis. Interestingly, the frequency of anergic phenotype insulin-specific T cells was significantly higher in the pancreatic LN of young NOD mice (5 weeks) compared to older NOD mice (20-26 weeks) ($p=0.0086$) (Figure 3-10 B), while effector phenotype cells concomitantly increased in older NOD mice compared to young NOD mice (data not shown). These data support a model where tolerance is induced in a large fraction of insulin-specific CD4$^+$ T cells early in life in the antigen-containing pancreatic LN, but since insulin-specific cells can be detected in the pancreas at this age (Figure 3-3 C), tolerance is notably incomplete and diabetes may only require a small fraction of self-reactive T cells escaping or reversing peripheral

tolerance. Furthermore, these data suggest that tolerance is not maintained over time, which may contribute to enhanced infiltration of the pancreas as NOD mice age. We did not find a significant population of anergic $CD44^{high}$ $Foxp3^{-}$ cells in the B6.g7 mice, despite the possibility that these mice could have been protected from diabetes due to efficient anergy induction. Rather, these cells maintain a naïve phenotype (Figure 3-4 and 3-6) and subsequently these mice do not develop disease.

Previous work defined the anergic phenotype as $CD44^{high}$, since antigen encounter is a requisite for anergy induction (209). Since the $CD44^{low}$ population in NOD mice contained some cells that were chronically stimulated ($CD44^{dim}$ $CD45RB^{dim}$), we evaluated FR4 and CD73 expression on $Foxp3^{-}$ $CD44^{low}$ cells. A small frequency of $FR4^{+}$ $CD73^{+}$ cells were found in the $CD44^{low}$ compartment of a few NOD mice (Figure 3-10 B), though it was incredibly modest compared to the $CD44^{high}$ compartment, with the majority of mice having no $CD44^{low}$ $FR4^{+}$ $CD73^{+}$ cells. We therefore predict that anergy and exhaustion are separate functional states corresponding to unique phenotypes in NOD mice. Whether anergic cells can eventually become exhausted is unclear, but would be interesting to pursue. Lastly, $FR4^{+}$ $CD73^{+}$ cells were completely absent from the $CD44^{low}$ $Foxp3^{-}$ population of insulin-specific $CD4^{+}$ T cells in B6.g7 mice, further suggesting that these cells were naïve. Collectively these data suggest that anergy is induced following antigen encounter in the pancreatic LN of NOD mice but not B6.g7, where the majority of cells remain naïve.

DISCUSSION

The importance of the insulin-specific adaptive immune response during Type 1 Diabetes is well established (59, 70, 74, 80, 84, 85). Although it was known that these cells became activated early in life and infiltrated the pancreas, the work presented herein was the first to track the endogenous insulin-specific $CD4^+$ T cell population in vivo during diabetes progression. Our work confirmed that these cells became activated early and infiltrated the pancreas, but extended the current knowledge by demonstrating that in NOD a large proportion of the population failed to encounter autoantigen, and of the population that did encounter autoantigen a large fraction did not infiltrate the pancreas. We speculate that anergy induction in the pancreatic LN contributes to holding the insulin-specific $CD4^+$ T cell population in the secondary lymphoid organs, and that inhibiting trafficking to the pancreas acts as an important mechanism of peripheral tolerance. Lastly, our data comparing CD45RB and CD44 expression in NOD mice over time suggested that insulin-specific $CD4^+$ T cells chronically engaged autoantigen, which could lead to exhaustion in older mice. The notion of exhaustion contributing to loss of function during autoimmunity provides a novel regulatory mechanism that remains to be explored.

Use of the insulin tetramer reagent allowed a direct comparison of an endogenous diabetogenic T cell response in diabetes-susceptible (NOD) and diabetes-resistant (B6.g7) mice for the first time. While the mechanisms that limit autoimmunity in B6.g7 mice that ultimately fail in NOD mice remain to be fully elucidated, our data support a model where there is a substantial difference in presentation of $insB_{10\text{-}23}$/MHC II complexes between the two mouse strains. In NOD, insulin-specific $CD4^+$ T cells

encountered autoantigen in the pancreatic LN between weeks 3-5 of life, and a subset became activated, differentiated into IFNγ-producing effector cells, and began infiltrating the pancreas, while another subset became anergic. Despite the induction of anergy in a large fraction of the insulin-specific population in the pancreatic LN, insulin-specific $CD4^+$ T cells in young NOD mice are capable of driving disease pathology. Therefore, we speculate that only a small number of activated effector cells are required for diabetes progression in NOD mice. On the contrary, these cells fail to encounter autoantigen in B6.g7 mice and remain naïve, allowing ignorance to pancreatic antigen to be the main mechanism of peripheral tolerance in these mice rather than a more active process such as anergy.

Our work utilizing the modified $insB_{10\text{-}23r3}$ peptide bound to $I\text{-}A^{g7}$ has revealed important differences between the insulin-specific $CD4^+$ T cell response in diabetes-susceptible and –resistant mice. However, we were limited in this study by the diversity of insulin-specific $CD4^+$ T cells that our tetramer could detect. While there is growing evidence that this modified register 3 epitope binds a critical population of insulin-specific $CD4^+$ T cells both in vitro and in vivo (84, 86, 87, 99), there is new evidence that the glutamic acid at B21 (position 8) is inhibitory to tetramer binding for a subset of insulin-specific clones (99). Therefore, a subpopulation of cells was likely excluded from this analysis. Future studies incorporating tetramers containing other $insB_{9\text{-}23}$ registers may allow us to gain a deeper perspective on the dynamics of the insulin-specific $CD4^+$ T cell population in vivo. It would be interesting to determine if other $insB_{9\text{-}23}$ registers have the same bias towards anergy induction as the register 3 epitope used in this study,

or if these cells are more pathogenic. Understanding how peptide/MHC binding impacts the effector potential of diabetogenic $CD4^+$ T cells may shed important insight into how these cells become activated during diabetes and how they can be targeted therapeutically to restore tolerance in patients.

In conclusion, we have developed a peptide/MHC class II tetramer reagent that allows tracking of ins$B_{10\text{-}23r3}$-specific $CD4^+$ T cells in vivo. This reagent can be used not only to track $CD4^+$ T cell responses in NOD mice, but is also useful for studying diabetes-resistant strains including B6.g7. The critical differences between the diabetes-prone and -susceptible mice were activation of the cells, differentiation into either functionally non-responsive anergic cells or pathogenic IFN-γ-producing Th1 cells, and effector cell infiltration of the pancreas. Despite the presence of pathogenic $CD4^+$ T cells in NOD mice, an anergic population emerged specifically in the pancreatic LN. In B6.g7 mice, an anergic phenotype did not develop: Tolerance was more likely maintained by the absence of antigen encounter. These studies provide important insight into the differences in the regulation of a known autoreactive $CD4^+$ T cell specificity in autoimmune-prone versus –susceptible hosts. Future work aimed at identifying factors that contribute to the breakdown of tolerance in NOD will be informative to developing better therapeutic approaches for diabetic patients. Additionally, work using B6.g7 mice could be informative to determine if therapies designed to enhance immunity will promote autoimmunity in a host with limited risk of developing the disease.

FOOTNOTES

[1]Portions of this work will be submitted for publication in 2013.

[2]J. Linehan constructed the $insB_{10\text{-}23r3}$:I-Ag7 tetramer reagent.

[3]J. Linehan and J. Spanier contributed to Figure 3-1 C and D priming of NOD and B6.g7 mice with $insB_{10\text{-}23}$ or whole insulin protein.

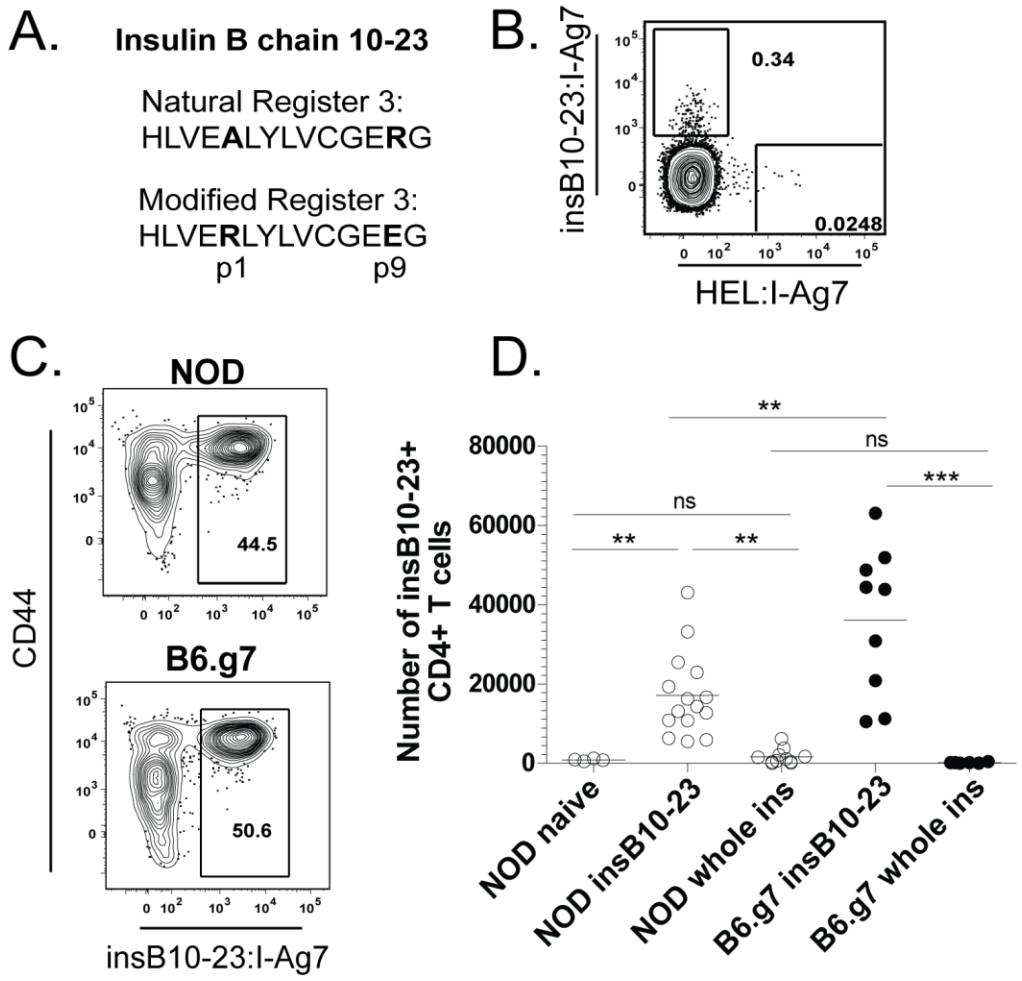

Figure 3-1: Development and validation of the $insB_{10\text{-}23r3}$:I-Ag7 tetramer reagent.

(A) Sequences for the $InsB_{10\text{-}23}$ peptide natural register 3 epitope (top) and modified register 3 epitope that contains substitutions (indicated in bold) at p1 (A to R) and p9 (R to E) (bottom). (B) Representative FACS contour plot from the spleen and non-draining LN bound fraction of a non-diabetic NOD mouse showing $InsB_{10\text{-}23r3}$:$I\text{-}A^{g7}$ and $HEL_{11\text{-}25}$:$I\text{-}A^{g7}$ staining. The plot is gated $CD4^+$ cells and is representative of nine NOD mice from two experiments. (C) Representative FACS contour plots of CD44 (y-axis) and $insB_{10\text{-}23r3}$:I-Ag7 tetramer (x-axis) from NOD or B6.g7 mice eight days following immunization with the modified $InsB_{10\text{-}23}$ peptide in CFA. Plots are gated on $CD4^+$ T cells. (D) Quantification of the number of $InsB_{10\text{-}23}$-specific $CD4^+$ T cells in NOD or B6.g7 mice 7-14 days post-immunization with the modified $InsB_{10\text{-}23}$ peptide in CFA. Individual unimmunized NOD n=4, immunized NOD with $InsB_{10\text{-}23}$ n=15 or whole insulin n=11, and immunized B6.g7 with $InsB_{10\text{-}23}$ n=9 or whole insulin n=6. Data are compiled from at least five experiments.

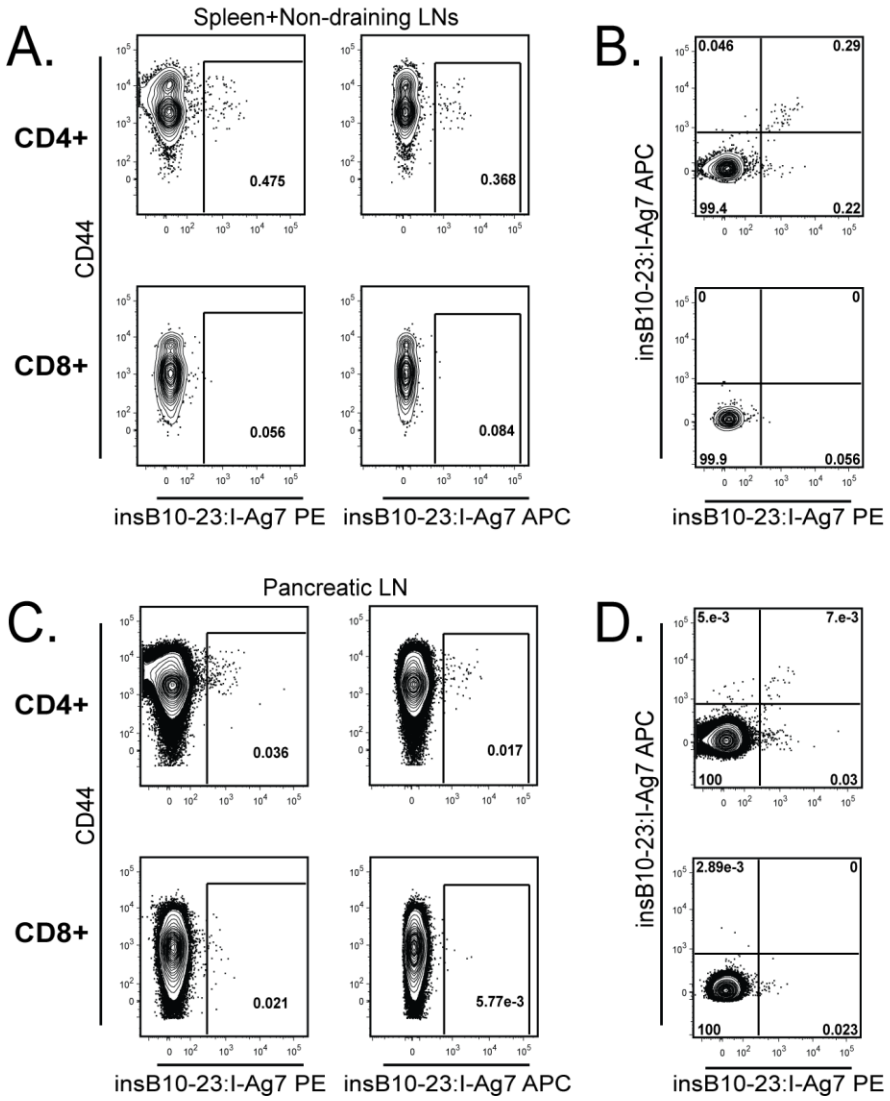

Figure 3-2: Staining samples for flow cytometry with the insB$_{10\text{-}23r3}$:I-Ag7 tetramer reagent in two different fluorochromes improves sensitivity and is optimal for detecting rare insulin-specific cells.

(A and C) Representative FACS contour plots showing insB$_{10\text{-}23r3}$:I-Ag7 tetramer staining (x-axis) using the PE-conjugated tetramer (left panels) compared to the APC-conjugated tetramer (right panels) on CD4$^+$ T cells (top panels) compared to CD8$^+$ T cells (bottom panels) in (A) the spleen and non-draining LNs bound fraction and (C) the pancreatic LN. The y-axis shows CD44 expression. (B and D) Representative FACS counter plots from the mouse shown in panel A or C gated on total CD4$^+$ or CD8$^+$ T cells comparing the PE-conjugated (x-axis) to APC-conjugated (y-axis) insB$_{10\text{-}23r3}$:I-Ag7 tetramer reagent in the spleen and non-draining LNs (B) and pancreatic LN (D). Data are representative of fifty-two non-diabetic mice analyzed from fifteen independent experiments.

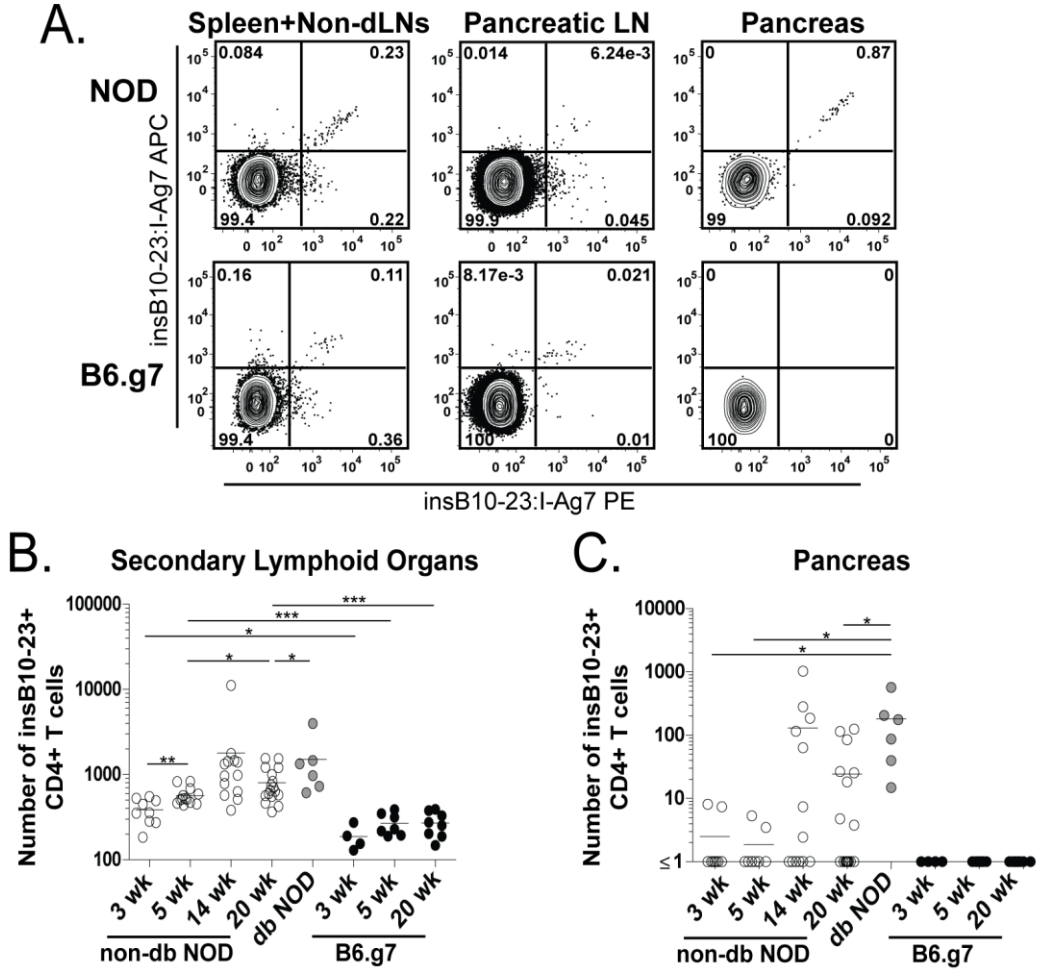

Figure 3-3: Insulin-specific CD4$^+$ T cells are detectable in the secondary lymphoid organs of NOD and B6.g7 mice, but only infiltrate the pancreas in NOD mice.

(A) Representative FACS contour plots from the spleen and non-draining LNs pooled, pancreatic LN, and pancreas of non-diabetic NOD (twenty weeks non-diabetic, representative of eight mice from five independent experiments) and B6.g7 (twenty-one weeks of age, representative of eight mice from three independent experiments). (B) Quantification of the number of $InsB_{10\text{-}23r3}$:I-A^{g7} cells that bind both the PE and APC tetramer (double positive) in the pancreatic LN, spleen, and non-draining LNs (referred to as "Secondary Lymphoid Organs") from individual NOD and B6.g7 mice. Cells are gated on CD4$^+$ $InsB_{10\text{-}23r3}$:I-A^{g7} PE$^+$ and APC$^+$. (C) Quantification of the number of cells in the pancreas of the mice displayed in panel B. For panels B and C data represent 9 mice for 3 weeks, 13 for 5 weeks, 13 for 14 weeks, and 17 for 20 weeks non-diabetic NOD, 6 mice for diabetic NOD, and 4 mice for 3 weeks, 7 for 5 weeks, and 8 for 20 weeks of age for B6.g7. Data for panels B and C are compiled from 15 experiments.

NOD **Total Numbers**	**3 wks Non-db**	**5 wks Non-db**	**14 wks Non-db**	**20 wks Non-db**	**Diabetic**
Total secondary lymphoid organs	385 ± 42	567 ± 37	1793 ± 786	800 ± 90	1511 ± 509
Pancreatic LN	185 ± 35	211 ± 19	377 ± 71	183 ± 33	356 ± 64
Spleen+non-draining LNs	200 ± 26	355 ± 36	1416 ± 739	618 ± 72	1156 ± 486
Pancreas	2.5 ± 1	1.8 ± 0.6	130 ± 79	24 ± 10	182 ± 83
NOD **CD44 High Numbers**	**3 wks Non-db**	**5 wks Non-db**	**14 wks Non-db**	**20 wks Non-db**	**Diabetic**
Total secondary lymphoid organs	117 ± 27	253 ± 26	1080 ± 619	270 ± 51	745 ± 361
Pancreatic LN	48 ± 18	132 ± 19	201 ± 49	53 ± 10	139 ± 43
Spleen+non-draining LNs	69 ± 16	122 ± 16	878 ± 588	218 ± 47	605 ± 336
NOD **CD44 Low Numbers**	**3 wks Non-db**	**5 wks Non-db**	**14 wks Non-db**	**20 wks Non-db**	**Diabetic**
Total secondary lymphoid organs	269 ± 33	314 ± 26	714 ± 174	530 ± 67	766 ± 152
Pancreatic LN	137 ± 29	80 ± 8	176 ± 27	130 ± 30	216 ± 40
Spleen+non-draining LNs	132 ± 14	234 ± 24	538 ± 155	400 ± 46	550 ± 153

Table 3-1: Quantification of insulin-specific $CD4^+$ T cells in the secondary lymphoid organs and pancreas of NOD mice.

Quantification of the number of $InsB_{10\text{-}23r3}$:I-A^{g7} cells that bind both the PE and APC tetramer in the pancreatic LN, spleen, and non-draining LNs (Total Secondary Lymphoid Organs) of NOD mice. The total cell numbers are also reported separately for the pancreatic LN and pooled spleen and non-draining LNs. Total numbers are gated on $singlet^+$, $CD3^+$ $B220^-$, $CD11b^-$, $CD11c^-$, $CD4^+$, $CD8a^-$, $InsB_{10\text{-}23r3}$:I-A^{g7} PE^+ and $InsB_{10\text{-}23r3}$:I-A^{g7} APC^+. Cell numbers are further subdivided into $CD44^{high}$ or $CD44^{low}$. Non-diabetic data are from 9 mice at 3 weeks, 13 mice at 5 weeks, 13 mice at 14 weeks, and 17 mice at 20 weeks of age. Diabetic data are from 6 mice.

B6.g7 **Total Cell Numbers**	**3 wks** **Non-db**	**5 wks** **Non-db**	**20 wks** **Non-db**
Total secondary lymphoid organs	187 ± 31	268 ± 30	270 ± 32
Pancreatic LN	28 ± 6	102 ± 18	86 ± 27
Spleen+non-draining LNs	159 ± 36	166 ± 19	184 ± 22
Pancreas	0 ± 0	0 ± 0	0 ± 0
B6.g7 **CD44 High Numbers**	**3 wks** **Non-db**	**5 wks** **Non-db**	**20 wks** **Non-db**
Total secondary lymphoid organs	10 ± 3	16 ± 5	17 ± 5
Pancreatic LN	3 ± 1	6 ± 3	5 ± 1.5
Spleen+non-draining LNs	8 ± 2.5	11 ± 3.5	13 ± 4
B6.g7 **CD44 low Numbers**	**3 wks** **Non-db**	**5 wks** **Non-db**	**20 wks** **Non-db**
Total secondary lymphoid organs	177 ± 29	252 ± 28	253 ± 28
Pancreatic LN	25 ± 7	97 ± 17	82 ± 27
Spleen+non-draining LNs	151 ± 34	155 ± 17	171 ± 19

Table 3-2: Quantification of insulin-specific $CD4^+$ T cells in the secondary lymphoid organs and pancreas of B6.g7 mice.

Quantification of the number of $InsB_{10\text{-}23r3}$:I-A^{g7} cells that bind both the PE and APC tetramer in the pancreatic LN, spleen, and non-draining LNs (Total Secondary Lymphoid Organs) of B6.g7 mice. Numbers are also reported separately for the pancreatic LN and pooled spleen and non-draining LNs. Total numbers are gated on singlet$^+$, $CD3^+$ $B220^-$, $CD11b^-$, $CD11c^-$, $CD4^+$, $CD8a^-$, $InsB_{10\text{-}23r3}$:I-A^{g7} PE^+ and $InsB_{10\text{-}23r3}$:I-A^{g7} APC^+. Cell numbers are further subdivided into $CD44^{high}$ or $CD44^{low}$. Data from are from 4 mice at 3 weeks, 7 mice at 5 weeks, and 8 mice at 20 weeks of age.

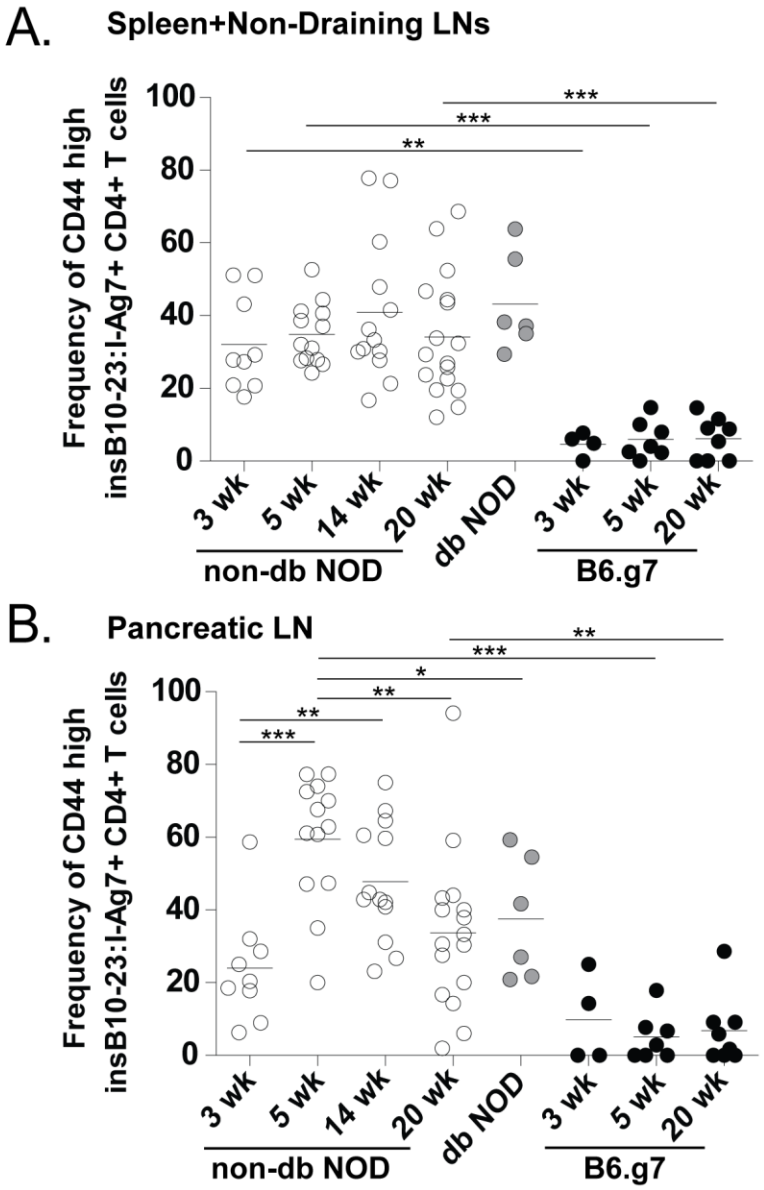

Figure 3-4: There is a higher frequency of antigen-experienced insulin-specific CD4$^+$ T cells in the secondary lymphoid organs of NOD mice compared to B6.g7 mice.

(A) Quantification of the frequency of CD44^{high} insulin-specific CD4$^+$ T cells in the spleen and non-draining LNs and (B) the pancreatic LN from the NOD and B6.g7 mice shown in Figure 3-3 gated on CD4$^+$ InsB$_{10\text{-}23r3}$:I-A^{g7} PE$^+$ and APC$^+$, CD44^{high} cells. Data represent 9 mice for 3 weeks, 13 for 5 weeks, 13 for 14 weeks, and 17 for 20 weeks non-diabetic NOD, 6 mice for diabetic NOD, and 4 mice for 3 weeks, 7 for 5 weeks, and 8 for 20 weeks of age for B6.g7. Data for panels B and C are compiled from 15 experiments.

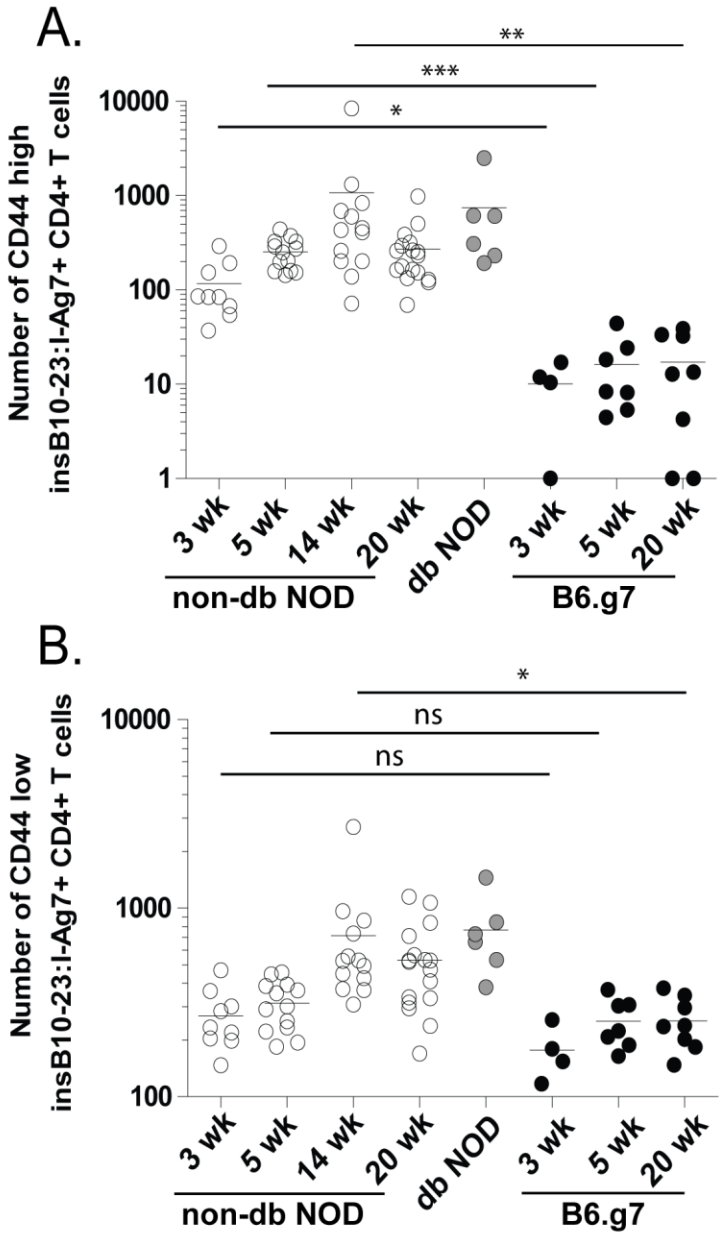

Figure 3-5: There is an increase in the number both CD44 high and low insulin-specific CD4$^+$ T cells in older NOD mice compared to B6.g7 mice.

(A) Quantification of the number of CD44^{high} insulin-specific CD4$^+$ T cells in the spleen and non-draining LNs and (B) the pancreatic LN from the NOD and B6.g7 mice shown in Figure 3-3 gated on CD4$^+$ InsB$_{10\text{-}23r3}$:I-A^{g7} PE$^+$ and APC$^+$, CD44^{high} cells. Data represent 9 mice for 3 weeks, 13 for 5 weeks, 13 for 14 weeks, and 17 for 20 weeks non-diabetic NOD, 6 mice for diabetic NOD, and 4 mice for 3 weeks, 7 for 5 weeks, and 8 for 20 weeks of age for B6.g7. Data for panels B and C are compiled from 15 experiments.

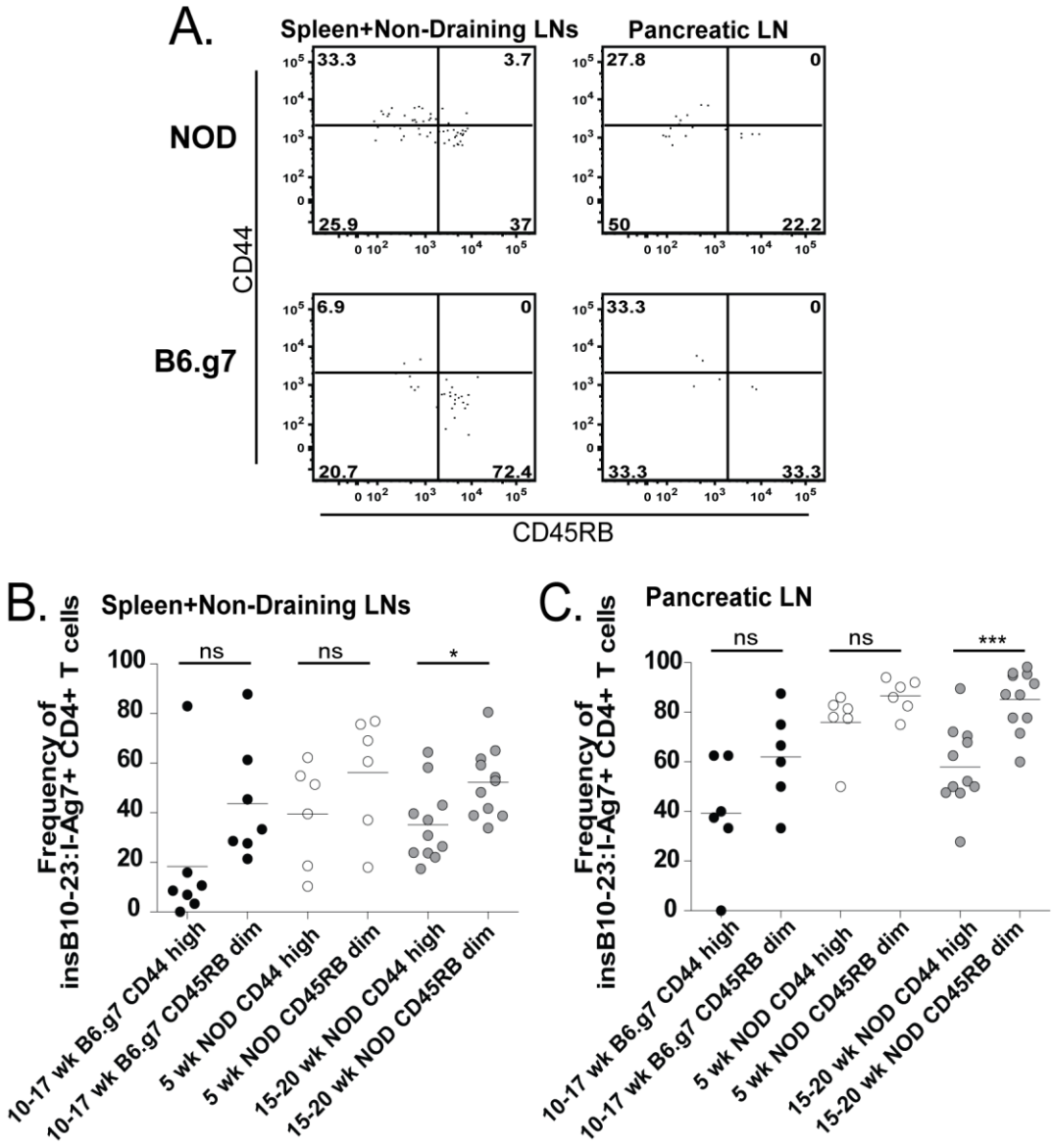

Figure 3-6: CD44 is not accurately reporting antigen experience in older NOD mice.

(A) Representative FACS dot plots showing CD44 (Y-axis) and CD45RB (X-axis) expression on insulin-specific $CD4^+$ T cells in NOD (18 weeks, non-diabetic) and B6.g7 (10 weeks) mice. Plots are gated on $CD4^+$ insB10-23:I-Ag7 PE^+ and APC^+ cells. Quantification of the frequency of $CD44^{high}$ or $CD45RB^{dim}$ insulin-specific $CD4^+$ T cells in the (B) the spleen and non-draining LNs and (C) the pancreatic LN of NOD and B6.g7 mice. Date represent 7 B6.g7 at 10-17 weeks, 6 non-diabetic NOD mice at 5 weeks, and 11 non-diabetic NOD mice at 15-20 weeks. Data are compiled from three experiments.

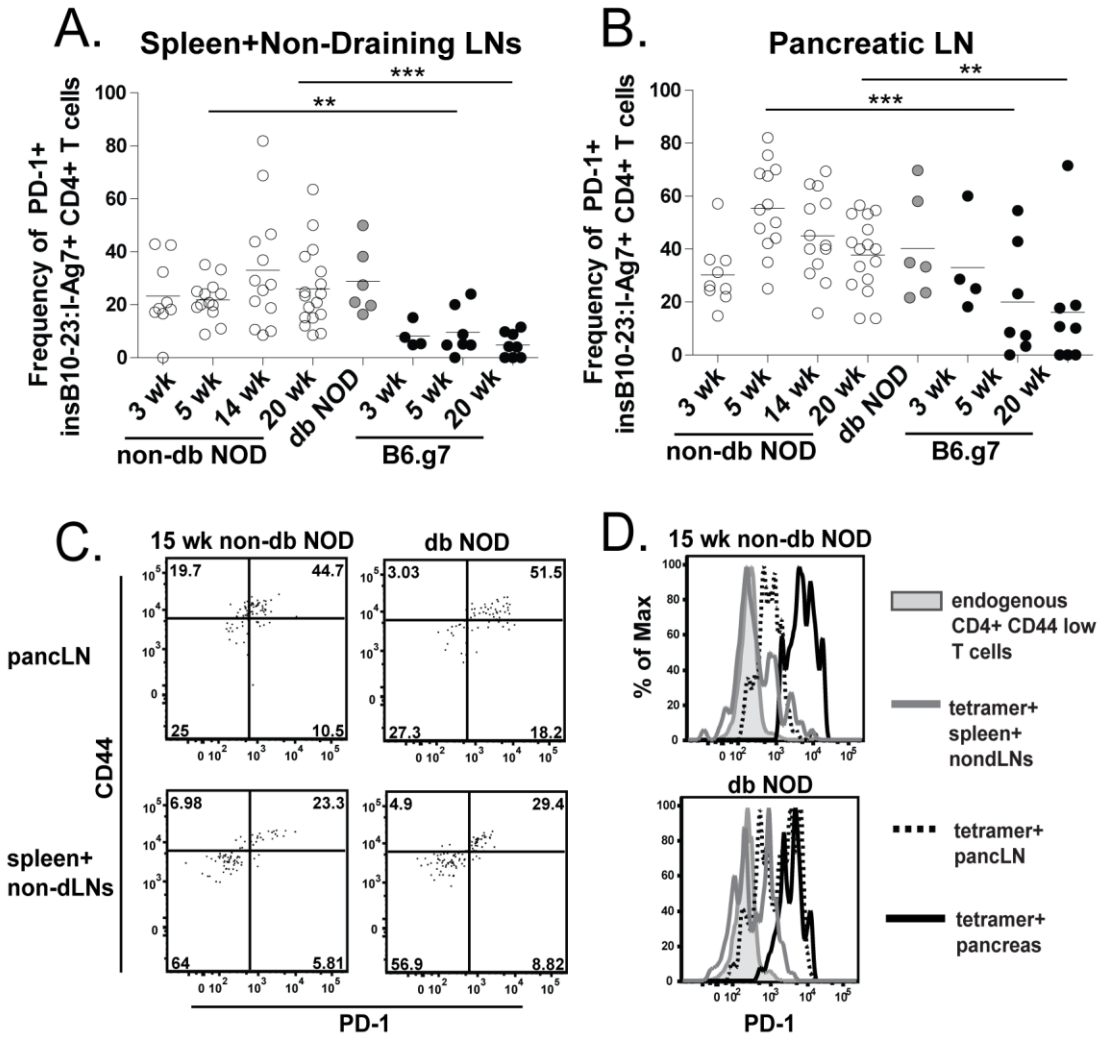

Figure 3-7: A higher frequency of insulin-specific CD4$^+$ T cells expresses the inhibitory receptor PD-1 in NOD mice than B6.g7 mice.

Quantification of the frequency of insulin-specific CD4$^+$ T cells expressing PD-1 in the (A) spleen and non-draining LNs and (B) the pancreatic LN of NOD and B6.g7 mice gated on CD4$^+$ InsB$_{10\text{-}23r3}$:I-A^{g7} PE$^+$ and APC$^+$ cells. Data represent 9 mice for 3 weeks, 13 for 5 weeks, 13 for 14 weeks, and 17 for 20 weeks non-diabetic NOD, 6 mice for diabetic NOD, and 4 mice for 3 weeks, 7 for 5 weeks, and 8 for 20 weeks of age for B6.g7. Data for panels B and C are compiled from 15 experiments. (C) Representative FACS dot plots showing PD-1 and CD44 expression on insulin-specific CD4$^+$ T cells from NOD mice. Data are representative of 13 mice for 14 weeks non-diabetic and 6 mice for diabetic NOD. (D) Representative FACS histograms showing PD-1 expression in the spleen and non-draining LNs, pancreatic LN, and pancreas. Data are representative of 7 non-diabetic 14-15 week old and 6 diabetic NOD mice.

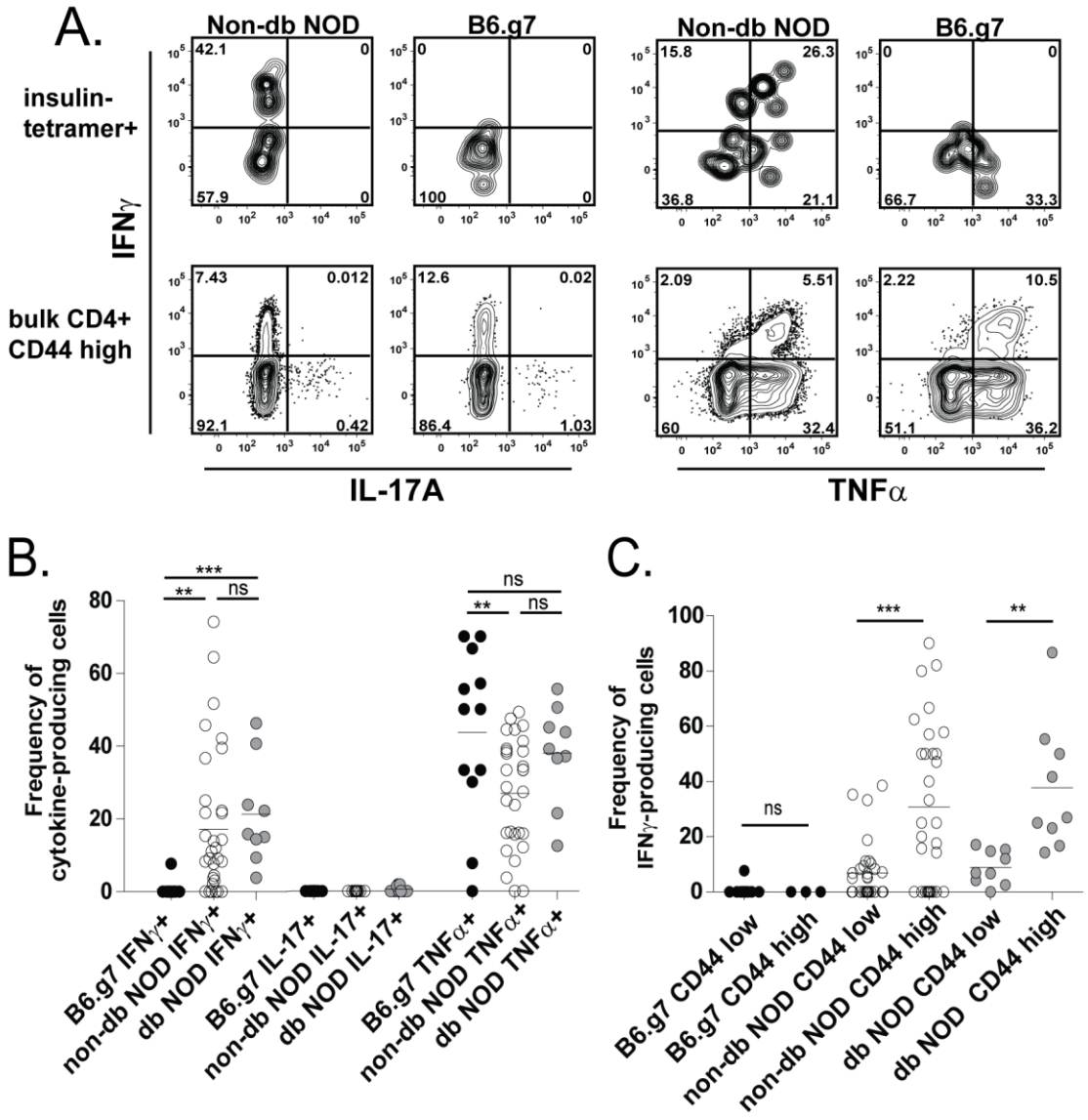

Figure 3-8: Insulin-specific CD4$^+$ T cells are capable of producing IFNγ in NOD mice but not B6.g7 mice.

(A) Representative FACS plots showing intracellular cytokine staining from insulin-specific CD4$^+$ T cells (top) compared to polyclonal bulk CD4$^+$ CD44^{high} cells (bottom). Cells were pooled from the pancreatic LN, spleen, and non-draining LNs. (B) Quantification of the frequency of insulin-specific CD4$^+$ T cells producing IFNγ, IL-17A, or TNFα from B6.g7, non-diabetic NOD, and diabetic NOD mice gated on CD4$^+$ InsB$_{10\text{-}23r3}$:I-A^{g7} PE$^+$ and APC$^+$ cells. (C) Quantification of the frequency CD44^{high} or CD44^{low} IFNγ$^+$ insulin-specific CD4$^+$ T cells from NOD and B6.g7 mice. Data are compiled from 9 experiments with 12 B6.g7, 28-32 non-diabetic NOD, and 9 diabetic NOD mice.

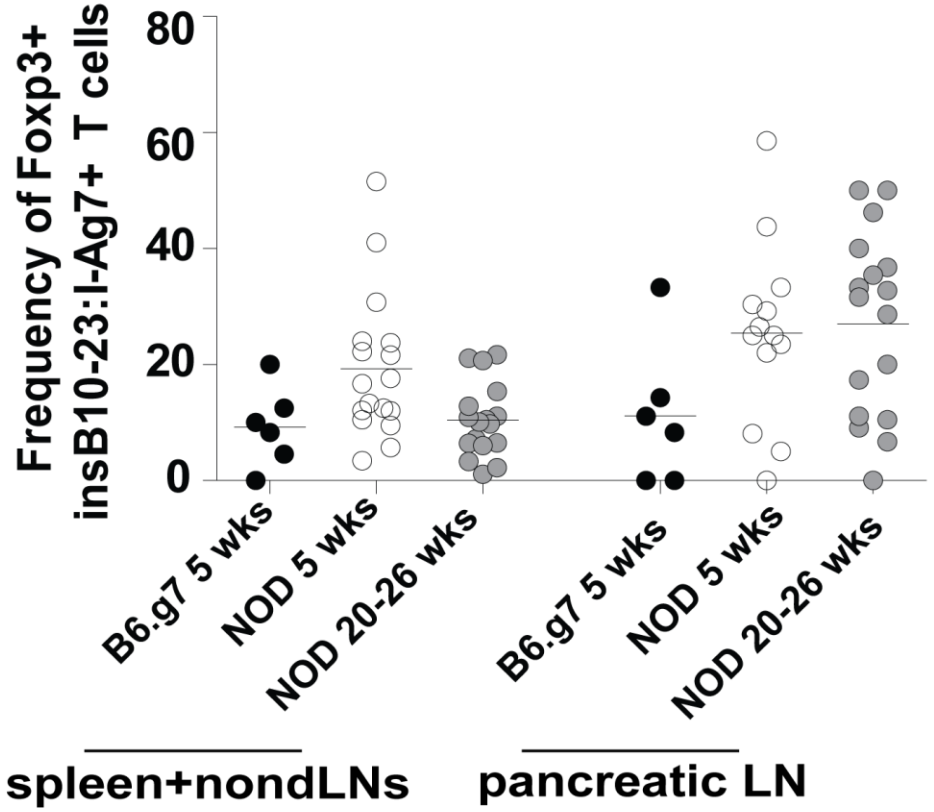

Figure 3-9: A subset of insulin-specific CD4+ T cells develop into Foxp3-expressing Treg cells in both NOD and B6.g7 mice.

Quantification of the frequency of Foxp3-expressing insulin-specific $CD4^+$ T cells in individual NOD or B6.g7 mice. Data show 6 B6.g7 at 5 weeks, 17 NOD at 5 weeks, and 17 NOD mice at 20-26 weeks. Data compiled from 6 experiments.

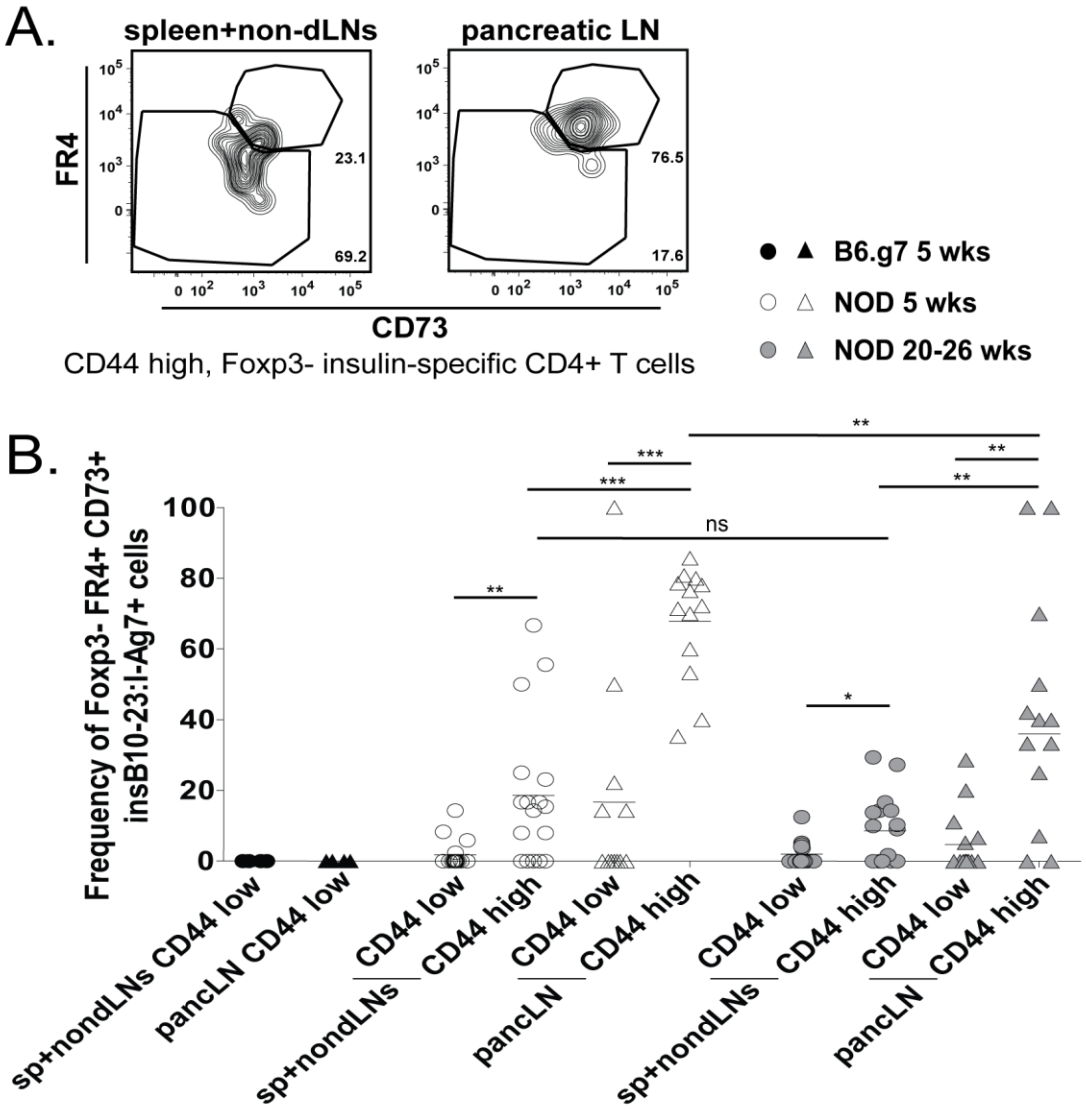

Figure 3-10: A large fraction of insulin-specific CD4$^+$ T cells in the pancreatic LN of NOD mice display an anergic phenotype, while the majority of these cells in B6.g7 mice are naïve.

(A) FACS contour plots showing FR4 and CD73 expression on insulin-specific CD4$^+$ T cells from a 5 week old non-diabetic NOD mouse. Plots are gated on CD4$^+$ InsB$_{10\text{-}23r3}$:I-A^{g7} PE$^+$ and APC$^+$, CD44^{high}, Foxp3$^-$ cells. (B) Quantification of the frequency of anergic phenotype (Foxp3$^-$ FR4$^+$ CD73$^+$) insulin-specific CD4$^+$ T cells in the spleen and non-draining LNs (circles) and the pancreatic LN (triangles) from 5 week old B6.g7 mice (black symbols, n=6), 5 week old non-diabetic NOD mice (white symbols, n=17), and 20-26 week old non-diabetic NOD mice (grey symbols, n=17). Insulin-specific CD4$^+$ T cells are further gated on CD44^{high} versus CD44^{low} cells in each organ compartment from individual mice. Data are compiled from 6 independent experiments.

CHAPTER 4:

The PD-1 pathway is critical for suppressing islet-specific effector CD4$^+$ T cell functions, but is not required for the induction of peripheral tolerance following exposure to endogenous autoantigen.[1]

INTRODUCTION

The inhibitory receptor Programmed Death-1 (PD-1) interacting with PD-L1 is critical for suppressing diabetes since disrupting PD-1/PD-L1 interactions accelerates diabetes in NOD mice (137, 138, 174). Polymorphisms in PD-1 have also been associated with human Type 1 Diabetes (181). Previous studies demonstrated roles for the PD-1 pathway in inhibiting CD4$^+$ T cell survival, proliferation, and cytokine production using in vitro and in vivo systems (59, 169, 174, 176, 177). Additionally, PD-1 interacting with PD-L1 has been shown to be critical for both the induction and maintenance of T cell anergy in islet-reactive CD4$^+$ T cells (59, 110). The ability of PD-1 to maintain tolerance in this model was at least partially due to its ability to prevent the T cell stop signal required for CD4$^+$ T cells to execute their effector functions (110). Importantly, PD-1 acted on tolerant islet-reactive T cells in the pancreatic LN as well as pancreatic islets, highlighting the role for this pathway in mediating tolerance in the autoimmune target organ (110). However, since many of the in vivo studies relied on adoptive transfer of non-physiologically high numbers of T cell receptor transgenic T cells, the cellular mechanisms by which PD-1 constrains diabetogenic CD4$^+$ T cells in hosts with a normal T cell repertoire remain unclear.

We re-examined the role of PD-1 in regulating $CD4^+$ T cells in vivo using the new adoptive transfer model described in Chapter 2 that more closely mimics the normal naïve pre-immune repertoire, as well as the endogenous insulin-specific $CD4^+$ T cell population described in Chapter 3 using the $insB_{10\text{-}23r3}$:$I\text{-}A^{g7}$ tetramer reagent. In NOD mice, anti-PD-L1 resulted in an increase in the number of islet-reactive $CD4^+$ T cells in the spleen, pancreatic LN, and pancreas. This increase in cell number was at least partially due to the ability of PD-1 to regulate cell cycle progression in the secondary lymphoid organs both early during initial priming (Day 7) as well as late after the cells became quiescent (Day 42). Loss of PD-1 also resulted in increased expression of the chemokine receptor CXCR3, which is involved in Th1 cell homing to the pancreas during Type 1 Diabetes (219, 220) and aids in optimizing Th1 cell differentiation in antigen-containing LNs (221). Additionally, loss of PD-1 altered localization of islet-reactive T cells within the pancreas, causing them to move from a more benign portion of the islet infiltrate into the islet core. These results confirm and extend results from previous studies showing that loss of PD-1 enhances T cell effector functions (174, 182).

Interestingly, our results showed that while the PD-1/PD-L1 pathway is important for controlling a variety of effector functions performed by $CD4^+$ T cells, only a specific subpopulation of islet-reactive cells was susceptible to anti-PD-L1 treatment. Contrary to our prediction based on our previous work (59, 110), PD-1 was not required for the induction or maintenance of T cell anergy, and blocking PD-1 did not restore functionality to anergic T cells. Rather, PD-1 was more important for regulating effector phenotype cells. These data suggest that PD-1 is required to suppress effector $CD4^+$ T

cell proliferation and infiltration of the pancreas, but is largely dispensable for tolerance when T cells are primed under physiological conditions. These findings have important clinical implications for exploiting the PD-1 pathway to modulate T cell effector functions.

MATERIALS AND METHODS

Mice

Mice were housed and bred in specific-pathogen free conditions in animal barrier facilities at the University of Minnesota. Female NOD mice were purchased from Taconic (Germantown, NY). NOD BDC2.5 TCR transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and crossed to NOD Thy1.1$^+$ mice. C57BL/6 PD-1-deficient mice (222) were backcrossed thirteen generations to the NOD background and PD-L1-deficient mice (174) were backcrossed fifteen generations to the NOD background. The mice were bred with BDC2.5 mice, and the resulting offspring that were heterozygous for PD-1 or PD-L1 expression were subsequently intercrossed to produce NOD.BDC2.5 PD-1 KO or PD-L1 KO mice. Wild type, PD-1- and PD-L1-deficient BDC2.5 mice were used for donors between 4-6 weeks of age. Pre-diabetic NOD mice (Thy1.2$^+$) were used as recipients for Thy1.1$^+$ BDC2.5 T cells between 7-12 weeks of age. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Detection of islet antigen-specific CD4$^+$ T cells

Naïve $CD4^+$ T cells from NOD.BDC2.5.$Thy1.1^+$ mice were isolated as described in Chapter 2 (216). Briefly, naïve $CD4^+$ T cells were enriched from single cell suspensions from spleen and non-antigen draining LNs (inguinal, axillary, brachial, cervical, periaortic) by depleting cells based on CD8α, MHC class II, CD117, CD24, CD25, B220, CD19, Ter119, Gr-1 and CD44 (eBioscience). Seventy-five hundred naïve BDC2.5 T cells were transferred intravenously into prediabetic NOD mice. Mice were harvested 3-6 weeks post-transfer and analyzed by flow cytometry as described in Chapter 2 (216). Briefly, BDC2.5 T cells from spleen and/or non-draining LNs were enriched using positive selection over magnetic columns (Miltenyi Biotech) by incubating cells with APC conjugated anti-Thy1.1 (eBioscience) followed by anti-APC microbreads. Pancreatic LN and pancreas samples were not subjected to magnetic enrichment due to the low number of total cells in these samples. Leukocytes were enriched from the pancreas using collagenase P digestion (Roche) and discontinuous percoll gradients as described (Pauken et at. 2013).

Insulin-specific $CD4^+$ T cells were detected using the $insB_{10\text{-}23r3}$:I-A^{g7} tetramer reagent described in Chapter 3. Briefly, single cell suspensions were incubated for one hour at room temperature with 10 nM of tetramer conjugated to PE and APC in medium containing Fc receptor block (2.4G2) and azide. Pancreatic LN and pancreas samples were then subjected to surface staining for FACS without subsequent enrichment. Spleen and/or non-draining LN samples were subjected to magnetic enrichment by incubating with both anti-PE and anti-APC microbeads at 4^oC for 30 minutes followed by elution over magnetic columns (Miltenyi Biotech).

Flow cytometry

Surface staining was performed for 30 minutes at 4^{o}C following enrichment protocols described above (magnetic enrichment or percoll). Surface staining was performed post-tetramer staining. Antibodies used from eBioscience included anti-Thy1.1 (HIS51 clone for FACS), CXCR3, PD-1 (clone J43), CD4, CD3, B220, CD11b, CD11c, CD44, CD45RB, IL-2, IFNγ, and TNFα. Antibodies from other companies included CD44 (V500 conjugate) and Ki67 from BD Bioscience and anti-CD8α (BV650 conjugate) from Biolegend. Samples were acquired using BD LSRII and Fortessa instruments (BD) and analyzed using Flow Jo software (Treestar). The gating strategy for BDC2.5 T cells was singlet^{+}, CD3^{+} lineage^{-} (B220^{-}, CD11b^{-}, CD11c^{-}) CD4^{+} Thy1.1^{+}. The gating strategy for insulin-specific CD4^{+} T cells was singlet^{+}, CD3^{+} lineage^{-} (B220^{-}, CD11b^{-}, CD11c^{-}) CD4^{+} CD8α^{-}, ins$B_{10\text{-}23r3}$: I-A^{g7}-PE and -APC tetramer double positive.

Immunofluorescence Microscopy

Pancreata were harvested for immunofluorescence and prepared as described in Chapter 2 (Pauken 2013 Diabetes). Briefly, O.C.T. embedded pancreata were cut at seven μm and fixed using acetone. Antibodies used to stain the tissue sections included guinea pig anti-swine inulin (Dako, Denmark) and donkey anti-guinea pig AF488 (Jackson ImmunoResearch), Thy1.1-PE (clone OX7, Biolegend, CA), and CD3e-APC (clone 145-2C11, eBioscience). Slides were mounted using Prolong Gold with DAPI (Life Technologies). Slides were imaged on a Leica epifluorescent DM5500 microscope

(Germany) and MetaMorph software was used to compile the data (Molecular Devices, CA).

Administration of Anti-PD-L1 antibody

Anti-PD-L1 antibody (clone M1H6) was generated and produced as described (164). Rat immunoglobulin G (IgG) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Mice were injected intraperitoneally with 200-250 μg/mouse anti-PD-L1 or Rat IgG antibody every other day prior to harvest for 2-3 injections or diabetes onset. Glucose levels were assessed by urine readings during the treatment period and blood glucose was measured at the time of harvest (LifeScan, Inc., Milpitas, CA). A reading above 250 mg glucose/dL of blood was considered diabetic.

Proliferation assays

To investigate proliferation at early time points post-transfer (day 7), purified BDC2.5 T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) prior to transfer. Following purification as described above, cells were resuspended in PBS with 5 μM CFSE (Life Technologies) for 5 minutes at room temperature. The labeling was quenched by adding an equal volume of fetal bovine serum and the cells were washed prior to transfer. To investigate proliferation at late time points (day 42), intracellular staining for the proliferative marker Ki67 was used instead of CFSE. Cells were permeabilized by resuspending in Foxp3 fixation/permeablization buffer according to manufacturer's recommendations (eBioscience). FITC-conjugated anti-Ki67 (B56, BD

Biosciences) was added following permeabilization of cells at doses recommended by the manufacturer (20 μl/sample).

Assessment of cytokine production

To compare cytokine production following anti-PD-L1 treatment on the bulk BDC2.5 T cell population, single cell suspensions from the pancreatic LN and pancreas were stimulated in DMEM containing 10% fetal bovine serum (Hy-clone), 2 mM L-glutamax, 100 U/ml penicillin-streptomycin, 0.1 M non-essential amino acids, 10 mM HEPES and 50 μM 2-mercaptoethanol (Life Technologies, Grand Island, NY) with 10 μg/ml brefeldin A, 100 ng/ml phorbol 12-myristate 13-acetate (PMA), and 1000 ng/ml ionomycin (Sigma, MO) for four hours at 37^{o}C and 5% CO_2. The BD cytofix/cytoperm fixation/permeabilization kit was used for intracellular cytokine staining. Cells were fixed overnight at 4^{o}C in the cytofix/cytoperm buffer to enhance detection of IL-2. To assess cytokine production from anergic and effector BDC2.5 T cells, 500 μg of acetylated p31 peptide (YVRPLWVRME) (Genemed Sythesis, San Antonio, TX) was injected intravenously and cells were harvested directly into medium containing brefeldin A 4 hours later. Subsequent enrichment and staining steps were also conducted in medium containing brefeldin A and azide.

Statistics

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, La Jolla, CA). We performed either unpaired two-tailed Student's t tests with a 95%

confidence interval or Mann-Whitney tests. Asterisks indicating statistical significance correspond with: not significant >0.05 (ns), significant 0.01 to 0.05 (*), very significant 0.001 to 0.01 (**), and extremely significant <0.001 (***).

RESULTS

Anti-PD-L1 accelerates Type 1 Diabetes onset and causes an increase in the number of islet-reactive $CD4^+$ T cells in NOD mice

We first determined whether blocking PD-1 signaling impacted the BDC2.5 T cells in the low adoptive transfer model described in Chapter 2. This model was utilized because the majority of the BDC2.5 T cells encountered autoantigen in a narrow time frame, providing a more synchronous population to identify the effect of PD-1 blockade than the insulin-specific population detected with the $insB_{10\text{-}23r3}$: $I\text{-}A^{g7}$ tetramer. Three weeks post-transfer anti-PD-L1 antibody was administered to block the interaction between PD-1 and PD-L1. An isotype antibody was administered as a control. Anti-PD-L1 injection resulted in a substantial increase in diabetes incidence (Figure 4-1 A, 0% diabetic in isotype-treated vs. 63.6% diabetic in anti-PD-L1 treated) consistent with previous work (137). This accelerated disease onset correlated with an increase in BDC2.5 T cell numbers that was statistically significant in the spleen (4.66-fold increase with anti-PD-L1, $p=0.0009$), pancreatic LN (2.46-fold increase with anti-PD-L1, $p<0.0001$), and pancreas (8-fold increase with anti-PD-L1, $p=0.0118$) (Figure 4-1 B). There was not a statistically significant increase in BDC2.5 T cell numbers in the

inguinal LN following PD-1 blockade (p=0.4166) (Figure 4-1 B), which was consistent with PD-1 regulating T cells in an antigen-dependent manner (166). We were surprised to observe an increase in BDC2.5 T cells numbers in the spleen, since the presence of pancreatic antigen in this organ has not been documented to our knowledge. We speculate that the increase in BDC2.5 T cells in the spleen reflected increased trafficking from the pancreatic LNs through blood circulation en route to the pancreas.

Loss of PD-1 signaling accelerates autoimmunity in a variety of mouse strains; however, the nature of the autoimmune disease induced is dependent on genetic background (138, 155, 156). PD-1 KO mice on the NOD genetic background develop accelerated Type 1 Diabetes (138), while PD-1 KO B6 develop a lupus-like nephritis (155). Based on these data, we hypothesized that blocking PD-1 through administration of anti-PD-L1 would not induce diabetes in B6.g7 mice, which do not develop Type 1 Diabetes spontaneously (223). An alternative possibility would be that since NOD MHC class II (H-2^{g7}) is the highest genetic risk factor for developing Type 1 Diabetes, B6.g7 would be susceptible to developing diabetes following PD-1 blockade. We determined that administering anti-PD-L1 dramatically accelerated Type 1 Diabetes onset in NOD mice, consistent with previous reports (137); however; anti-PD-L1 did not induce diabetes in B6.g7 mice (Figure 4-2). This result demonstrated that the presence of the H-2^{g7} allele alone was not sufficient to induce diabetes even when a key inhibitory pathway was blocked.

We next investigated the impact of anti-PD-L1 treatment on insulin-specific $CD4^+$ T cells in 15 week old NOD and B6.g7 mice. Since we observed an increase in the

number of BDC2.5 T cells in the spleen, pancreatic LN and pancreas in our low adoptive transfer model (Figure 4-1 B) (216), we hypothesized that anti-PD-L1 would cause an increase in the number of insulin-specific $CD4^+$ T cells in these organs in NOD mice. In NOD mice, we did not observe a substantial increase in the number of insulin-specific $CD4^+$ T cells on a population level in the secondary lymphoid organs (Figure 4-3 A). There was a trend towards more of these cells in the pancreas following anti-PD-L1 treatment, and substantially more of the pancreata analyzed with anti-PD-L1 contained insulin-specific cells compared to isotype control-antibody treated mice. However, this increase was not statistically significant. One critical difference between the population detected in the BDC2.5 T cell transfer model and with the $insB_{10\text{-}23r3}$: $I\text{-}A^{g7}$ reagent was the frequency of $CD44^{high}$ cells: Nearly 100% of BDC2.5 T cells encountered autoantigen in the pancreatic LN within 28 days post-transfer (Figure 2-2), whereas a large fraction of insulin-specific $CD4^+$ T cells remained $CD44^{low}$ in NOD mice (Figure 3-4). Since PD-1 expression correlated with CD44 expression (Figure 3-7), we predicted that anti-PD-L1 would specifically impact the $CD44^{high}$ population. Indeed, when the number of $CD44^{high}$ cells was compared, anti-PD-L1 treatment resulted in significantly increased numbers of insulin-specific $CD4^+$ T cells in both the spleen ($p=0.0069$) (Figure 4-3 B) and pancreatic LN ($p=0.0216$) (Figure 4-3 C). There was not a statistically significant difference in the number of $CD44^{low}$ insulin-specific $CD4^+$ T cells in the spleen ($p=0.7865$) or pancreatic LN ($p=0.6483$) (Figure 4-3 B and C). This result was expected since the majority of CD44 low cells do not express PD-1 (Figure 3-7 and data not shown). However, since the $CD44^{low}$ population contains a mixture of naïve cells ($CD45RB^{high}$) and potentially

chronically stimulated cells ($CD45RB^{dim}$), we speculate that PD-1 blockade was only capable of reversing a subset of antigen-experienced cells. Which insulin-specific T cell populations are susceptible to PD-1 blockade enhancing functions is not addressed in this study, but would be interesting to pursue considering the heterogeneity in this population.

Lastly, we determined how PD-1 blockade impacted insulin-specific $CD4^+$ T cells in B6.g7 mice. We hypothesized that anti-PD-L1 would have less of an effect on insulin-specific $CD4^+$ T cells in B6.g7 mice than NOD mice because the frequency of these cells that expressed PD-1 was lower in B6.g7 mice compared to NOD mice (Figure 3-7), and therefore a smaller fraction of the population was likely susceptible to PD-1 blockade. Additionally, anti-PD-L1 accelerated diabetes in NOD but not B6.g7 (Figure 4-2). When the insulin-specific $CD4^+$ T cell population was analyzed in the secondary lymphoid organs, there was not a statistically significant increase in the number of these cells following anti-PD-L1 treatment (Figure 4-4 A). Most importantly, there was no infiltration of the pancreas with anti-PD-L1 (Figure 4-4 A). Unexpectedly, there was a significant increase in the number of cells in the pancreatic LN in the $CD44^{high}$ population of insulin-specific $CD4^+$ T cells (Figure 4-C). As described earlier, a higher frequency of insulin-specific cells expressed PD-1 in the pancreatic LN than CD44 in B6.g7 mice (Figure 3-7). Therefore, expression of PD-1 in this organ could render these cells susceptible to PD-1 blockade. Nonetheless, PD-1 blockade does not result in an increase in these cells in the $CD44^{high}$ population in the spleen or increase infiltration of the pancreas. Therefore, the significance of this increase was likely minor in terms of Type 1 Diabetes pathogenesis.

Anti-PD-L1 causes sustained proliferation early during the priming phase of the response and causes cells to re-enter cell cycle late during the persistent phase of the response

We next focused on mechanisms by which PD-1 regulated islet-reactive $CD4^+$ T cells to slow Type 1 Diabetes progression. Due to the advantage of the BDC2.5 transfer model providing a synchronous population of cells in terms of antigen encounter and activation, the remainder of these studies were performed using this model instead of tracking insulin-specific $CD4^+$ T cells using tetramer. Previous work had identified a role for PD-1 in regulating T cell proliferation, survival, and cytokine production (169, 174, 184, 185, 224). We first focused on cell proliferation as a potential mechanism regulating the BDC2.5 T cell population considering the profound cellular increase following PD-1 blockade. We hypothesized that the cell number increase was due to increased proliferation rather than enhanced survival due to the stability of the BDC2.5 T cell population observed in the clonal expansion curve (Figure 2-3). To test this hypothesis we transferred naïve CFSE-labeled BDC2.5 T cells, treated with anti-PD-L1 at days 4 and 6 post-transfer and measured proliferation at day 7. Anti-PD-L1 treatment resulted in a statistically significant increase in the frequency of BDC2.5 T cells that had fully diluted CFSE in the spleen (47.83±4.41% with isotype vs. 61.18±2.62% with anti-PD-L1, $p=0.0405$) and pancreatic LN (24.00±4.86% with isotype vs. 38.68±2.60% with anti-PD-L1, $p=0.037$) at day 7 post-transfer (Figure 4-5 A and B). There was not a statistically significant difference in the frequency of CFSE-diluted BDC2.5 T cells in the inguinal

LN or the pancreas between isotype- and anti-PD-L1-treated mice (Figure 4-5 A and B). In the inguinal LN the majority of the BDC2.5 T cells in both conditions were undiluted (6.28±2.36% $CFSE^-$ with isotype vs. 17.21±4.64% $CFSE^-$ with anti-PD-L1, p=0.081), which was consistent with most cells being naïve at this early time point. Conversely, the majority of the BDC2.5 T cells in the pancreas at day 7 had completely diluted the CFSE dye with or without anti-PD-L1 treatment (84.25±2.45% with isotype vs. 87.67±6.51% with anti-PD-L1, p=0.7177). This result was consistent with the notion that rapidly proliferating BDC2.5 T cells were the predominant population entering the pancreas at early time points following activation.

We next tested whether PD-1 regulated cell cycle entry late post-transfer at a time point when the BDC2.5 T cell population had reached a plateau in the clonal expansion curve. We transferred naïve BDC2.5 T cells, waited 6 weeks, and treated with anti-PD-L1 or isotype control antibody and measured expression of Ki67, a molecule associated with cell cycle entry. The majority of the BDC2.5 T cells from isotype control antibody-treated animals were quiescent ($Ki67^{neg}$) from the inguinal LN (90.67±5.21%), spleen (68.57±5.01%), pancreatic LN (75.13±4.77%), and pancreas (70.17±5.66%) 6 weeks post-transfer (Figure 4-6 A and B). However, PD-1 blockade resulted in a substantial and significant increase in the frequency of the BDC2.5 population that had recently entered cell cycle ($Ki67^+$) in the spleen (31.43±5.01% with isotype vs. 70.83±4.61% with anti-PD-L1, p=0.0044), pancreatic LN (24.87±4.77% with isotype vs. 57.60±9.04% with anti-PD-L1, p=0.0328), and pancreas (29.83±5.66% with isotype vs. 61.37±6.84% with anti-PD-L1, p=0.0238) (Figure 4-6 A and B). PD-1 blockade did not result in a significant

increase in $Ki67^+$ BDC2.5 T cells in the inguinal LN as expected since this organ lacks pancreatic antigen (9.33±5.21% with isotype vs. 23.86±13.51% with anti-PD-L1, p=0.3724) (Figure 4-6 A and B). Collectively these data suggest that PD-1 regulates $CD4^+$ T cell proliferation both early during the initial priming and expansion phase (Figure 4-5) as well as late during the persistent phase of the response (Figure 4-6).

PD-1 blockade causes increased expression of the chemokine receptor CXCR3

Our results indicated that PD-1 blockade resulted in a substantial increase in BDC2.5 T cells in the pancreas (Figure 4-1 B). Therefore, we next determined whether PD-1 regulated expression of chemokine receptors associated with T cell trafficking to the pancreas. We hypothesized that PD-1 blockade would result in increased expression of CXCR3, a chemokine receptor known to promote effector Th1 cell trafficking to pancreatic islets during Type 1 Diabetes progression (219). CXCL10, a ligand for CXCR3, is highly expressed within pancreatic islets and is involved in the recruitment of pathogenic CXCR3-expressing T cells to the pancreas during disease progression (219, 220). Additionally, recent work showed that CXCR3 expression is important for optimizing Th1 cell differentiation in antigen-containing LNs due to the ability to promote homing of cells to locations in the LN with optimal antigen presentation (221). We examined CXCR3 expression on BDC2.5 T cells three weeks post-transfer because we consistently observed BDC2.5 T cell accumulation in the pancreas at this time point. As predicted, PD-1 blockade resulted in the up-regulation of CXCR3 (based on MFI) on BDC2.5 T cells in the spleen and pancreatic LN that was highly significant (2.49-fold

increase with anti-PD-L1 in the spleen, $p<0.0001$ and 3.15-fold increase with anti-PD-L1 in the pancreatic LN, $p<0.0001$). There was also an increase in CXCR3 in the inguinal LN (3.66-fold increase with anti-PD-L1, $p=0.0288$), but the biological significance of this increase was unclear (Figure 4-7 A and B). The levels of CXCR3 were low in the pancreas in both isotype- and anti-PD-L1-treated animals, and we speculate this was due to receptor down-regulation following exposure to CXCR3 ligands (Figure 4-7 A and B). PD-1 blockade also resulted in a significant increase in the frequency of CXCR3-expressing BDC2.5 T cells in the inguinal LN (7.92±4.3% with isotype vs. 33.44±9.13% with anti-PD-L1, $p=0.0249$), spleen (40.25±2.73% with isotype vs. 66.93±4.42% with anti-PD-L1, $p<0.0001$), and pancreatic LN (18.75±3.72% with isotype vs. 48.86±5.46% with anti-PD-L1, $p=0.0002$) (Figure 4-7 C). This up-regulation of CXCR3 may account for enhanced Th1 differentiation in the pancreatic LN and increased trafficking of islet-reactive $CD4^+$ T cells to the pancreas during Type 1 Diabetes progression.

Anti-PD-L1 does not increase the per cell capacity to produce effector cytokines, but causes an increase in the number of cytokine-producing cells in the pancreatic LN and pancreas

We previously determined that the BDC2.5 T cells were programmed to become IFNγ- and IL-2-producing Th1 effector cells following exposure to autoantigen (Figure 2-6). We hypothesized that anti-PD-L1 treatment would enhance the capacity for BDC2.5 T cells to produce effector cytokines to accelerate diabetes progression. To test this, we examined the effect of PD-1 blockade on the effector cytokine potential of BDC2.5 T

cells in the pancreatic LN and pancreas. Mice were treated mice with anti-PD-L1 or an isotype control antibody approximately one month post-transfer of naïve BDC2.5 T cells and IFNγ, TNFα, and IL-2 were measured following ex vivo stimulation with PMA and ionomycin. Figure 4-8 panels B and C and Table 4-1 show the frequencies of BDC2.5 T cells capable of producing one, two, or three effector cytokines. Contrary to our prediction, PD-1 blockade did not significantly alter the frequency of the BDC2.5 T cell population programmed to produce IFNγ, TNFα, and IL-2 (Figure 4-8 A-C and Table 4-1). However, PD-1 blockade resulted in a significant increase in the number of single, double, and triple cytokine-producing BDC2.5 T cells in both the pancreatic LN (Figure 4-8 D) (with anti-PD-L1 single$^+$ 3.9-fold increase, p=0.0008; double$^+$ 4.1-fold increase, p=0.0013; and triple$^+$ 3.7-fold increase, p=0.0011) and pancreas (Figure 4-8 E) (with anti-PD-L1 single$^+$ 13-fold increase, p=0.0004; double$^+$ 8.3-fold increase, p=0.0004; triple$^+$ 5.9-fold increase, p=0.004). These data suggest that PD-1 blockade does not alter the capacity for BDC2.5 T cells to produce effector cytokines on a per cell basis, but has a significant effect on the quantity of cytokine-producing effector BDC2.5 T cells due to increased proliferation.

Neutralizing PD-L1 results in more destructive insulitis and causes BDC2.5 T cells to re-localize from the more benign portion of the islet infiltrate into the islet core

Our data demonstrated the importance of PD-1/PD-L1-mediated regulation of islet-reactive CD4$^+$ T cells temporally, both early during priming and late when the cells were mostly quiescent as well as spatially, resulting in enhanced migration from the

secondary lymphoid organs to the pancreas. We next determined whether PD-1 was regulating the spatial organization of BDC2.5 cells within the pancreatic infiltrate. At three weeks post-transfer, the majority of pancreas infiltrate was peri-insulitis (Figure 4-9 A and C). However, anti-PD-L1 treatment caused a substantial conversion from peri-insulitis to destructive insulitis (Figure 4-9 B and C), consistent with previous work (137). Importantly, BDC2.5 cells were present within the islet core following PD-1 blockade (Figure 4-9 B). These data suggest that PD-1 is actively regulating BDC2.5 T cells within the pancreas itself, and that blocking PD-1 has a direct effect on cells within the tissue rather than simply enhancing migration from the secondary lymphoid organs. These data extend our understanding of the spatial regulation PD-1 implements on islet-reactive $CD4^+$ T cells.

PD-1 expressed by the BDC2.5 T cell is required to regulate the early expansion in the secondary lymphoid organs and infiltration of the pancreas, while PD-L1 expressed by the BDC2.5 T cell is dispensable

Our results demonstrated that PD-1/PD-L1 blockade enhanced islet-reactive $CD4^+$ T cell proliferation and infiltration of the pancreas. Previous work had demonstrated roles for both PD-1 and PD-L1 expressed by effector $CD8^+$ T cells in regulating function (184, 225); however, the specific requirement for PD-1 or PD-L1 on antigen-specific $CD4^+$ T cells during Type 1 Diabetes has not been studied. Therefore, we generated BDC2.5 mice deficient for PD-1 or PD-L1. We first confirmed that PD-1 was absent from the cell surface of BDC2.5 T cells from NOD.BDC2.5.PD-$1^{-/-}$ mice, and PD-L1 was absent from

the cell surface of BDC2.5 T cells from NOD.BDC2.5.PD-L1$^{-/-}$ mice (Figure 4-10 A). NOD.BDC2.5.PD-1$^{-/-}$ and NOD.BDC2.5.PD-L1$^{-/-}$ mice developed normally (data not shown), but both strains displayed accelerated Type 1 Diabetes compared to WT BDC2.5 mice (Figure 4-10 B). However, disease onset was delayed when compared to reports of non-T cell receptor transgenic NOD.PD-1$^{-/-}$ and NOD.PD-L1$^{-/-}$ mice (138, 174). Importantly, all donor mice used for these experiments were euglycemic at the time of BDC2.5 cell transfer.

We next tested the role of PD-1 and PD-L1 on BDC2.5 cells in the low cell transfer model in NOD recipients. This approach allowed us to determine the requirement for PD-1 and PD-L1 on the T cell in a wild type environment. We transferred 7500 naïve T cells from either WT or KO BDC2.5 mice into pre-diabetic NOD mice. The park rates between WT and KO BDC2.5 cells were comparable (data not shown). At day 7 post-transfer we observed a statistically significant increase in PD-1-deficient BDC2.5 cells in the spleen, pancreatic LN, and pancreas compared to PD-1-sufficient controls (Figure 4-11 A). There was not a significant increase in PD-1-deficient BDC2.5 cells in the inguinal LN. Conversely, there was no difference in the number of PD-L1-deficient BDC2.5 cells compared to PD-L1-sufficient controls in any organ (Figure 4-11 B). These data suggested that PD-1, not PD-L1, expressed by the BDC2.5 cell was required to suppress proliferation and infiltration of the pancreas.

Anti-PD-L1 treatment caused an increase in CXCR3 expression and altered the organization of BDC2.5 T cells in the pancreatic infiltrate. We hypothesized that PD-1 on the BDC2.5 T cell was mediating these effects rather than a bystander effect of anti-PD-

L1 altering the inflammatory environment. To begin to test this hypothesis, we next examined CXCR3 expression on PD-1 KO BDC2.5 cells after low transfer. We found significantly higher levels of CXCR3 in the spleen and pancreatic LN compared to WT (Figure 4-12 A and B). We observed slightly elevated levels of CXCR3 in the inguinal LN (Figure 4-12 A and B) similarly to what was observed with neutralizing antibody (Figure 4-7 A and B), but the significance of this expression was unclear. We observed the same trend in terms of frequency of $CXCR3^+$ cells, with elevated frequencies in the inguinal LN, spleen, and pancreatic LN (Figure 4-12 C). Importantly, CXCR3 was not elevated in the intact PD-1 KO BDC2.5 mouse compared to WT BDC2.5 mice (data not shown). Additionally, since naïve cells were transferred, all BDC2.5 cells were $CXCR3^-$ at the time of transfer.

Lastly, we determined how the loss of PD-1 selectively on the BDC2.5 cells impacted localization within the pancreas. Interestingly, PD-1 KO BDC2.5 cells could be detected both around and deep within the islet core (Figure 4-13 A). Mice that received PD-1 KO BDC2.5 cells also displayed more severe insulitis compared to mice that received WT BDC2.5 (Figure 4-13 B). This result suggested that loss of PD-1 on the BDC2.5 cells promoted the ability to penetrate into the core of the islet. Collectively these data support a model where PD-1 expressed by islet-reactive $CD4^+$ T cells regulates proliferation, CXCR3 expression, and pancreas infiltration.

PD-1 is required to suppress functions of effector phenotype BDC2.5 T cells, but is not required for the induction or maintenance of anergy induced following exposure to endogenous autoantigen

Our data support a model where PD-1 expressed by the islet-reactive $CD4^+$ T cell regulates proliferation and infiltration of the pancreas. However, these studies focused on a population level. We described two unique subsets of islet-reactive $CD4^+$ T cells in Chapter 2 (BDC2.5) and 3 ($insB_{10\text{-}23r3}$-specific): anergic cells defined as $FR4^+$ $CD73^+$ $CD44^{high}$ $Foxp3^-$, and effector cells defined as $FR4^-$ $CD73^-$ $CD44^{high}$ $Foxp3^-$. We were interested in determining whether anti-PD-L1 treatment differentially impacted these distinct subsets of islet-reactive $CD4^+$ T cells. Since previous work identified a critical role for PD-1 signaling in both inducing and maintaining T cell tolerance (59, 110), we hypothesized that loss of PD-1 would restore functionality to the anergic BDC2.5 T cell population. To test this hypothesis, we treated mice with anti-PD-L1 antibody three weeks post-transfer of 7500 naïve BDC2.5 T cells and quantified FR4 and CD73 expression. Loss of PD-1 signaling resulted in a reduction in the frequency of anergic phenotype BDC2.5 T cells in the pancreatic LN (Figure 4-14 A and B) as expected based on our previous findings (59, 110). Interestingly, loss of FR4 and CD73 only occurred in the pancreatic LN and not inguinal LN, suggesting that antigen is involved in the maintenance of tolerance (Figure 4-14 B). Unexpectedly, when the absolute cell number was quantified, it became evident that the decrease in the frequency of anergic BDC2.5 T cells in the pancreatic LN was not due to a loss of the anergic population, but rather a substantial and significant ($p=0.0053$) expansion of the effector BDC2.5 population

(Figure 4-14 C). This result suggested that PD-1 blockade did not cause anergy reversal, but rather had selectively targeted the effector population that arose due to other defects in peripheral tolerance in NOD mice.

The conclusion thus far that anti-PD-L1 did not reverse the anergic state was based on co-expression of FR4 and CD73. However, it was possible that during the antibody treatment period, FR4 and CD73 may not have been down-modulated from the cell surface; therefore, these cells may have acquired effector functions without loss of the anergy markers. For this reason we analyzed cytokine production by anergic and effector phenotype BDC2.5 T cells following anti-PD-L1 treatment. Anti-PD-L1 was capable of restoring some cytokine production by anergic phenotype BDC2.5 T cells that was statistically greater than isotype control-antibody treated animals (isotype vs anti-PD-L1: geometric MFI of IFNγ 118.8±2.46 versus 202.6±13.88, p=0.0012 and frequency of IFNγ-producing cells 16.83±1.40% versus 27.23±2.77%, p=0.0177) (Figure 4-15 A and B). However, the cytokine production from anergic cells remained blunted compared to the effector population in the isotype-control treated animals (anergic+aPD-L1 geometric MFI=202.6±13.88 and frequency=27.23±2.77% versus effector+isotype geometric MFI=460±48.73 and frequency=46.48±4.58%), and incredibly blunted compared to the effector population in the anti-PD-L1 treated animals (effector+aPD-L1 geometric MFI=729.8±33.24 and frequency=59.84±2.74%) (Figure 4-15 A and B). These data suggest while PD-1 blockade had a small effect on anergic BDC2.5 T cells, it was not capable of fully restoring function and had a much more substantial effect on enhancing functions of effector phenotype $CD4^+$ T cells.

Our previous work had identified a role for PD-1 in both the induction and maintenance of $CD4^+$ T cell anergy (59, 110). However, considering that in the low transfer model PD-1 was not required for the maintenance of tolerance (Figure 4-14 and 4-15), we re-evaluated the role for PD-1 during the induction phase of tolerance. We transferred 7500 naïve WT or PD-1 KO BDC2.5 T cells into pre-diabetic NOD mice and quantified FR4 and CD73 expression three weeks post-transfer. We found that PD-1 was not required for the induction of the anergic phenotype since the frequency of $FR4^+$ $CD73^+$ BDC2.5 T cells was not significantly different between WT and PD-1 KO in the pancreatic LN (anergic frequency for WT=74.45±4.29% and PD-1 KO=67.52±2.65%) (Figure 4-16 A and B). PD-1 KO BDC2.5 T cells also did not accelerate Type 1 Diabetes in this low transfer system (data not shown), suggesting that the host was capable of controlling this population when provided at a physiologically relevant precursor frequency. These data support a model where PD-1 is important for regulating islet-reactive $CD4^+$ T cells during Type 1 Diabetes by suppressing effector cells, not anergic cells. Rather, loss of tolerance and progression to clinical disease likely correlates with loss of PD-1 signaling by the pathogenic effector population, not the reversal of the anergic population (Figure 4-17). The difference between this finding and our previous findings (59, 110) highlights the importance of studying islet-reactive $CD4^+$ T cells under conditions that accurately recapitulate the natural course of Type 1 Diabetes in NOD mice.

DISCUSSION

The critical link between the PD-1/PD-L1 signaling pathway and Type 1 Diabetes is well established (137, 138, 174). However, the mechanism of how this pathway regulates autoreactive $CD4^+$ T cells during diabetes is not fully understood. In this thesis we developed model systems to investigate the effect of PD-1/PD-L1 interactions on antigen-specific $CD4^+$ T cells. Using these models, we determined that PD-1 predominantly regulated proliferation; however PD-1 blockade also resulted in enhanced infiltration of the pancreas, which may be due to increased CXCR3 expression following PD-1/PD-L1 blockade (Figure 4-17). PD-1 expressed by islet-reactive $CD4^+$ T cells was required to suppress effector functions in a cell-intrinsic manner, while PD-L1 expressed by the effector $CD4^+$ T cell was dispensable. Contrary to previous findings, the enhancement of effector functions associated with PD-1 blockade was primarily due to the impact on effector phenotype cells rather than reversing T cell anergy. These data suggest that under normal conditions PD-1 acts to limit Type 1 Diabetes progression by regulating effector islet-reactive $CD4^+$ T cell proliferation and infiltration of the pancreas, not by controlling anergic cells (Figure 4-17).

The role for PD-1/PD-L1 interactions in regulating T cells has been investigated previously in vitro and in vivo (59, 166, 169, 174-177, 184, 224). Current knowledge of how PD-1 regulates $CD4^+$ T cells during Type 1 Diabetes in vivo is largely limited to the secondary lymphoid organs, while less is known about the pancreas. Work from Guleria and colleagues investigated mechanisms of PD-1-mediated regulation of antigen-specific $CD4^+$ T cells in NOD mice (182), including BDC2.5 cells. However, a high number of BDC2.5 cells were transferred into recipient mice and a large fraction of these cells failed

to proliferate in the pancreatic LN even following anti-PD-L1 treatment. In our low transfer model, the majority of the BDC2.5 cells in the pancreatic LN diluted CFSE following anti-PD-L1 administration, highlighting an important difference between low and high T cell transfer numbers during diabetes pathogenesis. We extended the results from the previous study and demonstrated that PD-1 blockade caused quiescent cells to re-enter cycle during a later and chronic stage of disease. During this time, $Ki67^+$ BDC2.5 cells increased in the pancreas; however, it is unclear whether this increase correlated with trafficking or proliferation within the pancreas. It is interesting to speculate that islet-reactive $CD4^+$ T cells could enter cell cycle in the pancreas since T cells persist in this organ in NOD mice for weeks to months prior to diabetes onset. Additionally, we showed that loss of PD-1 signaling correlated with increased expression of CXCR3. Not only is CXCR3 involved in diabetogenic T cell homing to the pancreas, but also optimizes Th1 differentiation in antigen-containing LNs (221). These data support a model where PD-1 expressed by the islet-reactive $CD4^+$ T cell suppresses proliferation and limits CXCR3 expression, thereby inhibiting optimal Th1 differentiation and infiltration of the pancreas (Figure 4-17).

Utilizing a genetic approach, we determined that the effects measured with anti-PD-L1 were due to a direct effect of PD-1 on $CD4^+$ T cells, not PD-L1 on the T cell or indirect effects such as alternative ligand binding (e.g. PD-1/PD-L2 or B7-1/PD-L1). While the PD-1-KO result underscores the importance of PD-1 on the $CD4^+$ T cell in regulating diabetes, there are important differences between anti-PD-L1 treatment and the selective loss of PD-1 on BDC2.5 cells. Anti-PD-L1 induced rapid onset of diabetes

consistent with reports in intact NOD mice (137). Paradoxically, PD-1 KO BDC2.5 cells did not accelerate Type 1 Diabetes in NOD mice (data not shown) despite the overwhelming number of cells in the pancreas and direct invasion of the islet early post-transfer. The lack of diabetes observed with PD-1 KO BDC2.5 cell transfers differs from previous work using PD-1 KO OT-I T cell transfers into RIP-mOVA mice (184). We speculate that this discordance reflects differences in $CD4^+$ and $CD8^+$ T cells during Type 1 Diabetes. Since $CD8^+$ T cells are responsible for direct killing of beta cells, it stands to reason that PD-1 KO diabetogenic $CD8^+$ T cells could have a more substantial pathogenic effect on disease than PD-1 KO diabetogenic $CD4^+$ T cells. Our histological data suggest that anti-PD-L1 is impacting other cells within islets in addition to BDC2.5 cells (Figure 4-9). Additional work is needed to define the role of anti-PD-L1 on the dynamic interplay between diabetogenic $CD4^+$ and $CD8^+$ T cells, dendritic cells and Treg cells in the pancreas.

Our initial studies focused on the role of PD-1 in regulating islet-reactive $CD4^+$ T cells on a population level. While the data presented herein showed a general increase in proliferation (Figure 4-5 and 4-6) and CXCR3 expression (Figure 4-7), there was always a population of cells in the anti-PD-L1 treated mice that did not follow that trend but rather appeared unaffected by PD-1 blockade. This was particularly evident at late time points (Day 42) when cell cycle entry is assessed (Figure 4-6). Previous work in chronic LCMV infection had demonstrated that while PD-1 blockade could restore functionality to virus-specific $CD8^+$ T cells, there was a subset of cells that were resistant to the effects of anti-PD-L1 (180). It was speculated that this subset was the most functionally

exhausted or terminally differentiated (180, 226). Since we observed a subset of cells that did not appear to be responding to anti-PD-L1 treatment, we were interested in determining whether PD-1 blockade differentially impacted the two main subsets of BDC2.5 T cells we had identified in Chapter 2 (anergic versus effector). The most surprising and controversial finding of this work was that loss of PD-1 signaling had a more substantial impact on enhancing effector cell functions than reversing T cell anergy (Figure 4-17). Work from our own lab had suggested that PD-1 was important for both the induction and maintenance of T cell anergy when antigen-coupled ECDI fixed cells were used to induce tolerance. However, in this model, a high number (approximately $5x10^6$) of BDC2.5 T cells that have been activated in vitro using the high affinity mimetope p31 were used to induce diabetes in NOD mice. Since these cells were activated in vitro, they expressed high levels of PD-1 upon entering the mouse, and therefore were more likely to require PD-1 to induce tolerance than naïve cells becoming anergic following endogenous autoantigen encounter. Additionally, work from Keir and colleagues showed using OT-1 transfers into mice expressing incredibly low levels of OVA from the rat insulin promoter (RIP-mOVA^{low} mice) that these cells did not proliferate, and PD-1-deficiency was not sufficient to induce proliferation in this context (184). If sufficient antigen was provided (by activating the cells in vitro), PD-1 KO OT-1 could induce diabetes in RIP-mOVA^{low} mice, showing that these cells were not intrinsically impaired. Rather, these data suggest that loss of PD-1 was not capable of enhancing function when antigen presentation was suboptimal, which was also likely occurring in the low transfer BDC2.5 system. Additionally, the inflammatory contexts

were completely different between the high transfer of in vitro activated BDC2.5 and the low transfer of naïve BDC2.5. In the high transfer setting, the BDC2.5 T cells were sufficient to induce diabetes and destroy the pancreas within one week post-transfer (59). On the contrary, in the low transfer setting, the BDC2.5 T cells were not sufficient to induce diabetes (data not shown) and infiltration of the pancreas occurred gradually (Figure 2-4). Therefore, it is reasonable to predict that tolerance induction in the low transfer setting would be less dependent on PD-1 than the high transfer setting. Additionally, anergy induction by antigen-coupled ECDI-fixed cells is atypical compared to how classical anergy is induced in other models, particularly in vivo (134). Traditionally anergy induction is associated with naïve T cells becoming primed in the context of low levels or low affinity antigen in the absence of costimulatory molecules or other inflammatory molecules needed for optimal T cell activation (134). In the high transfer model with p31-coupled cells, the BDC2.5 T cells were characterized as anergic based on their impaired proliferative capacity and cytokine production following antigen challenge. However, these cells differ from classical anergy in the sense that they are encountering antigen for a second time after being optimally activated by a high affinity ligand in vitro (59) (Figure 4-18). Additional studies investigating the differences in the requirements for the induction and maintenance of tolerance between these two forms of anergic cells will be important to determine the efficacy of using antigen-coupled cells to induce tolerance clinically. Antigen-fixed ECDI-coupled cells do not induce FR4 and CD73 expression (data not shown), so these markers clearly are not requisite for defining anergy and do not demarcate all anergic cells.

The idea that PD-1 alone is not sufficient to induce or maintain tolerance when autoreactive T cells encounter autoantigen under the natural inflammatory conditions present during spontaneous diabetes development provides hope for patients receiving anti-PD-1 therapy to enhance immunity during chronic viral infections or malignancy, suggesting that unless an activated effector population of autoreactive T cells is pre-existing in the patient, the likelihood of developing autoimmunity is minimal. The risk of developing autoimmunity following PD-1 blockade due to either the breakdown of peripheral tolerance or the amplification of a pre-existing autoimmune response that had already broken tolerance by a different mechanism remains a critical question in the field. There are currently over thirty clinical trials blocking PD-1 in humans to enhance immunity (clinicaltrials.gov), and the data generated from these trials will help to inform the answer that the aforementioned question. By understanding how the PD-1 pathway functions during normal Type 1 Diabetes development we will strengthen our ability to target this pathway in humans.

FOOTNOTES

[1]Portions of this work have been previously published. Reprinted from *Diabetes*. Volume 62. Kristen E. Pauken, Marc K. Jenkins, Miyuki Azuma, and Brian T. Fife. **PD-1, but not PD-L1, expressed by islet-reactive CD4$^+$ T cells suppresses infiltration of the pancreas during Type 1 Diabetes**. p. 2859-2869. Copyright 2013, with permission from the American Diabetes Association (license number 3196070044473).

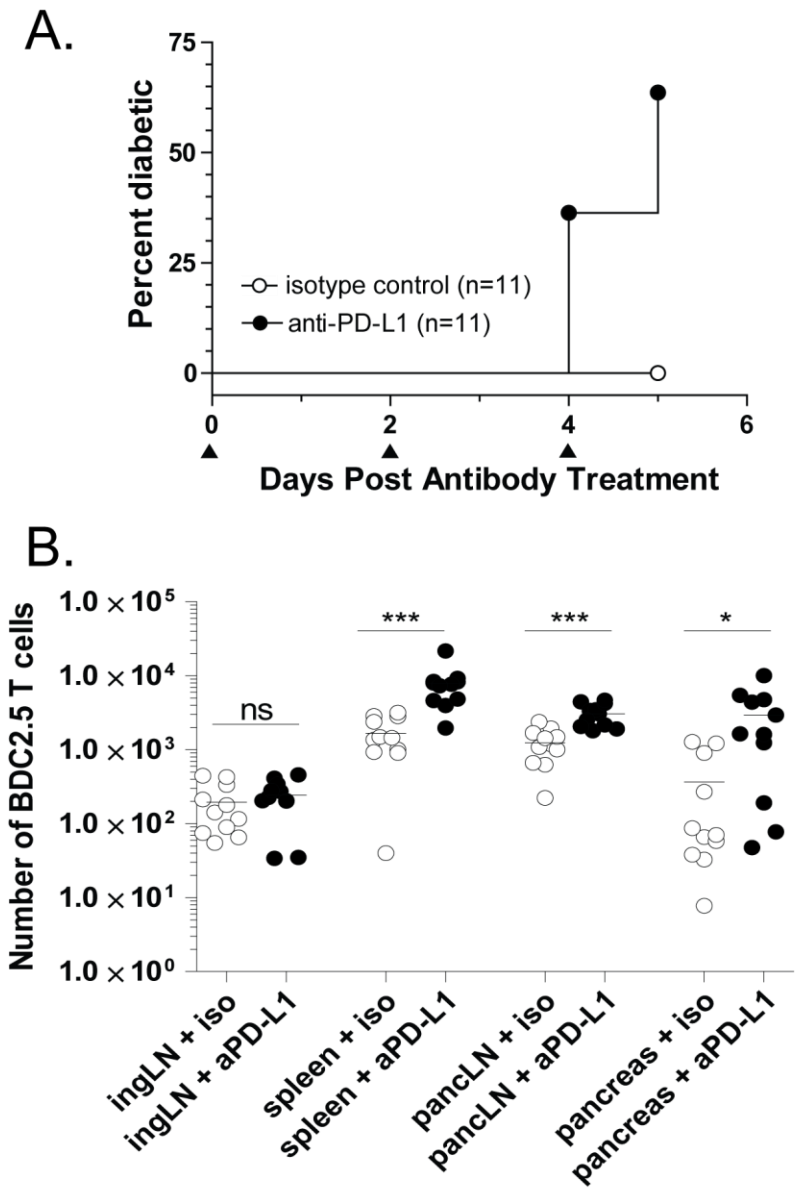

Figure 4-1: PD-1 blockade causes an increase in the number of BDC2.5 T cells in the spleen, pancreatic LN, and pancreas of NOD mice.

(A) Diabetes incidence three weeks post-transfer following anti-PD-L1 or isotype control treatment. Mice were harvested for FACS analysis five days after anti-PD-L1 treatment and 64% (n=11) were diabetic at this time compared to 0% (n=11) in the isotype control group. (B) Absolute number of BDC2.5 Thy1.1^{+} T cells three weeks post-transfer from the mice shown in panel A. Data from spleen, pancreatic LN, and pancreas are representative of 7 experiments, 4 of which contained inguinal LNs, with at least 3 mice per treatment condition per experiment. Data shown are combined from 3 experiments.

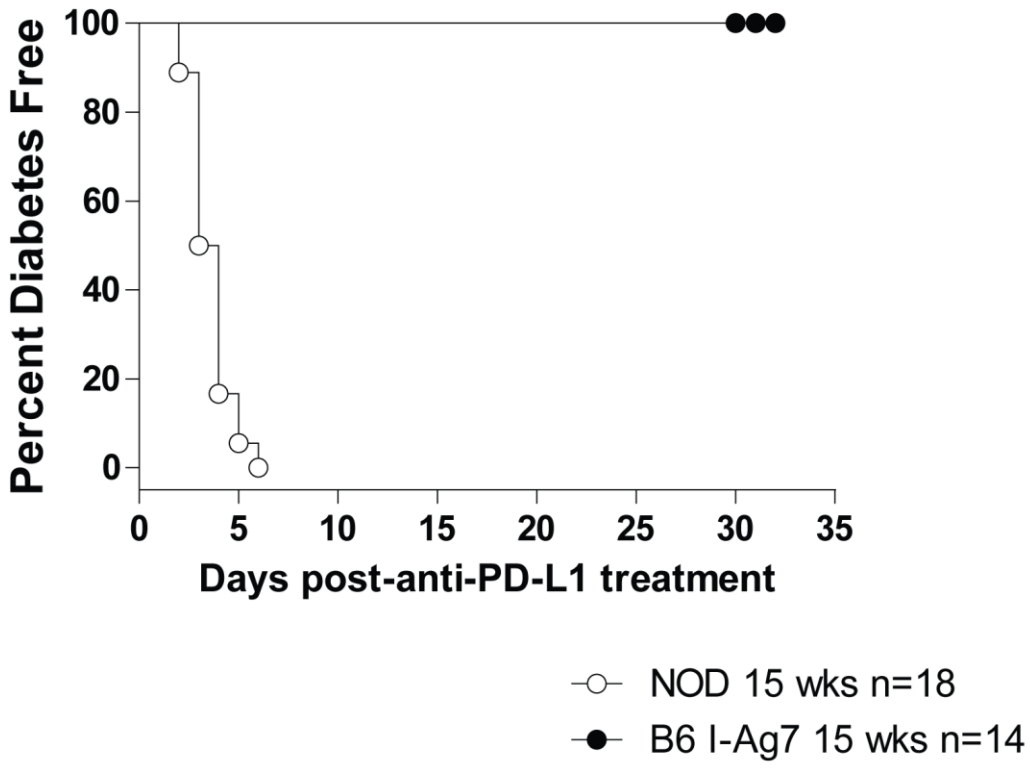

Figure 4-2: Anti-PD-L1 treatment accelerates Type 1 Diabetes onset in NOD mice but not B6.g7 mice.

Diabetes incidence of 15 week old NOD or B6.g7 mice treated with 250 μg anti-PD-L1 every other day for five injections (1.25 mg antibody total). Data show eighteen anti-PD-L1-treated NOD mice and fourteen anti-PD-L1-treated B6.g7 mice compiled from four independent experiments.

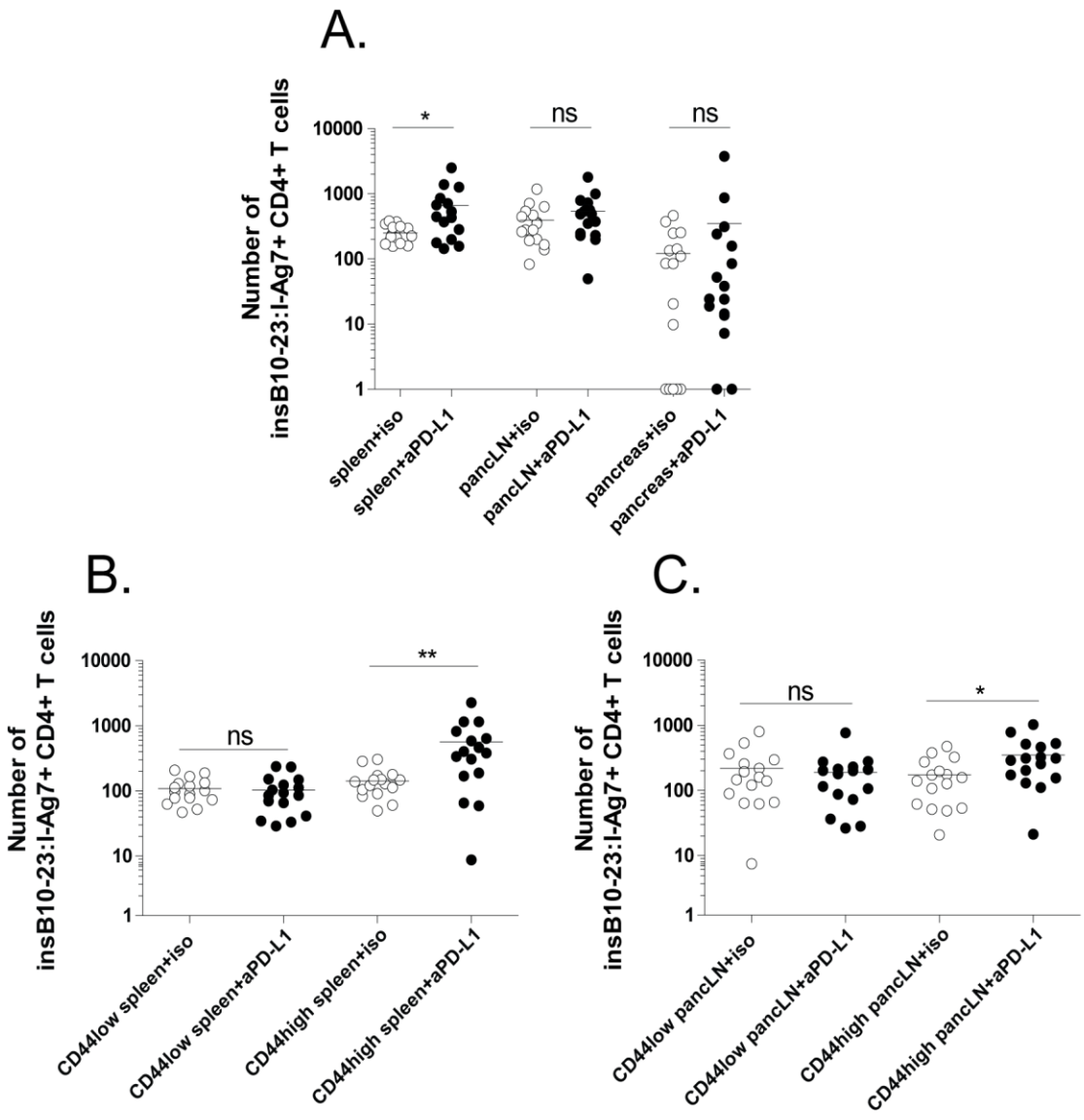

Figure 4-3: Anti-PD-L1 causes an increase in the number of CD44 high insulin-specific CD4$^+$ T cells in NOD mice.

(A) Absolute number of ins$B_{10\text{-}23r3}$-specific CD4$^+$ T cells in spleen, pancreatic LN, and pancreas of 15 week old NOD mice treated with 250 μg of anti-PD-L1 antibody at days -3 and -1 prior to harvest. CD44 low and CD44 high cell numbers from the (B) spleen and (C) pancreatic LN from the mice displayed in panel A following isotype-control or anti-PD-L1 antibody treatment. Data represent 16 mice from each treatment group compiled from 4 experiments.

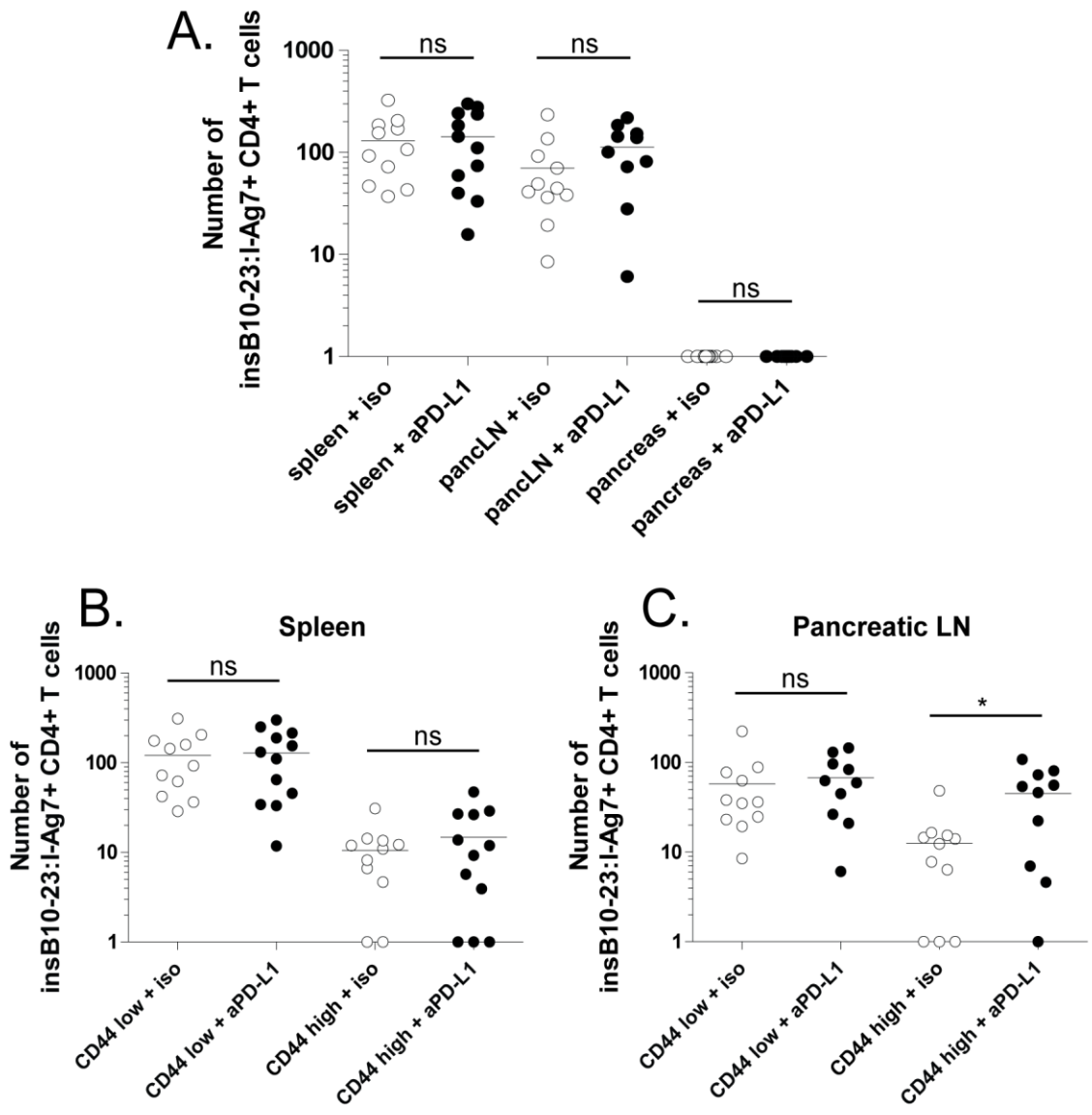

Figure 4-4: Blocking PD-1 does not cause an increase in the number of insulin-specific CD4$^+$ T cells in the spleen or pancreas of B6.g7 mice.

(A) Number of ins$B_{10\text{-}23r3}$-specific CD4$^+$ T cells in spleen, pancreatic LN, and pancreas of 11-16 week old B6.g7 mice treated with isotype control or anti-PD-L1 antibody. CD44 low and CD44 high cell numbers from the (B) spleen and (C) pancreatic LN from the mice in panel A. Data show 11 mice treated with an isotype-control antibody and 12 mice treated with anti-PD-L1 compiled from 3 experiments. One experiment received 250 μg antibody at days -3 and -1 and the other two received 250 μg antibody at -5, -3, and -1.

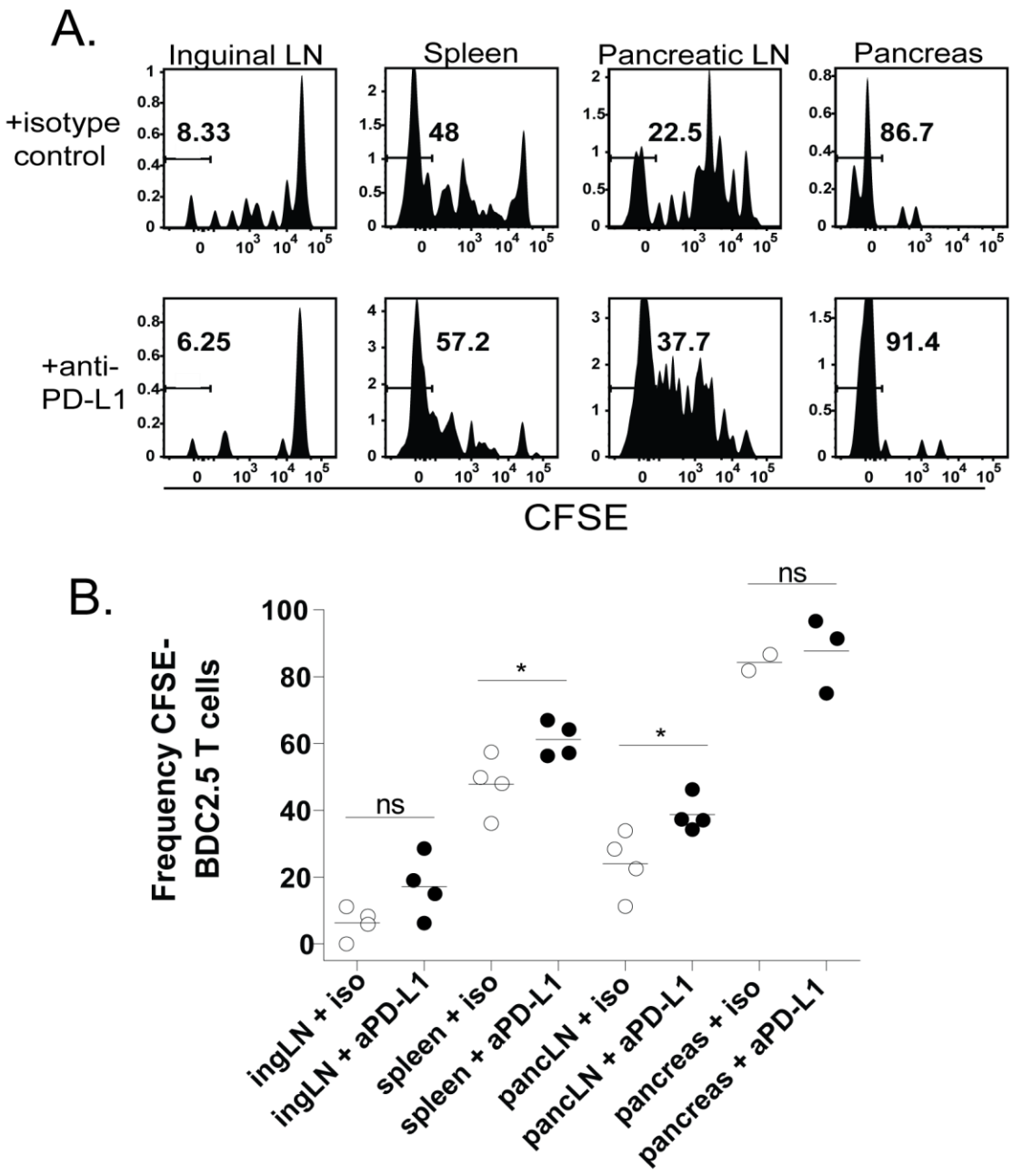

Figure 4-5: Anti-PD-L1 causes sustained proliferation early during the priming phase of the response.

(A) Representative FACS histograms illustrating cell proliferation determined by CFSE dye dilution gated on Thy1.1^{+} BDC2.5 T cells from mice that received 250 μg/mouse isotype control or anti-PD-L1 antibody at days -3 and -1 prior to harvest at day 7 post-transfer. (B) Frequency of BDC2.5 T cells that had fully diluted CFSE from panel A. Data are representative of 3 experiments with 4 mice in each treatment group.

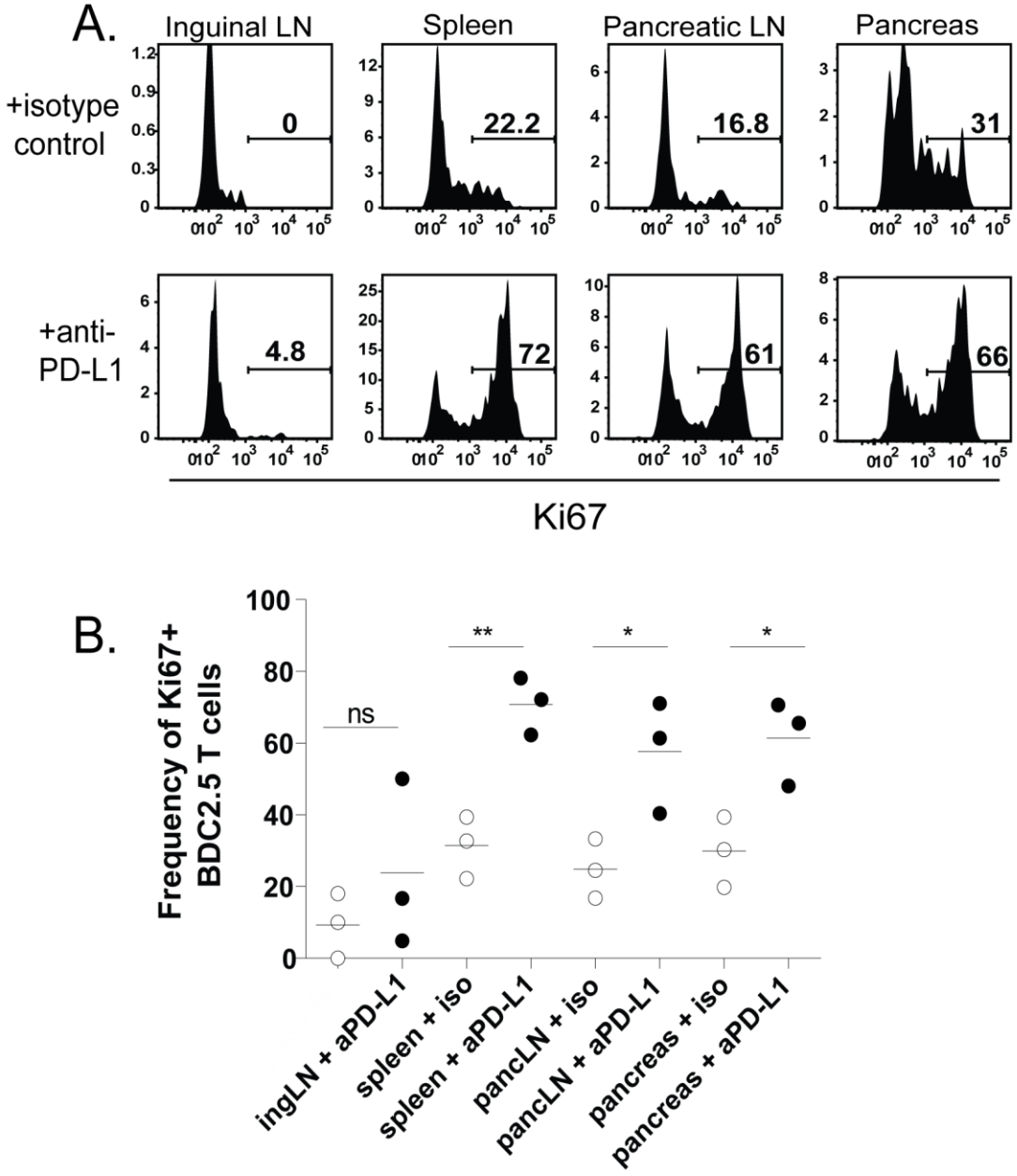

Figure 4-6: Loss of PD-1 causes islet-reactive CD4$^+$ T cells to re-enter cell cycle late during the persistent phase of the response.

(A) Ki67 expression from mice treated with 250 μg/mouse isotype control or anti-PD-L1 antibody at days -3 and -1 prior to harvest at day 42 post-transfer. (B) Quantification of the frequency of Ki67$^+$ BDC2.5 T cells from panel A. Data are representative of at least 4 independent experiments per organ with at least 3 mice per treatment condition.

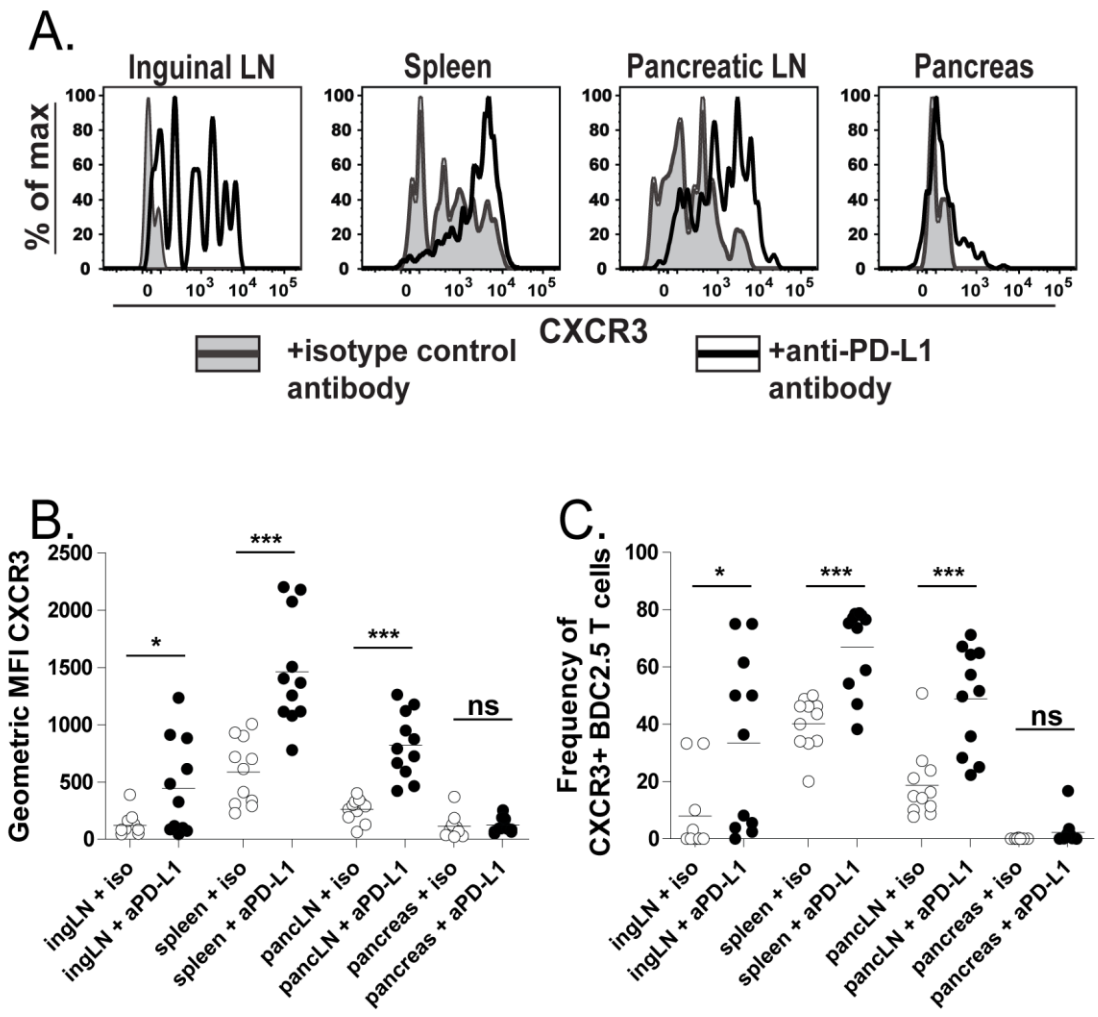

Figure 4-7: PD-1 blockade causes increased expression of the chemokine receptor CXCR3 on BDC2.5 T cells in the secondary lymphoid organs.

(A) Representative FACS histogram plots showing CXCR3 expression on Thy1.1^{+} BDC2.5 T cells from mice that received 200 μg of isotype control or anti-PD-L1 antibody at days -5, -3, and -1 prior to harvest. Mice were harvested 3 weeks post-transfer. Quantification of panel A showing (B) geometric MFI of CXCR3 and (C) the frequency of BDC2.5 T cells expressing CXCR3. Data are compiled from 3 experiments with at least 3 mice per treatment condition.

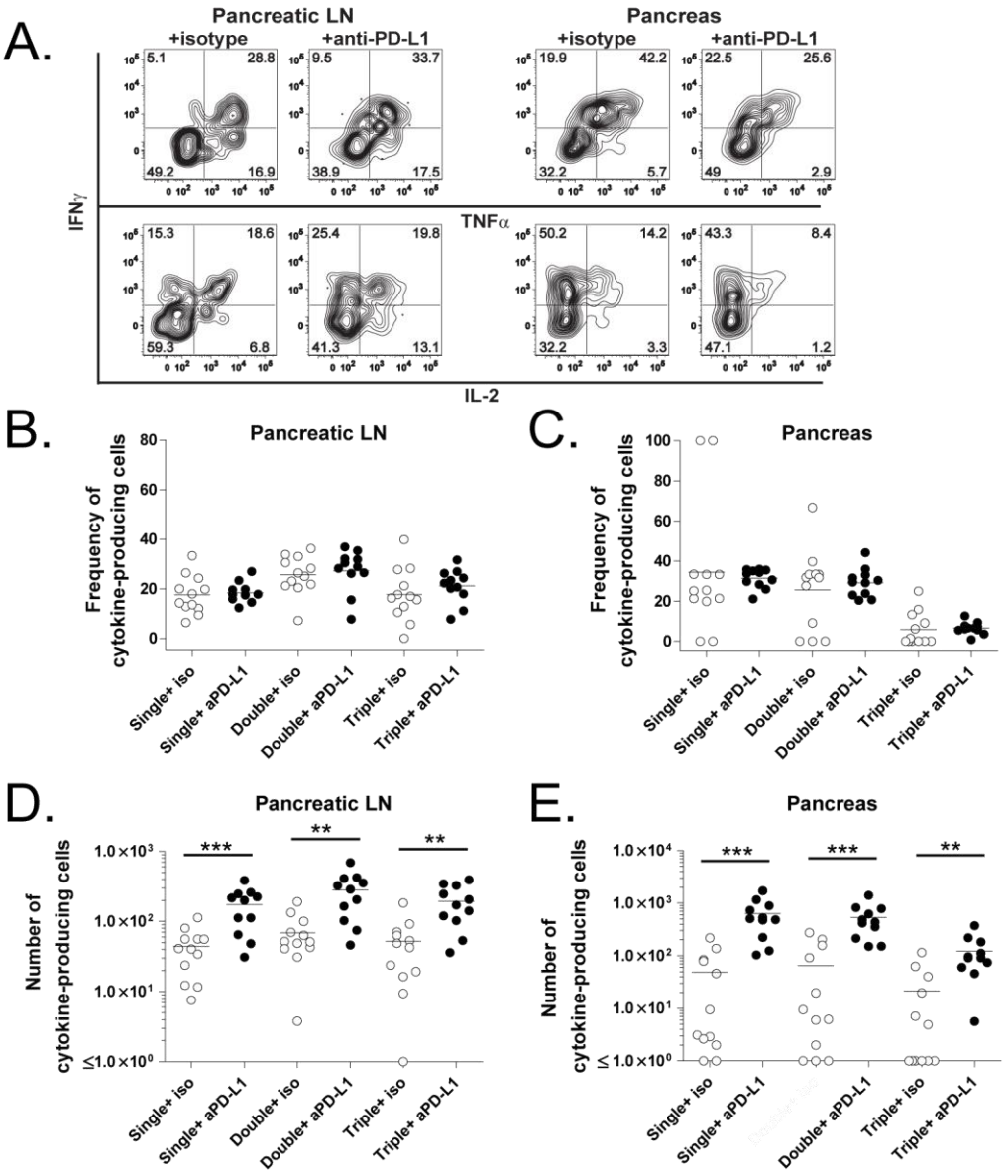

Figure 4-8: Anti-PD-L1 increases the number of cytokine-producing BDC2.5 cells in the pancreatic LN and pancreas.

(A) Intracellular IFNγ, TNFα, and IL-2 were measured in Thy1.1$^+$ BDC2.5 T cells isolated from the pancreatic LN and pancreas following ex vivo stimulation with PMA and ionomycin. Quantification of (B and C) frequency and (C and E) the number of cytokine-producing BDC2.5 T cells in (B and D) the pancreatic LN and (C and E) pancreas. Data in B-E show twelve mice treated with isotype control and eleven mice treated with anti-PD-L1. Data are representative of four independent experiments. Single$^+$ refers to cells that produce IFNγ, TNFα, or IL-2 only. Double$^+$ refers to cells that produce IFNγ+IL-2, IFNγ+TNFα, or TNFα+IL-2. Triple$^+$ refers to cells that produce IFNγ, TNFα, and IL-2.

A.

Organ: Pancreatic LN	Frequency of cytokine producing BDC2.5 T cells		
Number of cytokines produced	+Isotype antibody	+Anti-PD-L1	p value
One (IFNγ only, IL-2 only, TNFα only)	17.50±2.22%	18.42±1.21%	0.7283
Two (IFNγ+IL-2, IFNγ+TNFα, or IL-2+ TNFα)	25.52±2.26%	27.21±2.59%	0.6298
Three (IFNγ, IL-2, and TNFα)	17.67±3.08%	21.09±2.08%	0.3775

B.

Organ: Pancreas	Frequency of cytokine producing BDC2.5 T cells		
Number of cytokines produced	+Isotype antibody	+Anti-PD-L1	p value
One (IFNγ only, IL-2 only, TNFα only)	34.42±9.41%	31.43±1.45%	0.7672
Two (IFNγ+IL-2, IFNγ+TNFα, or IL-2+ TNFα)	25.54±5.76%	29.91±2.15%	0.5712
Three (IFNγ, IL-2, and TNFα)	5.92±2.40%	6.56±0.90%	0.8124

Table 4-1: Frequencies of cytokine-producing BDC2.5 T cells following isotype-control or anti-PD-L1 antibody treatment.

(A) The frequency of BDC2.5 T cells expressing one, two, or three cytokines in the pancreatic LN following anti-PD-L1 treatment. (B) The frequency of BDC2.5 T cells expressing one, two, or three cytokines in the pancreas following anti-PD-L1 treatment. These data are graphically illustrated in Figure 4-8. Single+ refers to cells that produce IFNγ only, TNFα only, or IL-2 only. Double+ refers to cells that produce IFNγ and IL-2, IFNγ and TNFα, or TNFα and IL-2. Triple+ refers to cells that produce IFNγ, TNFα, and IL-2. Data were combined from 3 independent experiments conducted between days 35 and 39 post-transfer and represent PMA and ionomycin stimulated cells from 12 mice treated with isotype control and 11 mice treated with anti-PD-L1. Data are representative of 4 independent experiments.

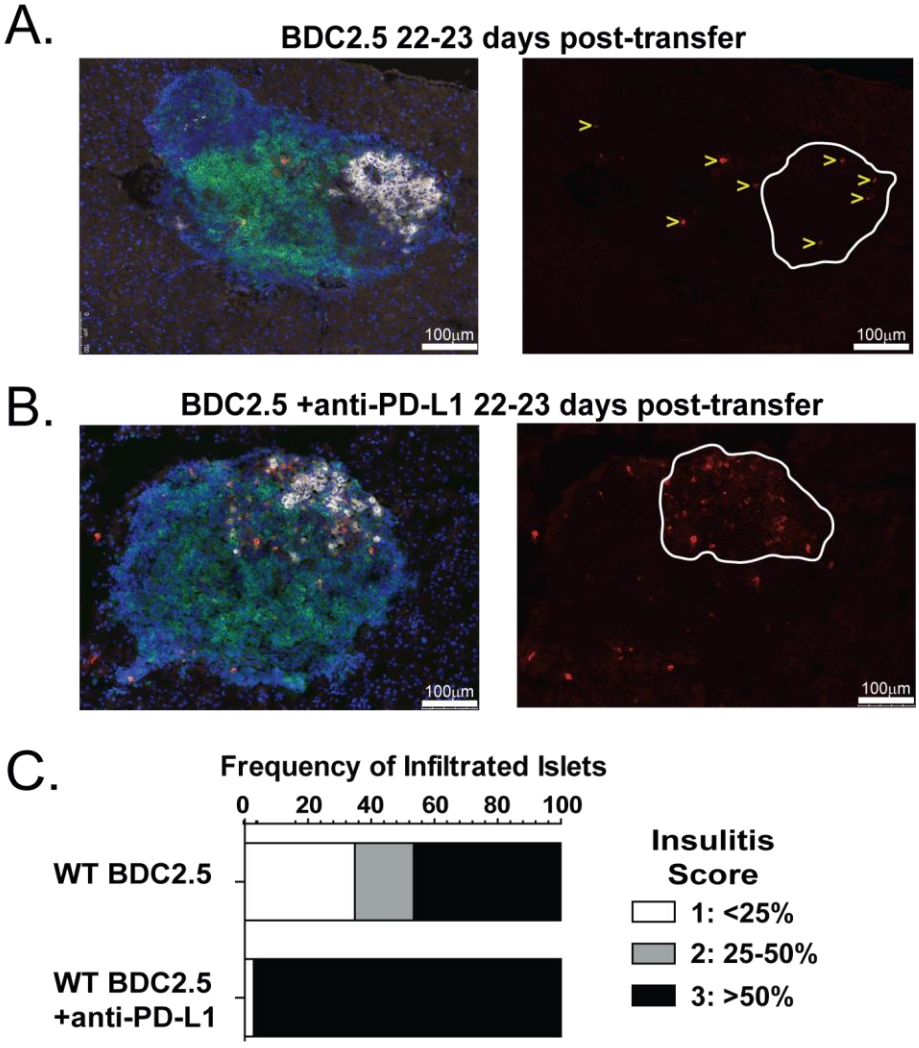

Figure 4-9: Neutralizing PD-L1 results in more destructive insulitis and causes BDC2.5 T cells to re-localize from the more benign portion of the islet infiltrate into the islet core.

(A and B) Histological analysis of the pancreas 3 weeks post-transfer with (B) or without (A) anti-PD-L1 treatment. Islets are representative of 4-6 mice per group. Grey is insulin, red is Thy1.1^{+} BDC2.5 T cells, green is CD3^{+} T cells, and DAPI marking nuclei is blue. Single color images showing BDC2.5 T cells in red are to the right of the overlaid image containing all four colors. White lines in the red single color image outline the islet body containing the insulin-producing cells. Select BDC2.5 T cells are marked with yellow arrows. Scale bar =100µm. (C) Insulitis from mice 3 weeks post-transfer with or without anti-PD-L1 administration. Scores indicate 1: peri-insulitis and/or <25 infiltrated, 2: 25-50% infiltrated, and 3: >50%. Forty-three islets from WT without anti-PD-L1 at day 22-23 and seventy islets from WT with anti-PD-L1 at day 22-23 were scored.

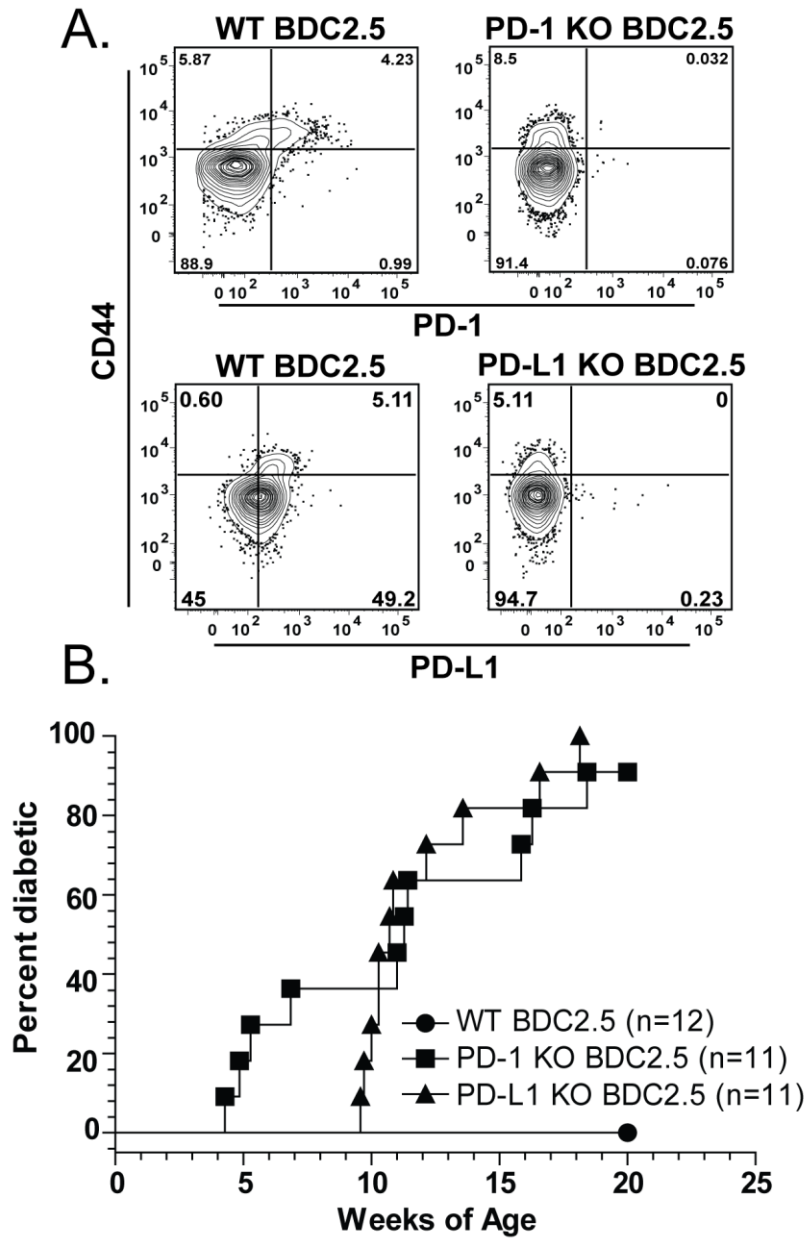

Figure 4-10: BDC2.5 mice lacking PD-1 or PD-L1 develop accelerated Type 1 Diabetes compared to wild type mice.

(A) FACS contour plots gated on BDC2.5 T cells at time of transfer showing PD-1 expression from the spleen and LNs of wild type and PD-1 KO BDC2.5 donors and PD-L1 expression on wild type and PD-L1 KO BDC2.5 donors. Data shown are representative of six independent experiments (PD-1 KO) and three independent experiments (PD-L1 KO). (B) Spontaneous diabetes incidence from WT BDC2.5 (n=12), PD-1 KO BDC2.5 (n=11), and PD-L1 KO BDC2.5 (n=11) determined by blood glucose measurements.

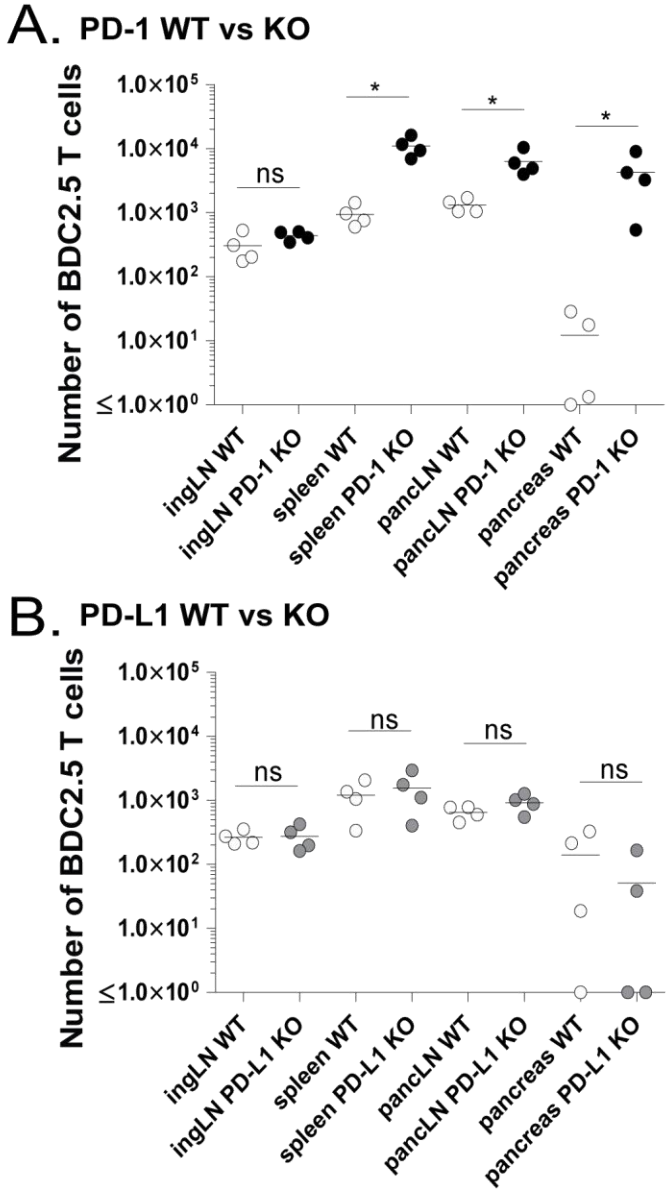

Figure 4-11: PD-1 expressed by the BDC2.5 T cell is required to regulate the early expansion in the secondary lymphoid organs and infiltration of the pancreas, while PD-L1 expressed by the BDC2.5 T cells is dispensable.

(A) The absolute number of WT or PD-1 KO BDC2.5 T cells was measured 7 days post-transfer of 7500 BDC2.5 T cells. Data are representative of 6 experiments containing spleen, pancreatic LN and pancreas, 5 of which also contained inguinal LNs, with 3-4 mice for each genotype. (B) The absolute number of WT or PD-L1 KO BDC2.5 T cells 8 days post-transfer. Data are representative of 3 experiments containing spleen, pancreatic LN and pancreas, 2 of which also contained inguinal LNs, with 3-4 mice per genotype.

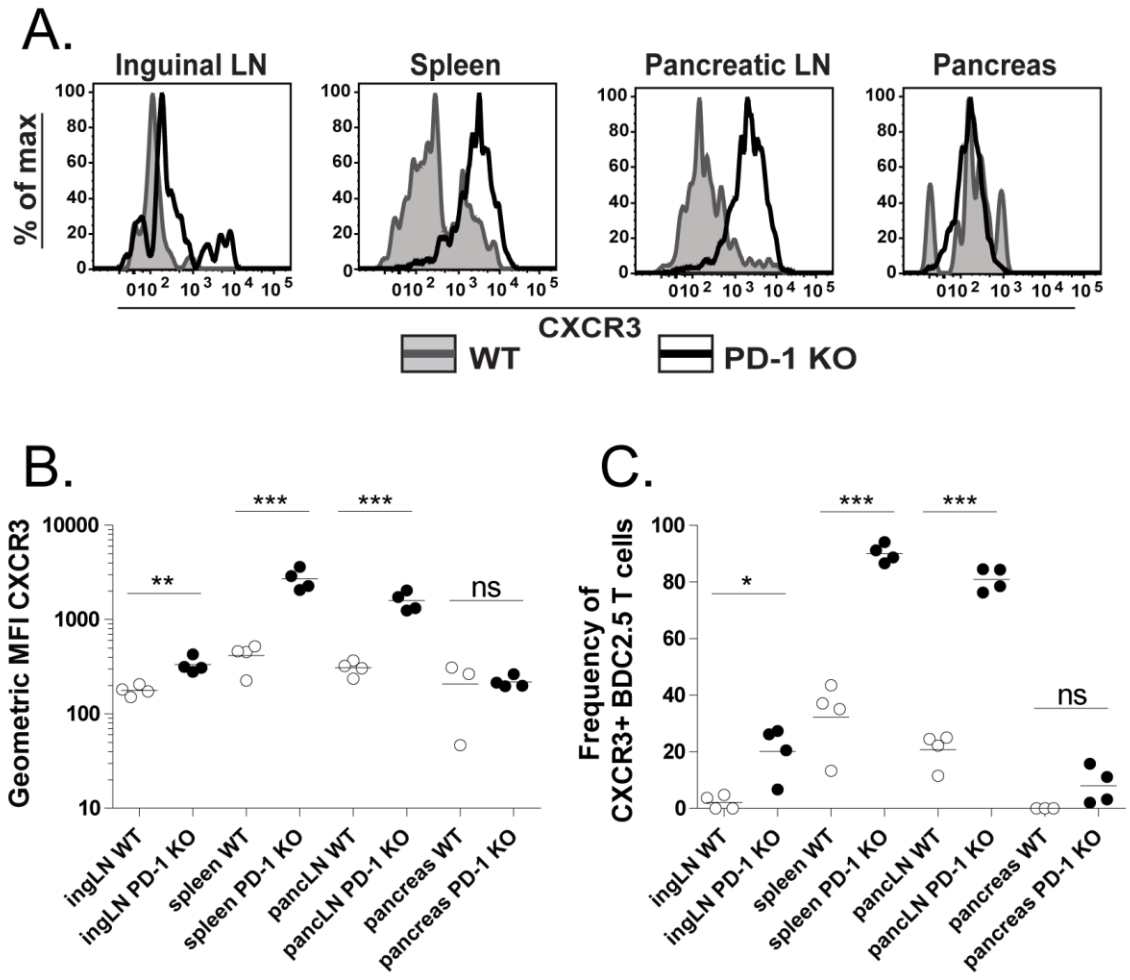

Figure 4-12: PD-1-deficient BDC2.5 T cells display elevated levels of CXCR3 in the secondary lymphoid organs.

(A) Representative FACS histograms showing CXCR3 expression of WT or PD-1 KO BDC2.5 T cells 7 days post-transfer. Quantification of (B) the geometric MFI of CXCR3 and (C) the frequency of $CXCR3^{+}$ WT or PD-1 KO BDC2.5 T cells 7 days post-transfer. Data are representative of 2 independent experiments with 4 mice per genotype.

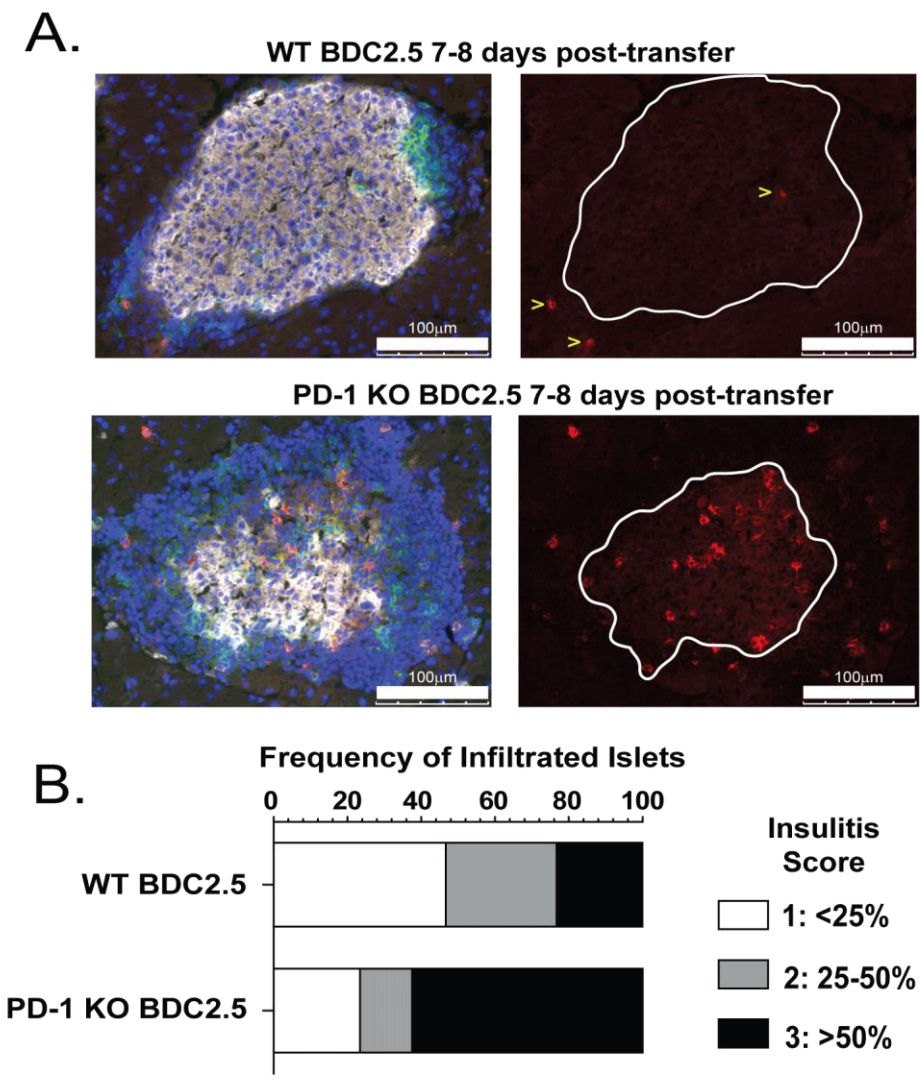

Figure 4-13: PD-1-deficient BDC2.5 T cells show altered localization within the pancreatic islets compared to PD-1-sufficient cells.

(A) Histological analysis of pancreas from mice 7-8 days post-transfer of WT or PD-1 KO BDC2.5 T cells. Grey is insulin, red is Thy1.1$^+$ BDC2.5 T cells, green is CD3$^+$ T cells, and DAPI marking nuclei is blue. Single color images showing BDC2.5 T cells in red are to the right of the overlaid image containing all four colors. White lines in the red single color image show the outline of the islet body containing the insulin-producing cells. BDC2.5 T cells are marked with a yellow arrows in the WT group to aid in the visualization of the cells. Scale bar =100μm. (B) Insulitis was scored blinded from mice 7-8 days post-transfer of WT or PD-1 KO BDC2.5 T cells. Thirty islets from WT and sixty-four islets from PD-1 KO transfers were scored blinded. Insulitis scores refer to; 1: peri-insulitis and/or <25% infiltrated, 2: 25-50% infiltrated, and 3: >50% infiltrated. Data are representative of two independent experiments with four mice per group.

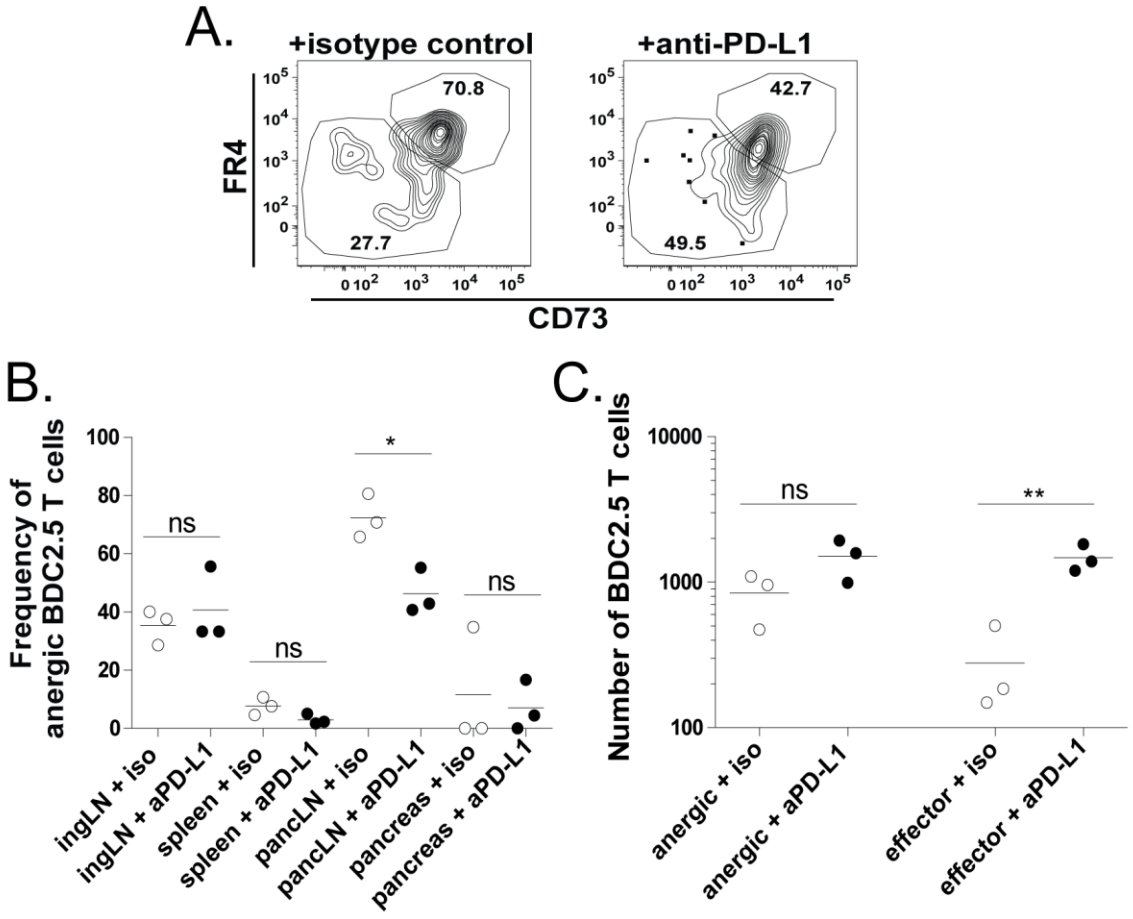

Figure 4-14: Blocking PD-1 preferentially restores proliferation to effector phenotype BDC2.5 T cells, not anergic cells.

(A) Representative FACS contour plots showing FR4 and CD73 expression on BDC2.5 T cells in the pancreatic LN three weeks post-transfer. Plots are gated on CD44^{high} Foxp3^{-} BDC2.5 T cells. (B) Quantification of the frequency of effector phenotype (CD44^{high} Foxp3^{-} FR4^{-} CD73^{-}) or anergic phenotype (CD44^{high} Foxp3^{-} FR4^{+} CD73^{+}) BDC2.5 T cells in the inguinal LN, spleen, pancreatic LN, and pancreas following antibody blockade. (C) Quantification of the number of effector or anergic phenotype BDC2.5 T cells in the pancreatic LN following antibody blockade. Each group received 200 μg of isotype-control or anti-PD-L1 antibody at days -5, -3, and -1 prior to harvest. Data are representative of 4 experiments with spleen, pancreatic LN, and pancreas, 3 of which also contained inguinal LNs, with 3-4 mice per group.

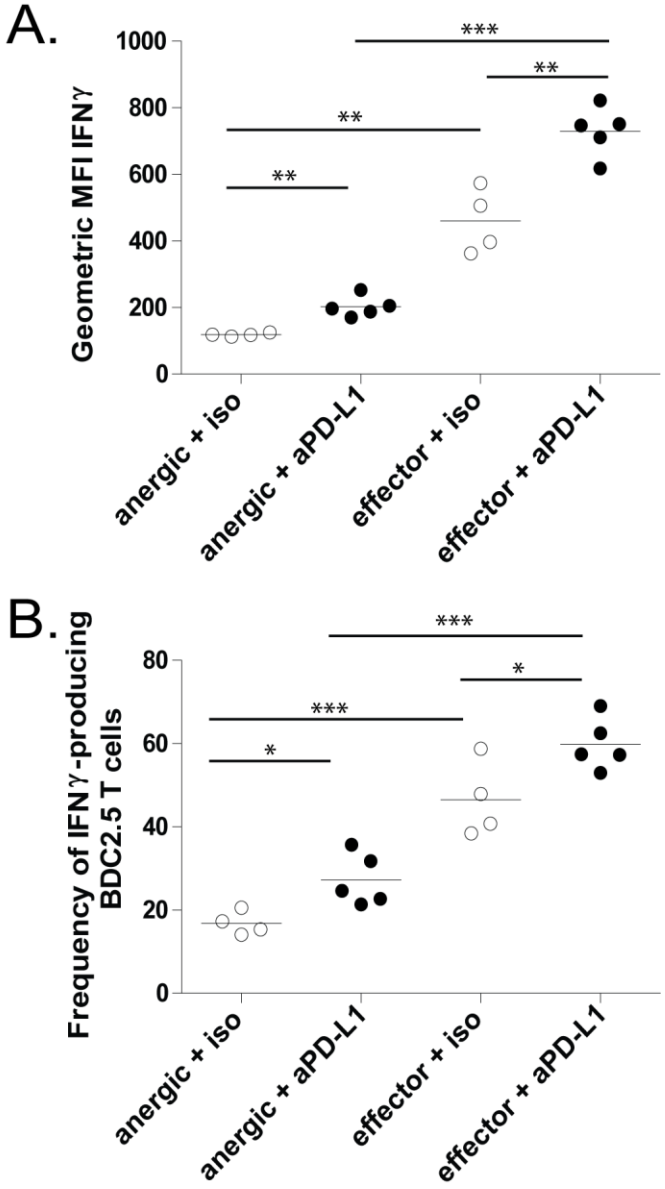

Figure 4-15: Loss of PD-1 does not restore cytokine production to anergic phenotype BDC2.5 T cells.

(A) Quantification of IFNγ production by effector ($CD44^{high}$ $Foxp3^{-}$ $FR4^{-}$ $CD73^{-}$) and anergic phenotype ($CD44^{high}$ $Foxp3^{-}$ $FR4^{+}$ $CD73^{+}$) BDC2.5 T cells in the pancreatic LN 49 days post-transfer into non-diabetic NOD mice. IFNγ production following 4 hours of stimulation with acetylated-p31 peptide intravenously is compared to a no stimulation control that received PBS intravenously. (B) Quantification of the frequency of IFNγ-producing effector and anergic phenotype BDC2.5 T cells in the pancreatic LN 49 days post-transfer into non-diabetic NOD mice. Data are representative of 3 experiments with 3-4 mice that received peptide stimulation per experiment.

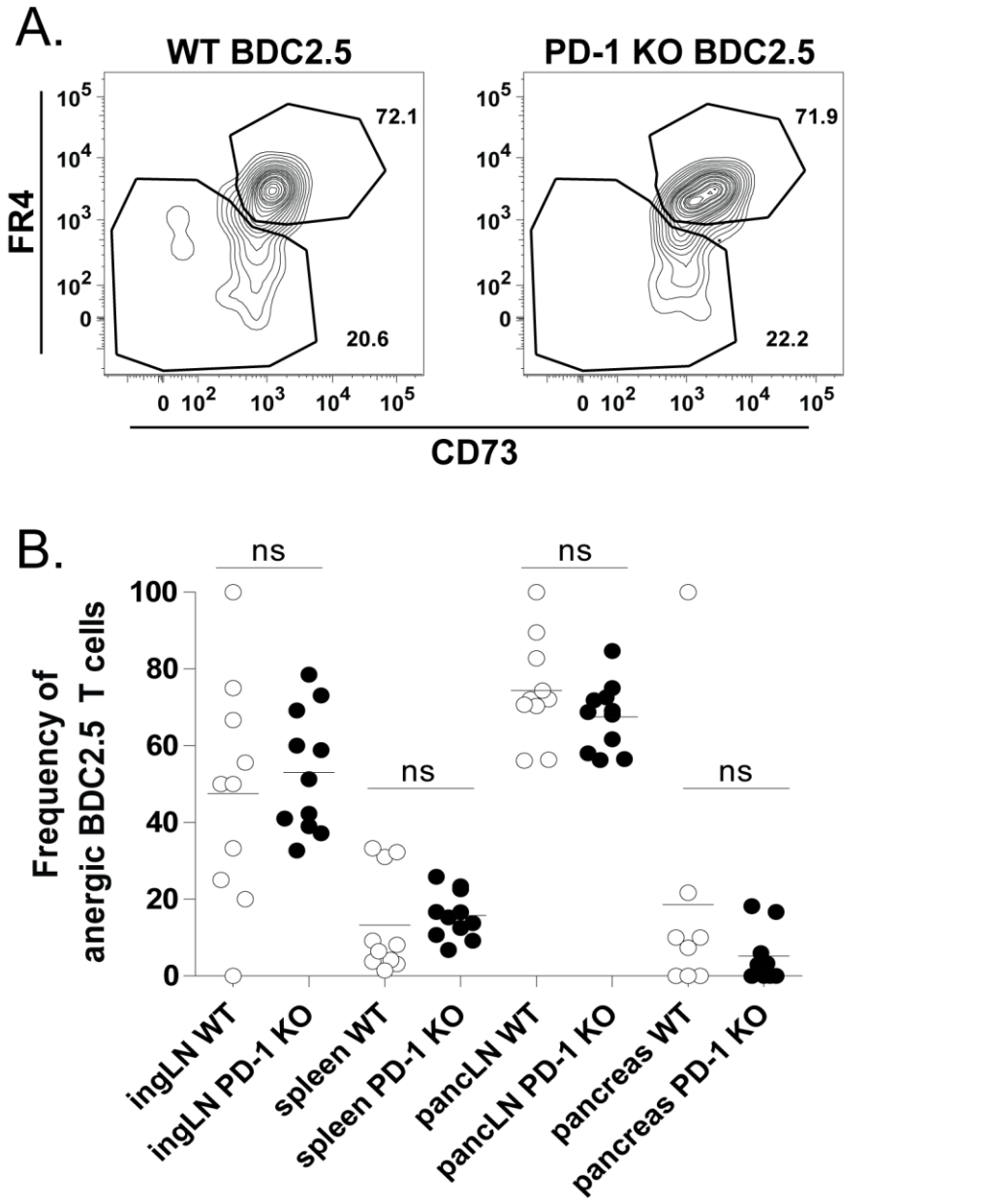

Figure 4-16: PD-1 expressed by the BDC2.5 T cell is not required for the induction of the anergic ($FR4^+$ $CD73^+$) population.

(A) Representative FACS contour plots showing FR4 and CD73 expression on wild type and PD-1 KO BDC2.5 T cells three weeks post-transfer into pre-diabetic NOD mice. Plots are gated on $CD44^{high}$ BDC2.5 T cells. (B) Quantification of the frequency of $FR4^+$ $CD73^+$ BDC2.5 T cells three weeks post-transfer into pre-diabetic NOD. Data represent 10 mice that received wild type and 11 mice that received PD-1 KO BDC2.5 T cells compiled from 3 independent experiments.

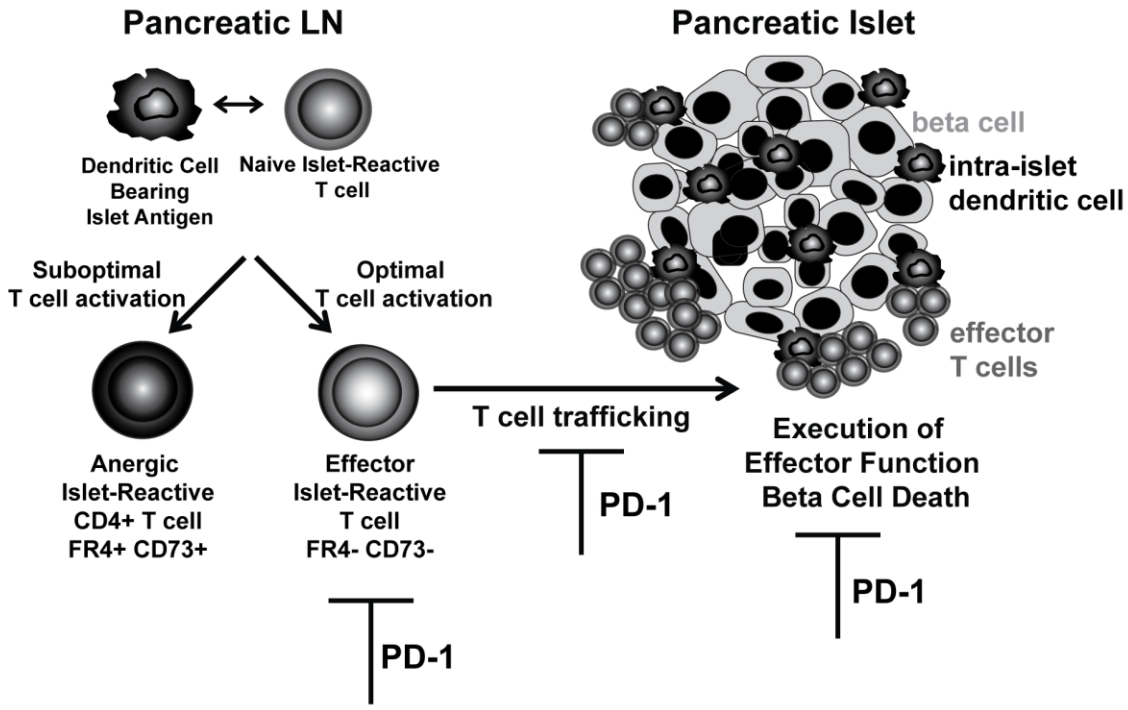

Figure 4-17: Model for PD-1-mediated regulation of islet-reactive $CD4^+$ T cells during Type 1 Diabetes progression

PD-1 becomes expressed on islet-reactive $CD4^+$ T cells following encounter with a cognate antigen-bearing dendritic cell. PD-1 acts to suppress effector $CD4^+$ T cells ($FR4^-$ $CD73^-$) in the pancreatic LN, preventing trafficking to the pancreas. Blocking PD-1 causes effector $CD4^+$ T cells to enter cell cycle, up-regulate CXCR3, traffic to the pancreas, and perform effector functions within the pancreas including secretion of IFNγ. Blocking PD-1 does not restore functionality to anergic $CD4^+$ T cells ($FR4^+$ $CD73^+$) in the pancreatic LN, and these cells do not traffic to the pancreas or contribute to disease pathology.

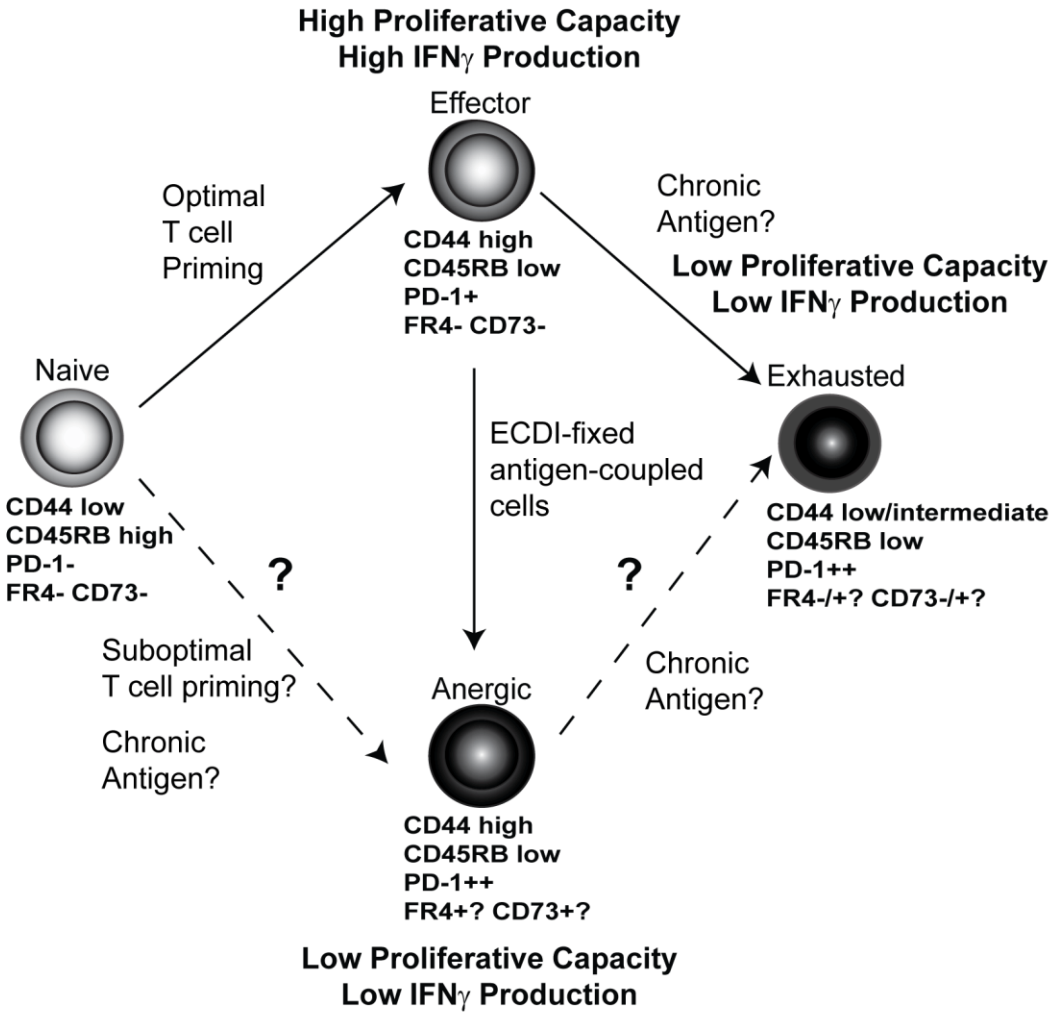

Figure 4-18: Model for lineage relationships between naïve, effector, anergic, and exhausted CD4$^+$ T cells during Type 1 Diabetes progression

Following encounter with endogenous autoantigen, two distinct fates were observed after transfer of naïve islet-reactive CD4$^+$ T cells: effector phenotype cells identified as FR4$^-$ CD73$^-$ and anergic phenotype cells identified as FR4$^+$ CD73$^+$. Effector phenotype cells have a greater functional capacity than anergic phenotype cells, producing more IFNγ following peptide challenge. We speculate that effector phenotype cells develop due to optimal T cell priming conditions, whereas anergic phenotype cells develop due to suboptimal T cell priming conditions. The factors that promote effector versus anergic differentiation remain unclear, but PD-1 is not required for the induction of anergy marked by FR4 and CD73. ECDI-fixed antigen-coupled cells can induce anergy, but these anergic cells do not express FR4 and CD73. Chronic antigen stimulation can cause effector cells to gradually lose functionality. Whether anergic CD4$^+$ T cells can also become exhausted or whether these phenotypes represent distinct lineages is unclear.

CHAPTER 5:

Clinical opportunities and the prospective of targeting the PD-1/PD-L1 pathway to modulate T cell responses in patients[1]

INTRODUCTION

The role of the PD-1/PD-L1 pathway in limiting autoimmunity by suppressing the effector functions of pathogenic autoreactive T cells is well established. The focus of this work has been further delineating how this pathway regulates islet-reactive $CD4^+$ T cells during Type 1 Diabetes development with the ultimate goal of determining whether this pathway can be targeted therapeutically to restore peripheral tolerance in patients. There were two main themes throughout the thesis: (1) Understanding the dynamics of islet-reactive $CD4^+$ T cells during the physiological progression of Type 1 Diabetes in NOD mice, including the development of pathogenic effector populations and well as tolerant populations, and (2) which populations of islet-reactive $CD4^+$ T cells were susceptible to PD-1-mediated regulation. This last chapter will discuss these findings in the context of clinical opportunities and potential therapeutic applications in terms of inducing tolerance to inhibit autoreactive T cells in an antigen-specific manner as well as targeting the PD-1 pathway to modulate host immunity. For inducing tolerance, the current clinical trials involving anti-CD3 (teplizumab) will be discussed, as well as preclinical models involving the adoptive transfer of Treg cells and antigen-coupled ECDI-fixed cells. For targeting the PD-1/PD-L1 pathway to modulate host immunity, I will discuss both the possibility of engaging PD-1 on autoreactive T cells to induce an inhibitory signal to

suppress effector functions, as well as issues regarding administering neutralizing antibodies against PD-1 to enhance immunity during chronic antigen settings such as persistent viral infections and malignancies. The overall goal of this section is to provide an assessment of the potential efficacy of inducing tolerance in Type 1 Diabetic patients, whether engaging PD-1 could be useful in tolerance induction, and the risk of eliciting autoimmunity as an unwanted side effect of anti-PD-1 therapy to enhance immunity. Ultimately, with a better understanding of the basic biology of tolerance and the PD-1 pathway we will be able to help patients make more informed decisions about treatments and develop a cure for Type 1 Diabetes.

THERAPEUTIC APPROACHES TO INHIBIT ANTI-ISLET IMMUNITY IN PATIENTS WITH TYPE 1 DIABETES

Addressing the anti-islet immune response is required to cure patients with Type 1 Diabetes

Developing a cure for Type 1 Diabetes in patients involves two critical events. First, the insulin-producing beta cells that have been destroyed by the immune system must be replaced in order for patients to regulate their own glucose metabolism independently of exogenous insulin therapy. There are methods that are currently being used successfully in the clinic to restore beta cell mass, including whole pancreas or islet transplantation (227). There are also approaches that are still in the development stage but hold great promise for the diabetes field, including production of insulin-producing cells derived from induced pluripotent stem cells (228). However, it was appreciated early

after pancreas transplantation entered the clinic that restoring beta cells was not sufficient to cure diabetes. In a patient that received a pancreas transplant from an identical twin, and subsequently had no allograft rejection issues, the recurrent autoimmune response destroyed the graft (229). These data have been replicated in murine models of Type 1 Diabetes, supporting a model where diabetogenic T cells persist long term following destruction of the pancreas. Following islet transplantation, these cells become re-activated and subsequently destroy the graft (230). The requirement for high doses of broad spectrum immunosuppressive drugs to suppress the autoimmune response following islet transplantation has greatly hindered use of islet transplant therapy in humans to cure Type 1 Diabetes. Therefore, in order to develop a more viable cure for Type 1 Diabetes, a second critical event must occur involving the specific suppression of the anti-islet autoimmune response. Our lab and others are currently focused on developing antigen-specific therapies to inhibit the diabetogenic T cell response during Type 1 Diabetes. The goal of antigen-specific therapies is to avoid broad spectrum immunosuppressive drugs which will render the patient highly susceptible to infections and malignancies. The following sections will discuss both antigen-specific therapies that are in clinical trials as well as prospective therapies that are still in preclinical models.

Clinical trials using anti-CD3 (teplizumab) to induce tolerance in patients with Type 1 Diabetes have shown mixed results

The most promising antigen-specific therapy currently in clinical trials is teplizumab, a humanized FcR-non-binding antibody specific for human CD3 (231, 232).

Anti-CD3 treatment in NOD mice can reverse new onset diabetes following a short 5 day treatment regiment (230). Anti-CD3 has a variety of effects on the host immune system, ultimately resetting the balance between effector and regulatory T cells in favor of regulation. Anti-CD3 preferentially induces apoptosis in activated T cells, which in a diabetic patient lacking concurrent infections will predominantly be the autoreactive T cell population. This treatment can also induce anergy, resulting in induction of a hyporesponsive state, or antigenic modulation, which prevents the T cells from encountering antigen based on physical blocking of the T cell receptor or internalization or shedding of the T cell receptor (233). The most dramatic effect of anti-CD3 is a clearing of islet-reactive T cells from the pancreas within the first two days after treatment. However, the effect of anti-CD3 on the effector population is transient, and these cells eventually repopulate the pancreas. Therefore, the most significant effect of anti-CD3 in terms of long lasting therapeutic potential is its ability to restore regulation in the immune system. Treatment with anti-CD3 causes a loss of both effector T cells and Treg cells, but Treg cells recover much more quickly, shifting the balance of the immune system from deregulated autoimmunity to a state of immune homeostasis. Anti-CD3 is considered an antigen-specific therapy because the majority of T cells that are activated in a diabetic mouse or patient and subsequently would be depleted are islet-antigen specific (233). However, the benefit of this therapy is that knowledge of the antigens to be targeted is not required, making treatment easily generalizable between patients. Unfortunately, while preclinical data in NOD mice and phase I and II clinical trials were promising, preliminary data from the first year of the phase III clinical trial have been

disappointing (234). The initial interpretation of the data from the phase III clinical trial suggested that anti-CD3 did not improve on the standard insulin therapy after one year (234). However, these interpretations have been heavily challenged, with many claiming that the parameters used to assess efficacy were misleading and not validated in the earlier clinical trials (235). Many are still hopeful that anti-CD3 will be an effective immunotherapeutic agent since in this trial it had a positive impact on C-peptide production and reduced patients' exogenous insulin needs (235). An active area of research is directed at identifying the mechanism of anti-CD3-mediated tolerance in humans and determining ways to increase efficacy by promoting more stable tolerance induction.

Preclinical models aimed at inducing tolerance in Type 1 Diabetic patients

Considering the mixed results obtained with anti-CD3 in the clinic, several groups have begun focusing on alternative approaches to induce antigen-specific tolerance to cure Type 1 Diabetes. Two of these approaches involve the adoptive transfer of Treg cells to induce better extrinsic control over effector autoreactive T cell populations and antigen-coupled ECDI-fixed cells as a mechanism to directly target the pathogenic T cell population to induce cell intrinsic tolerance. While both approaches aim to induce tolerance and suppress pathogenic diabetogenic T cell responses, they both have complicating factors that have slowed their entry into the clinic. Both require manipulating human cells ex vivo, which makes these protocols expensive, labor intensive, and have to be individualized for each patient rather than being mass produced

like anti-CD3. Additionally, the notion of antigen specificity complicates both approaches. These issues continue to be heavily considered as these therapies move closer to the clinic.

Adoptive transfer of Treg cells to suppress autoimmunity is an attractive therapeutic approach to treat Type 1 Diabetic patients because preclinical data using NOD mice suggested that adoptive transfer of either polyclonal or islet-antigen specific Treg cells could prevent disease (133, 140, 236). Several groups, including those led by Bluestone, June, and Blazar, have developed ways to expand Treg cells ex vivo to generate large numbers of cells for infusion back into the host (133, 237, 238). This approach, which utilizes exposure of highly purified Treg cells to anti-CD3 and anti-CD28 coated beads in conjugation with high levels of IL-2, generates cells which are capable of suppressing autoimmunity when transferred into Treg cell-deficient mice (133). However, incredibly high numbers of polyclonal Treg cells were needed for protection, reducing the feasibility of this approach in humans. Substantially fewer Treg cells were needed if the expanded population was specific for an islet-antigen (BDC2.5) (133); however, the requirement for antigen-specific Treg cells inevitably complicates translation into the clinic. Antigen-specificity requires more specialized therapy because the antigens may be different between patients, increasing the risk of Treg cells failing to protect. Additionally, the less generalizable the therapy, the more expensive it becomes. Furthermore, there is no evidence to suggest that islet-antigen specific Treg cells will not have a more general suppressive effect on host immunity, rendering the patient immunocompromised. An added layer of complexity is that new evidence suggests that

there are different varieties of Treg cells similarly to how there are different subsets of conventional $CD4^+$ T cells, and expression of the same master transcription factors between Treg cells and effector T cells makes these Treg cells more effective at suppressing than non-polarized Treg cells (239). Lastly, one of the greatest concerns with Treg cell therapy is the stability of the regulatory functions of the cells being transferred. Zhou and colleagues recently showed that there is a subpopulation of Treg cells that can lose Foxp3 expression in vivo and convert to pathogenic effector cells capable of inducing autoimmunity (204). This issue is particularly concerning for transferring islet-antigen specific Treg cells into patients, since the loss of regulatory functions and acquisition of effector functions could severely exacerbate the pre-existing autoimmune disease. The stability of ex vivo expanded human Treg cells and the conditions that could promote loss of regulatory properties should be thoroughly explored before adopting this therapy to attempt to treat Type 1 Diabetic patients. These issues have slowed translation of Treg therapy to the clinic. Additionally, issues of feasibility in humans have impeded the progress of moving this therapy forward, including: a lack of unique surface markers defining human Treg cells makes this population difficult to efficiently expand, the frequency of Treg cells in human peripheral blood is very low, and the number of Treg cells that would likely be needed to suppress autoimmunity is too high to easily be achieved by current methods. Despite these draw backs, Phase I clinical trials have been initiated transferring allogeneic Treg cells into patients with hematopoietic stem cell transplants using methods to expand human Treg cells (142, 240, 241), and the data

generated during these trials will likely be used to determine whether this approach could be useful for Type 1 Diabetic patients.

Antigen-coupled ECDI-fixed cells are an attractive therapeutic platform because they are one of very few therapies that can reverse Type 1 Diabetes in NOD mice (59). As discussed in Chapter 1, antigen-coupled ECDI-fixed cells have been used extensively in NOD mice to delineate the epitopes involved early in disease initiation as well as late during pre-clinical disease and at diabetes onset (59, 85). In NOD, ins$B_{9\text{-}23}$ is a critical initiating epitope, since treating 4 week old mice with this peptide coupled to splenocytes prevents diabetes. Treating older NOD mice with ins$B_{9\text{-}23}$-coupled cells failed to prevent diabetes, but whole insulin- coupled cells could prevent disease (85). Additionally, whole insulin-coupled cells also reversed diabetes in NOD mice (59), though the individual insulin peptides have not been tested in this context. The use of whole proteins rather than peptides may be advantageous therapeutically, since multiple epitopes can be targeted at once. However, priming NOD mice with whole insulin is not capable of generating an immune response, which is likely because the major pathogenic T cell population selectively recognizes peptides that are not natural cleavage products of insulin produced through the MHC class II processing pathway (57). Therefore, immunizing with different peptides versus proteins could be problematic in terms of antigen recognition. Additionally, Type 1 Diabetes is a complex autoimmune disease that involves a variety of antigens at different stages of the disease. The heterogeneity in antigens recognized in patients and how these antigens change as disease develops is not well understood, and could make it so that coupled cell therapy has to be highly

personalized if it is going to be efficacious. Additionally, providing a source of cells to be coupled to antigen may be difficult and expensive. Miller and colleagues have developed biodegradable microparticles that can be used as platforms for coupled cells antigens to circumvent this problem (242), though whether these particles will be nearly as tolerogenic as apoptotic cells in humans with high levels of autoimmune-mediated inflammation remains to be tested.

Lastly, additional studies are needed to determine the stability of the tolerance induced by Treg cells and antigen-fixed ECDI-coupled cells. Work from our lab has shown that PD-1 is required for both the induction and maintenance of tolerance following coupled cell therapy (59, 110). This could be problematic, since over 30 SNPs have been identified in the human PD-1 gene that are associated with enhanced susceptibility to several autoimmunity diseases (discussed in Chapter 1), and it is possible that these SNPs render the PD-1 pathway less effective in these patients. However, the data presented in this thesis argue that PD-1 is not required for the induction or maintenance of tolerance marked by FR4 and CD73 co-expression, but rather is selectively important for suppressing the functionality of the pre-existing effector population. It could therefore be reasoned that coupled cells induce a less stable form of tolerance than whatever is inducing tolerance following encounter with endogenous autoantigen. Indeed, coupled cells do not induce FR4 and CD73 (data not shown) even though they induce anergy based on functional impairment following antigen rechallenge. Whether there are differences in the stability of the tolerance phenotype between the $FR4^+$ $CD73^+$ population in the low transfer (defined in Chapter 2) and the ECDI-fixed

antigen-coupled cell model remains to be tested. However, since PD-1 is important for regulating the anergic population following antigen coupled cell therapy, combination therapies designed to engage PD-1 on T cells following coupled cell therapy may be possible to promote more stable tolerance in patients. Understanding the lineage relationships between effector, anergic, and exhausted cells, what factors drive the development of each population, and the stability of each population will be critical for determining whether durable tolerance can be induced in patients to cure Type 1 Diabetes (Figure 4-18).

POTENTIAL OBSTACLES INVOLVED IN EXPLOITING THE PD-1 PATHWAY TO INDUCE TOLERANCE IN TYPE 1 DIABTES

Identifying which cell type(s) to target to engage the PD-1/PD-L1 pathway in patients

The broad expression of PD-L1 on hematopoietic and non-hematopoietic cells suggests that PD-1/PD-L1 interactions are involved in regulating T cell functions in non-lymphoid tissues. This aspect of the PD-1 pathway makes it an attractive therapeutic target, since T cell functions can be regulated in the autoimmune target organ where the damage is occurring. However, addressing which cell type, whether hematopoietic or non-hematopoietic, must express PD-L1 in order to suppress T cell functions is particularly important since recent work using chronic viral infections showed that PD-L1 on both of these lineages was required for protective immunity, but that it played distinct roles on hematopoietic versus non-hematopoietic cells (243). Additionally, PD-L1 expressed by hematopoietic versus non-hematopoietic cells may drastically alter

which T cell subsets can be targeted for PD-1-mediated inhibition, which will be discussed in a later section.

Work by Keir and colleagues demonstrated that PD-L expressed by non-hematopoietic cells played an essential role in islet-specific tolerance (174), supporting the notion that inhibitory pathways can be engaged in the autoimmune target organ to inhibit T cell functions. In further support of this concept, work from our lab showed that PD-1 actively regulated tolerant T cells in pancreatic islets, since blocking PD-1 using neutralizing antibody to PD-L1 induced the T cell stop signal required to acquire effector functions at this site (110). Therefore, selective targeting of PD-L1 to beta cells is an attractive approach to treat Type 1 diabetes; however, two separate studies have yielded conflicting results. In one study, expression of PD-L1 from the human insulin promoter in NOD mice dramatically reduced spontaneous diabetes incidence (244), while expressing PD-L1 from the rat insulin promoter in C57BL/6 mice accelerated Type 1 Diabetes and islet allograft rejection (245). The differences between these two reports have not yet been reconciled. Genetic background, transgene promoter source, integration site, copy number, and protein expression levels and stability could account for the dramatic differences in the therapeutic efficacy observed in these two models. In addition to these possibilities, PD-L1 can also bind to B7-1, and the consequences of these interactions in pancreatic islets are unclear (149, 246). Data obtained using PD-L1 expression from the human insulin promoter in NOD is encouraging and warrants further investigation. Future work investigating the selective targeting of PD-L1 to intra-islet dendritic cells could also be informative. Subsequent work will be necessary to clarify the

role of intra-islet dendritic cell-specific PD-L1 and beta cell-specific PD-L1 expression in controlling diabetes.

In a second model of diabetes, PD-L1 expression was targeted to a different non-hematopoietic cell in the pancreas, the peri-islet Schwann cell (pSC). PD-L1 expression on pSC was achieved using the glial fibrillary acidic protein (GFAP) promoter (247). pSC expression of PD-L1 did not protect mice from developing diabetes (247). These failed attempts underscore the complexity of targeting inhibitory pathways in vivo to enhance islet-specific tolerance. Future work should be aimed at addressing these issues.

Lastly, a better understanding of how T cells receive the PD-1 signal in both lymphoid and non-lymphoid compartments will be essential to exploit this pathway to re-establish tolerance and treat autoimmunity since many autoreactive T cells reside in peripheral sites and not in secondary lymphoid organs. Work from Blackburn and colleagues have shown that PD-1^{high} cells can preferentially survive in the bone marrow compared to the spleen during chronic LCMV infection due to low levels of PD-L1 expressed in this location (248); therefore, understanding the dynamic expression of both PD-1 and PD-L1 in various tissues and how that impacts the efficacy of targeting this pathway are critical.

Approaches to engage PD-1 in patients to suppress T cell effector functions during autoimmunity

Strategies to engage inhibitory receptors to treat autoimmunity are highly sought after clinically due to the antigen-specific manner in which these receptors signal. T cells

must receive the PD-1 signal in conjunction with the antigen-specific T cell receptor signal to suppress T cell functions (166). Therefore, PD-L1 and peptide/MHC must be co-expressed by the same antigen presenting cell (in cis) to signal through PD-1 (166). While this signaling requirement is attractive when considering antigen-specific therapies because PD-1 will be engaged only on activated T cells and in an antigen dependent manner, the clinical feasibility of targeting antigen to the same cells expressing PD-L1 is complicated. Using a mouse model of multiple sclerosis (experimental autoimmune encephalomyelitis or EAE), Hirata and colleagues developed a strategy to provide the PD-1 signal to T cells in cis with T cell receptor engagement (249). In this study dendritic cells were engineered to express high levels of PD-L1 and myelin oligodendrocyte glycoprotein peptide presented in MHC class II. The engineered dendritic cells significantly reduced the severity of EAE and reduced the amount of cellular infiltrate in the spinal cord. The results of this study are encouraging and highlight the therapeutic potential of engineering dendritic cells as a strategy to engage PD-1 on autoreactive T cells in a clinical setting. However, the antigen-specific nature of the PD-1 inhibitory signal could also be an obstacle, since the immunodominant epitopes would need to be identified prior to treatment. Furthermore, epitope spreading could lead to a wide diversity of epitopes as disease progresses, making it difficult to identify newly emerging antigenic targets.

An additional approach to target these receptors is through membrane-bound agonists. Membrane-bound agonists are required to induce a signal through inhibitory receptors including PD-1 and CTLA-4 because these receptors must be cross-linked

through ligand to function. This concept has been most evident through work with CTLA-4. Soluble CTLA-4-Ig is not capable of cross-linking CTLA-4 on the surface of T cells in vivo; however, expressing a single-chain anti-CTLA-4 construct from the cell surface can engage CTLA-4 and deliver an inhibitory signal (250, 251). Using this approach, we demonstrated that B cell expression of anti-CTLA-4 single chain effectively reduced autoimmune diabetes incidence and decreased insulitis in NOD mice (251). This strategy could be pursued for PD-1 engagement to inhibit autoreactive T cells in vivo. Further development of this approach to engage PD-1 in clinical applications will depend upon efficient gene transfer technology and expression by antigen presenting cells in vivo. One strategy for ectopically expressing membrane-bound PD-1 agonists is delivery of genes via viral vectors. Ding and colleagues expressed full length murine PD-L1 in recombinant adenovirus to treat lupus-like syndrome in mice (252). Since the authors detected high levels of PD-L1 expression on renal proximal tubular epithelial cells, they postulated that PD-L1 was suppressing autoreactive T cells in the autoimmune target organ (252). One advantage of using viral vectors is that they can be engineered to be highly tissue specific by manipulating the viral capsid or using a tissue-restricted promoter to express virally-encoded transgenes. However, caution should be taken to ensure patient safety when considering the use of the viral vectors for clinical purposes. These findings highlight the complexities of engaging inhibitory receptors to inhibit T cell functions.

Can the PD-1 pathway be targeted to restore tolerance in Type 1 Diabetic patients?

In order to engage PD-1 on a T cell to deliver an inhibitory signal, PD-L1 and peptide/MHC must be expressed by the same cell, and that cell has to be capable of presenting antigen to the T cell of interest. It is likely that due to MHC restriction, $CD4^+$ T cells will be more limited in their ability to receive an inhibitory signal than $CD8^+$ T cells due to the more restricted expression of MHC class II compared to MHC class I (Figure 5-1). $CD4^+$ and $CD8^+$ T cells are both required during Type 1 Diabetes development; however, the specific timing and location in which these cells are required for pathogenesis may provide insight into how these cells should be targeted therapeutically. At least in NOD mice, $CD4^+$ T cells are critical for initiating Type 1 Diabetes, but $CD8^+$ T cells are more important at the later stages of diabetes for directly killing the insulin-producing cells. Therefore, it could be reasoned that targeting PD-L1 to beta cells would be sufficient to protect islets from $CD8^+$ T cell-mediated apoptosis since these cells also express high levels of MHC class I presenting diabetogenic peptides (Figure 5-1). Additional work is needed to define the temporal and spatial requirement for islet-reactive $CD4^+$ and $CD8^+$ T cells in humans to determine the efficacy of targeting these populations to protect pancreatic islets. Since immunotherapy will likely be used in conjunction with islet replacement therapy such as an islet transplant, PD-L1 or agonist anti-PD-1 could be expressed on the islets prior to transplant (e.g. through delivery of genes encoding these molecules by a virus) to protect the graft. These therapies hold promise for Type 1 Diabetic patients that someday we may have a cure.

AUTOIMMUNITY AS A SIDE EFFECT OF USING NEUTRALIZING ANTIBODIES TO BLOCK PD-1 IN PATIENTS TO RESTORE PROTECTIVE IMMUNITY

Our long term goal as a lab has been to understand how PD-1 operates to suppress T cell functions in the context of autoimmunity with the hope of exploiting this pathway therapeutically to suppress diabetogenic T cell responses in patients. However, due to the complexities of engaging PD-1 on a T cell to deliver an inhibitory signal, there has been limited progress in exploiting this pathway to restore tolerance in patients with autoimmunity. Rather, the greatest progress in terms of targeting this pathway for therapeutic benefit has been administering neutralizing antibodies to block PD-1 signaling during chronic viral infections and various malignancies (253-258). In both cases, chronic antigen engagement leads to a progressive loss of function and ultimately the failure to eliminate the virus or the tumor (148, 159). This loss of function, termed exhaustion, has been best defined for $CD8^+$ T cell responses, which are essential for direct killing of peptide/MHC class I-expressing virus-infected cells or tumor cells (179). While PD-1 blockade has been promising to revitalize exhausted $CD8^+$ T cells to aid in clearance of the tumor or virus, there is substantial concern that anti-PD-1 will elicit an autoimmune response in these patients. As immunologists studying the impact of PD-1 signaling on autoreactive T cells, we have a responsibility to address these issues and provide an assessment of risk involved in anti-PD-1 treatment. The goal of this section is to address this issue by first outlining how PD-1 blockade is thought to revive $CD8^+$ T cell responses focusing on chronic viral infections, then examining data from current

clinical trials targeting PD-1 and/or CTLA-4, and lastly discussing the risk of anti-PD-1 eliciting an aberrant autoimmune response in patients considering the data presented in Chapter 4 suggesting that PD-1 blockade more potently targets autoreactive effector T cells than anergic T cells.

Blocking PD-1 using neutralizing antibodies revitalizes CD8$^+$ T cell responses and enhances anti-tumor or anti-viral immunity in patients

In order for PD-1 to deliver an inhibitory signal to a T cell, the T cell receptor must simultaneously be engaged by peptide/MHC on the same cell expressing PD-L1 (166). During chronic viral infections and cancer, antigen-specific PD-1-expressing T cells encounter PD-L1 on cognate peptide/MHC-bearing infected cells or tumor cells (148). The chronic signaling through PD-1 leads to functional exhaustion of antigen-specific T cells (148, 158, 259). Because PD-1 and the T cell receptor are persistently engaged, administration of anti-PD-1 or anti-PD-L1 can block this inhibitory signal and release the cell from negative regulation. Evidence from both mouse and human systems has demonstrated that PD-1 blockade can restore function to CD8$^+$ T cells, leading to enhanced production of inflammatory cytokines and cytotoxic capacity and subsequently control of viral titers as well as reduced tumor burden (148, 158, 159, 260). These preclinical data have generated substantial interest in targeting this pathway to enhance immunity in patients with various malignancies or chronic viral infections.

Work in mouse models using chronic LCMV infection (Clone 13) has shown that while PD-1 blockade can restore T cell effector functions and ultimately lead to enhanced

control of the virus, there is a subset of cells that cannot be functionally restored (180). What factors dictate whether a cell will be susceptible to being invigorated by PD-1 blockade are unclear, but is currently an active area of research. One hypothesis is that as cells constantly encounter antigen and PD-1 signaling, they become progressively more exhausted and subsequently more terminally differentiated (179, 226). Once the cells reach a certain threshold, they can no longer be rescued by PD-1 blockade. One approach to overcome this limitation and restore function to a greater fraction of the population is blocking multiple inhibitory receptors, such as CTLA-4 or LAG-3 (259). Evidence in murine models has suggested that there is synergy between multiple inhibitory receptors including PD-1 and LAG-3, allowing blockade of multiple receptors to be more effective than blocking either molecule individually (259). It should be noted, however, that it remains to be determined whether those cells that cannot be rescued by PD-1 blockade alone are worth being rescued by combination therapy. It is possible that these cells are in such a dysfunctional state that restoration of function would be more detrimental than beneficial for the host. Gaining a deeper understanding of exhaustion from other stand points than simply effector function, such as metabolism and DNA damage, will be essential to determining the importance of these cells to anti-viral or anti-tumor immune responses.

Use of neutralizing antibodies to block PD-1 in patients to restore protective immunity during malignancies and chronic viral infections

Anti-PD-1 or anti-PD-L1 therapy to enhance immunity is currently in clinical trials for a variety of malignancies including advanced melanoma, non-small cell lung cancer, castration-resistant prostate cancer, renal cell cancer, and colorectal, ovarian, pancreatic, gastric, and breast cancers (253-255, 257, 258), and trials are beginning for chronic viral infections including hepatitis C (HCV) (256). Trials with anti-PD-1 in humans have been highly anticipated due to early successes targeting CTLA-4 with the monoclonal antibody ipilimumab (261). Based on work in murine models, PD-1 blockade was speculated to be more efficacious at restoring T cell functions than CTLA-4, particularly in non-lymphoid tissues where both PD-1 and importantly its ligand PD-L1 are highly expressed (262). While CTLA-4 is an important regulator of T cell functions, it currently hypothesized that CTLA-4 plays more of a role in suppressing functions in secondary lymphoid organs than non-lymphoid tissues (262). Early results using anti-PD-1 in tumors and HCV have been promising. In tumor trials anti-PD-1 has been well tolerated and has shown strong evidence of anti-tumor activity. Importantly, in one study where patients achieved objective responses during treatment, following cessation of therapy these responses were sustained for an additional three years (257). This result was exciting because it suggested that the effects of anti-PD-1 were durable and subsequently prolonged treatment could be avoided. In the HCV trial, anti-PD-1 led to a substantial reduction in viral load, even in patients that had failed to respond to Type I IFN therapy (256). In this study six patients showed signs of immune-related adverse events, including low levels of hyperthyroidism. Whether the hyperthyroidism was due to pre-existing autoimmunity or a new autoimmune response was unclear. A recent clinical

trial was conducted neutralizing both PD-1 (with nivolumab) and CTLA-4 (with ipilimumab) in patients with advanced melanoma. In this study, 53% of patients had an objective response, all of which experienced at least an 80% reduction in tumor burden (263). These results were superior to treating with either agent alone. Additionally, the safety profile of this combined therapy was manageable, with adverse effects not more severe than either therapy alone (263). These data have generated optimism that combination therapy to block inhibitory receptors in patients will be able to eradicate tumors and chronic viral infections.

Assessing the risk of eliciting an autoimmune response following anti-PD-1 therapy to enhance anti-viral or anti-tumor immunity in patients

While the early clinical trial data for PD-1 blockade have been promising, there is substantial concern for treatment eliciting autoimmunity. While autoimmunity will always be a potential risk for immunotherapies aimed at stimulating protective responses, relative risk assessments could be made to help patients make informed decisions on treatment options based on preclinical models. Based on the data presented in this thesis, I speculate that the risk of PD-1 eliciting autoimmunity is going to be very low. By studying islet-reactive $CD4^+$ T cells under physiological contexts, the role of PD-1 in the induction and maintenance of tolerance is not as critical as was originally anticipated based on work using highly non-physiological models of Type 1 Diabetes pathogenesis (110, 251). PD-1 is important for suppressing autoreactive T cell functions; however, it is uniquely involved in suppressing effector phenotype cells rather than anergic cells. I

further speculate that anti-PD-1 will not be able to restore functionality to chronically stimulated islet-reactive $CD4^+$ T cells that arise in the secondary lymphoid organs of older NOD mice, though this hypothesis remains to be fully tested. Therefore, I postulate that the only patients at risk of developing autoimmunity following anti-PD-1/PD-L1 treatment are those that have an activated, effector-like population of autoreactive T cells (Figure 5-2). All healthy individuals have autoreactive T cells in circulation (121-123). If these cells are naïve, blocking PD-1 will not have deleterious effects because it is not expressed on naïve T cells (Figure 5-2). If these cells have encountered antigen in a tolerogenic context and have been rendered anergic, blocking PD-1 will not have deleterious effects because PD-1 is not capable of restoring functionality to these cells (Figure 5-2). If these cells became activated but were persistently stimulated for several years without causing overt autoimmunity and eventually became functionally exhausted, I speculate that anti-PD-1 will also not restore functionality to those cells (Figure 5-2). Only if there are activated effector cells will therapeutic PD-1 blockade amplify the autoimmune response (Figure 5-2), and improvements on tetramer technology and the ability to track these cells in humans will greatly improve our ability to identify these patients prior to the initiation of therapy. One issue of particular concern that cannot be over-looked is immunopathology, since release of sequestered antigens due to enhanced killing by revitalized $CD8^+$ T cells may lead to activation of naïve autoreactive T cells, and these recently activated cells would be susceptible to enhanced functionality caused by PD-1 blockade. On-going results from clinical trials with anti-PD-1 will be informative in testing the hypotheses presented above. Ultimately clinical trials targeting

PD-1 should move forward despite the concern for eliciting autoimmunity in patients, since targeting one inhibitory pathway is unlikely to cause enough amplification of an autoimmune response to induce clinical symptoms of autoimmunity.

CONCLUSION

The importance of the inhibitory receptor PD-1 interacting with its ligand PD-L1 in limiting Type 1 Diabetes progression is well established. However, this pathway is not sufficient to prevent diabetes in the 60-80% of female mice that eventually develop the disease. In this work, I examined the role of PD-1 in regulating islet-reactive $CD4^+$ T cells using experimental systems that more accurately mimic physiological disease progression than previous models. By examining these $CD4^+$ T cell populations, it became evident that the frequency of pathogenic $CD4^+$ T cells in the islet-reactive pool was much smaller than originally expected. Following activation in the pancreatic LN, the majority of these cells were rendered anergic. The critical finding of this work was that PD-1 was not required to induce or maintain this tolerant state. Rather, PD-1 was more important for controlling the subset of effector phenotype cells that naturally arises in NOD mice. These results have important implications for immunotherapy. In terms of inhibiting the on-going islet-specific immune response, it is now appreciated that this population is numerically smaller than anticipated, providing renewed hope for patients undergoing anti-islet immunotherapy. In terms of targeting PD-1 to induce tolerance, it is unclear whether specifically ligating PD-1 on the islet-reactive T cells could provide therapeutic benefit. Considering the fact that PD-1 appears to be more important for

regulating pathogenic effector cells than anergic cells, PD-1 ligation in the pancreas may be effective to suppress the functions of this pathogenic subset. Lastly, I speculate that the risk of eliciting an autoimmune response when blocking PD-1 clinically to enhance immunity during chronic viral infections and tumors will be very low, since in order for therapeutic blockade of PD-1 to amplify an autoimmune response patients would require a pre-existing population of activated effector cells that were not being adequately restrained by peripheral tolerance (Figure 5-2). Future work addressing what factors contribute to the induction and maintenance of the anergic population independently of PD-1 will be informative to develop novel therapeutic approaches to promote tolerance clinically (Figure 4-18).

FOOTNOTES

[1]Portions of this work have been previously published. Reprinted from *Annuals of the New York Academy of Sciences*, Volume 1217, Brian T. Fife and Kristen E. Pauken, **The role of the PD-1 pathway in autoimmunity and peripheral tolerance**. p. 45-59. 2011. Permission pending.

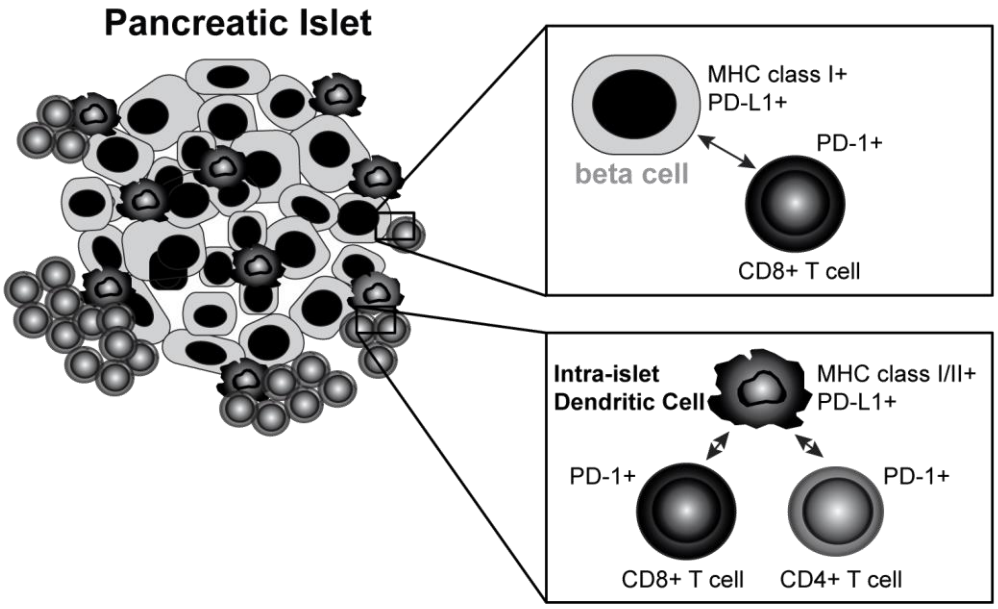

Figure 5-1: Model for differential regulation of PD-1/PD-L1 in pancreatic islets

PD-L1 is expressed on both intra-islet dendritic cells and insulin-producing beta cells. However, due to the requirement for PD-1 to be engaged in cis with the T cell receptor to deliver an inhibitory signal to T cells, PD-L1 and peptide/MHC must be expressed by the same cell to regulate T cells in the islet. Intra-islet dendritic cells express both MHC class II and MHC class I, so PD-L1-expressing dendritic cells could inhibit both MHC class II-restricted $CD4^+$ T cells and MHC class I-restricted $CD8^+$ T cells in the islet. Since insulin-producing beta cells only express MHC class I (but not MHC class II) in NOD mice, PD-L1 expressed by this cell type can only deliver an inhibitory signal to $CD8^+$ T cells in the islet.

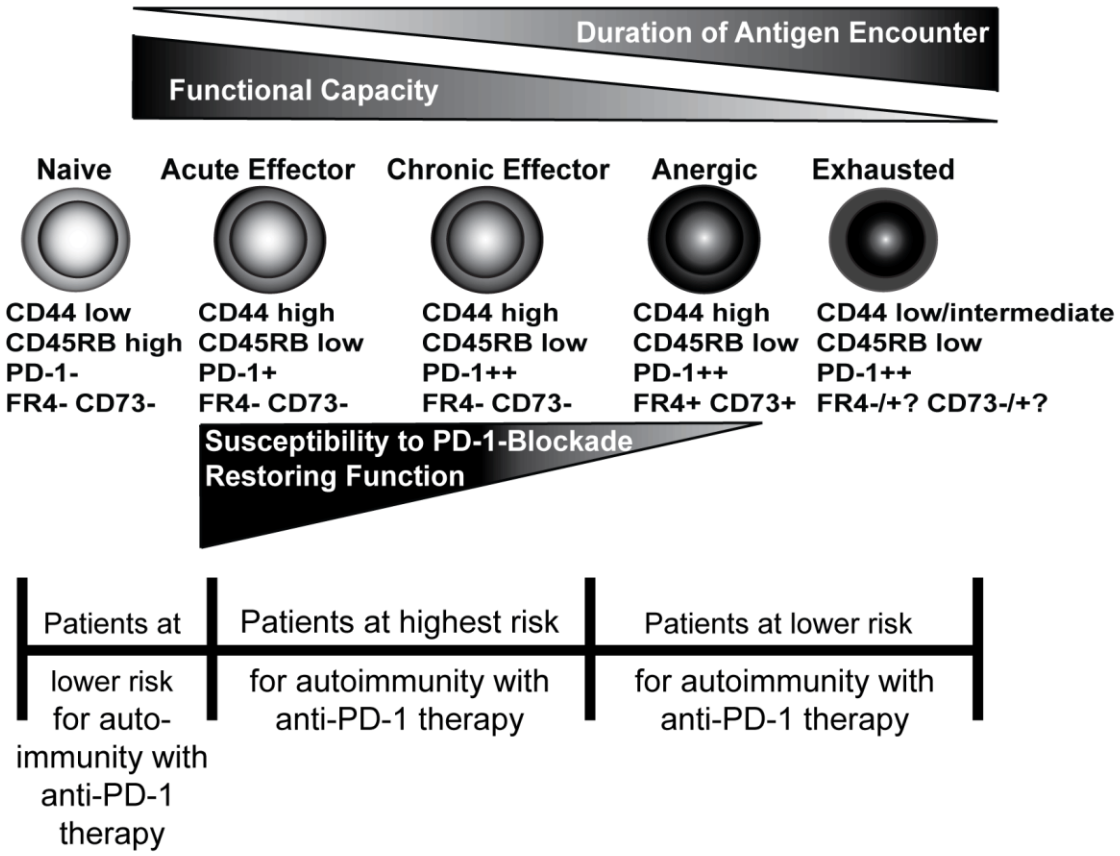

Figure 5-2: Predicted risk levels associated with therapeutic blockade of PD-1 in patients depending on the presence of different autoreactive T cell lineages

We have identified four main lineages of autoreactive $CD4^+$ T cells in diabetic hosts: naïve, effector, anergic, and exhausted. Effector cells can be further subdivided into "acute" or "chronic" effector based on the duration of time the cell has encountered antigen. Naïve T cells do not express PD-1, and therefore are not susceptible to PD-1 blockade. Following activation PD-1 becomes expressed, and remains expressed due to chronic antigen stimulation. Following optimal priming cells differentiate into effector phenotype cells ($FR4^-$ $CD73^-$) which are more functional than their anergic or exhausted counterparts, and remain susceptible to PD-1 blockade. PD-1 blockade leads to enhanced functionality in this population. Following suboptimal activation, anergic phenotype cells ($FR4^+$ $CD73^+$) develop which are functionally impaired. PD-1 blockade has a minimal effect on restoring the functions of these cells. Following persistent antigen engagement, autoreactive T cells can become exhausted, and PD-1 blockade has no effect on this population. Therefore, patients with a population of activated effector autoreactive T cells pose the highest risk for developing overt autoimmunity following therapeutic PD-1 blockade to enhance immune functions. Patients with only naïve, anergic, or exhausted autoreactive T cells have a limited risk of developing autoimmunity following PD-1 blockade.

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