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Therapeutic Interventions to Modulate the Anti-islet Response in Autoimmune Diabetes

by

Cristina Peñaranda Gomez

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

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by

Cristina Peñaranda Gomez

To my mom

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CONTRIBUTIONS OF CO-AUTHORS

Chapters I and V of this dissertation were written entirely by me under the supervision of Jeffrey A. Bluestone. The section entitled "Regulatory T cells in autoimmunity" in Chapter I is an excerpt of material to be published in Immunological Reviews (2011) entitled "Intrinsic and extrinsic control of peripheral T cell tolerance by costimulatory molecules of the CD28/B7 family".

Chapter II is co-authored with Qizhi Tang (UCSF), Helene Bour-Jordan (UCSF) and Jeffrey Bluestone (UCSF). Qizhi Tang performed experiments shown in Figure 5 A-D. I performed all other the experiments under the supervision of Jeffrey A. Bluestone and wrote the manuscript with the help of Helene Bour-Jordan.

Chapter III is based on material published in the journal Diabetes (2010, Jun;59(6):1461-8) entitled "Prevention of Diabetes by FTY720-Mediated Stabilization of Peri-Islet Tertiary Lymphoid Organs" with co-authors Qizhi Tang (UCSF), Nancy Ruddle (Yale University) and Jeffrey Bluestone (UCSF). I performed all the experiments and wrote the manuscript under the supervision of Jeffrey Bluestone.

Chapter IV is co-authored with Wilson Kuswanto (UCSF), Jerry Hofmann (UCSF), Rupert Kenefeck (University of Birmingham, UK), Parth Narendran (University of Birmingham, UK), Lucy S.K. Walker (University of Birmingham, UK), Jeffrey A. Bluestone (UCSF), Abul K. Abbas (UCSF), and Hans Dooms (UCSF). Rupert Kenefeck and Parth Narendran performed the experiments shown in Figure 22 under the supervision of Lucy S.K. Walker. Hans Dooms and I, with help from Wilson Kuswanto and Jerry Hofmann performed all other experiments. The study was designed by Hans

Dooms with input from Abul K. Abbas, Jeffrey A. Bluestone and me. Hans Dooms wrote the results section. I wrote the introduction and discussion sections.

ABSTRACT

Autoimmunity is caused by a loss of tolerance and results in an imbalance in immune homeostasis, particularly regulatory versus effector activities against self-antigens. Therapeutic approaches for the treatment of autoimmune diseases need to attack key components of the immune response to restore this balance. In this body of work I have worked toward this end using three distinct approaches to in the context of type 1 diabetes using the non-obese diabetic mouse model. First, I used a global approach to shift the balance of regulatory to effector T cells. I show that non-Fc receptor binding anti-CD3 monoclonal antibody treatment, which has been previously shown to reserve diabetes, alters the ratio of effector to regulatory T cells (Tregs) due to preferential depletion of activated effector T cells. In addition, treatment with anti-CD3 mAbs leads to increased expression of Helios in Tregs, suggesting stabilization of Tregs may account to the extended efficacy of this antibody. Second, I used FTY720 to "lock" lymphocytes in the lymph node and pancreas thereby isolating these locations. I show that continuous treatment with FTY720 prevents diabetes development but treatment withdrawal leads to rapid onset of disease. Furthermore, morphological changes that occur during the development of the disease in control and treated mice show that tertiary lymphoid organ development and their subsequent destruction correlate with disease progression suggesting that islet destruction maybe be due to loss of TLO integrity. Finally, I used a targeted approach to directly alter memory T cells. I show that treatment of NOD mice with anti-IL7Rα monoclonal antibodies prevents and cures diabetes and induces expression of the inhibitory receptor PD-1 on memory T cells. Moreover, inhibiting the

interaction of PD-1 with its ligand PD-L1 restores disease in cured mice. The data suggest that IL-7 contributes to the pathogenesis of autoimmune diabetes by keeping memory T cells in a functionally competent, tolerance-resistant state, and uncover a novel link between IL-7 and the PD-1/PD-L1 tolerance pathway. Together the data reveal biological the mechanisms at the basis of therapeutic interventions aimed at preventing or reversing diabetes through manipulating the immunological pathways underling the pathogenesis of the disease. The insights will improve our ability to translate these interventions to the treatment of patients with type 1 diabetes.

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CHAPTER I

Introduction

Tolerance and Autoimmunity

The immune system has the difficult task of mounting responses to thousands of potentially pathogenic antigens that come in contact with the host whilst not reacting to self-antigens. Autoimmunity is the result of an active immune response against self-antigens caused by the loss of tolerance. Two mechanisms of tolerance, central and peripheral, have evolved to prevent autoimmunity from occurring. Central tolerance involves the deletion of self-reactive T cells in the thymus and B cells in the bone marrow. However, some self-reactive lymphocytes are able to escape this selection and must be controlled by mechanisms of tolerance in the periphery. These include regulatory T cells, induction of anergy or deletion upon antigen encounter, production of suppressive cytokines and expression of inhibitory receptors on autoreactive T cells. One case of autoimmunity that is widely studied is type 1 diabetes (T1D) caused by an attack on the insulin producing β cells in the pancreas. In the United States type 1 diabetes accounts for 5-10% of all diagnosed cases of diabetes, about 1-2 million people, and the incidence is on the rise[1]. This disease is usually diagnosed in childhood to early adulthood and patients usually require exogenous insulin injections for the rest of their lives. The nonobese diabetic (NOD) mouse is a well-established spontaneous model of autoimmune type 1 diabetes, which closely mimics the human disease[2]. Makino and colleagues identified this mouse in Japan from a line of Jc1:ICR mice that were being bred to develop a cataract-prone strain. A subline from this screen was found to

spontaneously developed diabetes and has since been used as one of the main mouse models for type 1 diabetes. Although there are many similarities between disease development in humans and the NOD mice, as will be further discussed, two main differences are the onset of disease development and the sex skewing seen in the mouse model. In humans disease occurs before or during puberty while in NOD mice it occurs much later in adult mice. Sex skewing resulting in higher incidence in females versus male is seen in NOD mice, but not in humans. It is not understood what accounts for these differences.

Genetic and environmental factors that contribute to autoimmune diabetes

Both genetic and environmental factors contribute to development of type 1 diabetes. In fact, the concordance rate for identical twins is 40-50%[3] and only about 15% of patients have a first degree relative who also has the disease[4], highlighting that this is heterogeneous and multigenic disease. Genome-wide association studies (GWAS) have found that certain human leukocyte antigen (HLA) polymorphisms, in particular in the DR3 and DR4 genotypes[5], are the highest risk for development on T1D; interestingly other HLA alleles have been found to offer significant protection. However, there are over 50 other regions, many of which encode proteins involved in the immune system, that have also been shown to increase the risk for T1D[6, 7].

The genetic loci that contribute to diabetes susceptibility in NOD mice have been widely studied. So far, at least 20 chromosomal regions, termed Idd loci, have been shown to be necessary for disease development by outbreeding to diabetes resistant strains such as C57/B6; however the specific gene or mutation responsible for the

increased susceptibility in many of these regions has not been identified. Interestingly, many of these have also been identified in human patients through GWAS. Similar to findings in the human disease, it has been shown that the major histocompatibility complex (MHC) haplotype H2g7 is the single most important genetic contributor in the NOD mouse[8]. Crystal structure of this MHC suggests that MHC:peptide complexes are unstable and may result in poor negative selection in the thymus leading to an increase in auto-reactive T cells in the periphery[9, 10]. In addition, it has been suggested that the peptides presented by this MHC are different from those presented by other MHC molecules. One of the most studied and controversial Idd loci is Idd3, which located on chromosome 3 and includes the genes encoding IL-2 and IL-21. GWAS studies found single nucleotide polymorphisms (SNPs) in the syntenic region, which also includes the IL-2 and IL-21 genes, on human chromosome 4q27 that are significantly associated with T1D[11]. Interestingly, GWAS studies have also found SNPs in the IL-2Rα/CD25 gene as well as PTNP22[12], which likely influences IL-2 signaling, suggesting that dysregulation of the IL-2 signaling pathway may be a key factor in diabetes development.

The environmental factors that contribute to T1D development are poorly understood. The increase in incidence of T1D, as well as other autoimmune diseases, in industrialized countries has given rise to the "hygiene hypothesis" which suggests that lack of exposure to infectious agents, such as parasites, during childhood alters the immune system and predisposes humans to allergies and autoimmune diseases. It has been suggested that viral infections of β cells may lead to cell death, which starts the immune response; viral infections may also activate islet antigen specific T cells due to

antigenic mimicry. However, specific viruses capable of inducing type 1 diabetes have not been isolated.

Environmental factors have also been shown to affect disease incidence in NOD mice. Colonies maintained in specific pathogen free facilities have higher incidence than those kept in "dirtier" facilities[13]. In addition, mice exposed to microbial stimuli, such as infection with schistosoma, mycobacteria or viruses, also show decreased incidence[14]. It is not clear why this occurs, although it is thought that they lead to immune deviation towards a global Th2 response rather than the Th1 response that is seen, as will be discussed later. Interestingly, it was recently shown that NOD mice lacking the toll-like receptor adaptor protein MyD88 are protected from disease development. This effect was shown to depend on intestinal commensal microbes because germ-free mice had normal disease incidence[15]. It is unclear whether this is due to a specific species of commensal or whether these same effects are seen in human patients.

Pancreatic infiltration in autoimmune diabetes

Diabetes, both in humans and NOD mice, is characterized by an attack mediated by lymphocytes on the pancreatic insulin-secreting β cells that eventually leads to a lack of insulin production and hyperglycemia. Lymphocytes, as well as macrophages, infiltrate the islets of NOD mice as early as 3 to 4 weeks of age. The lymphocytes continue to accumulate surrounding the islets, form organized tertiary lymphoid organs (TLOs), and by 15 to 25 weeks of age, completely invade the islets and destroy them. Loss of blood sugar control occurs when approximately 70-80% of islets have been destroyed[16].

Analysis of pancreas infiltrates of human diabetic patients has been problematic since most studies have been done years after disease diagnosis. Recently, Willcox et al analyzed pancreas samples by immunohistochemistry from patients who died within 18 months of diagnosis[17]. In contrast to the large accumulations of lymphocytes found next to islets of diabetic NOD mice, only scattered infiltrating cells were found surrounding glucagon or insulin positive islets. $CD8^+$ cells were most abundant but $CD68^+$ macrophages, $CD20^+$ and $CD138^+$ B cells and $CD4^+$ T cells were also present. It is possible that even this time point is too late and most of the infiltrate has disappeared due to antigen clearance.

Although all NOD mice show some level of lymphocyte infiltration, only 60 to 80% of females and 20 to 30% of males actually show invasion and destruction of islets and go on to develop hyperglycemia[18]. Therefore, the autoimmune destruction of pancreatic islets in NOD mice has been suggested to include two checkpoints: checkpoint 1 or insulitis, which involves infiltration of lymphocytes into the pancreas, and checkpoint 2 or islet invasion, which involves killing of β cells in the islets and results in hyperglycemia[19]. Insulitis has been shown to be the result of high turnover of β cells, which leads to a release of self antigens into the circulation of mice at around 3 to 4 weeks of age[20]. However, what controls invasion of lymphocytes into pancreatic islets and their destruction, leading some mice to develop overt diabetes but not others, is not understood.

Interestingly, it has been shown that the pancreatic draining lymph node is not necessary after 4 weeks of age for disease development in the NOD mouse model[21]. Lymph node removal at 3 weeks of age greatly reduced diabetes incidence (from 100% to

20% at 28 weeks of age), while lymph node removal at 4 weeks of age only slightly reduced diabetes incidence (from 60% to 40% at 30 weeks of age). However, lymph node removal at 10 weeks of age had no effect on disease development. Importantly, protection from disease after early removal of the PLN was not due to modifications of the immune system such as removal of autoreactive T cells or increase in regulatory T cells. These data illustrate that, while the draining pancreatic lymph node is required for checkpoint 1 in disease (insulitis), it is dispensable for checkpoint 2 (islet destruction). Furthermore, they suggest that the events required to overcome checkpoint 2 are in the pancreas, presumably, in the tertiary lymphoid organs.

Immune cell subsets and their role in autoimmune diabetes

The role of B cells in the development of type 1 diabetes in both human and NOD mice is still controversial. Autoantibodies to islet antigens, such as insulin, GAD65 and insulinoma antigen-2 (IA-2), are found in patients at both preclinical and clinical stages of the disease and seropositivity to multiple antigens increases the risk of disease development[22]. Interestingly, NOD mice have also been shown to develop autoantibodies to the same antigens as humans suggesting that specificity may be important in disease development[4]. However, autoantibodies are also found in individuals and mice that never develop clinical symptoms, so it is unclear whether these autoantibodies are pathogenic.

NOD mice carrying a null immunoglobulin μ allele (NOD.Igμnull), which have a block in B cell development, are protected from both insulitis and diabetes[23]. In addition, deletion of B cells by antibody depletion prevents diabetes development[24]. However, transfer of B cells to lymphopenic recipients or serum from autoantibody positive mice is

not able to induce disease development. Furthermore, T cells isolated from diabetic mice transferred to lymphopenic recipients can cause disease in the absence of B cells, suggesting that B cells are not required at the effector stage of disease. Therefore, it has been suggested that the main role of B cells is as antigen-presenting cells during T cell activation. In fact, islet reactive T cells were present at lower levels in NOD.Igμnull compared to NOD mice suggesting a defect in T cell expansion[25]. In support of this hypothesis, NOD mice that lack MHC class II expression only on B cells are also protected from disease development[26].

Unlike B cells, T cells are both necessary and sufficient for disease development. $CD8^+$ T cells are considered to be more important early in disease by killing islets and allowing for epitope spreading and $CD4^+$ T cell priming. NOD mice deficient in MHC class I or NOD mice treated with depleting anti-CD8 monoclonal antibodies at 2-5 weeks of age are protected from both insulitis and diabetes[27, 28]. The TCR from the 8.3 TCR transgenic mouse, specific for islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP), was cloned from an islet infiltrating $CD8^+$ T cell[29]. These mice develop insulitis and diabetes and transfer of 8.3 TCR transgenic cells to a lymphopenic recipient also leads to diabetes[30]. A tetramer reagent, composed of four MHC class I molecules bound to a peptide recognized by the 8.3 TCR, shows reactivity in spleen, pancreatic lymph node and pancreas of NOD mice, particularly at disease onset, demonstrating that this antigen is one of the major targets of the polyclonal immune response[31]. $CD8^+$ T cells utilize cytolytic effector molecules, such as perforin and granzyme, as well as cytokines, such as IL-1, to kill their targets upon antigen

recognition. Of these mechanisms, perforin has been shown to be important for islet killing as perforin-deficient NOD mice develop insulitis but rarely diabetes[32].

CD4$^+$ T cells are necessary both early and late during disease as their depletion using antibody treatment prevents development and progression of diabetes. In contrast to MHC class I deficient mice, NOD mice deficient in MHC class II develop insulitis but not diabetes. MHC class II restricted TCR transgenic mice have also been developed. BDC2.5 TCR transgenic mice, which are now known to recognize chromogranin A[33], developed aggressive insulitis and accelerated diabetes when first crossed to the NOD background[34]. However, upon further backcrossing to the NOD background the accelerated diabetes was lost. Purified BDC2.5 TCR transgenic T cells can cause disease upon transfer to a lymphopenic recipient, but not to an immunocompetent recipient, unless they are previously activated *in vitro* with their cognate peptide[35].On a Rag sufficient background, BDC2.5 transgenic mice develop Foxp3$^+$ Tregs which are capable of preventing and curing disease in intact NOD mice[36]; on the other hand, on a Rag deficient background, BDC2.5 transgenic mice become diabetic very quickly. It has been suggested that this occurs due to a lack of Tregs or due to the inability of T cells to use alternative alpha chains, thereby increasing the proportion of bona fide transgenic T cells. MHC class II dimers specific for BDC2.5 cells have also been developed and are reactive against a population of CD4$^+$ cells in intact NOD mice[37, 38].

Like many organ-specific autoimmune diseases, T1D is considered to be caused primarily by Th1 cells. IL-12 production by antigen presenting cells such as dendritic cells is a potent inducer of Th1 cells and is necessary for proper Th1 skewing by inducing the expression of the transcription factor Tbet. Th1 cells produce IFNγ, which activates

macrophages and promotes differentiation of B cells and isotype switching to IgG2a. NOD mice deficient in Tbet are completely protected from insulitis and diabetes, likely due to defects in both T cells and dendritic cells[39]. Similarly, mice deficient in Stat 4, which mediates IL-12 signaling, are mostly protected from insulitis or diabetes[40]. Interestingly, IL-12, IFNγ or IFNγR deficient mice develop diabetes normally suggesting that other cytokines can compensate for their biological activities *in vivo*[41-43].

Regulatory T cells in autoimmunity

$CD4^+$ regulatory T cells (Tregs) are one of the mechanisms of peripheral tolerance developed by the immune system to control responses against invading pathogens and prevent self reactivity. Importantly, they represent a mechanism of lasting dominant tolerance that can be transferred to a new host. Their unique expression of the transcription factor Foxp3, constitutive high levels of CD25 and their functional ability to suppress T cell responses *in vivo* and *in vitro* are hallmarks of this T cell subset. Importantly, Tregs are dependent on IL-2 although they do not produce IL-2. In fact, Foxp3 in cooperation with Runx1 and NFAT binds to the IL-2 promoter and inhibits its transcription[44,45]. Mutations in the gene encoding the Foxp3 protein in humans result in Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome, a multi-organ autoimmune disease, and similar mutations in mice result in the scurfy phenotype[46]. Likewise, absence of Tregs is the consequence of the multi-organ autoimmunity seen in mice with defective IL-2 signaling as the transfer of WT Tregs to mice deficient in IL-2Rα or IL-2Rβ prevents lymphoproliferation and autoimmunity[47, 48].

Natural Tregs (nTregs) arise in the thymus during the CD4 single positive stage. They are thought to be the result of positively selected $CD4^+$ T cells that recognize self antigens; interestingly, their TCR repertoire differs from $CD4^+$Foxp3- conventional T cells[49] suggesting that commitment towards a Treg lineage is a programmed rather than a random event. The majority of Tregs exit the thymus between day 3-5 post-birth in the mouse. This explains the observation that day 3 thymectomy (d3Tx) results in multi-organ autoimmunity[50]. Few $Foxp3^+$ cells can be found in the periphery of d3 Tx mice and their disease can be prevented by transfer of WT $CD4^+CD25^+$ Tregs[51].

Interestingly, it has been shown that Treg development and homeostasis is critically dependent on costimulatory molecules, in particular CD28. The surprising finding that CD28 or B7-1/B7-2 deficient mice on the NOD background developed accelerated, rather than delayed, disease was due to a marked decrease in Tregs both in the thymus and the periphery likely due to defects in IL-2 production[52, 53]. These findings led to in depth investigation of Treg function in NOD mice. Co-transfer of $CD4^+CD25^+$ cells from 8-10 week old but not 16 week old mice was found to delay disease caused by transfer of diabetogenic splenocytes to lymphopenic hosts[54], suggesting that “old” or “diabetic” Tregs may be defective compared with “young” Tregs.

Nonetheless, the role of Tregs in human diabetes is still controversial. Although initially it was reported that new onset diabetic patients had reduced frequency of $CD4^+CD25^+$ compared to healthy controls, later reports showed no differences of either $CD4^+CD25^{hi}$ or $CD4^+Foxp3^+$ cells [55,56,57]. Defects in Treg suppressive function has also been reported with equally conflicting results. Multiple factors further complicate results from these studies[58]. First, most studies have used Tregs isolated from peripheral blood; it

is not known whether these Tregs are a good representation or behave similarly to Tregs isolated from lymph nodes, or more importantly, from the inflamed tissue. Second, for functional analysis it is impossible to isolate only true Foxp3$^+$ cells since this is an intracellular molecule. Although other surface markers, such as CD127, have been used to exclude activated or memory cells, it is possible that contaminating cells skew the results. In addition, many studies have used *in vitro* expanded Tregs; whether suppressive function post-expansion corresponds to suppressive function *in vivo* is now known. Third, *in vitro* suppressions assays may not be representative of Treg function *in vivo* as the cytokine milieu *in vivo* may affect Treg function. Finally, it is possible that Tregs are not different in numbers or function in patients, but that effector/memory T cells in these patients are resistant to Treg suppression. Indeed, memory cells have shown to be resistant to Treg suppression[59].

We have further studied the relationship between IL-2, Tregs and autoimmunity in the NOD model[60]. We found that at the time of disease onset, the percentage of Tregs preferentially increases in the pancreatic lymph node compared to the inguinal lymph node. Importantly, these Tregs are activated, based on decreased CD62L expression, and functional, based on their ability to prevent proliferation of naïve BDC2.5 transgenic T cells as well as their clustering and swarming behavior around dendritic cells observed by two-photon microscopy. However, analysis of islet infiltrating lymphocytes showed that the percentage of Tregs negatively correlates with the size of the infiltrate such that the larger the infiltrate surrounding an individual islet, the lower the percentage of Tregs. Further analysis of pancreas infiltrating Tregs revealed that they have lower expression of CD25 and Foxp3 compared to PLN or ILN Tregs and lower expression of Bcl-2, which is

an IL-2 driven anti-apoptotic effector molecule. $CD4^+$ T cells isolated from the islets produced significantly lower amounts of IL-2 mRNA in response to restimulation compared to $CD4^+$ T cells from the spleen suggesting that lack of IL-2 in the inflamed tissue may contribute to changes in the phenotype and function of Tregs. Importantly, treatment of prediabetic NOD mice with low dose anti-IL-2/IL-2 complexes prevented diabetes development and increased the percentage of Tregs in the pancreas as well as their CD25 expression. We theorized that decreased IL-2 production in the pancreas leads to decreased CD25 expression in pancreas resident Tregs which results in poor survival and/or function of these cells, contributing to deregulated autoreactive T cells and destruction of the tissue. In addition to being unable to control autoreactive T cells, CD25 low Tregs may become unstable. In fact, we have also showed that Foxp3 expression can be lost on Tregs, and since the Treg repertoire is skewed towards self-reactivity, this may result in a very pathogenic population[61].

Whether the decrease in CD25 expression seen in pancreas resident Tregs of NOD mice is specific for this model or it can be generalized for other autoimmune diseases is still controversial. Lazarski et al reported that Tregs in the dermis after intradermal OVA-CFA challenge also had lower CD25 expression and concluded that this was a general phenomenon in inflamed tissues rather than a characteristic of autoimmunity[62]. However, in double transgenic mice expressing OVA in the islets (RIP-OVA) and the OVA-reactive TCR DO11.10, which develop rapid diabetes, pancreas resident Tregs express normal levels of CD25[62]. Therefore, not all Tregs in the context of an inflammatory response lose CD25 expression and in some cases of reduced CD25 expression autoimmunity is not seen.

Therapies for type 1 diabetes

As previously discussed, patients diagnosed with type 1 diabetes go on to require exogenous insulin for the rest of their lives. This therapy does not have negative side effects per se, but some complications do arise because blood glucose needs to be very tightly controlled. Long-term diabetic patients can suffer from nerve and blood vessel damage leading to diabetic retinopathy, neuropathy, perivascular disease and high cholesterol. Furthermore, long-term use of exogenous insulin can be a hindrance on the quality of life, particularly in very young patients. Immunotherapies to treat type 1 diabetes, consequently, need to achieve a balance between suppressing the autoimmune response while allowing protective immune responses against invading pathogens and naturally arising tumors. Importantly, effective therapies will need to avoid the risks of broad immunosuppression, such as cancer and reactivation of latent viruses. Therefore, therapies to specifically control autoreactive T cells will be required to effectively develop a true cure for diabetes.

The first immunotherapies tried to treat type 1 diabetes were broad immunosuppressant therapies, but not surprisingly, these therapies were discontinued due to their limited efficacy and harmful side effects. For example, cyclosporine A treatment within 6 weeks diagnosis lasting up to a year eliminated the need for exogenous insulin[63]. However, effect was not long lasting such that there was a relapse when treatment was discontinued. Furthermore, there was major renal toxicity and the cost of the therapy was very high preventing additional trials. Anti-thymocyte globulin (ATG) plus prednisone was shown to reduce insulin requirements in some patients but severe, though transient, thrombocytopenia outweighed its clinical effects[64]. More directed approaches, such as

elimination of B cells using anti-CD20 monoclonal antibodies (Rituximab) showed a modest but significant improvement in terms of hemoglobin A1c (HbA1c) levels and insulin use[65]. However, the treatment did not seem to induce tolerance and although there was no difference in the rate of neutropenia or infection, there was a reduction in the levels of IgM. Finally, attempts to modulate the immune response by activating the innate immune system have also been done. Although initial studies with bacillus Calmette-Guerin (BCG) vaccination were promising[66], a double-blind clinical trial that included 94 patients within 4 months of onset of symptoms showed that C-peptide secretion, insulin requirements or HbA1c levels were not different between the vaccinated and control patients[67].

A number of clinical trials have been performed to directly affect the autoimmune response against insulin producing β cells. Induction of antigen-specific tolerance to whole insulin was successful in NOD mice but human results have been disappointing. Both oral [68] and nasal [69] administration of insulin to recently diagnosed patients had little or no effect on C-peptide secretion, insulin requirement or β cell function. However, the dose of insulin used in the human trials was 10-100-fold lower per administration relative to body weight compared the NOD studies. Therefore it is possible that this is still an attractive therapy. Indeed, a study in NOD mice showed low doses of subcutaneous insulin had no effect on disease, but a 20-fold increase in the dose protected from disease[70].

A recent review of therapies tried in the NOD mouse model identified 463 agents, or combinations of agents, published from 1980 to mid 2004[71]. Two main conclusions from this literature review are that most therapies had no effect and some even

exacerbated disease and that the timing, frequency and dose of treatment had a great impact on the efficacy of the therapy. The latter conclusion highlights that there are different mechanisms at the various stages of the disease, as has been discussed earlier. Importantly, this literature review also found many variables that confound comparative analysis of agents, including definition of “diabetic” mouse (200, 300 or 400 mg/dL blood glucose), time from diagnosis to treatment initiation, and definition of efficacy (blood glucose below 250mg/dL, independence from exogenous insulin). Therefore standardization of criteria is key to more efficiently and effectively translate these therapies to treatment of patients. It should also be pointed out that most therapies tried in the NOD mouse were aimed at preventing, rather than reversing, disease and most reagents that were tried were unable to reverse disease. Nevertheless, although animal models may not always be the best predictors for effective therapies, they have revealed many important insights into the pathogenesis of the disease and will likely continue to reveal novel pathways suitable for therapeutic interventions.

CHAPTER II

Anti-CD3 mAb therapy promotes tolerance by selectively depleting pathogenic cells while preserving regulatory T cells

INTRODUCTION

Immunotherapy targeting CD3 molecules has shown tremendous promise for the treatment of autoimmune diseases and the prevention of allograft rejection in humans and in murine pre-clinical models[72, 73]. In patients, the anti-CD3 monoclonal antibody (mAb) OKT3 was described as an efficient treatment to prevent acute renal allograft rejection more than twenty years ago[74]. However, the development of OKT3 and other anti-CD3 mAb therapeutics were hampered by serious side-effects associated with a "cytokine storm" released as a consequence of generalized T cell activation[75, 76]. Thus, novel anti-CD3 mAb reagents were developed to avoid the detrimental consequences secondary to the cross-linking of the T cell receptor (TCR)/CD3 complex by the OKT3 mAbs bound to Fc receptors (FcR) on antigen-presenting cells. Remarkably, these FcR-non-binding (FNB) anti-CD3 mAbs retained their immunosuppressive properties in pre-clinical models *in vivo* without the toxicity associated with the parental anti-CD3 mAb and they demonstrated clinical efficacy in the treatment of acute renal allograft rejection[77-81].

Although FNB anti-CD3 mAbs were initially referred to as non-mitogenic, it is now clear that these reagents are not passive blockers of TCR:MHC interactions but instead trigger altered TCR signaling, compared to classical anti-CD3 mAbs, that leads to functional consequences in T cells[82, 83]. Previous data from our laboratory showed that FNB anti-CD3 mAbs induced IL-4 production and IL-4-dependent proliferation of Th0

or Th2 clones but not Th1 clones *in vitro*[83]. Furthermore, restimulation of bulk T cells previously activated in the presence of FNB anti-CD3 mAbs resulted in decreased IL-2 production, but normal IL-4 production, suggesting that Th1 clones are more sensitive to FNB anti-CD3 mAbs than Th2 clones[83]. Immunoprecipitation of the CD3 ζ chain from T cell clones stimulated with FNB anti-CD3 mAbs showed that the p21 and p23 subunits as well as CD3ε and CD3-associated kinase ZAP70 were phosphorylated, albeit at lower levels than when anti-IgG was added to promote crosslinking[83]. MAP kinase phosphorylation and NFATc translocation to the nucleus were also evident[83], but PLCγ activation and calcium flux were absent[84], showing that some but not all components downstream of CD3 signaling are activated. Interestingly, Th1 clones showed more phosphorylation of all CD3 components than Th2 clones, further demonstrating their increased sensitivity to that FNB anti-CD3 mAbs[83]. These data reveal that FNB anti-CD3 mAbs delivers a partial signal that has different functional consequences depending on the Th phenotype of the cell.

The tolerogenic properties of FNB anti-CD3 mAb without accompanying long-term immunosuppression made these reagents an attractive therapy for T cell-mediated autoimmune diseases. Although over one hundred therapies can prevent the development of diabetes when administered to young pre-diabetic NOD mice, only a handful of treatments can reverse disease in new-onset diabetic mice[71]. Among the latter, administration of anti-CD3 mAb was shown to restore normoglycemia in newly diabetic animals and was unexpectedly more efficient after the clinical onset of disease than in pre-diabetic NOD mice[85, 86]. Importantly, both low dose FcR-binding and FNB anti-CD3 mAb could induce long-term remission in new-onset diabetic NOD mice[85].

Despite the great promises of FNB anti-CD3 mAb treatment in autoimmunity and transplantation, the mechanisms of action of these reagents *in vivo* are still not clearly defined. While both FcR-binding and FcR non-binding anti-CD3 mAb-induced transient T cell depletion in NOD mice, the depletion was more complete with FcR-binding anti-CD3 mAb[85]. Furthermore, FNB anti-CD3 mAb treatment has been proposed to result in the generation of regulatory T cells (Tregs) in some studies[87-89] but not in others[90-92]. $CD4^+CD25^+Foxp3^+$ Tregs can suppress effector T cell (Teff) responses *in vitro* and *in vivo* and they are crucial for the maintenance of peripheral tolerance and the prevention of autoimmunity[58, 93]. Given their potential to induce long-term tolerance and their ability to prevent and even reverse autoimmune diabetes and other autoimmune diseases[36, 52], it is critical to establish whether the mode of action of FNB anti-CD3 mAb involves the induction of Tregs *in vivo*. Potential changes in the relative frequency of effector and regulatory T cells following FNB anti-CD3 mAb treatment have made it difficult to evaluate the direct effect of the therapy on each cell subset and have contributed to the confusion in this field.

In this study, we examined the influence of FNB anti-CD3 mAb on effector versus regulatory T cells using defined models and therapeutic dosing. We demonstrate that although FNB anti-CD3 mAb induced an increase in the relative percentage of Tregs, this process was not due to de novo generation or expansion of Tregs. Instead, the increased Treg to Teff ratio was due to preferential depletion of activated effector T cells *in vivo* through Fas- and caspase 3-independent pathways. Furthermore, FNB anti-CD3 mAb treatment led to increased expression of Helios in Tregs, suggesting stabilization of Tregs which may account to the protracted efficacy of the drug.

MATERIALS AND METHODS

Mice

BALB/c, C57BL/6 mice were purchased from Charles River (Wilmington, MA), NOD mice were purchased form Taconic (Germantown, NY) and Caspase 3-deficient on the C57BL/6 background mice were purchased from Jackson Laboratories (Bar Harbor, ME). DO11.10 TCR-Tg mice, Bcl-2 transgenic mice on C57BL/6 background, Bim-deficient mice on C57BL/6 background, Fas-deficient lpr/lpr mice on a BALB/c background, FasL-deficient gld/gld mice on BALB/c background, Foxp3.GFP-Cre and Foxp3.GFP-Cre x Rosa26.flox.stop YFP on the NOD background were bred at our facility. All mice were housed in a specific pathogen-free facility at The University of California at San Francisco. All experiments complied with the Animal Welfare Act and the National Institutes of Health guidelines for the ethical care and use of animals in biomedical research and were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Antibodies and other reagents

Fc block (2.4G2) mAbs, FNB anti-CD3 mAb 145-2C11-γ3 (2C11-IgG3) mAbs were produced in our laboratory. 145-2C11-IgG2a-Ala-Ala mAbs were provided as a gift from Centocor/Johnson & Johnson and produced in our laboratory. Anti-CD3 mAb clone 145-2C11 (BioLegend), anti-CD4 clone RM4-5 (eBiosciences), anti-CD8 clone 53-6.7 (Southern Biotechnologies), anti-CD25 clone PC61 (eBiosciences), anti-FoxP3 clone FJK-16 (eBiosciences), anti-Thy1.1 clone OX-7 (BioLegend), anti-PD-1 clone J43 (eBiosciences), polyclonal anti-Neuropilin-1 (R&D), anti-Helios clone 22F6 (BioLegend)

were used. 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) was purchased from Molecular Probes Inc. (Eugene, OR). FTY720 provided by Novartis Pharmaceuticals (St. Louis, MO) was administered daily i.p. at a dose of 1mg/kg. EasySep mouse CD4 T cell enrichment kit was purchased from StemCell Technologies (Vancouver, BC, Canada). Mouse IgG whole molecule purchased from Rockland Immunochemicals for Research (Gilbertsville, PA) was used as control.

Flow cytometry and cell sorting

Single-cell suspensions were prepared from the spleen and LN of indicated mice using standard procedures and stained for 20-30 min on ice in staining buffer (2% FCS and 0.01% sodium azide). Cells were sorted on a Mo-Flo cytometer^{TM} (Cytomation, Fort Collins, CO) to greater than 95% purity. Flow cytometric analyses were performed on a BD LSRII flow cytometer with Diva software (BD/PharMingen).

Adoptive transfer experiments and in vivo treatments

Mice were treated intravenously (i.v.) with FNB anti-CD3 mAb at indicated doses every day for five days and control mice received whole mouse Ig using the same regimen. For adoptive transfer experiments, 9-10 x 10^6 sorted $CD4^+Foxp3/GFP^-$, 12 x 10^6 cells enriched $CD4^+$ CFSE-labeled T cells or 0.4-0.5 x 10^6 sorted $Helios^{lo/-}$ or $Helios^{hi}$ Tregs were transferred into syngeneic recipients via retro-orbital injection on day 0. On days 1-5, recipients were treated with FNB anti-CD3 mAb or control Ig as described above. Eight days after adoptive transfer, T cell proliferation was examined by flow cytometry. For adoptive transfer of $Helios^{lo/-}$ or $Helios^{hi}$ $Thy1.1^+$ Tregs cells were

enriched prior to flow cytometric analysis: a single cell suspension of all lymph nodes and spleen were harvested and incubated with 2µg/mL Thy1.1-APC in 200µl for 30 minutes at 4°C. Cells were washed, resuspended in 200µl and incubated with 50µl anti-APC magnetic beads (Miltenyi Biotec) 30 minutes at 4°C. Cells were washed and cell suspension was passed though a MACS Separation LS column (Miltenyi Biotec) to obtain positive fraction.

Cell death assays

DMEM-glutamax medium (Life Technologies, Gaithersburg, MD) supplemented with 5% heat-inactivated FCS (Summit Biotechnology, Ft. Collins, CO), 100 U/ml penicillin, 100 U/ml streptomycin, non-essential amino acids, 10 mM HEPES and 50 µM β-mercaptoethanol (all from Life Technologies) was used for cell culture.

To prepare Th1 effector T cells, single-cell suspensions were prepared from the spleen and LN of DO11.10 mice. DO11.10 cells were stimulated with 0.1 µg/ml ova peptide in the presence of 400 ng/ml IFNγ and 25 µg/ml anti-IL-4 for 7 days, with supplement of medium containing 20 U/ml recombinant human IL-2 on day after the initiation of the culture. The cells were restimulated with 0.1 µg/ml ova peptide without added IFNγ and anti-IL-4 on day 7. The DO11.10 cells were harvested one week later and restimulated with anti-CD3-IgG3 overnight and the numbers of viable cells in each well were determined using flow cytometry after anti-Thy-1 and PI staining.

For measurement of cell death after *in vivo* treatment, mice were treated with two 10µg doses of FNB anti-CD3 mAb or control Ig 24 hrs apart and harvested 38 hrs after the first injections. Single cell suspensions from whole lymph nodes were *incubated in*

vitro alone, or in the presence of 2 ng/ml rIL-2 (eBiosciences, San Diego, CA) or 20 ng/ml rIL-7 (Peprotech, Rock Hill, NJ). Cultures were harvest at 26 and 48 hrs and cell death was assessed by DAPI (Invitrogen, Carlsbad, CA) inclusion by flow cytometry.

Statistical analysis

The statistical significance of differences between groups was determined by the Mann-Whitney test using Prism software.

RESULTS

FcR non-binding anti-CD3 mAb does not promote the conversion of Treg cells or alter their stability

In agreement with what has been previously reported[85], 10 μg/day x 5 days FNB anti-CD3 mAb treatment induced a transient decrease in the percentage of total $CD4^+$ T cells in lymph nodes and an increase in the percentage of $CD4^+Foxp3^+$ Tregs in secondary lymphoid organs (**Figure 1A and 1B**). However, the total $CD4^+$ cell counts decreased (**Figure 1C**), mirroring the percentage decrease, and the $CD4^+Foxp3^+$ cell counts also decreased (**Figure 1D**). The decrease in $CD4^+Foxp3^+$ cells was not as pronounced as what was observed for total $CD4^+$ T cells potentially explaining the net percentage increase in the Treg subset. These results suggest that the FNB anti-CD3 mAb treatment may not induce the expansion of endogenous Treg cells, but transiently altered the proportions of Tregs due to a selective loss of $CD4^+Foxp3^-$ cells in secondary lymphoid organs.

To determine if FNB anti-CD3 mAb treatment might also promote the conversion of naïve Foxp3^{-} T cells into Foxp3^{+} Treg cells, we sorted and transferred CD4^{+}Foxp3^{-} cells from Foxp3-GFP reporter mice, and treated the recipient mice with FNB anti-CD3 mAb. The percentage of Foxp3^{+} cells within the transferred population was not statistically different between control and FNB anti-CD3 mAb-treated mice (**Figure 2A**) suggesting that stable Foxp3 expression was not induced by FNB anti-CD3 mAb. Moreover, to examine whether FNB anti-CD3 mAb induced Foxp3 expression transiently, we treated NOD.Foxp3.GFP-Cre x Rosa26.flox.stop.YFP mice[61] and analyzed the proportions of YFP^{+}GFP^{+} bona fide Tregs and YFP^{+}GFP^{-} "transient" Foxp3 expressing cells on day 14 after treatment. The percentage of CD4^{+} cells that expressed Foxp3 at any time (total YFP^{+}) was increased in FNB anit-CD3 treated mice, which we attribute to a loss of Foxp3^{-} cells (**Figure 2B**). Importantly, the percentage of bona fide Tregs (YFP^{+}GFP^{+}) did not change. Thus, we found no evidence that FNB anti-CD3 mAb promoted expansion of natural Treg cells, generation of adaptive Treg cells or transient expression of Foxp3 *in vivo*.

FcR non-binding anti-CD3 mAb does not promote expansion or survival of Treg cells

The previous experiments clearly demonstrated that FNB anti-CD3 mAb treatment does not induce Treg conversion. However, these data could not distinguish whether the increased frequency of Tregs was due solely to preferential loss of Foxp3^{-} cells or involved the concomitant expansion of Foxp3^{+} Tregs[87-89]. In order to determine the effects of FNB anti-CD3 mAb on the proliferation of Foxp3^{-} vs Foxp3^{+} CD4^{+} T cells, congenically marked CD4^{+} cells were CFSE-labeled and transferred to recipient mice

that were either treated with control antibody or FNB anti-CD3 mAb (**Figure 3A**). The ratio of $Foxp3^-$ to $Foxp3^+$ cells decreased in FNB anti-CD3 mAb-treated mice (**Figure 3B**), suggesting a preferential loss of $Foxp3^-$ cells as described above. Interestingly FNB anti-CD3 mAb treatment led to a decrease in the percentage of undivided $Foxp3^-$ cells and an increase in the percentage of cells that had divided only one time (**Figure 3C**). Importantly, there was no difference in the percentage of $Foxp3^+$ cells that had either not divided or divided one time (**Figure 3D**) demonstrating that FNB anti-CD3 mAb does not induce proliferation of existing $Foxp3^+$ Tregs.

FNB anti-CD3 mAb preferentially induces cell death of $CD4^+Foxp3^-$ cells in vivo

The loss of $CD4^+Foxp3^-$ cells could result from preferential migration out of lymph nodes or deletion of these cells induced by FNB anti-CD3 mAb. First, analysis of $CD4^+$ T cells counts in other tissues such as bone marrow, intestine, liver and lung in mice treated with FNB anti-CD3 mAb treatment revealed no significant increase in $CD4^+$ T cells that could account for the loss of these cells in the secondary lymphoid organs (data not shown). To examine the possibility of T cell migration more directly, we treated mice with FNB anti-CD3 mAb under the cover of FTY720 to block exit of T cells from LN and then examined the numbers of T cells and percentages of Treg cells in the LNs. The FTY720 treatment led to a significant drop in the lymphocyte count in the peripheral blood demonstrating that LN exit was blocked (data not shown). FNB anti-CD3 mAb induced a similar drop in the $CD4^+$ T cell count and an increase in Treg cell percentages in the FTY720 and control treated mice (**Figure 4A** and data not shown). Thus, the selective loss of $CD4^+Foxp3^-$ cells after FNB anti-CD3 mAb treatment was not due to

preferential trafficking out of lymph nodes, suggesting that the relative increase of $CD4^+Foxp3^+$ compared to $CD4^+Foxp3^-$ was due to preferentially deletion of $CD4^+Foxp3^-$ cells by FNB anti-CD3 mAb.

Mechanism of FNB anti-CD3 mAb-mediated cell death

In order to measure cell death caused by *in vivo* treatment with FNB anti-CD3 mAb, we incubated *in vitro* cells harvested from mice that had been treated with FNB anti-CD3 mAb *in vivo*. Cells harvested from FNB anti-CD3 mAb-treated mice showed increased cell death, as measure by DAPI uptake, compared to mice treated with control antibody (**Figure 4B**). Cell death was not prevented by addition of IL-2 or IL-7 (**Figure 6**) suggesting that FNB anti-CD3 mAb induced cell death is not a consequence of cytokine deprivation *in vitro* and may be the result of an apoptotic program that was initiated by FNB anti-CD3 mAb *in vivo*.

We next investigated the mechanism of FNB anti-CD3 mAb depletion of T cells *in vitro* and *in vivo*. T cell death is mediated often by either cell surface death receptors such as Fas and TNFαRI, or mitochondria death effector molecules such as Bim [94-96]. In fact, T cells deficient in Fas (*lpr*, not shown) or Fas ligand (*gld*) were resistant to FNB anti-CD3 mAb induced cell death *in vitro* (**Figure 5A**). However, *lpr* mice treated with FNB anti-CD3 mAb showed a similar level of T cell depletion as in WT mice (**Figure 5B**) suggesting that the *in vivo* mechanism of cell death distinct from that seen *in vitro*.

Previous studies by our group and others showed that *in vivo* depletion of naïve T cells by the parental anti-CD3 mAb or by superantigen is mediated by the pro-apoptotic protein Bim and blocked by over-expression of anti-apoptotic protein Bcl-2 or Bcl-

xL[53,96]. We investigated whether depletion of naïve T cells *in vivo* by FNB anti-CD3 mAb was mediated by a similar mechanism. We treated Bim-deficient and wild type (WT) control mice with FNB anti-CD3 mAb and compared the rate of CD4 depletion one week after the treatment. $CD4^+$ T cells were depleted to a similar extend in Bim-deficient and WT mice (**Figure 5C**). This depletion resulted in a similar rise in the percentages of Treg cells as observed in earlier experiments (data not shown). It was possible that other Bim-related mitochondria proapoptotic proteins, such as Bad and Bax, mediated FNB anti-CD3 mAb-induced T cell death. Cell death mediated by these proteins and Bim is blocked by over expression of anti-apoptotic protein Bcl-2. To determine potential roles of other mitochondria proapoptotic factors in FNB anti-CD3 mAb-induced T cell depletion, we compared T cell numbers in WT and Bcl-2 transgenic mice after FNB anti-CD3 mAb treatment. The result showed that the rate of $CD4^+$ T cell depletion was similar in WT and Bcl-2 transgenic mice (**Figure 5D**). Finally, we analyzed the role of caspase 3, which is the downstream caspase activated by both intrinsic and extrinsic apoptosis pathways and its deficiency has been shown to prevent anti-CD3 mAb-induced death of T cells *in vitro*[97]. Treatment of caspase 3 knockout mice with FNB anti-CD3 mAb resulted in depletion of $CD4^+$ T cell in the lymph nodes similarly to that seen in wild type mice (**Figure 5E**). Together, these data suggest FNB anti-CD3 mAb may activate multiple apoptotic pathways *in vivo* such that absence of one does not prevent cell death or that one main pathway is activated but in its absence there is compensation by the other pathways. Alternatively, it is possible that FNB anti-CD3 mAb activates previously unknown apoptotic pathways that are independent of caspase 3.

Induction of Helios expression in Tregs

Although the preferential deletion of effector T cells following anti-CD3 mAb therapy could account for short term remission, it remained possible, if not likely, that changes in Tregs (other than expansion or induction) might play a role in long term stable remission of diabetes in treated NOD mice. It was recently reported that the transcription factor, Helios, is expressed by thymus-derived natural Tregs but reduced or absent in adaptive Tregs induced *in vitro* by TGFβ[98]. Furthermore, $Helios^{lo/-}$ Tregs produce more IFNγ and IL-2 than $Helios^{hi}$ Tregs[98], suggesting that they may be "plastic" or unstable. We hypothesized that FNB anti-CD3 mAb may alter the relative proportion of $Helios^{hi}$ to $Helios^{lo/-}$ Tregs thereby increasing the proportion of stable Tregs. The treatment of mice with FNB anti-CD3 mAb led to an increase in the proportion and absolute number of $Helios^{hi}$ Tregs (**Figure 7A and B**). This increase could be due to proliferation or preferential survival of $Helios^{hi}$ Tregs, or induced expression of Helios in $Helios^{lo/-}$ cells. As shown in Figure 3, FNB anti-CD3 mAb did not induce proliferation of Tregs, therefore it is unlikely that proliferation of $Helios^{hi}$ cells was responsible for the increased percentage of this Treg subset in treated animals. In order to differentiate between preferential survival of $Helios^{hi}$ cells or induced expression in $Helios^{lo/-}$ cells, sorted Tregs were transferred to lympho-replete hosts that were then treated with FNB anti-CD3 mAb. To distinguish between $Helios^{hi}$ and $Helios^{lo/-}$ Tregs, we used the surface markers PD-1 and Neuropilin-1, which are preferentially expressed by $Helios^{hi}$ Tregs (Yadav and Bluestone, manuscript in preparation). We sorted $CD25^{+}PD\text{-}1^{-}Nrp1^{-}$ cells, of which 44-55% were $Helios^{hi}$, and $CD25^{+}PD\text{-}1^{+}Nrp1^{+}$ cells, of which 69-70% were $Helios^{hi}$. After control Ig treatment, 24% of transferred $Helios^{lo/-}$ Tregs expressed Helios, while 68% of

$Helios^{hi}$ Tregs expressed high levels of Helios consistent with a stable phenotype *in vivo*. FNB anti-CD3 mAb treatment resulted in an increased percentage of Helios expressing Tregs in both the sorted $Helios^{lo/-}$ (68%) and $Helios^{hi}$ (82%) populations.

DISCUSSION

FcR non-binding anti-CD3 mAbs effectively control autoimmunity and transplant rejection in pre-clinical murine models and clinical trials in patients[73]. The mechanisms underlying the tolerogenic outcome of FNB anti-CD3 mAb therapy are still ill-defined. In particular, the respective effects of FNB anti-CD3 mAbs on conventional T cells versus Treg cells are controversial. In this study, we addressed this issue directly by comparing the impact of FNB anti-CD3 mAbs on conventional $CD4^{+}Foxp3^{-}$ T cells $CD4^{+}$ $Foxp3^{+}$ Tregs. Our data showed that although all T cell subsets underwent partial deletion following FNB anti-CD3 mAb treatment, there was a relative enrichment of Tregs. Importantly, the increase in the frequency of Tregs was not associated with induction or expansion of adaptive Tregs, or alteration in the stability of natural $Foxp3^{+}$ Tregs. While killing of T cells induced by FNB anti-CD3 mAb was dependent on the Fas pathway *in vitro*, it occurred independently of Fas, Bim and caspase 3 *in vivo* and is likely not due to cytokine withdrawal. Most significantly, FNB anti-CD3 mAb treatment increased the proportion of Helios-expressing Tregs. Thus, our study suggests that FNB anti-CD3 mAb therapy promotes tolerance and prevents autoimmunity by restoring the balance between pathogenic autoreactive effector T cells and suppressive regulatory T cells and potentially increases the stability of the Treg population.

Our study clearly shows that the relative enrichment in Treg following FNB anti-CD3 mAb treatment was due to distinct sensitivity of conventional and regulatory T cells to T cell death. Considering the therapeutic applications of FNB anti-CD3 mAbs, this finding has important implications and greatly clarifies the mode of action of these reagents. It is notable that the current theories regarding the pre-clinical and clinical efficacy of FNB anti-CD3 mAb therapy often involve the generation of Tregs in addition to its effect on effector T cells. Indeed, although reversal of autoimmune disease by FNB anti-CD3 mAb treatment was associated with an increase in $CD4^+CD25^+$ Tregs in several murine models, this increase was an augmentation of the relative frequency of Tregs [87-89]. Similarly, Bisikirska et al reported an increase in the frequency of $CD8^+CD25^+Foxp3^+$ regulatory T cells in the peripheral blood of diabetic patients treated with OKT3-γ1(Ala-Ala)[99]. It is difficult to establish whether only the relative percentage of these cells had increased or whether there was an active induction of this cell population. In the mouse, we did not see transient or stable induction of Foxp3 expression on $CD8^+$ cells (data not shown). Conversely, other studies did not observe increased Treg percentages after FNB anti-CD3 mAb treatment *in vivo* [90-92]. There are a number of variables that could explain the differences between studies. In particular, different dosing regimen and the use of different forms of FNB anti-CD3 mAb that differ in their half-life *in vivo* and their molecular weight such that equal doses in micrograms could provide dissimilar numbers of binding sites could all affect the outcome of treatment. Thus, two of the aforementioned studies used high doses of FNB anti-CD3 reagents (100 μg/ml)[91, 92], which in our studies did not result in the relative enrichment in Tregs observed with lower doses (10 μg/ml or 50 μg/ml).

The recent report of Helios expression as a marker for thymus-derived natural Tregs[98], led us to ask whether FNB anti-CD3 mAb had preferential effects on natural versus adaptive Tregs. Our data shows increased expression of Helios after FNB anti-CD3 mAb treatment, suggesting that Helios expression can be induced on a previously $Helios^{lo/-}$ population, although we cannot rule out the possibility that $Helios^{lo/-}$ cells are preferentially depleted by the treatment. Helios expression does not seem to directly affect Treg function [98]; however, data from our laboratory suggests that $Helios^{lo/-}$ adaptive Tregs are not able to control autoimmune responses *in vivo* (Yadav, Bluestone, et al., manuscript in preparation). Since we have previously shown that Tregs can lose Foxp3 expression and become pathogenic[61] and a greater proportion of $Helios^{lo/-}$ Tregs produce cytokines, we hypothesize that Helios expression is a marker of stable and functional Tregs. Our laboratory also showed that at the time of disease onset, the Tregs in the pancreas of NOD mice express lower levels of Foxp3 and CD25, likely making them unstable and dysfunctional, and that treatment with IL-2/anti-IL-2 complexes can prevent disease by augmenting the percentage of Tregs in the pancreas[60]. Therefore, we hypothesize that in addition to restoring the balance between effector and regulatory T cells, FNB anti-CD3 mAb also affects the balance between stable and unstable Tregs.

Whether our results in mice can be extended to the human is still controversial. As previously mentioned, a population of $CD8^{+}Foxp3^{+}$ cells arises in human patients treated with OKT3-γ1(Ala-Ala) which we do not see in mice treated with FNB anti-CD3 mAb. Unlike murine T cells, human T cells transiently express Foxp3 protein upon activation[100]; however, it seems like this Foxp3 expression does necessarily not confer suppressive function. Therefore, in humans Foxp3 expression may not be a reliable

marker of regulatory T cells as it is in mice. In addition, the effect of FNB anti-CD3 mAb on human cells has been mostly studied *in vitro*; however it is possible that *in vitro* effects differ from *in vivo* effects, as we show that *in vitro*, T cell death is Fas mediated but deletion of Fas has no effect on T cell depletion *in vivo*.

In conclusion, our results suggest that FNB anti-CD3 mAb treatment reverses autoimmunity by depleting autoreactive pathogenic T cells and restoring the balance of effector versus regulatory T cells towards increased regulation. This outcome is achieved by the selective sensitivity of effector T cells versus relative resistance of Tregs to FNB anti-CD3 mAb-induced cell death but not by de novo induction of adaptive Tregs or expansion of natural Tregs. However, this process creates an immunological milieu that favors immune regulation. Our results have important implications for the design of FNB anti-CD3 mAb-based immunotherapies in autoimmune diseases and suggest the pertinence of developing combination therapies that would strengthen FNB anti-CD3 mAb modes of action.

CHAPTER III

Prevention of diabetes by FTY720-mediated stabilization of peri-islet tertiary lymphoid organs

INTRODUCTION

Multiple factors play a role in the development of diabetes in the NOD mouse model starting with the activation of T cells by antigen-presenting cells, leading to T cell differentiation and, ultimately, destruction of the target tissue. However, the location of these key events remains unclear. For example, what is the role of tertiary lymphoid organs (TLOs) that form in the pancreas in diabetes development? Specifically, is continuous recruitment of lymphocytes to the pancreas necessary or, once TLOs are established, are the cells in these structures sufficient to cause diabetes?

Tertiary lymphoid organs are accumulations of lymphoid cells that arise in the adult as a result of chronic inflammation in non-lymphoid locations[101]. TLOs have been described in microbial infection, chronic allograft rejection and autoimmunity, such the pancreas of NOD mice and the brain of mouse models of MS. TLOs share many morphological similarities to secondary lymphoid organs in respect to their cellular composition, chemokine expression and vasculature. They have compartmentalized T and B cell zones with follicular dendritic cell networks that can evolve into germinal centers [101]. In addition, other subsets of dendritic cells, macrophages and natural killer cells have been described in these structures[101]. TLOs have been found to express chemokines such as SLC (stromal cell-derived factor 1, also known as CXCL12) the ligand for CCR5 and BLC (B lymphocyte chemoattractant, also known as CXCR13) one

of the ligands for CCR7[101], which are responsible for T cell migration to the T cell zone and B cell migration to the B cell zone in lymph nodes respectively[102]. Finally, high endothelial venules (HEVs) that express functional peripheral node addressin (PNAd), an L-selectin ligand that facilitates lymphocyte recruitment into lymph nodes, and lymphatic vessels that express early lymphatic markers, such as the lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1) and the transcription factor propero-related homeobox 1 (Prox-1), have also been described[103].

However, a detailed analysis of TLOs in the pancreas of NOD mice has not been performed and could give insights into the development of the disease including whether antigen presentation occurs in the pancreas leading to epitope spreading and expansion of the immune response; whether continuous recruitment of lymphocytes to the site is necessary to cause disease and whether priming of T cells in these structures sufficiently activates them to be pathogenic.

Despite the associations with chronic inflammation, it is unclear whether TLOs directly contribute to the development of immune responses by serving as priming sites for lymphocytes or are simply a consequence of the chronic inflammation. In support of their direct role, there is evidence demonstrating that TLOs can support B cell selection. For instance, analysis of the BCR L chain genes in individual TLOs in the pancreas of NOD mice showed that each TLO contained oligoclonal B cells enriched for germinal center markers and for sequences containing mutations within the complementarity determining regions, suggesting antigen driven selection[104]. However, little data exists demonstrating that priming of naïve T cells can occur in these structures[101]. Regardless of their ability to prime T cells, it has been demonstrated that the maintenance of TLOs is

essential for disease development in NOD mice[105106]. Wu and coworkers first showed that treatment with lymphotoxin-β receptor-immunoglobulin fusion protein (LTβR-Ig) destroyed TLOs, leading to infiltrate clearing, prevention of beta cell destruction and long-term protection from disease[105]. Lee and coworkers then showed that this effect was due to the ability of LTβR-Ig to block the endogenous interaction of LTβR with one of its ligands LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for binding herpesvirus entry mediator on T cells)[106]. Expression of LIGHT mRNA was shown to increase with age in the pancreas of NOD mice, and overexpression of LIGHT in the pancreatic islets accelerated disease development in the NOD background. The authors of this study also suggest that priming in the pancreas is sufficient for disease development since transfer of islet specific BDC2.5 TCR transgenic splenocytes was able to transfer disease to pancreatic lymph node-ectomized mice; however, they did not explore this possibility further. In addition to the assessment of TLOs that develop during situations of chronic inflammation, the understanding of these structures has been extended by the development of transgenic models. In particular, the rat insulin promoter (RIP) driven expression of chemokines from the beta cells of the pancreas has been used by a number of groups[107-109].

FTY720 is a sphingosine-1-phosphate mimetic that alters lymphocyte trafficking resulting in sequestration of lymphocytes in lymph nodes[110]. This has now been shown to be due to its ability to bind and lead to the internalization of the S1P receptor 1, which is necessary for lymphocyte trafficking out of lymph nodes. FTY720 is being used clinically in transplant patients to prevent rejection and has been found to be effective in the treatment of murine autoimmune models such as systemic lupus myocarditis and type

1 diabetes[110]. It is thought that the inhibition of lymphocyte trafficking to the tissue is responsible for its effectiveness in transplantation; however, the mechanism of disease protection in models of autoimmunity is not understood.

For these studies, we used the S1P1 agonist, FTY720, which blocks T cell egress from lymphoid tissues[111], to "lock" cells in the draining pancreatic lymph node and the pancreas as a model to study the role of TLOs in the pathogenesis of diabetes in NOD mice. We show that continuous treatment with FTY720 prevents diabetes development regardless of when the treatment is begun. However, treatment withdrawal led to rapid onset of disease. This accelerated onset occurred only in older mice with pre-existent TLOs established in the pancreas and was independent of the draining pancreatic lymph node. Focused studies on the pancreatic TLOs showed that inhibition of T and B cell recruitment did not have dramatic effects on the composition of T cell subsets in the pancreas and that naïve T cells were able to traffic and proliferate in the pancreas. Furthermore, morphological changes occurred during the development of the disease suggesting that temporal development correlated with disease progression and subsequent destruction of the TLOs. Our results suggest that islet destruction maybe be due to loss of TLO integrity.

MATERIALS AND METHODS

Mice

NOD, NOD.BDC25[34] and NOD.Rag2 KO mice were bred at the University of California, San Francisco. NOD mice were also purchased from Taconic. All mice were housed under specific pathogen-free conditions at the University of California, San

Francisco Animal Barrier Facility. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. For diabetes incidence, urine glucose was followed twice weekly.

Human Pancreas Samples

Samples from human diabetic patients were obtained through JDRF's nPOD project. The age of the patients ranged from 12-22 years old and had been diagnosed with type 1 diabetes 1-8 years prior to death. Details regarding their collection and methods of preparation can be found at www.jdrfnpod.org.

FTY720 administration

FTY720 was obtained from Novartis Pharmaceuticals and was dissolved in normal saline. A dose of 20μg/day was administered by i.p. injection three times per week.

Pancreatic lymph node-ectomy

Pancreatic lymph nodes were removed as previously described [21].

Antibodies

For flow cytometry, labeled antibodies specific for CD4 clone RM4-5, CD8 clone 53-6.7, CD25 clone PC61, FoxP3 clone FJK-16s, CD45 clone I3/2.3, CD11c clone N418, CD86 cone GL1, CD40 clone 3/23, CD80 clone 16-10A1, I-A^{g7} clone 10.2.16 were used. Cells were fixed and permeabilized using Foxp3 Staining Kit per manufacturer's

protocol. For immunofluorescence, primary antibodies specific mCD4 clone L3T4, hCD4 clone RPA-T4, mCD45R/B220-biotin clone RA3.6B2, hCD19 clone H1B19, mCD31 clone 390, mMAdCAM clone MECA367, mLyve-1 clone 223322, and ERTR7 were used. MECA79 antibody was obtained from Dr. Steve Rosen (University of California, San Francisco).

Immunofluorescence

Pancreata were isolated and frozen in optimum cutting temperature embedding compound. Consecutive sections (7µm) were cut with a Leica CM 3050S cryomicrotome and fixed for 15 minutes in acetone at -20°C . Sections were blocked with 5% BSA and 3% normal serum corresponding to the secondary antibody and/or Avidin/Biotin Blocking Kit per manufacturer's protocol. Slides were incubated with primary and secondary antibodies for 1-2 hours at room temperature and counterstained with Gill's Hematoxylin. Digital images were captured with an Optiphot microscope equipped with an Axiocam digital camera and analyzed using Adobe Illustrator CS2.

Isolation of lymphocytes from pancreas

Pancreases were excised independently of lymph nodes and were incubated for 30 min at 37 °C in collagenase P (1 mg/ml; Roche), DNase (20 µg/ml) and 0.2% (wt/vol) BSA in RPMI medium. Digested tissue was strained through a 70-µM cell strainer (BD Biosciences) and was washed once with RPMI medium containing DNase. Lymphocytes were separated from low-density pancreatic cells by centrifugation through a 40%–60% (vol/vol) percoll gradient. Lymphocytes were washed and stained for flow cytometry.

Isolation of dendritic cells

Dendritic cells were isolated from lymph nodes and islets as previously described[112].

Adoptive transfers and CFSE dilution analysis

$CD4^+CD25^-CD62L^+$ cells were sorted from NOD.BDC2.5tg$^+$Thy1.1 mice. Cells were then labeled with CFSE and $1x10^6$ transferred to NOD.Thy1.2 mice. Immediately after transfer and daily until time of harvest, mice were injected with FTY720. On day 5 single cell suspensions from pancreas, spleen, PLN and ILN were stained for flow cytometric analysis. To calculate the percent of T cells that entered cell cycle, the original number of cells that entered cell cycle was determined by dividing the number of events at each division (i.e. CFSE peak) by the number of divisions that occurred. This number was divided by the total number of cells (original number of cells that entered cell cycle + number of cells that never entered cell cycle).

Statistical analysis

The statistical significance between Kaplan-Meier survival curves in Figure 8 and 14 was determined by a Logrank test using Prism software. The statistical significance of differences between groups in Table 1 and Figure 10 was determined by the two-tailed Student's *t* test using Excel. The statistical significance of differences between groups in Figure 11 was determined by the Mann-Whitney test using Prism software.

RESULTS

Continuous FTY720 treatment prevents diabetes but leads to rapid disease development upon withdrawal

FTY720 blocks T cell egress from lymph nodes and has been shown to be effective clinically in the treatment of autoimmunity and transplant rejection[113]. We set up a model using FTY720 that was designed to examine the effects of "locking" lymphocytes in the pancreatic lymph node (PLN) and pancreas. Treatment of female NOD mice with 20 μg/day 3 times per week starting at 10 or 14 weeks of age, when insulitis had already begun or was established, completely protected mice from diabetes development (**Figure 8A,B**). Withdrawal of drug treatment after 10 or 7 weeks, respectively, led to rapid development of diabetes. Seventy to eighty-eight percent of mice were diabetic 18 days post-treatment withdrawal in multiple independent experiments (**Figure 8A, B**). This compares to 48% incidence among untreated control mice. Similarly, continuous treatment until 25 weeks led to protection during treatment and acceleration of disease upon withdrawal (data now shown). In order to assess whether insulitis at the beginning of treatment was necessary for the rapid disease onset seen after withdrawal, we began treatment at 7 weeks of age, when insulitis is present but minimal (**Figure 8C**). Unlike the previous regimens, withdrawal 10 weeks later did not lead to rapid diabetes development. By 29 weeks of age, 12 weeks after FTY720 withdrawal, only 50% of FTY720 treated mice had become diabetic with similar kinetics as observed in control mice. As expected, continuous FTY720 treatment led to a marked decreased of circulating lymphocytes which returned to normal levels 7 days after

treatment withdrawal (data not shown). These results suggest that the disease acceleration was not due to direct effects of the drug on the pancreas.

Continuous FTY720 treatment does not clear pancreatic infiltrate

A possible explanation for disease protection during FTY720 treatment is clearance of pancreatic infiltrate. Histological analysis and scoring of islet infiltrates of 20-week old mice that had been treated with FTY720 from 10-12 to 20-22 weeks of age and age-matched controls showed that treatment had limited effects on islet infiltration (**Figure 8D**). Forty percent of the islets had some degree of peri-islet infiltration similar to that observed in untreated 10-12 week old NOD mice suggesting that FTY720 treatment did not clear tissue infiltrate but rather blocked trafficking of new cells from the periphery. Interestingly, the overall fraction of pancreatic islets exhibiting grades 1, 2 or 3 insulitis remained consistent between 10-week old and 20-week old FTY720-treated mice suggesting that FTY720 blocked further islet invasion.

FTY720 treatment leads to minor changes in infiltrating $CD4^+$ T cells

Next, we compared the infiltrate in 20-22 week old mice undergoing continuous FTY720 treatment from 10-12 weeks of age (as in Figure 8A) to aged-matched untreated controls. The total number of cells in the non-draining inguinal lymph node (ILN) and the spleen was 3-fold less in FTY720 treated mice (**Table 1**) likely due to sequestration of cells in the thymus (data not shown) and systemic lymphopenia, respectively. Interestingly, the total cell number in the PLN remained unchanged (**Table 1**); a result that could reflect continued expansion of cells specific for pancreatic antigens. Similar

results were observed when examining the $CD4^+$ and $CD8^+$ T cells (**Table 1**). In the pancreas, the percentage of $CD4^+$ T cells decreased 2-3 fold but there was no change in infiltrating $CD8^+$ T cells (**Table 1**). These results suggest that disease protection during treatment, but not the disease acceleration seen after withdrawal, may be due to a decrease in infiltrating $CD4^+$ T cells. Importantly, these data suggest that potentially pathogenic T cells were able to persist at the site of inflammation without continuous trafficking from peripheral lymphoid tissues.

Since FTY720 has been suggested to increase the proportion of Tregs [114, 115], we assessed whether a proportional increase in Tregs could account for the disease protection during treatment. Analysis of absolute numbers of Tregs showed that the total number of $CD4^+Foxp3^+$ cells decreased in the ILN and spleen but remained the same in the PLN (**Table 1**). The percentage of $CD4^+Foxp3^+$ cells in the pancreas remained the same; however, the absolute number of Tregs likely decreased in treated mice suggesting that changes in Tregs were unlikely to be responsible for disease prevention.

In addition to inhibiting the migration of lymphocytes, FTY720 has also been suggested to have effects on dendritic cells (DCs) [116]. To test this hypothesis, we treated 16-18 week old NOD mice with FTY720 for 10 days and analyzed the percentage of $CD11c^+$ cells in the islets, PLN and ILN as well as their expression of CD86, CD40, CD80 and I-Ag7 (MHC II). The treatment did not affect the percentage of DCs or their maturation state in any tissue analyzed (**Figure 9**), suggesting that disease protection observed during FTY720 treatment or disease acceleration observed after treatment withdrawal was not due to changes in DCs.

Analysis of tertiary lymphoid organs in the pancreas

Since the total cell number in the PLN was unaffected by FTY720 treatment and a significant number of cells remained in the pancreas, it was unlikely that disease protection during FTY720 treatment could be attributed solely to a reduction in infiltrating cells. Therefore, we analyzed the effect of long-term FTY720 treatment on the structure and characteristics of TLOs. Because FTY720 blocks lymphocyte egress from the lymph nodes, we hypothesized that disease was prevented by inhibiting lymphocyte egress from the TLOs in the pancreas to the site of tissue destruction - the β-cells. First, we characterized the TLOs in pre-diabetic 8-week old, non-diabetic 20-week old and naturally diabetic mice by immunofluorescent staining of individual markers on consecutive pancreas sections using markers described in the Materials and Methods. ERTR7 reactivity and $CD31^+$ vessels were observed surrounding all islets and throughout the acinar tissue at all the time points analyzed (**Figure 10A** and data not shown). In addition, ERTR7 reactivity was found within the infiltrate suggesting that these structures have a supporting stromal network, as is seen in T cell zones in the spleen and lymph nodes[117]. However, although the results trend towards an increase in the association of the presence of $MAdCAM^+$ or $Lyve^+$ vessels and disease progression, these differences were not statistically significant (**Figure 11**). In addition, most infiltrates in both pre-diabetic and naturally diabetic mice contained $PNAd^+$ HEVs (**Figure 10D-G**) suggesting that, like secondary lymphoid organs, these structures are capable of recruiting naïve T cells. Analysis of at least three consecutive sections showed that infiltrates of pre-diabetic 20-week old mice were usually, but not always, associated with insulin-producing islets (**Figure 10A and 10E)**. Although T and B cells were present in the pancreas of 8-week

old mice, only 15% (3/20) of the infiltrates showed clear compartmentalization whereas 89% (24/27) infiltrates in 20-week old mice showed distinct T and B cell zones. In the majority of these, the T cell zone was closer to the islet while the B cell zone was on the edge of the infiltrate, reminiscent of lymph nodes (**Figure 10A**). Only 15% of infiltrates (3/19) in naturally diabetic mice showed T/B cell compartmentalization, which correlated with an absence of insulin-positive islets that we attribute to destroyed β-cells (**Figure 10B and 10G**). However, β-cells may have been present but not producing insulin due to the inflammation [118]. True TLOs, defined as infiltrates with T/B compartmentalization and association with at least two of the vascular markers analyzed, were present in 15% (3/20) of infiltrates in 8-week old mice increasing to 81% (22/27) of infiltrates in 20-week old mice but decreasing to only 26% (5/19) of infiltrates in naturally diabetic mice (**Figure 10D-F**). Therefore, TLOs peaked as a proportion of the infiltrate at the "peak" of the inflammatory response before diabetes onset.

We analyzed NOD-Rag KO mice to assess whether the appearance of the TLO markers was due to inflammation or a normal characteristic of the NOD pancreas (Figure 2C). As in NOD mice, there was ERTR7 staining and $CD31^+$ vessels around islets and throughout the ascinar tissue. $MAdCAM^+$ and $Lyve\text{-}1^+$ vessels were associated with 30% and 59% of islets, respectively, which is significantly lower than in mice with an intact immune system (**Figure 11**). There was no PNAd staining suggesting an adaptive immune response was necessary for the development of HEVs in the pancreas (data not shown).

Analysis of FTY720 treated mice after 8-10 weeks of continuous treatment (20-weeks old) showed that the majority of the infiltration was associated with insulin-

producing islets (**Figure 10G**). Moreover, the infiltrates in FTY720 treated mice showed a similar degree of organization, based on T/B compartmentalization (19/21), as age-matched untreated controls (F**igure 10G**). Similar to pre-diabetic and naturally diabetic mice, the TLOs in these mice had $PNAd^+$ HEVs as well as Lyve-1^+ and $MAdCAM^+$ vessels (**Figure 11**). Analysis of mice on day 12 after FTY720 release, when 30-60% mice have become diabetic, showed that the infiltrates lose T/B compartmentalization and are similar to those of naturally diabetic mice; only 31% (5/16) of infiltrates were true TLOs based on our criteria (**Figure 10H**).

In an attempt to define TLOs in human diabetic patients, we analyzed pancreas sections from four diabetic patients obtained through Juvenile Diabetes Research Foundation's Network for Pancreatic Organ donors with Diabetes (nPOD). Although $CD4^+$ T cells and $CD19^+$ B cells were found surrounding $insulin^+$ islets (**Figure 12**), the cells were scattered and did not form large clusters as was observed in prediabetic and early-onset diabetic NOD mice. However, we could not distinguish whether the absence of TLOs in the human samples was due to temporal differences (the human tissues were harvested from cadavers with long term disease) or species differences.

Accelerated disease upon FTY720 withdrawal is independent of the draining pancreatic lymph node

A possible explanation for the accelerated disease following cessation of FTY720 is that the relevant antigen-specific cells continue to expand to sufficient numbers and activation status to cause rapid disease upon treatment withdrawal and restoration of trafficking. This would suggest that during long-term FTY720 treatment there are

reservoirs of islet-reactive cells that accumulate either within or outside of the pancreas. To determine in which lymphoid tissues islet antigens are presented that can lead to antigen-specific T cell expansion, we transferred 1 x 10^6 CFSE labeled naïve $CD4^+CD25^-$ $CD62L^+$ islet antigen-specific BDC2.5 TCR-Tg cells to 8-week old NOD mice and treated with FTY720 daily to prevent recirculation. After 5 days, cells were harvested and proliferation evaluated by CFSE dilution (**Figure 13**). In untreated mice, 37% and 50% of transferred cells had entered the cell cycle in the PLN and the pancreas, respectively. The proliferation was antigen dependent as only 8% and 14% of the TCR-Tg T cells went into cycle in the ILN and spleen, respectively. In FTY720 treated mice, a higher percentage of cells that entered the cell cycle was observed in the PLN (54%) and pancreas (75%). These results suggested that naïve cells were able to home directly to and proliferate in the pancreas and that FTY720 treatment did not prevent antigen presentation or antigen driven proliferation. Similar to untreated mice, 6% and 2% of the cells in the ILN and spleen, respectively, proliferated in FTY720 treated mice suggesting that the PLN is the only secondary lymphoid tissue where islet antigens are presented and that the proliferating cells seen in the spleens of untreated mice are a result of recirculation.

We reasoned that the cells causing rapid onset of diabetes upon FTY720 withdrawal originated from either the PLN or the pancreas. Thus, we tested the effect of surgical removal of the PLN in mice undergoing FTY720 treatment. FTY720 treatment was begun at 12 weeks of age, the PLN was surgically removed (PLNx) 9 weeks later, and treatment was withdrawn one week after the surgery. Forty percent of PLNx mice developed diabetes within 15 days of treatment withdrawal compared to 80% of sham

controls (**Figure 14A**). As our previous experiments had shown, the age at the beginning of treatment influences whether there is accelerated disease upon treatment withdrawal suggesting that although the PLN may be important early in disease progression, the requirement of this lymph node diminishes as disease progresses. We hypothesized that if we began treatment late in life, a greater percentage of mice would develop disease independently of the PLN due to the increased level of infiltration at the time of treatment. Therefore for the second series of experiments, we began treatment at 16 weeks of age, removed the PLN at 23 weeks of age and withdrew FTY720 one week later. Under these conditions, 50% of sham control mice and 65% of PLNx mice became diabetic within 15 days of FTY720 withdrawal (**Figure 14B**). These results suggest that the diabetes acceleration observed after FTY720 can be attributed to antigen-reactive T cells present in both pools. However, the PLN is not required for the accelerated disease in mice with the most progressed disease. In this setting, the cells present in the TLOs of the pancreas at the beginning of the treatment are sufficient to cause diabetes upon FTY270 withdrawal.

DISCUSSION

In this study, we used FTY720 treatment of NOD mice to prevent trafficking of lymphocytes into the pancreas and study the role of tertiary lymphoid organs (TLOs) in diabetes development. Our results show that continuous FTY720 treatment prevents diabetes development and, when withdrawn, leads to an accelerated disease that is independent of the PLN if treatment is begun when insulitis is established. Analysis of NOD mice at various stages of disease and during FTY720 treatment showed that

lymphocyte infiltration into the pancreas leads to the formation of organized TLOs characterized by T/B compartmentalization and specialized vasculature, including lymphatic vessels, HEVs and MAdCAM expressing vessels. Furthermore, our data showed that these structures do not exhibit T/B compartmentalization in naturally diabetic mice even though the specialized vasculature remains unchanged. We propose a model where cells that infiltrate the pancreas organize into TLOs that, like secondary lymphoid organs, have T/B compartmentalization and specialized vasculature. Once these structures are established the immune response is amplified and eventually is sufficient to destroy the neighboring islet. In doing so, T/B compartmentalization is lost and TLO integrity is lost.

Although there is agreement that the main mode of action of FTY720 is by blocking lymphocyte egress from the thymus and lymph nodes, some controversy remains concerning other effects on T cells. Indeed, protection from disease development even in the presence of significant infiltration in the pancreas suggested the possibility of direct effects of FTY720 on T cells. Previous studies have suggested that FTY720 induces Tregs or enhances their function[114, 115]. In our study, increased Tregs were only observed in the ILN, not in the PLN or in the pancreas, the locations where an increase in Tregs would be likely to have an effect on disease outcome. Interestingly, it is unclear whether this increase was due to preferential homing, proliferation or conversion from effectors to Tregs. FTY720 has also been suggested to induce apoptosis of T cells [113]. Similar to our results, Maki et al reported that continuous treatment of NOD mice with FTY720 prevented diabetes development during treatment[119]. They observed diabetes protection even after withdrawal in mice that started treatment at 4 weeks of age which

led to the suggestion that therapy eliminated autoreactive effector T cells by FTY720-mediated apoptosis[119]. Rather than previous interpretations, we propose that the lack of accelerated disease following treatment of young prediabetic NOD mice is a result of a low number of antigen-specific T cells in TLOs at the time of treatment initiation, which was not sufficient to develop a pathogenic T cell population capable of rapid disease induction once drug treatment was discontinued. This interpretation fits with the analysis of 8-week old mice, where TLOs were not yet organized into distinct T and B cells zones and therefore not fully established by this point. Moreover, in a model of allograft rejection, Habicht et al showed that FTY720 treatment had no effect on alloreactive T cell apoptosis, T cell activation, proliferation, cytokine production[120]. Our data are consistent with the documented mechanism of action of FTY720, namely, that FTY720 controls exit from primary and secondary lymphoid organs. Additionally, our data suggest that FTY720 also controls exit out of TLOs and entry into the neighboring pancreatic tissue thereby stabilizing these structures and preventing islet destruction and diabetes development.

Tertiary lymphoid organs have been previously described in the pancreas of NOD mice; however, a characterization at different stages of disease including association with specialized vessels had not been described. Our data show that Lyve1$^+$ and MAdCAM$^+$ vessels are present even in non-inflammatory conditions, such as the pancreas of NOD-RAG KO mice. Inflammation in NOD mice results in a higher percentage of islets associated with specialized vasculature. Whether this increase reflects neovascularization or expression of these markers on vessels that were previously present is not known.

The role of TLOs in the amplification of the immune response is unclear[81]. Our results expand on the role of TLOs in T cell responses. The adoptive transfer of naïve BDC2.5 Tg T cells during FTY720 treatment shows that naïve cells can in fact be recruited directly to the pancreas rather than having to be primed first in the PLN. In addition, proliferation of these cells in the pancreas suggests that there is local antigen presentation[121]. However, it is not known how the antigen reaches the TLOs. It is possible that islet-resident dendritic cells traffic to the TLO bringing antigen with them, antigen is picked up by TLO resident antigen presenting cells, such as macrophages and DCs, at the TLO/islet boundary or antigen travels through the lymph from the islet to the TLO.

The requirement of the lymphotoxin (LT) axis for TLO development has been explored because of its importance in lymph node development. Disruption of TLOs by over expression of LIGHT, a LTβR ligand, in the pancreas of NOD mice (NOD-RIP-LIGHT) results in accelerated diabetes compared to non-transgenic controls [106]. This suggests that during the natural course of disease and TLO break down additional signals are present that allow pathogenic cells to destroy the islets rather than instructing them to leave the pancreas. Therefore, preventing islet destruction by altering TLO maintenance will require a deeper understanding of the signals that control islet destruction. In addition, targeting of TLOs may be difficult since the same signals that induce, and presumably maintain, TLOs are also necessary for lymph node integrity[81].

Analysis of TLOs in type 1 diabetes patients has been challenging. In accordance with our findings, Willcox et al analyzed pancreas samples from patients who died within 18 months of diagnosis[17] and found scattered infiltrating cells surrounding glucagon or

insulin positive islets. We hypothesize that the lack of true TLOs in the nPOD samples is due to timing: analyzing long-term clinically diabetic patients likely misses the peak of the immune response in the pancreas. In fact, the NOD mouse data supports this interpretation since we found 81% of infiltrates were localized to TLOs in 20-week old prediabetic mice but only 26% of infiltrates were localized to TLOs in diabetic mice. This highlights the need for screening and analysis of pre-diabetic, autoantibody-positive individuals in order to elucidate the role of TLOs in type 1 diabetes.

CHAPTER IV

Interleukin-7 receptor blockade reverses autoimmune diabetes through PD-1-mediated inhibition of memory T cells

INTRODUCTION

One of the main differences between the adaptive and the innate branches of the immune system is the ability of the adaptive branch to develop memory such that secondary exposure to antigen results in a faster and stronger immune response. Upon encountering their cognate antigen, naïve T cells proliferate and develop into short-lived effector T cells that produce cytokines. Once the response is controlled or the antigen is cleared, most of the effector cells die and long-lived memory cells, phenotypically characterized by high expression of CD44 and IL-7Rα, remain at a higher precursor frequency than the corresponding naïve population. Whether memory cells differentiate linearly from effector cells or are a separate lineage is still controversial, particularly in $CD4^+$ T cells[123, 124]. Interestingly, the frequency of memory T cells positively correlates with the frequency of the naïve T cells prior to antigen exposure[125]. Memory cells can either be effector memory T cells, which downregulate CD62L, can home to non-lymphoid tissues and can rapidly produce cytokines, or central memory T cells, which express CCR7 and CD62L, reside primarily in the lymph nodes and quickly proliferate upon antigen encounter[126]. Unlike their naïve counterparts, memory T cells require lower levels of TCR and costimulation signaling[127], making them more sensitive to antigen stimulation. Indeed, memory T cells are able to proliferate and produce IL-2 *in vitro* in the absence of anti-CD28 or exogenous APCs[128].

Although memory T cells are long lived, low level of proliferation in the absence of antigen helps maintain the pool of memory cells [129]. The cytokines IL-7 and IL-15 have been shown to signal survival and proliferation of $CD4^+$ and $CD8^+$ memory T cells respectively. IL-7, produced primarily by stromal cells in lymph nodes, signals through a heterodimeric receptor composed of the IL7Rα, which is shared by the thymic stromal lymphopoietin (TSLP) receptor, and the common γ chain, which is shared by the IL-2, -4, -9, -15 and -21 receptors. IL7Rα expression in $CD4^+$ T cells is bimodal: in naïve T cells, IL7Rα expression is high; upon activation, IL-7Rα is rapidly downregulated and re-expressed, at higher levels than in naïve T cells, at the memory stage.

Memory T cells play an important role in autoimmune disease and are the main pathogenic cells perpetuating the self-reactive response. While interfering with T cell receptor and costimulatory signals required for activation of naïve self-reactive T cells has been successful to prevent autoimmunity in some models[97], it has typically not been effective once disease is established, likely because memory T cells are less sensitive to these interventions. The difficulty to control memory T cells underscores the need for novel approaches to target these cells. Since memory T cells are critically dependent on instructive signals from IL-7 for their generation and maintenance[130-132], interfering with this cytokine may represent a strategy for treating autoimmune disease.

In this study, we examined whether blocking the cytokine IL-7 has an effect on the pathogenesis of type 1 diabetes in the NOD mouse model. We treated NOD mice with anti-IL-7Rα blocking antibodies both before and at diabetes onset and found that IL-7 blockade prevented and cured diabetes through induction of the inhibitory molecule PD-1 on memory $CD4^+$ T cells. Our data suggest that IL-7 contributes to the pathogenesis of

autoimmune diabetes by keeping memory T cells in a functionally competent, tolerance-resistant state and uncover a novel link between IL-7 and the PD-1/PD-L1 tolerance pathway.

MATERIALS AND METHODS

Mice

Female NOD mice were purchased from The Jackson Laboratory or Taconic. NOD.*scid*, NOD.Thy1.1 and NOD.Foxp3GFP mice were bred in our facility [61]. Mice were housed in the specific pathogen-free facility of the University of California San Francisco. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco.

Diabetes assessment and histology

Diabetes incidence was followed by urine analysis (Diastix, Bayer) and measuring of blood glucose levels with a Contour glucose meter (Bayer). Mice with two consecutive readings between 250-400 mg/dl were considered new-onset diabetic and used for anti-IL-7Rα treatment. Histological analysis was performed by fixing pancreata in 10% buffered formalin and staining 5μm sections with hematoxylin and eosin; 10 sections/pancreas were blindly scored for insulitis. Scores are as follows: 0 = no infiltrate, 1 = 0-25%, 2 = 25-75%, 3 = > 75%.

Antibody treatment

Anti-IL-7Rα (rat IgG2a, clone A7R34) and anti-PD-L1 (rat IgG2a, clone MIH5) antibodies for *in vivo* blocking experiments were produced by the hybridoma cell lines and purified with Protein G Sepharose 4 Fast Flow (GE Healthcare) in our lab. Rat IgG (Jackson ImmunoResearch Laboratories) was used as a control. For IL-7Rα blockade, 0.5 mg anti-IL-7Rα antibodies in PBS were administered twice weekly intraperitoneally. Anti PD-L1 antibodies were administered as described previously[133].

Antibodies

Fc receptors were blocked with anti-CD16/CD32 antibodies, prepared in our lab from the hybridoma cell line, before all antibody staining procedures. The following antibodies coupled to the indicated fluorochromes were used for detection of murine activation markers and cytokine receptors: anti-CD3ε-Pacific Blue (17A2, eBioscience); anti-CD4-PerCP (RM4-5, BD) or -Alexa-700 (eBioscience); anti-CD8-PercP (53-6.7, BD); anti-CD11b-FITC (M1/70, BD); anti-CD11c-FITC (HL3, BD); anti-CD25–FITC, -APC (7D4, BD) or –APC-eFluor780 (PC61, eBioscience); anti-CD44–APC or –APC-eFluor780 (IM7, eBioscience); anti-CD45R/B220 (RA3-6B2, eBioscience); anti-CD127–biotin (A7R34, eBioscience); CD90.1-PercP (OX-7, BD); CD90.2-PercP (30-H12, Biolegend); CD279-PE (J43, eBioscience) or –PE-Cy7 (RMP1-30, Biolegend); anti-FoxP3-FITC (FJK-16s, eBioscience); streptavidin–PE and -APC (eBioscience); Armenian hamster control IgG2 PE (BD).

Tetramer staining, flow cytometry and cell sorting

Islet antigen-specific T cells present in secondary lymphoid organs were detected after enrichment with BDC2.5 pMHC tetramers (I-A^{g7} BDC 2.5 mimotope sequence 2 RTRPLWVRME (NIH tetramer core facility)) following a method described previously[125]. Tetramer-positive cells were quantified using percentages obtained by flow cytometry and absolute counts of enriched cells. Phenotypic analysis of cell populations was performed by multi-parameter flow cytometry. Fluorescence intensities were measured on an LSRII or Fortessa flow cytometer and data were analyzed with FACSDiva (BD Biosciences) or FlowJo software. For adoptive transfer studies and *in vitro* experiments, naïve ($CD25^{neg}CD44^{low}IL\text{-}7R\alpha^{high}$) and memory ($CD25^{neg}CD44^{high}IL\text{-}7R\alpha^{high}$) $CD4^+$ T cells from donors were labeled with antibodies and isolated with a high-speed cell sorter (MoFlo, DakoCytomation).

In vitro stimulation assays

Naive $PD\text{-}1^{neg}Foxp3^{neg}$ $CD4^+$ T cells were isolated from NOD.Foxp3GFP mice and stimulated with 10 μg soluble anti-CD3 mAb (145-2C11, BD) and 1 μg anti-CD28 mAb (37.51, BD) antibodies in the presence or absence of recombinant murine IL-7 (Peprotech). Cells were cultured in RPMI 1640 media (Invitrogen) supplemented with 1mM each of L-glutamine, non-essential amino acids, sodium pyruvate, HEPES, penicillin, streptomycin (Invitrogen), 50μM 2-ME, and 10% fetal calf serum (Omega Scientific) and incubated at 37° in 5% CO_2. Cell cultures were set up in round-bottom 96 well plates (BD Falcon) and harvested after 2, 4 and 6 days for flow cytometric analysis

of PD-1 expression. Death cells were excluded from the analysis by staining with 4’6-diamidino-2-phenylindole (Invitrogen).

PD-1 staining on T cells from peripheral blood of Type 1 Diabetes patients

Venous blood was drawn with ethical approval from patients attending the clinical Type 1 diabetes service at the University Hospital Birmingham National Health Service Foundation Trust, UK. Patients had been clinically diagnosed with type 1 diabetes according to the 1997 American Diabetes Association guidelines. Venous blood was also drawn from healthy volunteers, matched as closely as possible for age and sex. Mononuclear cells were isolated from fresh blood samples by buoyant density centrifugation and analyzed by multicolor flow cytometry using antibodies against CD3, CD4, CD45RA, CD25 and PD-1. Data were acquired using a Cyan ADP Analyzer and processed using FlowJo software.

Statistics

Statistically significant differences between groups were determined using the one-tailed Mann Whitney *u* test. Horizontal bars in graphs indicate statistical significance ($P< 0.05$) and P values are indicated.

RESULTS

Anti-IL7Rα treatment prevents and cures diabetes in NOD mice

One of the challenges in treating type 1 diabetes is that it is a slowly developing, chronic disease and the efficacy of most treatments highly depends on the stage of the

disease at which the treatment is given[71]. We initially asked whether blocking IL-7 signals in NOD mice, which spontaneously develop type 1 diabetes, would prevent disease development. We began treating pre-diabetic NOD mice at 10 weeks of age, when islet infiltration is established, with anti-IL-7Rα antibodies to block IL-7 cytokine activity. In control groups, mice started becoming diabetic at 14 weeks of age and, as expected, the incidence of diabetes gradually increased over the treatment period reaching 60-70% by 24 weeks. In contrast, only 10% of mice receiving anti-IL-7Rα antibodies developed diabetes during the treatment period (**Figure 15A**). Histological examination revealed significantly diminished islet infiltration in the treated animals (**Figure 15B**) showing that IL-7Rα blockade prevents accumulation of autoreactive cells in the islets.

While this result established a role for IL-7 in disease development, it is clinically more relevant to initiate treatment once hyperglycemia is apparent. To test whether blocking IL-7 could reverse established diabetes, we administered anti-IL-7Rα antibodies to a cohort of new-onset diabetic NOD mice and followed blood glucose levels. We found that this treatment restored normoglycemia in ~ 50% of treated animals (**Figure 15C**), adding IL-7Rα blockade to the list of only a handful of treatments capable of reversing the disease [71] and the first cytokine receptor blockade therapy for diabetes. Importantly, anti-IL-7Rα treated mice remained normoglycemic long after the treatment was stopped, far exceeding the estimated half-life of the antibodies. Two possible explanations for this long-lasting effect are that (1) T cell survival, particularly of islet antigen-specific cells, was significantly compromised leading to clearing of cellular infiltrate thereby “resetting” the disease to an early stage, or (2) the treatment induced

tolerance in the diabetogenic T cells. Given the well-established role of IL-7 in regulating T cell homeostasis [134], it is not surprising that total lymphocyte and $CD4^+$ T cells numbers showed a tendency to decrease after 2-3 weeks of anti-IL-7Rα treatment (**Figure 16**). However, histological analysis showed only a limited reduction of the islet infiltrates (**Figure 15D**) suggesting that depletion of diabetogenic T cells does not explain the effect of anti-IL-7Rα therapy. Furthermore, the numbers of islet-specific $CD4^+$ T cells, identified with BDC2.5 pMHC tetramers[125] were similar in the secondary lymphoid organs of cured and new-onset diabetic animals (**Figure 15E**). These data demonstrate that considerable numbers of islet-reactive T cells remain present in the anti-IL-7Rα-treated mice, indicating that poor T cell survival is not the main mechanism for the therapeutic effect.

Anti-IL7Ra treatment does not selectively deplete diabetogenic memory T cells

Diabetogenic $CD4^+$ T cells in the NOD mouse can be identified in adoptive transfer experiments[135] and are predominantly found in the $CD44^{high}$ effector/memory T cell population[136, 137] (our own unpublished observations). To rule out that IL-7Rα blockade led to selective deletion of $CD44^{high}$ memory T cells, we adoptively transferred congenically marked naïve and memory T cells to NOD mice and followed their persistence after IL-7Rα blockade. We found no differences in the percentages of naïve and memory T cells present in the draining pancreatic lymph nodes (**Figure 17A**) and other secondary lymphoid tissues (data not shown) of anti-IL-7Rα-treated and control mice. Hence, while absolute numbers of memory T cells decrease to some extent, their relative presence in the $CD4^+$ T cell population was not affected by IL-7 deprivation.

This observation prompted us to compare the diabetogenic capacity of memory T cells isolated from anti-IL-7Rα-treated and control mice by adoptively transferring equal numbers of these cells to NOD.*scid* recipients and following diabetes incidence. Strikingly, memory $CD44^{high}$ $CD4^{+}$ T cells isolated from anti-IL-7Rα-treated NOD mice failed to transfer diabetes to NOD.*scid* recipients, unlike transferred control cells (**Figure 17B**). The same difference was seen when total $CD4^{+}$ T cells (excluding $CD25^{+}$ cells to eliminate Tregs) isolated from anti-IL-7Rα–treated and control mice were transfered, albeit with slower disease kinetics (**Figure 17C**). The failure of these cell populations to cause diabetes was not due to poor survival or "grafting" after adoptive transfer since equal numbers of memory T cells were recovered from the lymphoid organs 8-10 weeks later (**Figure 18A**). Also, similar numbers of Tregs developed in recipients of treated vs untreated $CD4^{+}$ T cells (**Figure 18B**), indicating differential Treg development is not the underlying mechanism. Importantly, while the numbers and phenotype of the cells recovered from NOD.*scid* lymphoid organs was indistinguishable, cells derived from anti-IL-7Rα-treated mice failed to accumulate in the islets (data not shown), suggesting they were resistant to activation by islet antigens.

IL-7 controls PD-1 expression in vivo and in vitro

Two major tolerance mechanisms known to control pathogenesis in the NOD diabetes model are the cell-intrinsic inhibitory pathway PD-1/PD-L1[133, 138] and Tregs[60]. Therefore, we analyzed the impact of IL-7Rα blockade on the presence of PD-1-expressing $CD4^{+}$ T cells and Tregs in NOD mice. After 2-3 weeks of treatment, the percentage of PD-1^{+} $Foxp3^{neg}$ cells and $Foxp3^{+}$ Tregs was increased within the $CD4^{+}$ T

cell population (**Figure 19A-C, Figure 20**). Enhanced numbers of Tregs after IL-7 blockade have been reported previously[139] and can be attributed to lower IL-7Rα expression in this population and hence reduced dependency on IL-7 for survival. While the increase in Tregs may contribute to diabetes prevention and reversal in mice deprived of IL-7 signals, such bystander suppression cannot explain the inability of adoptively transferred $CD4^+$ T cells, from which Tregs were excluded, to cause diabetes in NOD.*scid* recipients (**Figure 17B and C**).

The cell surface receptor PD-1 is induced upon T cell activation and remains expressed in $CD44^{high}$ effector and memory cells that are subject to persistent antigen stimulation, such as in autoimmunity and chronic viral infection, resulting in attenuated responses[140, 141]. To assess whether enhanced expression of PD-1 on $CD4^+Foxp3^{neg}$ T cells explains the protective effect of IL-7Rα blockade, we treated a cohort of new-onset diabetic mice with anti-IL-7Rα and, once cured, and asked if blocking the PD-1/PD-L1 pathway would restore the disease. The rapid relapse (~ 4-5 days) seen after administration of anti-PD-L1 antibodies (**Figure 21A**) demonstrates that blocking IL-7 keeps diabetogenic effector/memory T cells in an unresponsive state that is dependent on the presence of PD-1. Importantly, PD-L1 blockade accelerated diabetes onset in NOD.*scid* mice that received $CD44^{high}$ memory T cells isolated from anti-IL-7Rα-treated mice, proving engagement of PD-1 tolerizes islet-reactive memory T cells in a cell–intrinsic manner (**Table 2**). Finally, to directly demonstrate a causal relationship between IL-7 signaling and absence of PD-1 expression, we isolated naive $CD4^+$ $PD\text{-}1^{neg}$ T cells from NOD mice and stimulated these cells *in vitro* with anti-CD3/CD28 antibodies in the absence or presence of recombinant IL-7. We found that IL-7 diminished PD-1

expression on activated T cells in a dose-dependent manner (**Figure 21B**). Although initial induction of PD-1 was similar in these cultures (data not shown), the presence of IL-7 suppressed surface expression of the receptor at the later stages of the response (days 4-6). Interestingly, comparison of PD-1 expression on $CD4^+$ T cells from peripheral blood of diabetic patients versus healthy controls showed decreased presence of $CD4^+PD1^+$ memory T cells in diabetic patients (**Figure 22**), providing a rationale for developing methods to increase expression of this inhibitory molecule.

DISCUSSION

In this study we show that treatment of NOD mice with anti-IL7Rα monoclonal antibodies can prevent and cure diabetes. Importantly, this effect is not due to preferential depletion of memory or antigen specific diabetogenic cells. Furthermore, cells isolated from anti-IL7Rα treated mice were unable to transfer disease to NOD.*scid* recipients suggesting that treatment established a mechanism of dominant tolerance that could be transferred to a new host independent of Tregs. Memory cells present in animals after anti-IL-7Rα treatment expressed the inhibitory receptor PD-1 and inhibiting the interaction of PD-1 with its ligand PD-L1 restored diseased in cured mice. *In vitro* activation of naïve T cells in the presence of recombinant IL-7 resulted in reduced PD-1 expression, suggesting that IL-7 may directly control PD-1 expression *in vivo*.

Recently Liu et al[139] showed that blockade of IL-7 at the onset of EAE resulted in reduction of disease severity due to a selective reduction of IL-17 production. This decrease was due to preferential apoptosis of Th17 cells, compared to Th1 cell and Tregs, via inhibition of the JAK/STAT5 pathway. Although we did not specifically look at the

survival of Th1 vs Th17 cells, this is likely not the mechanism of disease reversal we seen in NOD mice, as Th17 cells are not considered the pathogenic population in this model. In fact, Th17-skewed BDC2.5 transgenic effector cells become IFNγ producing Th1 cells after transfer to NOD.*scid* mouse and inhibition of IL-17 does not prevent diabetes in this transfer model[142]. Decreased survival of Th1 cells is also not likely the sole mechanism since for our adoptive transfer experiments equal numbers of cells were transferred in order to correct for decreased survival in the absence of IL-7. Even in this situation, cells isolated from anti-IL-7Rα treated mice failed to transfer disease to lymphopenic hosts.

The expression of PD-1 on $CD8^+$ memory T cells has been widely reported in the setting of chronic virus infections, such as HIV, HBV and HCV, where antigen exposure is prolonged because the infection is not effectively cleared. Blockade of the PD-1/PDL-1 interaction results in increased numbers of cytokine-producing virus-specific $CD8^+$ T cells and a reduction in viral titers[141]. Therefore, PD-1 expression on memory $CD8^+$ T cells has been a feature of functional impairment or exhaustion. Although exhaustion of memory $CD4^+$ T cells has not been suggested, we propose that autoimmune settings can give rise to such a population, since there is also chronic exposure to antigen.

Our data reveal a novel role for IL-7 in T cell responses by regulating antigen responsiveness. It has recently been shown that high doses of IL-7 enhance anti-tumor responses by counteracting inhibitory mechanisms in T cells [143]. Our results suggest a physiological role of IL-7 in reducing PD-1 expression and maintaining effector/memory T cells in a functionally responsive state. We speculate that in chronic viral infection IL-7 deprivation, due to low IL-7Rα expression, may be responsible for the emergence of

"exhausted" memory $CD8^+$ T cells expressing high levels of PD-1[141], suggesting that methods to increase IL-7 signals in these cells may enhance anti-viral immunity. We hypothesize that in NOD mice and diabetic patients, access to IL-7 prevents PD-1 upregulation on pathogenic autoreactive T cells that would otherwise by inhibited by PD-L1 expressed on the islet itself. In support of this hypothesis, we show that patients with type 1 diabetes have a decreased proportion of PD-1^+ memory cells.

Although we do not know at this time whether IL-7 plays a role in regulating PD-1 expression in islet-reactive T cells in human diabetes, our study identifies IL-7 as an attractive target for therapy. Importantly, IL-7 blockade did not have a negative impact on the survival of Tregs. Therefore, a combination therapy that involves IL-7 blockade to control memory cells and IL-2 to boost Tregs would be an attractive approach.

CHAPTER V

CONCLUSION

In this body of work I, along with my collaborators, investigated three immunotherapies for the treatment of type 1 diabetes in the NOD mouse model (**Figure 23**): Fc receptor non-binding (FNB) monoclonal anti-CD3 antibodies, FTY720 and anti-IL7Rα monoclonal antibodies. Our findings not only expand our understanding of the mechanism of action of each therapy, but also uncovered previously unrecognized characteristics of the immune response during autoimmune diabetes. First, our studies of FNB anti-CD3 mAb revealed that, contrary to previous interpretations, this therapy does not induce Tregs, but rather alters the balance of conventional T cells to regulatory T cells. Furthermore, it also alters the balance of stable to unstable Tregs, a newly emerging concept in the field of Tregs. Second, we show that FTY720 treatment of NOD mice prevents diabetes, but accelerates disease development upon withdrawal. Analysis of tertiary lymphoid organs (TLOs) in the pancreas of untreated mice revealed that the structures evolve from an unorganized accumulation of cells into organized B and T cell compartments that mirror the organization in secondary lymphoid tissues. TLOS in FTY720 treated mice were compartmentalized suggesting that this drug stabilizes TLOs. Just 12 days after drug withdrawal, disorganization resembled that seen in naturally diabetic mice. Therefore, islet destruction correlates with lack of compartmentalization, suggesting that TLOs may be protective structures. Third, we show that treatment of NOD mice with anti-IL7Rα antibody prevents and cures diabetes through upregulation of PD-1 on $CD4^+$ memory cells since blockade of PD-1/PD-L1 interactions in cured mice,

restores diabetes. This finding suggests that in addition to being a survival factor for memory cells, IL-7 also maintains effector/memory T cells in a functionally responsive state by down regulating PD-1; in the absence of this cytokine, cells express PD-1 and become tolerant.

One of the most promising therapeutic interventions for the treatment of type 1 diabetes has been anti-CD3 mAbs because of their long lasting effects without the need for continuous treatment. As is discussed in Chapter II, a short treatment of NOD mice with anti-CD3 mAbs was found to permanently reverse disease [86] and modifications of this antibody were developed to minimize its side effects. A clinical trial using hOKT3γ1(Ala-Ala) (Teplizumab), a humanized version of the anti-CD3 mAb used in our studies, showed that treatment of newly diagnosed patients improved C-peptide responses and HbA1c levels and reduced insulin usage with only mild side effects[144]. In a second trial using ChAglyCD3 (Otelixizumab), there were more adverse effects and, although HbA1c levels were similar between treated and control patients, treated patients showed increased insulin secretion and reduced insulin usage[145]. However, the one-year safety and efficacy data of the Phase 3 clinical trial of Teplizumab showed no efficacy based on a composite of insulin usage and HbA1c levels (J.A. Bluestone, personal communication). It is not clear why this trial showed no efficacy while the previous trial with Teplizumab and trials with other anti-CD3 mAbs derivates showed such promise. Although it had been assumed that FNB anti-CD3 mAbs induced Tregs in mice, our data shows that this treatment actually alters the balance of Tregs and Teffs as well as adaptive versus natural Tregs. Therefore, further investigation into the modes of action of these mAbs may give further insights into their mode of action. For example, it is

possible that different dose regimens affect Tregs and Teffs differently, potentially explaining the conflicting results between the various clinical trials.

Promising clinical trials using FTY720 as a treatment for multiple sclerosis are underway[146] suggesting its possible use in treating other autoimmune diseases such as type 1 diabetes. However, our data suggests that if there is a significant pool of autoreactive cells at the beginning of treatment this pool continues to expand during treatment and becomes highly pathogenic, such that if treatment were advertently or inadvertently discontinued the stealth T cells might rapidly destroy the target tissue. Therefore it may be necessary to combine this therapy with one that eliminates potentially pathogenic autoreactive T cells like anti-CD3 or anti-IL-7R.

Our finding that IL-7Rα blockade in NOD mice reverses diabetes and leads to increased PD-1 expression on memory cells, together with the finding that patients with type 1 diabetes patients have a lower percentage of PD-1^{hi} memory cells, suggests that autoreactive T cells may overcome tolerance by downregulating this inhibitory surface receptor. Further research confirming that autoreactive T cells in humans are indeed PD-1^{neg} in patients with type 1 diabetes, as well as other autoimmune diseases, is justified. If this is the case, developing therapeutic approaches to restore the PD-1 tolerance pathway, such as blockade of IL-7Rα signaling, will become a new area of research for the treatment autoimmune diseases.

As discussed in the introduction, one of the major concerns about treating type 1 diabetes with immunomodulatory therapies is the risk of general immunosuppression such as reactivation of latent viruses or lack of protection against cancer. Although we did not directly address this issue in any of our studies, our data suggest that these

therapies do not completely inhibit immune responses. For example, we showed that antigen-specific T cells are still present after anti-IL7Rα treatment, suggesting that immune responses are complex using a variety of complementary pathways that overcome blockade of individual components. Likewise, we show that dendritic cells are able to traffic during FTY720 treatment, suggesting that this drug does not impair presentation of tissue antigens in lymph nodes. Nevertheless, the ability to mount protective immune responses during the treatments needs to be directly addressed. Importantly, as the targets of these drugs are mostly expressed on cells of the adaptive immune system, it is likely that the innate branch of the immune system is unaffected.

The future of immunomodulatory therapies for autoimmune disease likely lies in the use of multiple agents to achieve the best response and decrease deleterious side effects. Our data suggest that combining FNB anti-CD3 mAb and FTY720 may be a successful combination: cells activated during FTY720 treatment would be depleted by FNB anti-CD3 mAb increasing the specificity for islet antigen specific T cell deletion. Another successful combination may be anti-IL-7Rα and IL-2/anti-IL-2 complexes to tolerize autoreactive memory T cells and boost Tregs respectively. Further investigation into the mode of action of these and other therapies will allow us to predict which combination therapies will be most successful.

Finally, advances in transplantation and stem cell therapy may make new immunotherapies useful in a larger population of patients with type 1 diabetes. The first pancreas transplant was performed in 1966 and since then significant advances have been made with current 1-year survival rates over 75%[147, 148]. Islet transplants have also been successful, with 80% of recipients achieving insulin independence 1 year post

transplant[149]. However, these transplants require immunosuppression and the complications from these drugs can be problematic and thus not widely useful for the bulk of people with T1D. Recently, advances in developing induced pluripotent stem cells suggest another possible therapy[150]. In addition to embryonic stem cells, pancreatic and non-pancreatic adult stem cells are being used as potential sources to replenish beta cells. The major hurdle in this area of research is being able to produce beta cells that are responsive to glucose levels. Moreover, in the absence of immunosuppression, the newly regenerated islets would likely be targeted by the immune system since autoreactive memory T cells would still be present in these patients. Therefore, for transplantation or islet regeneration to become the main therapeutic approaches for the treatment of type 1 diabetes, the ability to control anti-islet immune responses will be critical and research into achieving tolerance will continue to be active area of investigation.

		Pancreas	PLN	ILN	Spleen
Total cell number (x 10^6)	Control	n/d	2.73 ± 1.40	9.97 ± 3.50	79.6 ± 25.9
			*	*	*
	FTY720 treated	n/d	3.16 ± 0.80	3.65 ± 1.03	31.7 ± 15.2
Percent CD4$^+$ of CD45$^+$ cells	*Control*	*26.2 ± 9.6*	*48.5 ± 1.5*	*51.4 ± 7.5*	*28.3 ± 4.7*
		*	*	*	*
	FTY720 treated	*9.3 ± 1.9*	*32.4 ± 1.8*	*27.8 ± 3.3*	*13.1 ± 2.6*
Total CD4$^+$ cell number (x 10^6)	Control	n/d	1.32 ± 0.67	4.99 ± 1.47	21.6 ± 4.48
				*	*
	FTY720 treated	n/d	1.02 ± 0.26	1.00 ± 0.25	4.07 ± 1.95
Percent Foxp3$^+$ of CD4$^+$ cells	*Control*	*17.3 ± 6.8*	*21.6 ± 5.7*	*11.8 ± 2.6*	*17.9 ± 4.2*
				*	
	FTY720 treated	*26.5 ± 9.8*	*24.3 ± 3.3*	*27.2 ± 2.3*	*20.0 ± 3.1*
Total CD4$^+$Foxp3$^+$ cell number (x 10^6)	Control	n/d	0.28 ± 0.15	0.60 ± 0.23	3.91 ± 1.33
				*	*
	FTY720 treated	n/d	0.25 ± 0.07	0.27 ± 0.07	0.84 ± 0.45
Percent CD8$^+$ of CD45$^+$ cells	*Control*	*7.2 ± 5.3*	*19.8 ± 2.6*	*20.5 ± 2.4*	*12.5 ± 2.0*
			*	*	*
	FTY720 treated	*4.0 ± 4.1*	*31.9 ± 3.3*	*31.5 ± 3.4*	*4.0 ± 1.0*
Total CD8$^+$ cell number (x 10^6)	Control	n/d	0.54 ± 0.29	2.02 ± 0.73	9.86 ± 3.31
			*	*	*
	FTY720 treated	n/d	1.01 ± 0.28	1.13 ± 0.26	1.25 ± 0.59

Table 1. Analysis of T cells during FTY720 treatment. NOD female mice were treated for 10 weeks with FTY720 beginning at 10-12 weeks of age. Mice were analyzed on the last day of treatment. Total cell number and percentages of CD4$^+$ and CD8$^+$ lymphocytes amongst CD45$^+$ cells and CD4$^+$Foxp3$^+$ cells amongst CD4$^+$ cells was determined by flow cytometry. Mean values of 3 independent treatment experiments are shown. Control n=8. FTY720 treated n=11. n/d: not determined. PLN: draining pancreatic lymph node. ILN: non-draining inguinal lymph node. *p<0.01 between control and FTY720 treated in the same tissue.

Donor treatment (NOD)	Recipient treatment (NOD.*scid*)	Days until hyperglycemic
new-onset/untreated	rat IgG	35, 67
	anti-PD-L1	32, 39
new-onset + anti-IL7Rα/cured	rat IgG	63, 88, > 170
	anti-PD-L1	42, 44, 81

Table 2. PD-1 expression on CD44^{high} memory T cells controls their diabetogenicity. CD44^{high} CD4^{+} memory T cells were isolated from new-onset diabetic NOD mice that were left untreated or that were cured with anti-IL-7Rα blockade. 5 x 10^{5} memory cells isolated from each individual mouse were split in two and adoptively transferred to two NOD.*scid* recipients. Matching recipients were treated with rat IgG or anti-PD-L1 antibodies (0.25 mg every other day for two weeks, followed by twice weekly) and diabetes incidence was followed. Numbers shown represent individual mice.

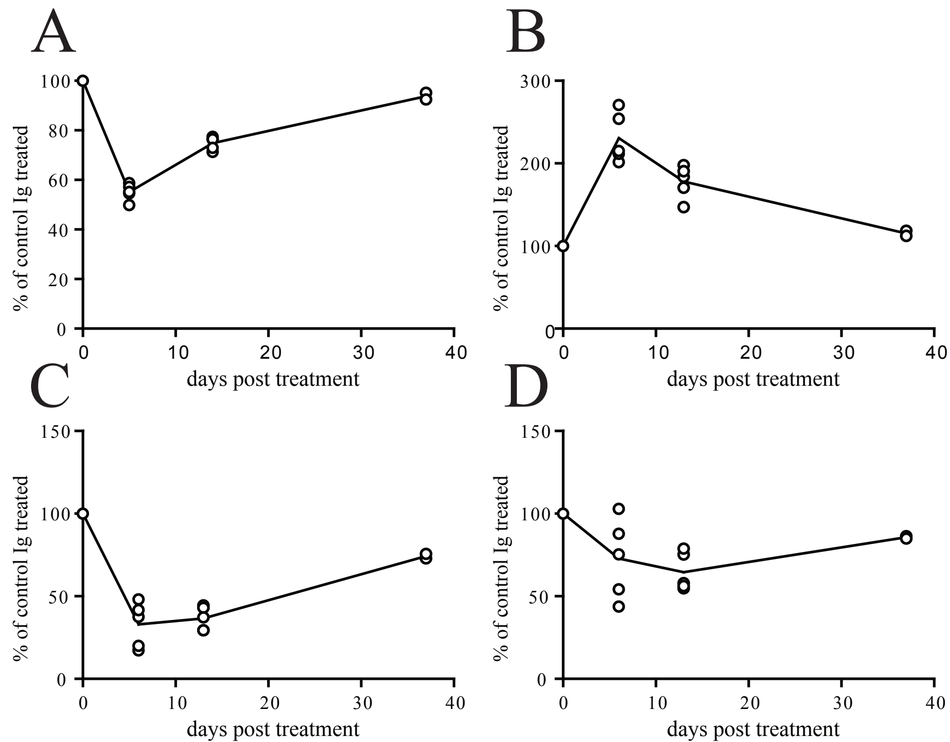


Figure 1. Anti-CD3 mAb treatment increased the percentage but not the absolute number of $CD4^+Foxp3^+$ T cells. Mice received 10μg FNB anti-CD3 mAb on 5 consecutive days. At the indicated times peripheral LNs were harvest, total cell numbers were determined and T cells subsets were analyzed by flow cytometry. Data shown is normalized to Ig treated control mice harvested on the same day. (A) Percent of $CD4^+$ cells within lymphocyte gate. (B) Percent of $Foxp3^+$ within $CD4^+$ gate. (C) Total $CD4^+$ cells. (D) Total $CD4^+Foxp3^+$ cells. Each dot represents an individual mouse.

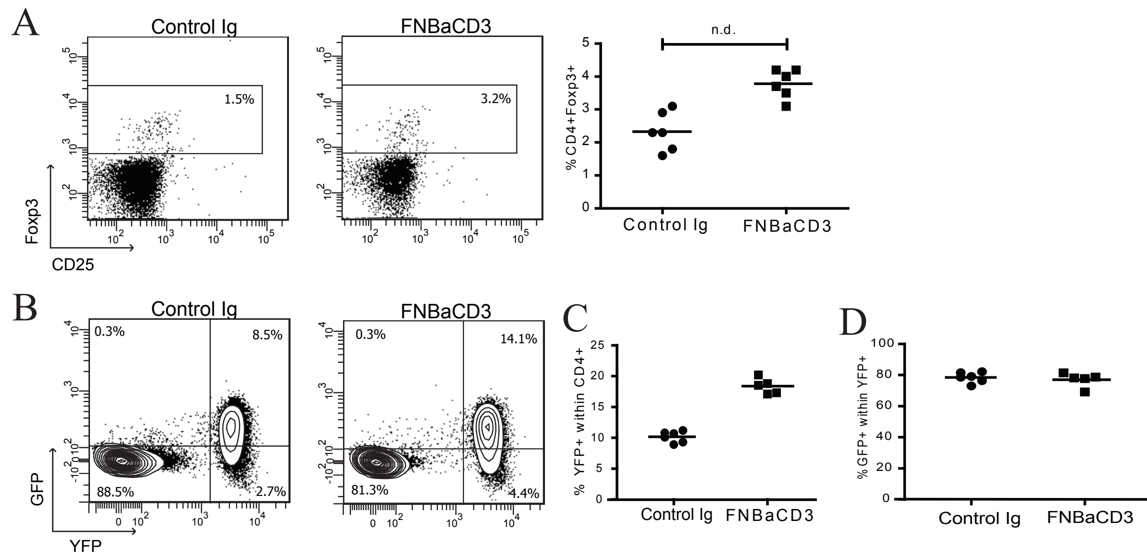


Figure 2. FNB anti-CD3 mAb does not induce conversion of $CD4^+Foxp3^+$ Treg cells from $CD4^+Foxp3^-$ T cells or affect Treg stability. (A) Congenically marked $CD4^+GFP^-$ cells sorted from Foxp3-GFP reporter mice were transferred to wild type recipients that were treated with 10μg FNB anti-CD3 mAb on 5 consecutive days. On day 7 expression of Foxp3 was analyzed on the transferred cells by flow cytometry. (B) Foxp3.GFP-Cre x Rosa26.flox.stop mice were treated with 10μg FNB anti-CD3 on 5 consecutive days and analyzed on day 14 by flow cytometry. Representative FACS plots gated on $CD4^+$ T are shown. (C) Percentage of YFP^+ cells within the $CD4^+$ population (sum of upper and lower right quadrants on plots shown in B). (D) Percentage of GFP^+ cells within the YFP^+ population (upper right quadrant divided by sum of upper and lower right quadrants on plots shown in B). Data shown is pooled from two independent experiments.

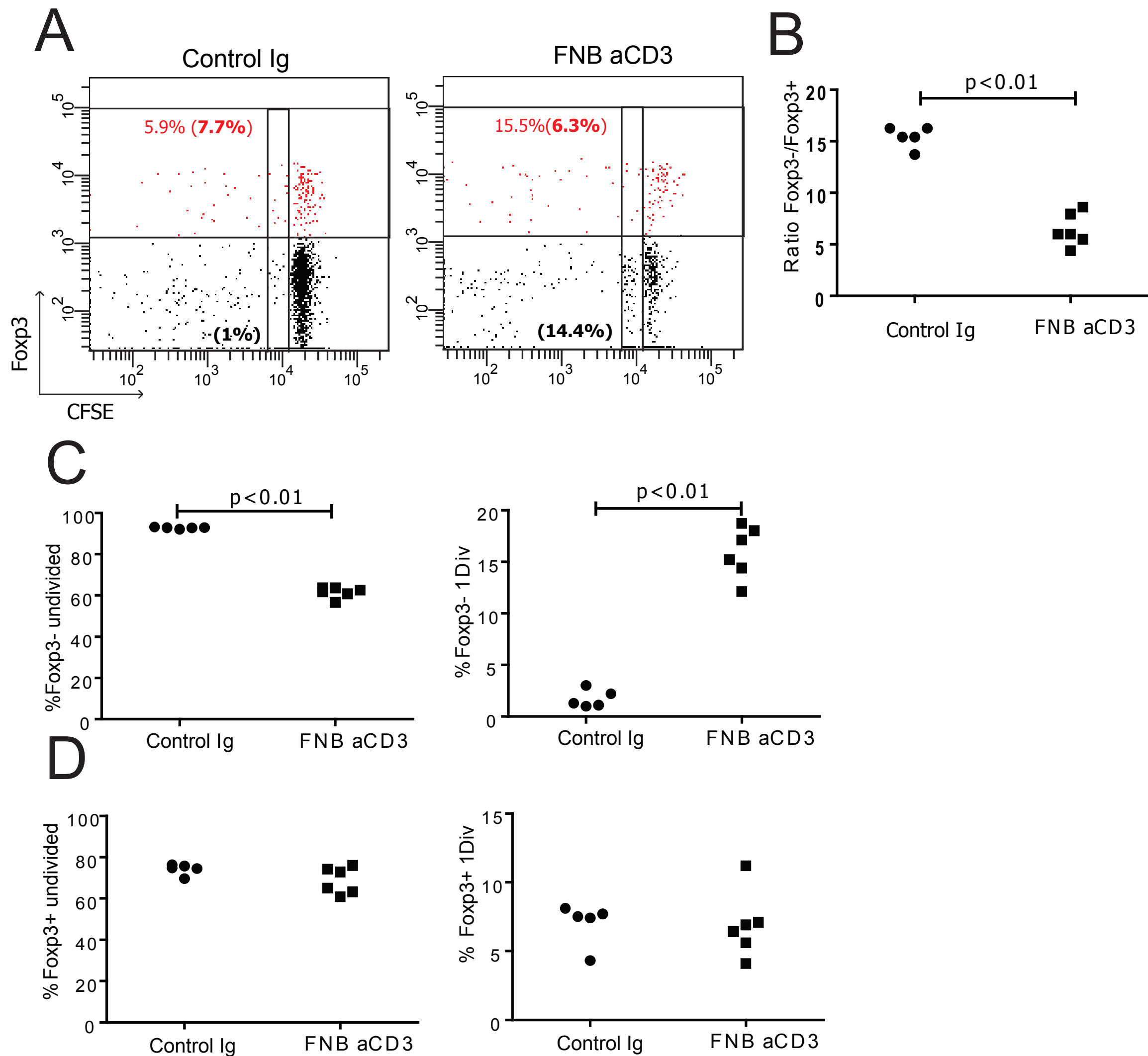


Figure 3. FNB Anti-CD3 mAbs preferentially depletes $CD4^+Foxp3^-$ T cells.

Congenically marked purified $CD4^+$ T cells were labeled with CFSE and transferred to recipient mice that were treated with 10μg FNB anti-CD3 on 5 consecutive days and analyzed on day 7 by flow cytometry. (A) Representative FACS plots showing CFSE dilution and Foxp3 expression on transferred cells. (B) Ratio of $Foxp3^-$ to $Foxp3^+$ cells. (C) Percentage of $Foxp3^-$ cells that remained undivided (left) or divided one time (right). (D) Percentage of $Foxp3^+$ cells that remained undivided (left) or divided one time (right). Data shown is pooled from two independent experiments.

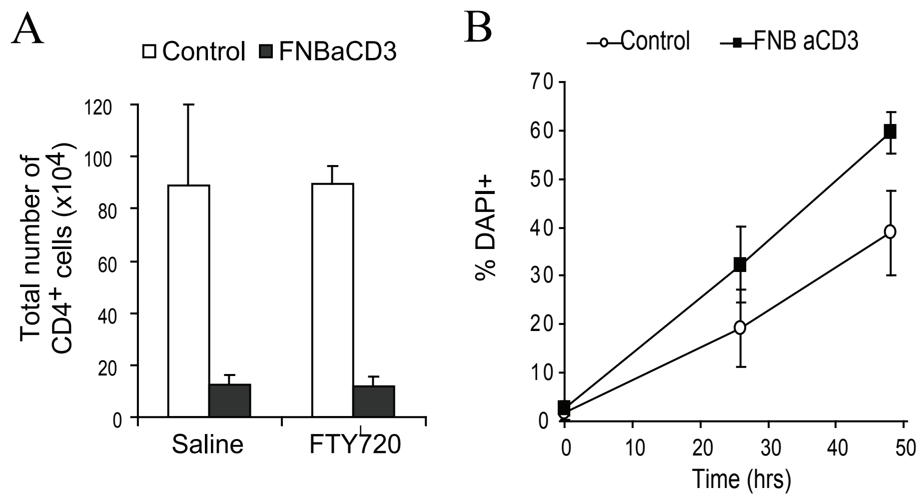


Figure 4. Depletion of T cells induced by FNB anti-CD3 mAbs is due to cell death *in vivo*. (A) Mice were treated with FTY720 daily on days 1-8 to block lymphocytes from exiting LNs and with 10μg FNB anti-CD3 mAb on days 2-6. The total numbers of $CD4^+$ T cells in LN of saline and FTY720 treated NOD mice were analyzed on day 9. (B) Mice were treated twice with 10μg FNB anti-CD3 mAb 24 hrs apart. 38hrs after first injection LNs were harvested and cells were cultured *in vitro* for the times specified. Viability was analyzed by flow cytometry by measuring DAPI inclusion. Data shown represents average of 5-6 mice pooled from at least two independent experiments. Bars represent standard deviation between samples.

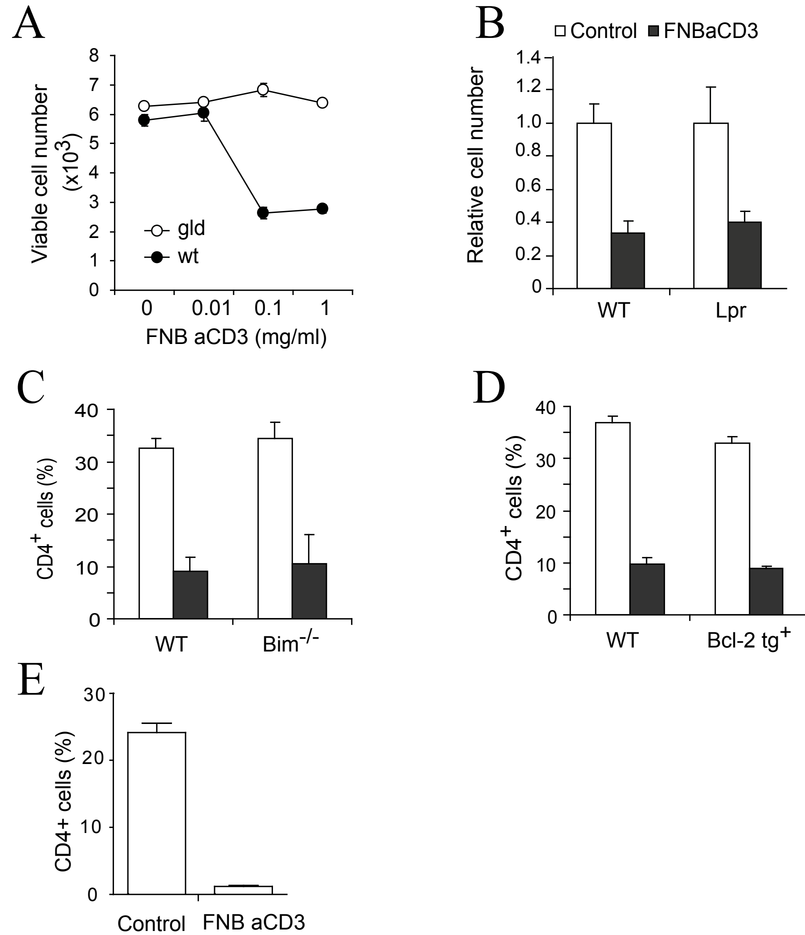


Figure 5. FNB anti-CD3 mAbs induce T cell death via distinct mechanisms *in vitro* **and** *in vivo*. (A) Th1 cell lines were generated from WT or Fas ligand-deficient *gld* DO11.10 TCR transgenic mice. Th1 cells were exposed to a titrated dose of FNB anti-CD3 mAb *in vitro* and the number of viable cells after overnight culture were determined by flow cytometry by counting CD4$^+$Annexin$^-$PI$^-$ cells. (B-D) WT, Lpr, Bim-deficient mice or Bcl-2 transgenic mice were given a 5-day course of FNB anti-CD3 mAb or PBS as control and the percentage of CD4$^+$ cells in the LNs was determined one week after the initiation of the treatment. (E) Caspase-3 deficient mice were treated with 10μg control Ig or FNB aCD3 on 5 consecutive days. On day 7, the percentage of CD4$^+$ cells was determined by flow cytometry.

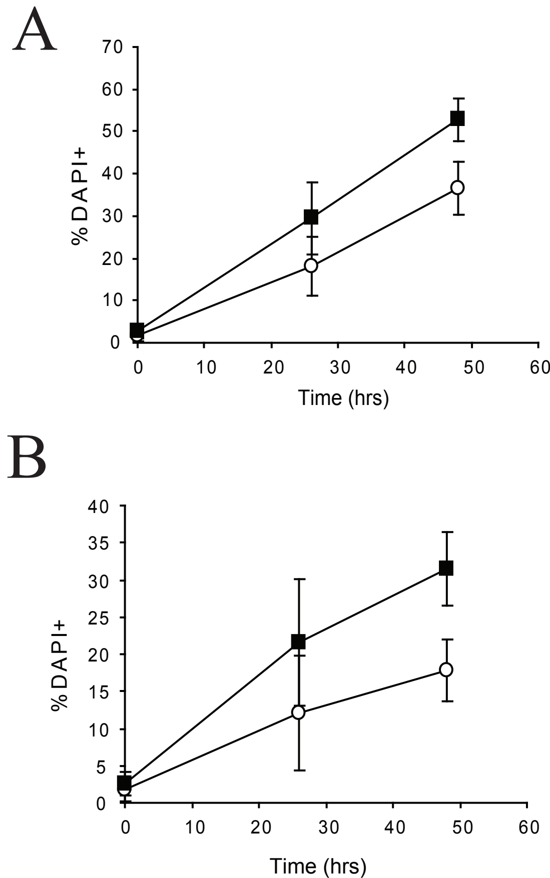


Figure 6. FNB anti-CD3 mAb-induced death cannot be overcome by IL-2 or IL-7 ***in vitro*****.** Mice were treated twice with 10µg control Ig (empty symbols) or FNB anti-CD3 mAb (filled symbols) 24 hrs apart. 38hrs after first injection LNs were harvested and cells were cultured *in vitro* with (A) 1ng/mL IL-2 or (B) 10ng/mL IL-7. Viability was analyzed by flow cytometry at the indicated times by measuring DAPI inclusion. Data shown represents average of 5-6 mice pooled from at least two independent experiments. Bars represent standard deviation between samples.

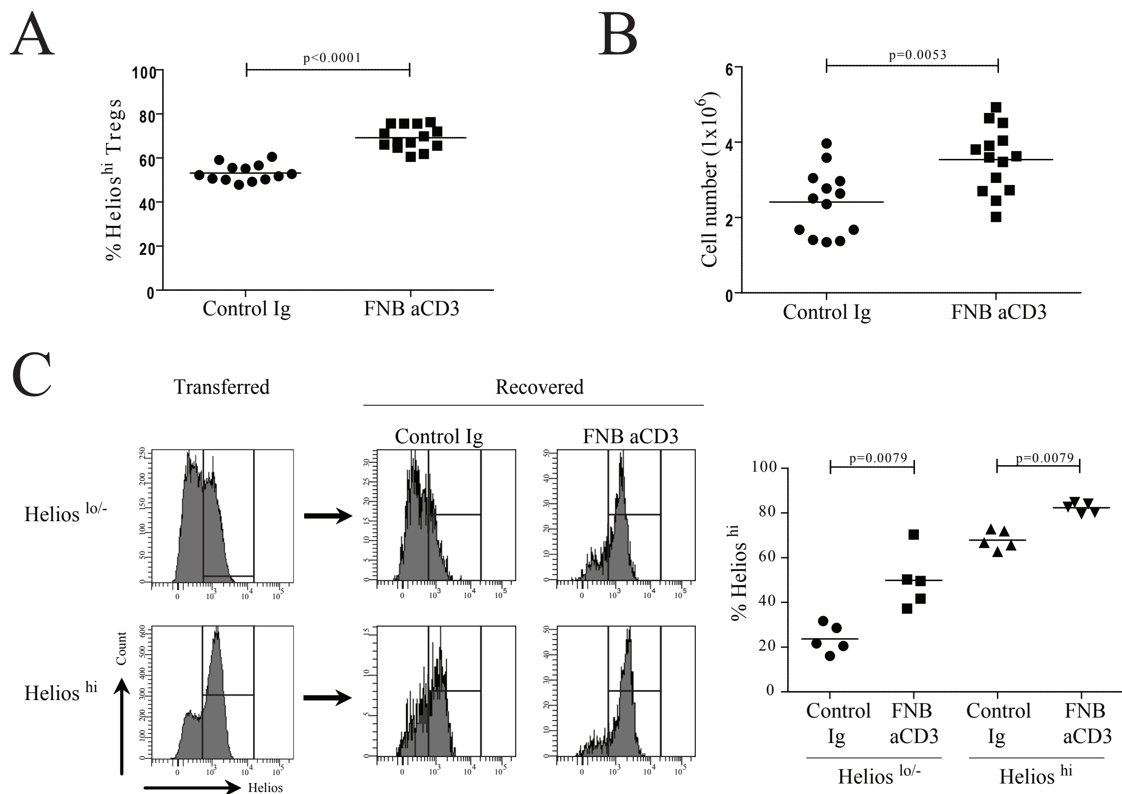


Figure 7. FNB anti-CD3 mAb treatment induces Helios expression on Tregs. (A-B) NOD mice were treated with 10 μg FNB anti-CD3 mAb on 5 consecutive days. On day 7 all lymph nodes and spleen were harvested and the percentage and total number of $CD4^{+}Foxp3^{+}Helios^{hi}$ were calculated. Data shown are a summary of three independent experiments. (C) $Thy1.1^{+}$ $CD25^{+}PD\text{-}1^{-}Nrp1^{-}$ ($Helios^{lo/-}$) and $CD25^{+}PD\text{-}1^{+}Nrp^{+}$ ($Helios^{hi}$) Tregs were sorted and transfered to $Thy1.2^{+}$ mice. Recipients were treated with 10μg aFNB anti-CD3 mAb on 5 consecutive days. All lymph nodes and spleen were harvested and Thy1.1 cells were enriched. Helios expression on transferred $Foxp3^{+}$ cells was analyzed. Representative histograms are shown on left and pooled data from two independent experiments are shown on right.

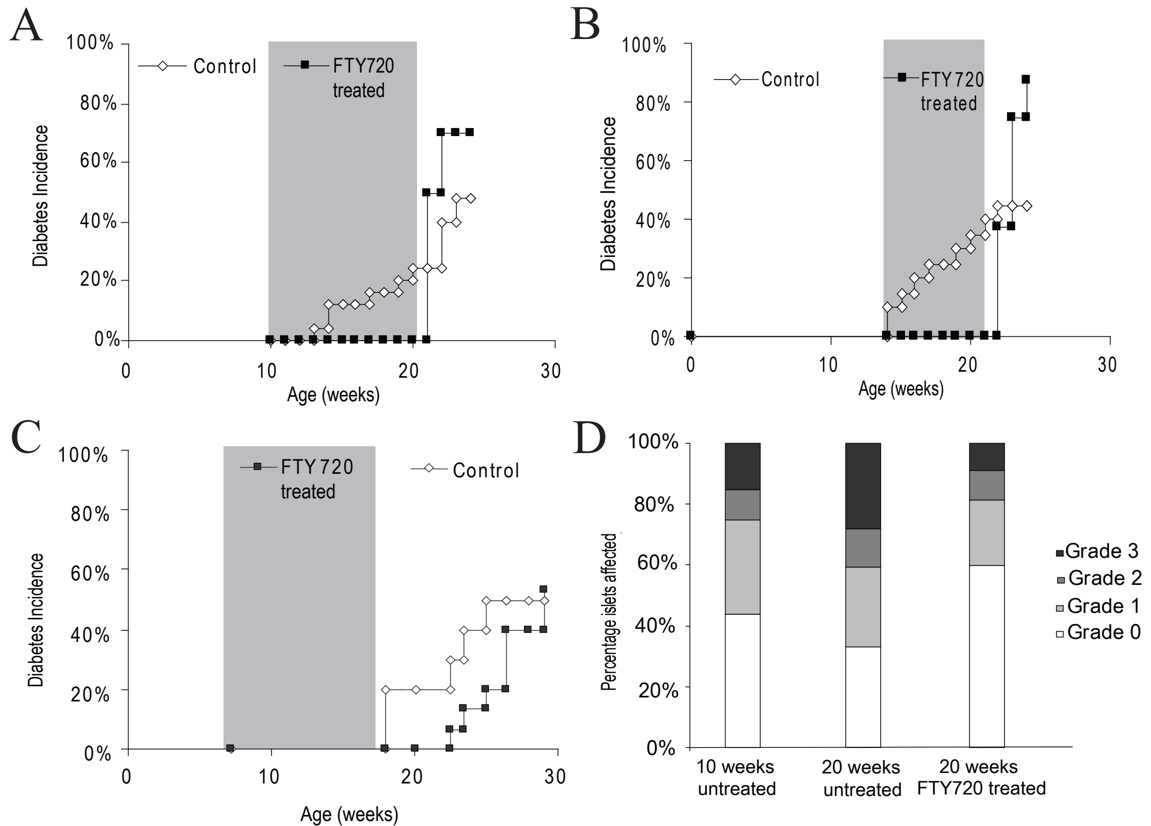


Figure 8. FTY720 treatment of NOD mice. (A) Female NOD mice treated three times per week with 20 µg/day FTY720 from 12 to 22 weeks of age (shaded). Control n=25, FTY720 treated n=10. p=0.0091 after FTY720 withdrawal. (B) Female NOD mice treated as in (A) starting at 14 weeks of age for 7 weeks. Control n=20, FTY720 treated n=8. p=0.0004 after FTY720 withdrawal. (C) Female NOD mice treated as in (A) starting at 7 weeks of age for 9 weeks (shaded). Control n=10, FTY720 treated n=15 pooled from two independent experiments. p=0.7723 after FTY720 withdrawal. (D) Histological analysis of pancreas sections at the end of FTY720 treatment in panel A. 10 weeks untreated n= 6 mice, 1001 individual islets; 20 weeks untreated n=6 mice, 576 individual islets; 20 weeks FTY720 treated n= 7 mice from two independent treatments, 816 individual islets. Grade 0=no infiltrate. Grade 1=<25% of islet infiltrated. Grade 2=25-75% of islet infiltrated. Grade 3=>75% of islet infiltrated.

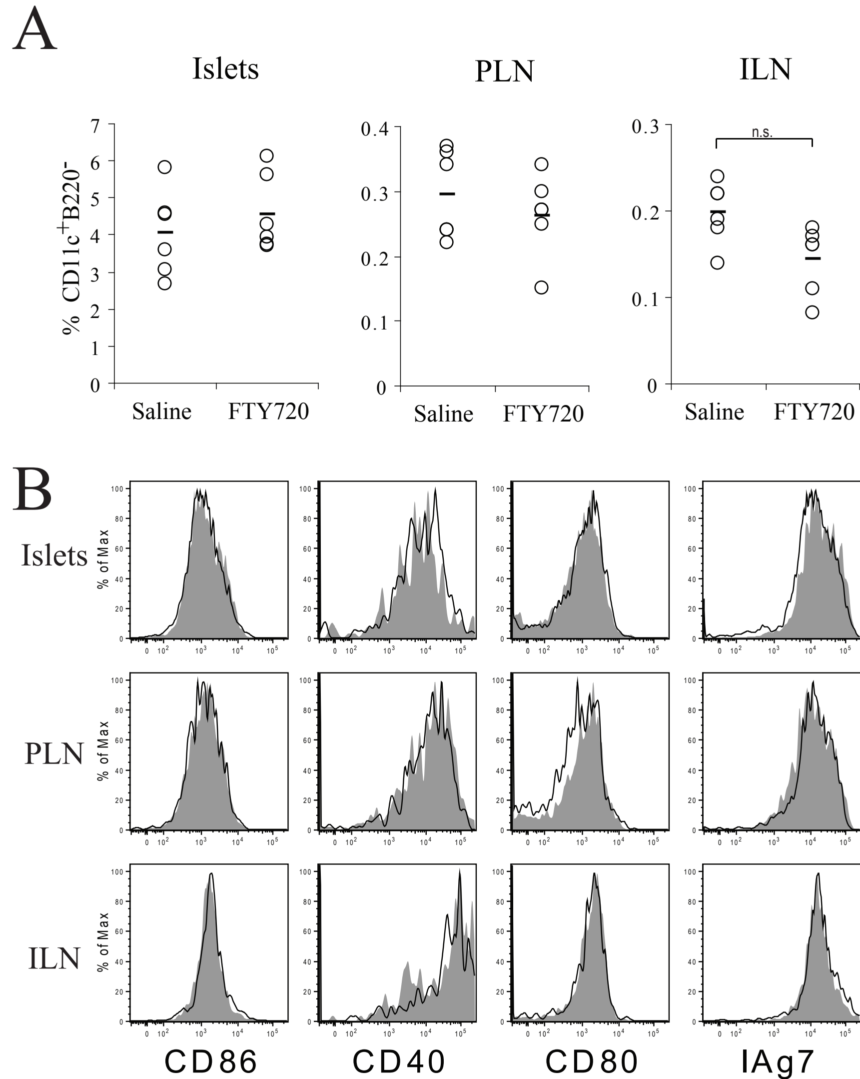


Figure 9. Analysis of dendritic cells during FTY720 treatment. Prediabetic 16-18 week old female NOD mice were treated with saline or 20μg FTY720 every other day for 10 days. On day 11 DCs were isolated and analyzed by flow cytometry. (A) Percent of $CD11c^{+}B220^{-}$ dendritic cells within the $CD45^{+}$Live gate. Data pooled from two independent experiment. (B) Representative expression of costimulatory and maturation markers on $CD11c^{+}B220^{-}$ dendritic cells. Shaded histogram represents saline treated mice. Solid line represents FTY720 treated mice. PLN: pancreatic lymph node. ILN: Inguinal lymph node

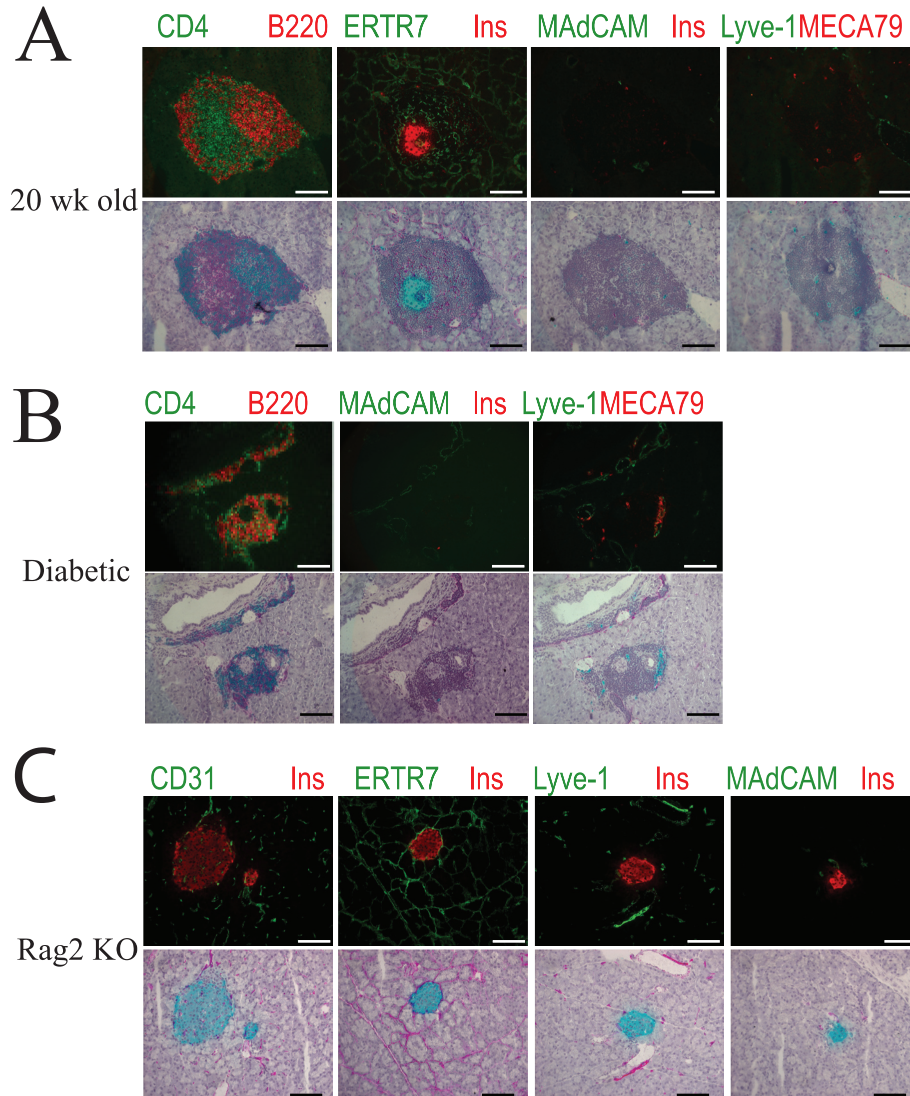


Figure 10. Analysis of TLOs in the pancreas of NOD mice. Pancreas sections from (A) 20 week old, (B) naturally diabetic and (C) Rag2-KO NOD mice were co-stained with anti-insulin and anti-CD31, anti-ERTR7 and anti-insulin, anti-Lyve-1 and anti-insulin, anti-MAdCAM and anti-insulin, anti-CD4 and anti-B220 or anti-Lyve1 and MECA-79 antibodies. Representative immunofluorescence images of consecutive sections (except for C) stained with the indicated antibodies are shown on the top and are superimposed on hematoxilin counterstain bright field images on the bottom (green fluorophore appears as pink, red fluorophore appears as blue). Bar represents 100µm.

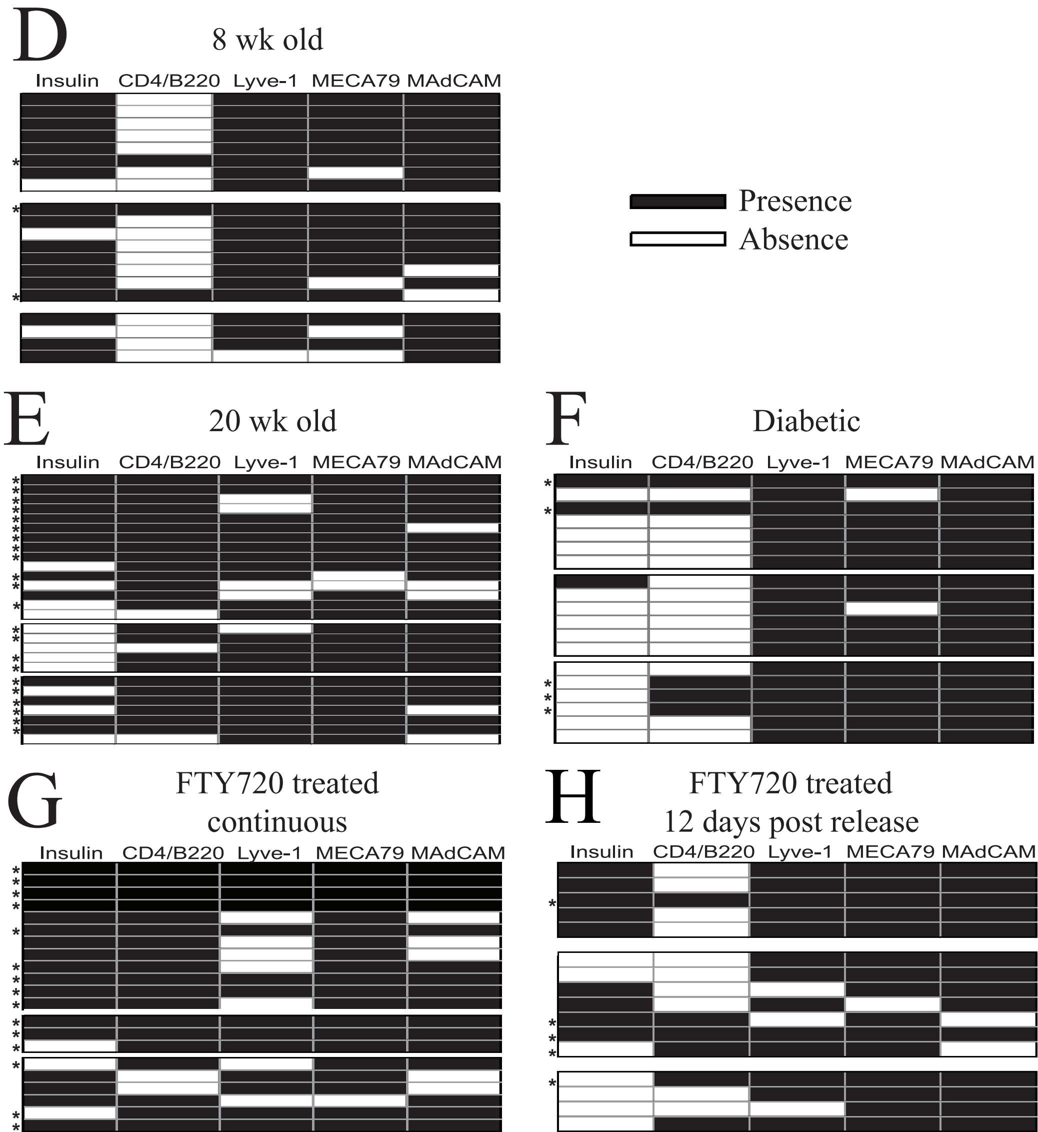


Figure 10. Analysis of TLOs in the pancreas of NOD mice (continued). (D-H) Infiltrates were scored based on their association with insulin producing islets (Column 1), whether there was T/B cell zone compartmentalization (Column 2), and association with Lyve-1$^+$ (Column 3), MECA79/PNAd$^+$ (Column 4) or MAdCAM$^+$ (Column 5) vessels. Each row indicates a separate infiltrate/islet analyzed. Each block indicates a separate mouse. * indicates infiltrates considered to be true TLOs based on T/B compartmentalization and association with at least two of the vascular markers analyzed.

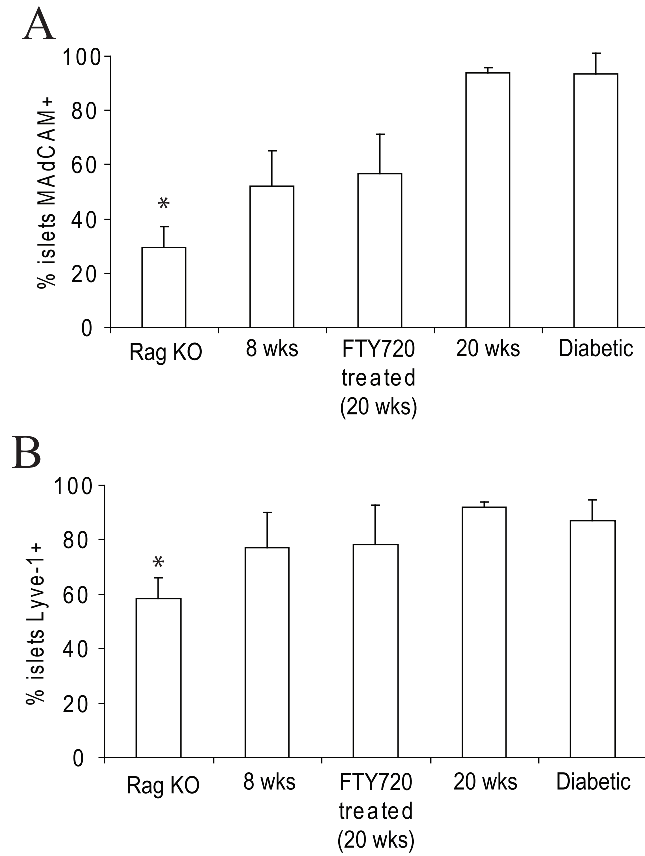


Figure 11. Quantification of islet association with MAdCAM and Lyve-1 positive vessels. Pancreas sections were co-stained with anti-insulin and anti-MAdCAM or anti-insulin and anti-Lyve-1 antibodies. Insulin positive islets were scored as positive or negative based on their association with (A) $MAdCAM^+$ or (B) $Lyve\text{-}1^+$ vessels. 2-4 sections from 3-6 mice per group were analyzed. Error bars represent standard deviation. * $p<0.05$ between RAG KO and all other groups.

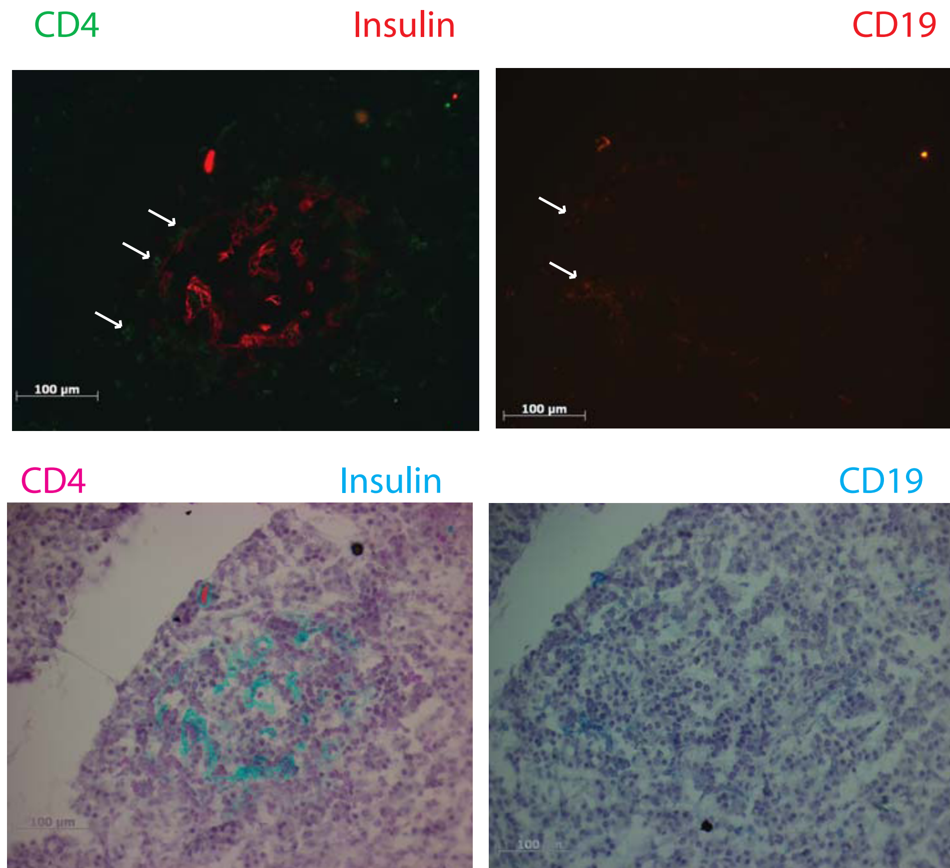


Figure 12. Analysis of pancreatic infiltrates in human diabetic patients. Pancreas sections obtained through nPOD were stained with anti-CD4 and anti-insulin or anti-CD19 antibodies. Representative immunofluorescence images of consecutive sections stained with the indicated antibodies are shown on the top and are superimposed on hematoxilin counterstain bright field images on the bottom (green fluorophore appears as pink, red fluorophore appears as blue). Arrows point to individual cells.

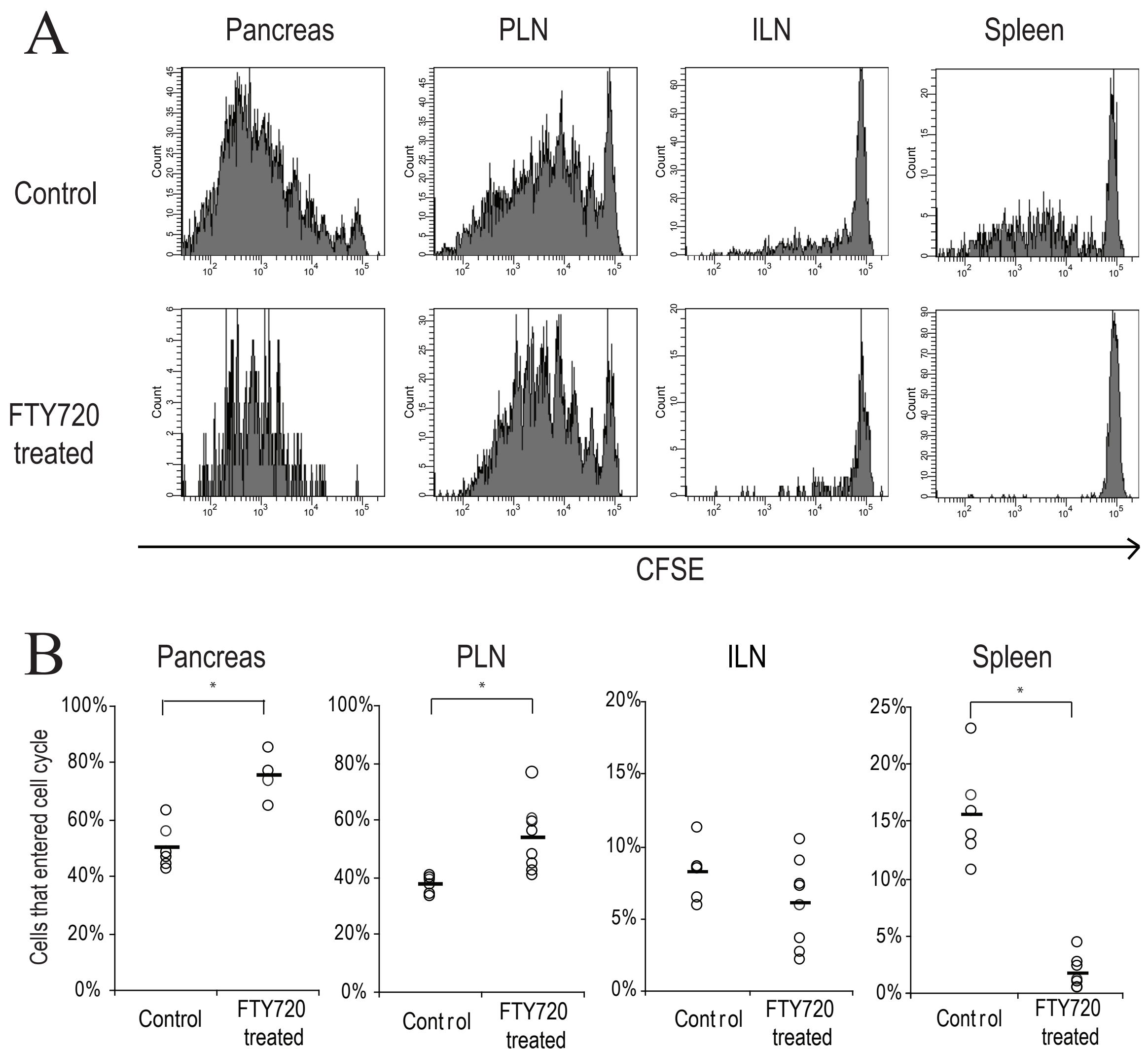


Figure 13. Proliferation of BDC2.5tg T cells in NOD mice. 1x10^6 $CD25^-CD62L^+$ naïve BDC2.5 TCR transgenic cells were CFSE labeled and transferred to 8-10-week old NOD females. After transfer mice were treated with 20μg FTY720 daily. On Day 5 BDC2.5 proliferation was analyzed by CFSE dilution in the pancreas, draining PLN, non-draining ILN and spleen. (A) Representative histograms of control (top) and FTY720 treated (bottom) mice. (B) Percentage of cells that entered the cell cycle was calculated as described in the materials and methods section. Data pooled from two independent experiments. * $p<0.01$

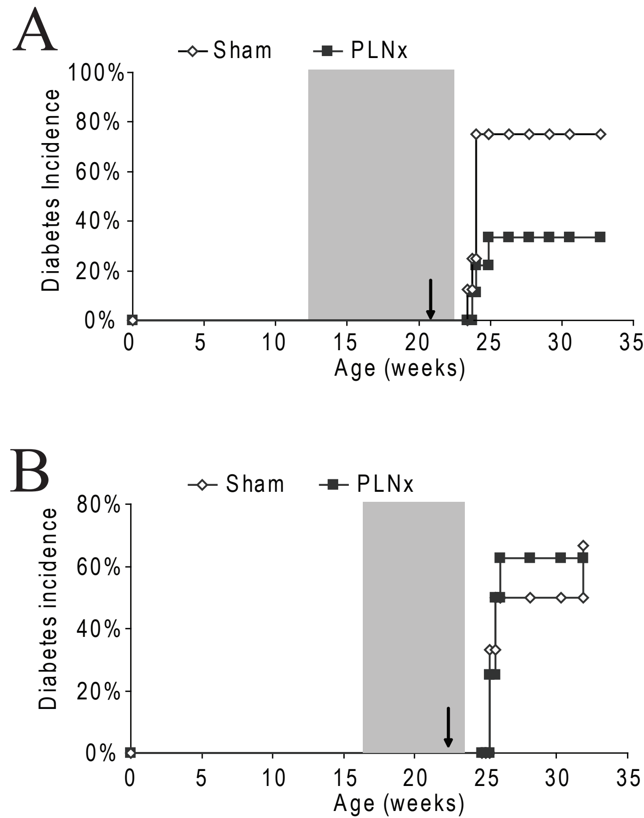


Figure 14. Role of draining pancreatic lymph node on FTY720-induced diabetes. (A) NOD female mice were treated for 9-10 weeks (shaded) with FTY720 beginning at weeks 11-13. During the last week of treatment the pancreatic lymph nodes were surgically removed. Disease incidence was monitored after FTY720 withdrawal. Data are pooled from two independent experiments. Sham n= 8, PLNx n=9. p=0.0817 (B) NOD female mice were treated for 8 weeks (shaded) with FTY720 beginning at week 16. During the last week of treatment the pancreatic lymph nodes were surgically removed. Disease incidence was monitored after FTY720 withdrawal. Sham n= 6, PLNx n=8. p= 0.8456.

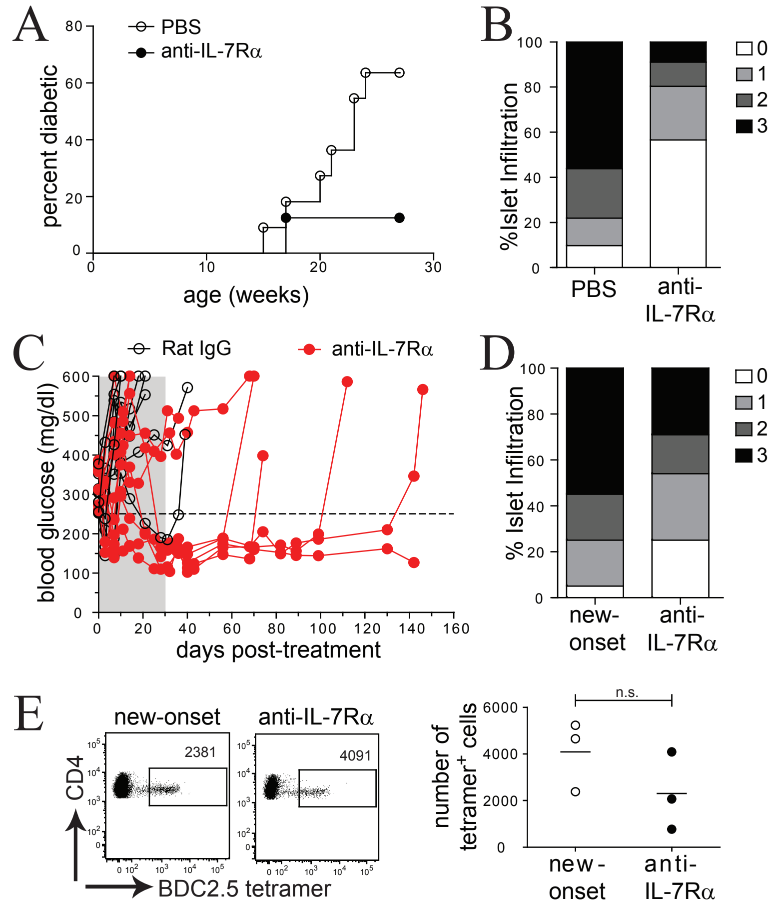


Figure 15. IL-7Rα blockade prevents and reverses diabetes in NOD mice.

(A) Female NOD mice were treated with anti-IL-7Rα monoclonal antibodies (n=8) or PBS (n=11), starting at 10 weeks of age, and diabetes incidence was followed. (B) Infiltration of pancreatic islets in 24 week old, non-diabetic mice from panel A quantified as percent of islets showing the indicated histological scores (PBS, n=3; anti-IL-7Rα, n=6). (C) New-onset diabetic NOD mice (blood glucose > 250 mg/dl (dotted line)) were treated with anti-IL-7Rα antibodies (n=10) or rat IgG (n=9) for 4 weeks (shaded area). Blood glucose levels were followed for up to 5 months. (D) Histological scores of new-onset NOD mice that became normoglycemic after anti-IL-7Rα treatment, compared to untreated new-onset mice. (E) Quantification of islet antigen-specific $CD4^+$ T cells present in new-onset and anti-IL-7Rα-cured mice, determined by tetramer staining.

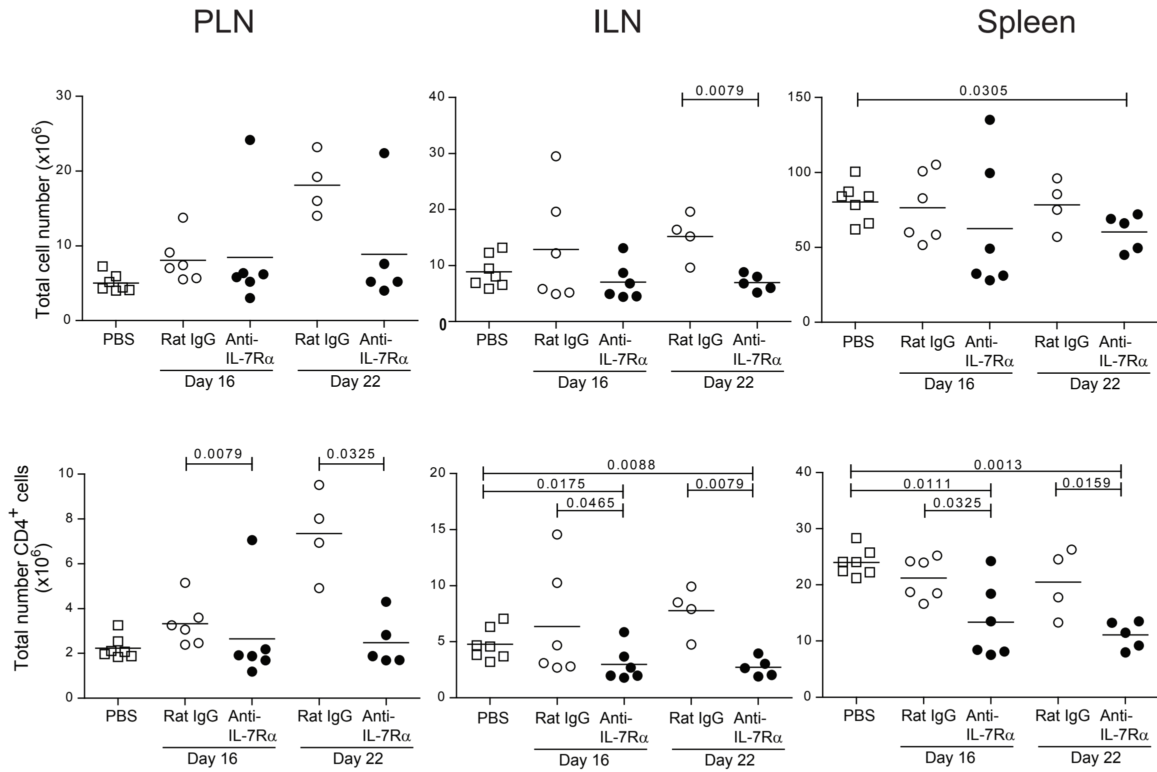


Figure 16. Lymphocyte numbers after anti-IL-7Rα treatment. Pre-diabetic NOD mice (10-12 weeks) were treated with anti-IL-7Rα or rat IgG antibodies for 16 or 22 days. Total cell numbers (upper panels) in pancreatic (PLN) and inguinal (ILN) lymph nodes and spleen are shown and $CD4^+$ T cell numbers (lower panels) derived from these cell numbers and percentages of $CD4^+$ T cells determined by flow cytometry.

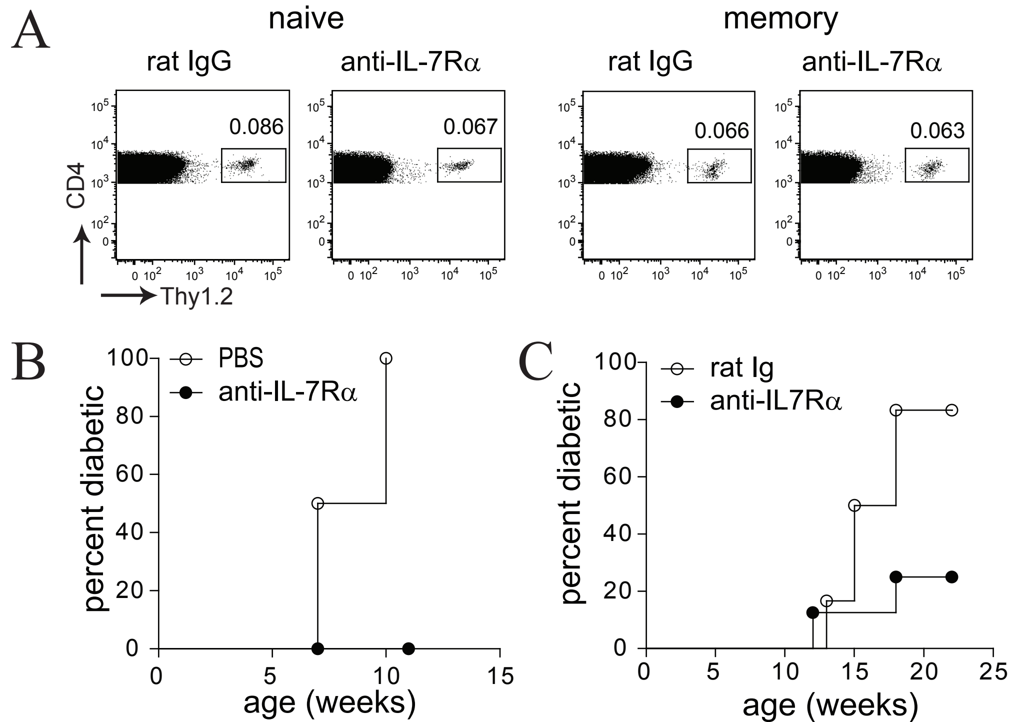


Figure 17. CD4$^+$ T cells isolated from anti-IL-7Rα-treated mice fail to transfer diabetes to NOD-*scid* recipients. (A) 7.5 x 10^5 CD44^{low} (naïve) or CD44^{high} (memory) CD4$^+$Thy1.2$^+$ T cells were transferred to NOD.Thy1.1 recipients and treated with rat IgG or anti-IL-7Rα. Dot plots show % of CD4$^+$Thy1.2$^+$ cells present within the CD4$^+$ population after 4 weeks of treatment. Data is representative of two mice.

(B) CD25^{neg}CD44^{high}CD4$^+$ memory T cells were isolated from lymph nodes and spleen of 24-week old, non-diabetic NOD mice that were treated with anti-IL-7Rα antibodies (n=3) or PBS (n=2) as in Figure 15A, and 2.5 x 10^5 cells from individual mice were transferred to NOD-*scid* recipients and diabetes incidence was followed. (C) Total CD25^{neg}CD4$^+$ T cells were isolated from pre-diabetic (14 week old) NOD mice that were treated for 2-4 weeks with anti-IL-7Rα(n=8) or rat IgG (n=6) and 3.7 x 10^6 cells from individual mice were transferred to a NOD-*scid* recipients and diabetes incidence followed. Graph shows pooled data from two independent experiments.

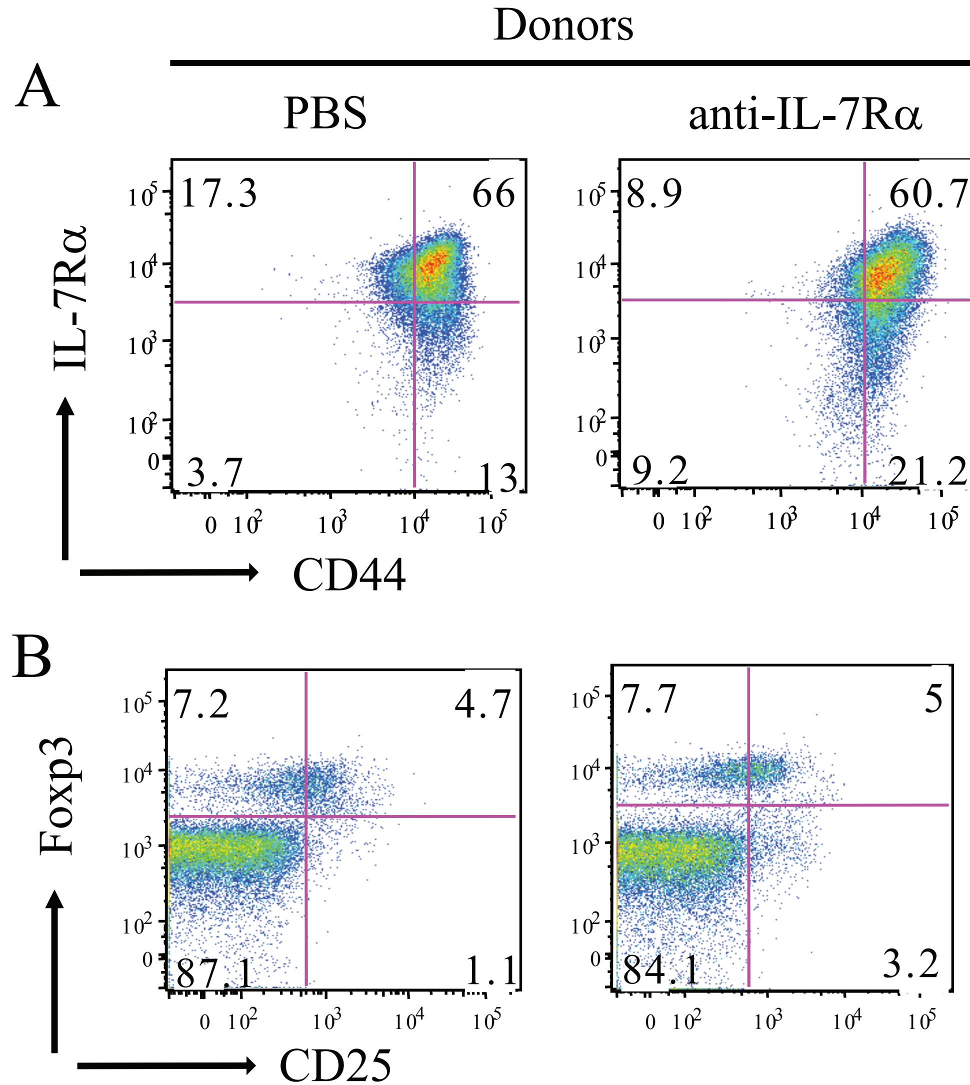


Figure 18. Failure of memory T cells from anti-IL-7Rα-treated mice to cause diabetes is not due to impaired T cell survival or increased Treg generation. Memory T cells were isolated from anti-IL-7Rα- or PBS-treated NOD donors as in Figure 17 and transferred to NOD.*scid* recipients. Pancreatic lymph nodes were harvested 8-10 weeks later and histograms show (A) $CD44^{high}IL\text{-}7Ra^{high}$ memory T cells and (B) $Foxp3^{+}CD25^{+}$ Tregs present within the $CD4^{+}$ T cell gate.

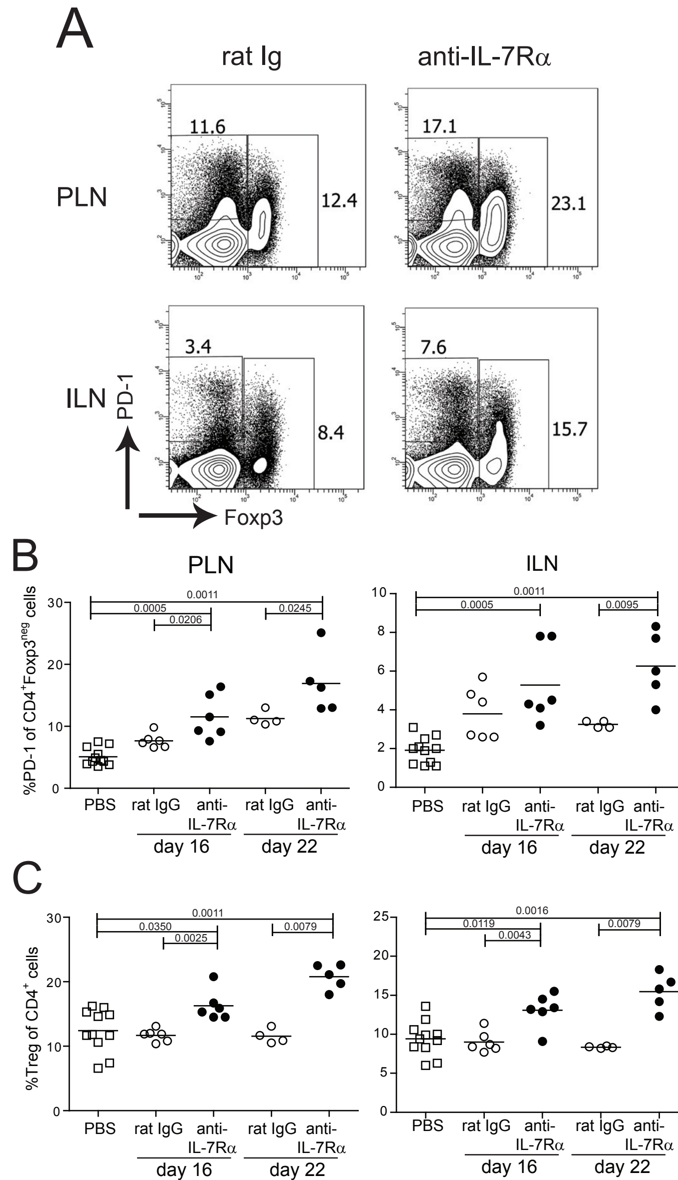


Figure 19. Absence of IL-7 signals increases numbers of PD-1$^+$ and Foxp3$^+$ CD4$^+$ T cells. Pre-diabetic NOD mice (10-12 weeks) were treated with anti-IL-7Rα or rat IgG antibodies for 16 or 22 days and the pancreatic (PLN) and inguinal lymph nodes (ILN) were stained for CD4, Foxp3 and PD-1. (A) Histograms show the gates used to determine the percentages (values indicated) of Foxp3$^+$CD4$^+$ Tregs, and PD-1$^+$ cells within the Foxp3^{neg}CD4$^+$ T cell population. (B) and (C) Summary of percentages of PD1$^+$ and Foxp3$^+$ CD4$^+$ T cells respectively. Each symbol represents an individual mouse.

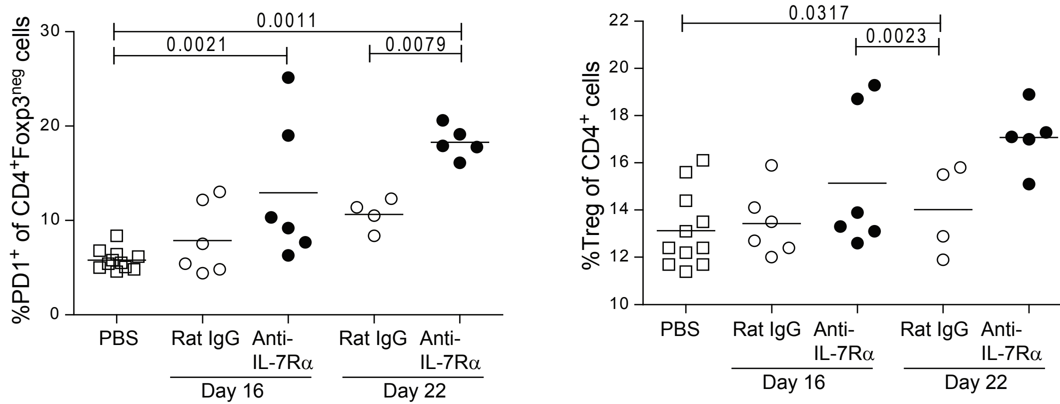


Figure 20. Percentages of PD1$^+$ and Foxp3$^+$ CD4$^+$ T cells in the spleen. Pre-diabetic NOD mice (10-12 weeks) were treated with anti-IL-7Rα or rat IgG antibodies for 16 or 22 days. Summary of percentages of PD1$^+$ (left) and Foxp3$^+$ CD4$^+$ T (right) cells in the spleen using gates shown in Figure 19A. Each symbol represents an individual mouse.

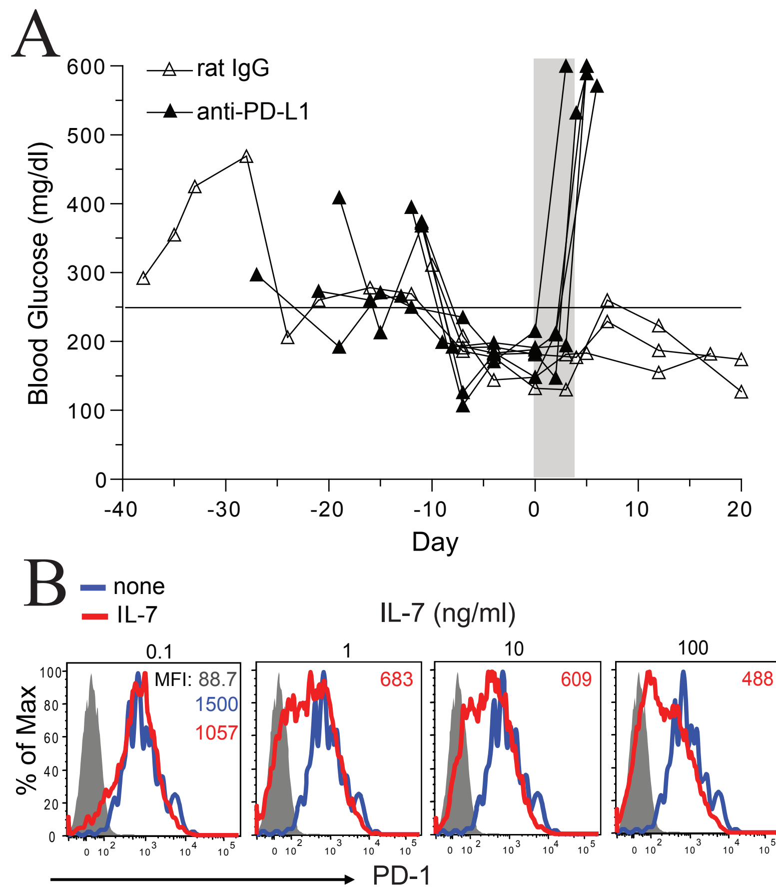


Figure 21. IL-7Rα blockade reverses diabetes by promoting PD-1-dependent tolerance. (A) New-onset diabetic NOD mice were treated as in Figure 15A; cured mice (3 consecutive blood glucose readings < 250 mg/dl for 1 week) were treated with anti-PD-L1 antibodies or rat IgG every other day (starting on day 0). Graph shows blood glucose levels from individual mice. (B) Naive PD-1^{neg}Foxp3^{neg} CD4^{+} T cells (gray, filled) were isolated from NOD.Foxp3-GFP mice and stimulated *in vitro* with anti-CD3 (10 μg/ml) + anti-CD28 (1 μg/ml) antibodies with (red) or without (blue) the indicated amounts of recombinant IL-7. Cultures were harvested on day 6 and histograms show PD-1 expression. MFI is indicated and data are representative for three independent experiments.

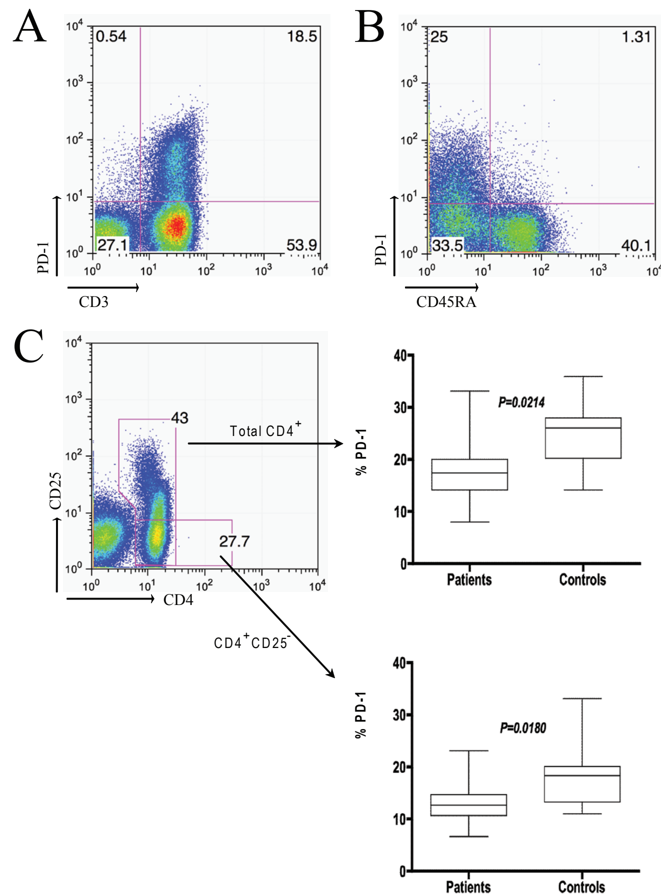
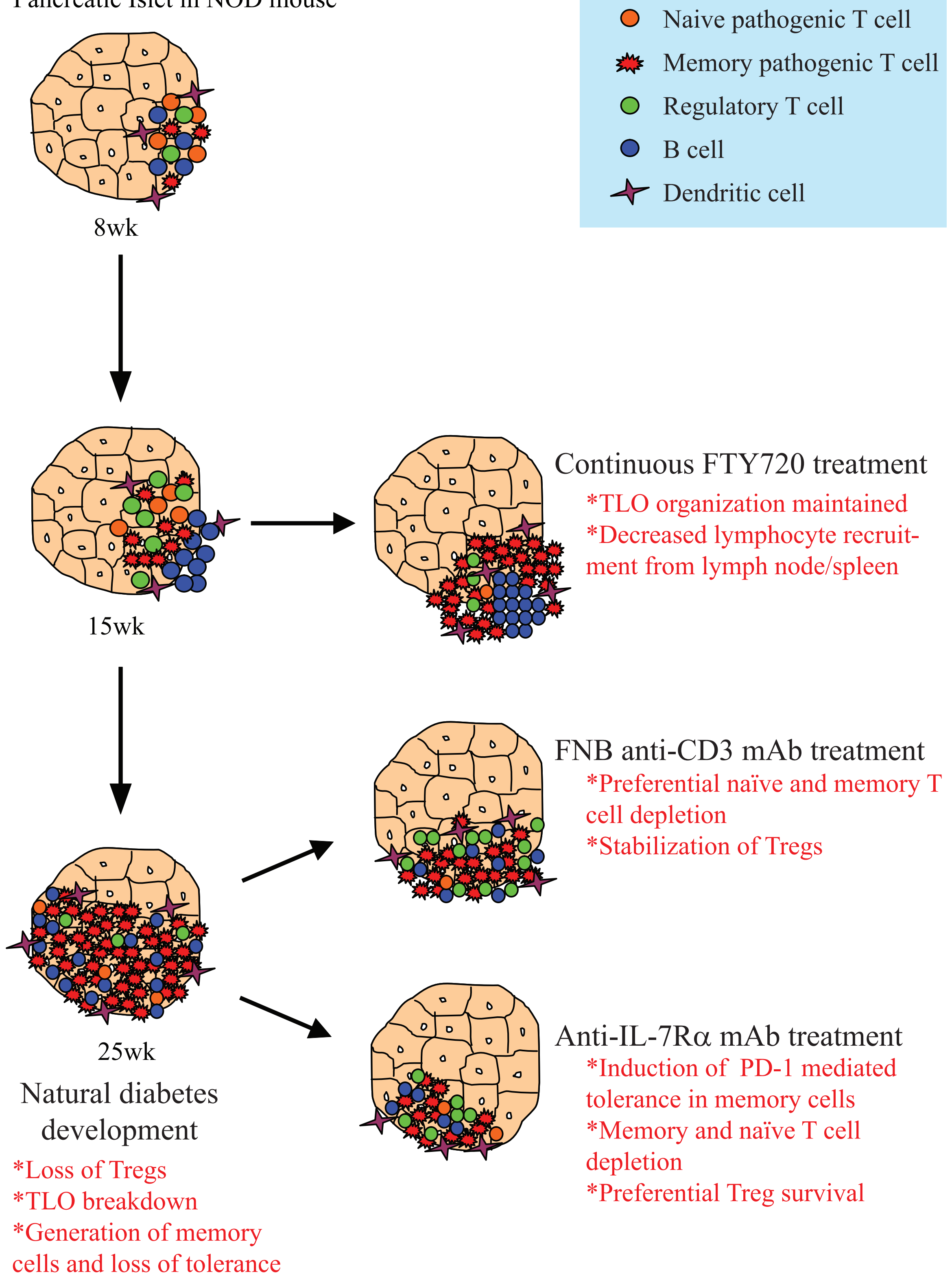


Figure 22. Reduced PD-1 expression in type 1 diabetes patients. Fresh *ex-vivo* PBMCs, isolated from type 1 diabetes patients or healthy controls were analyzed by FACS analysis. (A) Representative PD-1 staining on whole PBMC. (B) Representative CD45RA staining on gated CD4$^+$ lymphocytes showing PD-1 expression is largely restricted to memory cells. (C) Comparison of PD-1 staining on CD4$^+$ T cells from type 1 diabetes patients and healthy controls. The difference seen in the total CD4$^+$ population (p=0.0214) is not attributable to PD-1 expression on Treg since the difference is maintained after gating on CD4$^+$CD25^{neg} cells (p=0.0180). Presented data derive from 16 type 1 diabetes patients and 13 healthy controls with mean ages of 29 and 27 respectively. There was no correlation of PD-1 levels with donor age, haemoglobin A1C or blood glucose levels.



Pancreatic Islet in NOD mouse
Naive pathogenic T cell
Memory pathogenic T cell
Regulatory T cell
B cell
Dendritic cell
8wk
15wk
Continuous FTY720 treatment
*TLO organization maintained
*Decreased lymphocyte recruit-
ment from lymph node/spleen
FNB anti-CD3 mAb treatment
*Preferential naïve and memory T
cell depletion
*Stabilization of Tregs
25wk
Natural diabetes
development
*Loss of Tregs
*TLO breakdown
*Generation of memory
cells and loss of tolerance
Anti-IL-7Rα mAb treatment
*Induction of PD-1 mediated
tolerance in memory cells
*Memory and naïve T cell
depletion
*Preferential Treg survival

Figure 23. Model of therapeutic interventions of type 1 diabetes in the NOD mouse model. Islet infiltration in the NOD mouse model begins around 3-4 weeks of age and by 8 weeks of age is composed of T cells, B cells, dendritic cells and other cells of the innate immune system. Tertiary lymphoid organs characterized by compartmentalized T and B cell zones and specialized vasculature, such as lymphatic and high endothelial vessels, develop surrounding individual islets by 15 weeks of age. At this time point, the percentage of regulatory T cells in the infiltrate is equal to or higher than in the draining pancreatic lymph node and memory T cells have developed after activation of naïve T cells that recognize islets self-antigens. By 25 weeks of age, islets are destroyed and insulin production is lost resulting in hyperglycemia. The percentage of regulatory T cells in these infiltrates is lower than in the pancreatic lymph resulting in an imbalance between regulatory and effector functions in the pancreas. Furthermore, tertiary lymphoid organs loose their compartmentalization suggesting that loss of tertiary lymphoid organ stability is either the cause or consequence of islet destruction. We investigated three therapeutic interventions to modulate the immune system with the aim of restoring the balance between regulatory and effector function to prevent or reverse diabetes. First, we show that continuous treatment of prediabetic NOD mice with the S1P1 agonist FTY720 prevents diabetes development by stabilizing tertiary lymphoid organs, thereby inhibiting pancreas resident T cells from attacking and destroying the neighboring islet, and by inhibiting continuous recruitment of lymphocytes from the spleen and lymph nodes. Upon treatment withdrawal, pancreas resident T cells quickly destroy surrounding islets resulting in diabetes and a similar loss of tertiary lymphoid organ organization that is seen in natural diabetes development. Second, we show that the reversal of diabetes by

Fc receptor non-binding anti-CD3 monoclonal antibody treatment is due to preferential depletion of effector cells and induction of Helios expression on regulatory T cells leading to the stabilization and/or induction of natural regulatory T cells. Finally, we show that anti-IL-7Rα monoclonal antibodies reverse diabetes by partially depleting memory T cells while preserving regulatory T cells and, importantly, inducing PD-1 mediated tolerance in the remaining cells.

REFERENCES

1. Prevention CfDCa. National diabetes fact sheet: general information and national estimates on diabetes in the United States, 2007. Atlanta, GA: US Department of Health and Human Services, Centers for Disease Control and Prevention 2008.
2. Makino, S., Kunimoto, K., Muraoka, Y., Mizushima, Y., Katagiri, K., Tochino, Y. Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu 1980;29(1):1-13.
3. Barnett, A.H., Eff, C., Leslie, R.D., Pyke, D.A. Diabetes in identical twins. A study of 200 pairs. Diabetologia 1981;20(2):87-93.
4. Csorba, T.R., Lyon, A.W., Hollenberg, M.D. Autoimmunity and the pathogenesis of type 1 diabetes. Crit Rev Clin Lab Sci 2010;47(2):51-71.
5. Erlich, H., Valdes, A.M., Noble, J., Carlson, J.A., Varney, M., Concannon, P., Mychaleckyj, J.C., Todd, J.A., Bonella, P., Fear, A.L., Lavant, E., Louey, A., Moonsamy, P. HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk: analysis of the type 1 diabetes genetics consortium families. Diabetes 2008;57(4):1084-92.
6. Barrett, J.C., Clayton, D.G., Concannon, P., Akolkar, B., Cooper, J.D., Erlich, H.A., Julier, C., Morahan, G., Nerup, J., Nierras, C., Plagnol, V., Pociot, F., Schuilenburg, H., Smyth, D.J., Stevens, H., Todd, A. J, Walker, N.M., Rich, S.S. Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. Nat Genet 2009;41(6):703-7.
7. Concannon, P., Rich, S.S., Nepom, G.T. Genetics of type 1A diabetes. N Engl J Med 2009;360(16):1646-54.

8. Wicker, L.S., Todd, J.A., Peterson, L.B. Genetic control of autoimmune diabetes in the NOD mouse. Annu Rev Immunol 1995;13:179-200.

9. Kanagawa, O., Martin, S.M., Vaupel, B.A., Carrasco-Marin, E., Unanue, E.R. Autoreactivity of T cells from nonobese diabetic mice: an I-Ag7-dependent reaction. Proc Natl Acad Sci U S A 1998;95(4):1721-4.

10. Cucca, F., Lampis, R., Congia, M., Angius, E., Nutland, S., Bain, S.C., Barnett, A.H., Todd, J.A. A correlation between the relative predisposition of MHC class II alleles to type 1 diabetes and the structure of their proteins. Hum Mol Genet 2001;10(19):2025-37.

11. Chistiakov, D.A., Voronova, N.V., Chistiakov, P.A. The crucial role of IL-2/IL-2RA-mediated immune regulation in the pathogenesis of type 1 diabetes, an evidence coming from genetic and animal model studies. Immunol Lett 2008;118(1):1-5.

12. Bottini, N., Musumeci, L., Alonso, A., Rahmouni, S., Nika, K., Rostamkhani, M., MacMurray, J., Meloni, G.F., Lucarelli, P., Pellecchia, M., Eisenbarth, G.S., Comings, D., Mustelin, T. A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nat Genet 2004;36(4):337-8.

13. Pozzilli, P., Signore, A., Williams, A.J., Beales, P.E. NOD mouse colonies around the world--recent facts and figures. Immunol Today 1993;14(5):193-6.

14. McInerney, M.F., Pek, S.B., Thomas, D.W. Prevention of insulitis and diabetes onset by treatment with complete Freund's adjuvant in NOD mice. Diabetes 1991;40(6):715-25.

15. Wen, L., Ley, R.E., Volchkov, P.Y., Stranges, P.B., Avanesyan, L., Stonebraker, A.C., Hu, C., Wong, F.S., Szot, G.L., Bluestone, J.A., Gordon, J.I., Chervonsky, A.V. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 2008;455(7216):1109-13.

16. Alanentalo, T., Hornblad, A., Mayans, S., Nilsson K, A., Sharpe, J., Larefalk, A., Ahlgren, U., Holmberg, D. Quantification and three-dimensional imaging of the insulitis-induced destruction of beta-cells in murine type 1 diabetes. Diabetes 2010;59(7):1756-64.

17. Willcox, A., Richardson, S.J., Bone, A.J., Foulis, A.K., Morgan, N.G. Analysis of islet inflammation in human type 1 diabetes. Clin Exp Immunol 2009;155(2):173-81.

18. Anderson, M.S., Bluestone, J.A. The NOD mouse: a model of immune dysregulation. Annu Rev Immunol 2005;23:447-85.

19. Andre, I., Gonzalez, A., Wang, B., Katz, J., Benoist, C., Mathis, D. Checkpoints in the progression of autoimmune disease: lessons from diabetes models. Proc Natl Acad Sci U S A 1996;93(6):2260-3.

20. Hoglund, P., Mintern, J., Waltzinger, C., Heath, W., Benoist, C., Mathis, D. Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. J Exp Med 1999;189(2):331-9.

21. Gagnerault, M.C., Luan, J.J., Lotton, C., Lepault, F. Pancreatic lymph nodes are required for priming of beta cell reactive T cells in NOD mice. Journal of Experimental Medicine 2002;196(3):369-77.

22. Achenbach, P., Bonifacio, E., Koczwara, K., Ziegler, A.G. Natural history of type 1 diabetes. Diabetes 2005;54 Suppl 2:S25-31.

23. Serreze, D.V., Chapman, H.D., Varnum, D.S., Hanson, M.S., Reifsnyder, P.C., Richard, S.D., Fleming, S.A., Leiter, E.H., Shultz, L.D. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice. J Exp Med 1996;184(5):2049-53.

24. Hu, C.Y., Rodriguez-Pinto, D., Du, W., Ahuja, A., Henegariu, O., Wong, F.S., Shlomchik, M.J., Wen, L. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest 2007;117(12):3857-67.

25. Serreze, D.V., Fleming, S.A., Chapman, H.D., Richard, S.D., Leiter, E.H., Tisch, R.M. B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Immunol 1998;161(8):3912-8.

26. Noorchashm, H., Lieu, Y.K., Noorchashm, N., Rostami, S.Y., Greeley, S.A., Schlachterman, A., Song, H.K., Noto, L.E., Jevnikar, A.M., Barker, C.F., Naji, A. I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet beta cells of nonobese diabetic mice. J Immunol 1999;163(2):743-50.

27. Katz, J., Benoist, C., Mathis, D. Major histocompatibility complex class I molecules are required for the development of insulitis in non-obese diabetic mice. Eur J Immunol 1993;23(12):3358-60.

28. Wang, B., Gonzalez, A., Benoist, C., Mathis, D. The role of CD8+ T cells in the initiation of insulin-dependent diabetes mellitus. Eur J Immunol 1996;26(8):1762-9.

29. Nagata, M., Santamaria, P., Kawamura, T., Utsugi, T., Yoon, J.W. Evidence for the role of CD8+ cytotoxic T cells in the destruction of pancreatic beta-cells in nonobese diabetic mice. J Immunol 1994;152(4):2042-50.

30. Verdaguer, J., Yoon, J.W., Anderson, B., Averill, N., Utsugi, T., Park, B.J., Santamaria, P. Acceleration of spontaneous diabetes in TCR-beta-transgenic nonobese diabetic mice by beta-cell cytotoxic CD8+ T cells expressing identical endogenous TCR-alpha chains. J Immunol 1996;157(10):4726-35.

31. Krishnamurthy, B., Dudek, N.L., McKenzie, M.D., Purcell, A.W., Brooks, A.G., Gellert, S., Colman, P.G., Harrison, L.C., Lew, A.M., Thomas, H.E., Kay, T.W. Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP. J Clin Invest 2006;116(12):3258-65.

32. Kagi, D., Odermatt, B., Seiler, P., Zinkernagel, R.M., Mak, T.W., Hengartner, H. Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. J Exp Med 1997;186(7):989-97.

33. Stadinski, B.D., Delong, T., Reisdorph, N., Reisdorph, R., Powell, R.L., Armstrong, M., Piganelli, J.D., Barbour, G., Bradley, B., Crawford, F., Marrack, P., Mahata, S.K., Kappler, J.W., Haskins, K. Chromogranin A is an autoantigen in type 1 diabetes. Nat Immunol 2010;11(3):225-31.

34. Katz, J.D., Wang, B., Haskins, K., Benoist, C., Mathis, D. Following a diabetogenic T cell from genesis through pathogenesis. Cell 1993;74(6):1089-100.

35. Suri, A., Katz, J.D. Dissecting the role of CD4+ T cells in autoimmune diabetes through the use of TCR transgenic mice. Immunol Rev 1999;169:55-65.

36. Tang, Q., Henriksen, K.J., Bi, M., Finger, E.B., Szot, G., Ye, J., Masteller, E.L., McDevitt, H., Bonyhadi, M., Bluestone, J.A. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med 2004;199(11):1455-65.

37. Masteller, E.L., Warner, M.R., Ferlin, W., Judkowski, V., Wilson, D., Glaichenhaus, N., Bluestone, J.A. Peptide-MHC class II dimers as therapeutics to modulate antigen-specific T cell responses in autoimmune diabetes. J Immunol 2003;171(10):5587-95.

38. Stratmann, T., Martin-Orozco, N., Mallet-Designe, V., Poirot, L., McGavern, D., Losyev, G., Dobbs, C.M., Oldstone, M.B., Yoshida, K., Kikutani, H., Mathis, D., Benoist, C., Haskins, K., Teyton, L. Susceptible MHC alleles, not background genes, select an autoimmune T cell reactivity. J Clin Invest 2003;112(6):902-14.

39. Esensten, J.H., Lee, M.R., Glimcher, L.H., Bluestone, J.A. T-bet-deficient NOD mice are protected from diabetes due to defects in both T cell and innate immune system function. J Immunol 2009;183(1):75-82.

40. Yang, Z., Chen, M., Ellett, JD., Fialkow, L.B., Carter, J.D., McDuffie, M., Nadler, J.L. Autoimmune diabetes is blocked in Stat4-deficient mice. Journal of Autoimmunity 2004;22(3):191-200.

41. Trembleau, S., Penna, G., Gregori, S., Chapman, H.D., Serreze, D.V., Magram, J., Adorini, L. Pancreas-infiltrating Th1 cells and diabetes develop in IL-12-deficient nonobese diabetic mice. J Immunol 1999;163(5):2960-8.

42. Serreze, D.V., Post, C.M., Chapman, H.D., Johnson, E.A., Lu, B., Rothman, P.B. Interferon-gamma receptor signaling is dispensable in the development of autoimmune type 1 diabetes in NOD mice. Diabetes 2000;49(12):2007-11.

43. Hultgren, B., Huang, X., Dybdal, N., Stewart, T.A. Genetic absence of gamma-interferon delays but does not prevent diabetes in NOD mice. Diabetes 1996;45(6):812-7.

44. Ono, M., Yaguchi, H., Ohkura, N., Kitabayashi, I., Nagamura, Y., Nomura, T., Miyachi, Y., Tsukada, T., Sakaguchi, S. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. Nature 2007;446(7136):685-9.

45. Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A.D., Stroud, J.C., Bates, D.L., Guo, L., Han, A., Ziegler, S.F., Mathis, D., Benoist, C., Chen, L., Rao, A. FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell 2006;126(2):375-87.

46. Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., Ochs, H.D. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001;27(1):20-1.

47. Kramer, S., Schimpl, A., Hunig, T. Immunopathology of interleukin (IL) 2-deficient mice: thymus dependence and suppression by thymus-dependent cells with an intact IL-2 gene. J Exp Med 1995;182(6):1769-76.

48. Suzuki, H., Zhou, Y.W., Kato, M., Mak, T.W., Nakashima, I. Normal regulatory alpha/beta T cells effectively eliminate abnormally activated T cells lacking the interleukin 2 receptor beta in vivo. J Exp Med 1999;190(11):1561-72.

49. Hsieh, C.S., Zheng, Y., Liang, Y., Fontenot, J.D., Rudensky, A.Y. An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. Nat Immunol 2006;7(4):401-10.

50. Nishizuka, Y., Sakakura, T. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. Science 1969;166(906):753-5.

51. Asano, M., Toda, M., Sakaguchi, N., Sakaguchi, S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med 1996;184(2):387-96.

52. Salomon, B., Lenschow, D.J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., Bluestone, J.A. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 2000;12(4):431-40.

53. Tang, Q., Henriksen, K.J., Boden, E.K., Tooley, A.J., Ye, J., Subudhi, S.K., Zheng, X.X., Strom, T.B., Bluestone, J.A. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. J Immunol 2003;171(7):3348-52.

54. Gregory, S.G., Schmidt, S., Seth, P., Oksenberg, J.R., Hart, J., Prokop, A., Caillier, S.J., Ban, M., Goris, A., Barcellos, L.F., Lincoln, R., McCauley, J.L., Sawcer, S.J., Compston, D.A., Dubois, B., Hauser, S.L., Garcia-Blanco, M.A., Pericak-Vance, M.A., Haines, J.L. Interleukin 7 receptor alpha chain (IL7R)

shows allelic and functional association with multiple sclerosis. Nat Genet 2007;39(9):1083-91.

55. Kukreja, A., Cost, G., Marker, J., Zhang, C., Sun, Z., Lin-Su, K., Ten, S., Sanz, M., Exley, M., Wilson, B., Porcelli, S., Maclaren, N. Multiple immuno-regulatory defects in type-1 diabetes. J Clin Invest 2002;109(1):131-40.

56. Putnam, A.L., Vendrame, F., Dotta, F., Gottlieb, P.A. CD4+CD25high regulatory T cells in human autoimmune diabetes. J Autoimmun 2005;24(1):55-62.

57. Brusko, T., Wasserfall, C., McGrail, K., Schatz, R., Viener, H.L., Schatz, D., Haller, M., Rockell, J., Gottlieb, P., Clare-Salzler, M., Atkinson, M. No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. Diabetes 2007;56(3):604-12.

58. Brusko, T.M., Putnam, A.L., Bluestone, J.A. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. Immunol Rev 2008;223:371-90.

59. Hill, J.A., Hall, J.A., Sun, C.M., Cai, Q., Ghyselinck, N., Chambon, P., Belkaid, Y., Mathis, D., Benoist, C. Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. Immunity 2008;29(5):758-70.

60. Tang, Q., Adams, Y. J, Penaranda, C., Melli, K., Piaggio, E., Sgouroudis, E., Piccirillo, C.A., Salomon, B.L., Bluestone, J.A. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. Immunity 2008;28(5):687-97.

61. Zhou, X., Bailey-Bucktrout, S.L., Jeker, L.T., Penaranda, C., Martinez-Llordella, M., Ashby, M., Nakayama, M., Rosenthal, W., Bluestone, J.A. Instability of the

transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nat Immunol 2009;10(9):1000-7.

62. Lazarski, C.A., Hughson, A., Sojka, D.K., Fowell, D.J. Regulating Treg cells at sites of inflammation. Immunity 2008;29(4):511; author reply 2.

63. Bougneres, P.F., Landais, P., Boisson, C., Carel, J.C., Frament, N., Boitard, C., Chaussain, J.L., Bach, J.F. Limited duration of remission of insulin dependency in children with recent overt type I diabetes treated with low-dose cyclosporin. Diabetes 1990;39(10):1264-72.

64. Eisenbarth, G.S., Srikanta, S., Jackson, R., Rabinowe, S., Dolinar, R., Aoki, T., Morris, M.A. Anti-thymocyte globulin and prednisone immunotherapy of recent onset type 1 diabetes mellitus. Diabetes Res 1985;2(6):271-6.

65. Pescovitz, M.D., Greenbaum, C.J., Krause-Steinrauf, H., Becker, D.J., Gitelman, S.E., Goland, R., Gottlieb, P.A., Marks, J.B., McGee, P.F., Moran, A.M., Raskin, P., Rodriguez, H., Schatz, D.A., Wherrett, D., Wilson, D.M., Lachin, J.M., Skyler, J.S. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009;361(22):2143-52.

66. Shehadeh, N., Calcinaro, F., Bradley, J. B, Bruchim, I., Vardi, P., Lafferty, J. K. Effect of adjuvant therapy on development of diabetes in mouse and man. Lancet 1994;343(8899):706-7.

67. Allen, F. H, Gay, C. E, Klingensmith, J. G, Hamman, F. R. Identification and treatment of cystic fibrosis-related diabetes. A survey of current medical practice in the U.S. Diabetes Care 1998;21(6):943-8.

68. Pozzilli, P., Pitocco, D., Visalli, N., Cavallo, M.G., Buzzetti, R., Crino, A., Spera, S., Suraci, C., Multari, G., Cervoni, M., Bitti M, M.L., Matteoli, M.C., Marietti, G., Ferrazzoli, F., Faldetta C, M.R., Giordano, C., Sbriglia, M., Sarugeri, E., Ghirlanda, G. No effect of oral insulin on residual beta-cell function in recent-onset type I diabetes (the IMDIAB VII). IMDIAB Group. Diabetologia 2000;43(8):1000-4.

69. Harrison, L.C., Honeyman, M.C., Steele, C.E., Stone, N.L., Sarugeri, E., Bonifacio, E., Couper, J.J., Colman, P.G. Pancreatic beta-cell function and immune responses to insulin after administration of intranasal insulin to humans at risk for type 1 diabetes. Diabetes Care 2004;27(10):2348-55.

70. Karounos, D.G., Goes, S.E. Utilization of NOD mice in the study of type 1 diabetes. Methods Mol Med 2003;83:81-90.

71. Shoda, L.K., Young, D.L., Ramanujan, S., Whiting, C.C., Atkinson, M.A., Bluestone, J.A., Eisenbarth, G.S., Mathis, D., Rossini, A.A., Campbell, S.E., Kahn, R., Kreuwel, H.T. A comprehensive review of interventions in the NOD mouse and implications for translation. Immunity 2005;23(2):115-26.

72. Chatenoud, L. CD3-specific antibody-induced active tolerance: from bench to bedside. Nat Rev Immunol 2003;3(2):123-32.

73. Chatenoud, L., Bluestone, J.A. CD3-specific antibodies: a portal to the treatment of autoimmunity. Nat Rev Immunol 2007;7(8):622-32.

74. Cosimi, A.B., Burton, R.C., Colvin, R.B., Goldstein, G., Delmonico, F.L., LaQuaglia, M.P., Tolkoff-Rubin, N., Rubin, R.H., Herrin, J.T., Russell, P.S.

Treatment of acute renal allograft rejection with OKT3 monoclonal antibody. Transplantation 1981;32(6):535-9.

75. Abramowicz, D., Schandene, L., Goldman, M., Crusiaux, A., Vereerstraeten, P., Pauw D, L., Wybran, J., Kinnaert, P., Dupont, E., Toussaint, C. Release of tumor necrosis factor, interleukin-2, and gamma-interferon in serum after injection of OKT3 monoclonal antibody in kidney transplant recipients. Transplantation 1989;47(4):606-8.

76. Chatenoud, L., Ferran, C., Reuter, A., Legendre, C., Gevaert, Y., Kreis, H., Franchimont, P., Bach, J.F. Systemic reaction to the anti-T-cell monoclonal antibody OKT3 in relation to serum levels of tumor necrosis factor and interferon-gamma [corrected]. N Engl J Med 1989;320(21):1420-1.

77. Alegre, M.L., Collins, A.M., Pulito, V.L., Brosius, R.A., Olson, W.C., Zivin, R.A., Knowles, R., Thistlethwaite, J.R., Jolliffe, L.K., Bluestone, J.A. Effect of a single amino acid mutation on the activating and immunosuppressive properties of a "humanized" OKT3 monoclonal antibody. J Immunol 1992;148(11):3461-8.

78. Friend, P.J., Hale, G., Chatenoud, L., Rebello, P., Bradley, J., Thiru, S., Phillips, J.M., Waldmann, H. Phase I study of an engineered aglycosylated humanized CD3 antibody in renal transplant rejection. Transplantation 1999;68(11):1632-7.

79. Woodle, E.S., Thistlethwaite, J.R., Ghobrial, I.A., Jolliffe, L.K., Stuart, F.P., Bluestone, J.A. OKT3 F(ab')2 fragments--retention of the immunosuppressive properties of whole antibody with marked reduction in T cell activation and lymphokine release. Transplantation 1991;52(2):354-60.

80. Woodle, E.S., Xu, D., Zivin, R.A., Auger, J., Charette, J., O'Laughlin, R., Peace, D., Jollife, L.K., Haverty, T., Bluestone, J.A., Thistlethwaite, J.R.Jr. Phase I trial of a humanized, Fc receptor nonbinding OKT3 antibody, huOKT3gamma1(Ala-Ala) in the treatment of acute renal allograft rejection. Transplantation 1999;68(5):608-16.

81. Motallebzadeh, R., Bolton, E.M., Pettigrew, G.J. Lymphoid tissue formation in allografts: innocent until proven guilty. Transplantation 2008;85(3):309-11.

82. Herold, K.C., Burton, B. J, Francois, F., Poumian-Ruiz, E., Glandt, M., Bluestone, J.A. Activation of human T cells by FcR nonbinding anti-CD3 mAb, hOKT3gamma1(Ala-Ala). J Clin Invest 2003;111(3):409-18.

83. Smith, J.A., Tang, Q., Bluestone, J.A. Partial TCR signals delivered by FcR-nonbinding anti-CD3 monoclonal antibodies differentially regulate individual Th subsets. J Immunol 1998;160(10):4841-9.

84. Smith, J.A., Tso, . JY, Clark, M.R., Cole, . MS, Bluestone, J.A. Nonmitogenic Anti-CD3 Monoclonal Antibodies Deliver a Partial T Cell Receptor Signal and Induce Clonal Anergy Journal of Experimental Medicine 1997;185(8):1413-22.

85. Chatenoud, L., Primo, J., Bach, J.F. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. J Immunol 1997;158(6):2947-54.

86. Chatenoud, L., Thervet, E., Primo, J., Bach, J.F. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci U S A 1994;91(1):123-7.

87. Belghith, M., Bluestone, J.A., Barriot, S., Megret, J., Bach, J.F., Chatenoud, L. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. Nat Med 2003;9(9):1202-8.

88. Bresson, D., Togher, L., Rodrigo, E., Chen, Y., Bluestone, J.A., Herold, K.C., Herrath v, M. Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. J Clin Invest 2006;116(5):1371-81.

89. Kohm, A.P., Williams, J.S., Bickford, A.L., McMahon, J.S., Chatenoud, L., Bach, J.F., Bluestone, J.A., Miller, S.D. Treatment with nonmitogenic anti-CD3 monoclonal antibody induces CD4+ T cell unresponsiveness and functional reversal of established experimental autoimmune encephalomyelitis. J Immunol 2005;174(8):4525-34.

90. Chen, G., Han, G., Wang, J., Wang, R., Xu, R., Shen, B., Qian, J., Li, Y. Essential roles of TGF-beta in anti-CD3 antibody therapy: reversal of diabetes in nonobese diabetic mice independent of Foxp3+CD4+ regulatory T cells. J Leukoc Biol 2008;83(2):280-7.

91. Herrath v, M.G., Coon, B., Wolfe, T., Chatenoud, L. Nonmitogenic CD3 antibody reverses virally induced (rat insulin promoter-lymphocytic choriomeningitis virus) autoimmune diabetes without impeding viral clearance. J Immunol 2002;168(2):933-41.

92. Yang, W., Hussain, S., Mi, Q.S., Santamaria, P., Delovitch, T.L. Perturbed homeostasis of peripheral T cells elicits decreased susceptibility to anti-CD3-

induced apoptosis in prediabetic nonobese diabetic mice. J Immunol 2004;173(7):4407-16.

93. Tang, Q., Bluestone, J.A. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 2008;9(3):239-44.

94. Green, D.R. Overview: apoptotic signaling pathways in the immune system. Immunol Rev 2003;193:5-9.

95. Hildeman, D., Jorgensen, T., Kappler, J., Marrack, P. Apoptosis and the homeostatic control of immune responses. Curr Opin Immunol 2007;19(5):516-21.

96. Hildeman, D.A., Zhu, Y., Mitchell, T.C., Bouillet, P., Strasser, A., Kappler, J., Marrack, P. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. Immunity 2002;16(6):759-67.

97. Woo, M., Hakem, R., Soengas, M.S., Duncan, G.S., Shahinian, A., Kagi, D., Hakem, A., McCurrach, M., Khoo, W., Kaufman, S.A., Senaldi, G., Howard, T., Lowe, S.W., Mak, T.W. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev 1998;12(6):806-19.

98. Thornton, A.M., Korty, P.E., Tran, D.Q., Wohlfert, E.A., Murray, P.E., Belkaid, Y., Shevach, E.M. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol 2010;184(7):3433-41.

99. Bisikirska, B., Colgan, J., Luban, J., Bluestone, J.A., Herold, K.C. TCR stimulation with modified anti-CD3 mAb expands CD8+ T cell population and induces CD8+CD25+ Tregs. J Clin Invest 2005;115(10):2904-13.

100. Walker, M.R., Kasprowicz, D.J., Gersuk, V.H., Benard, A., Landeghen V, M., Buckner, J.H., Ziegler, S.F. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. J Clin Invest 2003;112(9):1437-43.

101. Drayton, D.L., Liao, S., Mounzer, R.H., Ruddle, N.H. Lymphoid organ development: from ontogeny to neogenesis. Nat Immunol 2006;7(4):344-53.

102. Cyster, J.G. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol 2005;23:127-59.

103. Oliver, G. Lymphatic vasculature development. Nat Rev Immunol 2004;4(1):35-45.

104. Kendall, P.L., Yu, G., Woodward, E.J., Thomas, J.W. Tertiary lymphoid structures in the pancreas promote selection of B lymphocytes in autoimmune diabetes. J Immunol 2007;178(9):5643-51.

105. Wu, Q., Salomon, B., Chen, M., Wang, Y., Hoffman, L.M., Bluestone, J.A., Fu, Y.X. Reversal of spontaneous autoimmune insulitis in nonobese diabetic mice by soluble lymphotoxin receptor. J Exp Med 2001;193(11):1327-32.

106. Lee, Y., Chin, R.K., Christiansen, P., Sun, Y., Tumanov, A.V., Wang, J., Chervonsky, A.V., Fu, Y.X. Recruitment and activation of naive T cells in the islets by lymphotoxin beta receptor-dependent tertiary lymphoid structure. Immunity 2006;25(3):499-509.

107. Luther, S.A., Lopez, T., Bai, W., Hanahan, D., Cyster, J.G. BLC expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis. Immunity 2000;12(5):471-81.

108. Luther, S.A., Bidgol, A., Hargreaves, D.C., Schmidt, A., Xu, Y., Paniyadi, J., Matloubian, M., Cyster, J.G. Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. J Immunol 2002;169(1):424-33.

109. Chen, C. S, Vassileva, G., Kinsley, D., Holzmann, S., Manfra, D., Wiekowski, M.T., Romani, N., Lira, S.A. Ectopic expression of the murine chemokines CCL21a and CCL21b induces the formation of lymph node-like structures in pancreas, but not skin, of transgenic mice. J Immunol 2002;168(3):1001-8.

110. Simpson, P.B., Mistry, M.S., Maki, R.A., Yang, W., Schwarz, D.A., Johnson, E.B., Lio, F.M., Alleva, D.G. Cuttine edge: diabetes-associated quantitative trait locus, Idd4, is responsible for the IL-12p40 overexpression defect in nonobese diabetic (NOD) mice. Journal of Immunology 2003;171(7):3333-7.

111. Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G.J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C.L., Rupprecht, K., Parsons, W., Rosen, H. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science 2002;296(5566):346-9.

112. Melli, K., Friedman, R.S., Martin, A.E., Finger, E.B., Miao, G., Szot, G.L., Krummel, M.F., Tang, Q. Amplification of autoimmune response through induction of dendritic cell maturation in inflamed tissues. J Immunol 2009;182(5):2590-600.

113. Brinkmann, V., Lynch, K.R. FTY720: targeting G-protein-coupled receptors for sphingosine 1-phosphate in transplantation and autoimmunity. Curr Opin Immunol 2002;14(5):569-75.

114. Daniel, C., Sartory, N., Zahn, N., Geisslinger, G., Radeke, H.H., Stein, M. J. FTY720 ameliorates Th1-mediated colitis in mice by directly affecting the functional activity of CD4+CD25+ regulatory T cells. J Immunol 2007;178(4):2458-68.

115. Sawicka, E., Dubois, G., Jarai, G., Edwards, M., Thomas, M., Nicholls, A., Albert, R., Newson, C., Brinkmann, V., Walker, C. The sphingosine 1-phosphate receptor agonist FTY720 differentially affects the sequestration of CD4+/CD25+ T-regulatory cells and enhances their functional activity. J Immunol 2005;175(12):7973-80.

116. Lan, Y.Y., Creus D, A., Colvin, B.L., Abe, M., Brinkmann, V., Coates, P.T., Thomson, A.W. The sphingosine-1-phosphate receptor agonist FTY720 modulates dendritic cell trafficking in vivo. Am J Transplant 2005;5(11):2649-59.

117. Vliet V, E., Melis, M., Foidart, J.M., Ewijk V, W. Reticular fibroblasts in peripheral lymphoid organs identified by a monoclonal antibody. J Histochem Cytochem 1986;34(7):883-90.

118. Sherry, N.A., Tsai, E.B., Herold, K.C. Natural history of beta-cell function in type 1 diabetes. Diabetes 2005;54 Suppl 2:S32-9.

119. Maki, T., Gottschalk, R., Monaco, A.P. Prevention of autoimmune diabetes by FTY720 in nonobese diabetic mice. Transplantation 2002;74(12):1684-6.

120. Habicht, A., Clarkson, M.R., Yang, J., Henderson, J., Brinkmann, V., Fernandes, S., Jurewicz, M., Yuan, X., Sayegh, M.H. Novel insights into the mechanism of action of FTY720 in a transgenic model of allograft rejection: implications for therapy of chronic rejection. J Immunol 2006;176(1):36-42.

121. Pabst, R. Plasticity and heterogeneity of lymphoid organs. What are the criteria to call a lymphoid organ primary, secondary or tertiary? Immunol Lett 2007;112(1):1-8.

122. O'Connor, P., Comi, G., Montalban, X., Antel, J., Radue, E.W., Vera d, A., Pohlmann, H., Kappos, L. Oral fingolimod (FTY720) in multiple sclerosis: two-year results of a phase II extension study. Neurology 2009;72(1):73-9.

123. Chang, J.T., Palanivel, V.R., Kinjyo, I., Schambach, F., Intlekofer, A.M., Banerjee, A., Longworth, S.A., Vinup, K.E., Mrass, P., Oliaro, J., Killeen, N., Orange, J.S., Russell, S.M., Weninger, W., Reiner, S.L. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. Science 2007;315(5819):1687-91.

124. Wu, C.Y., Kirman, J.R., Rotte, M.J., Davey, D.F., Perfetto, S.P., Rhee, E.G., Freidag, B.L., Hill, B.J., Douek, D.C., Seder, R.A. Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo. Nat Immunol 2002;3(9):852-8.

125. Moon, J.J., Chu, H.H., Pepper, M., McSorley, S.J., Jameson, S.C., Kedl, R.M., Jenkins, M.K. Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity 2007;27(2):203-13.

126. Sallusto, F., Geginat, J., Lanzavecchia, A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745-63.

127. London, C.A., Lodge, M.P., Abbas, A.K. Functional responses and costimulator dependence of memory CD4+ T cells. J Immunol 2000;164(1):265-72.

128. Croft, M., Bradley, L.M., Swain, S.L. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. J Immunol 1994;152(6):2675-85.

129. Tough, D.F., Sprent, J. Turnover of naive- and memory-phenotype T cells. J Exp Med 1994;179(4):1127-35.

130. Li, J., Huston, G., Swain, S.L. IL-7 promotes the transition of CD4 effectors to persistent memory cells. J Exp Med 2003;198(12):1807-15.

131. Kondrack, R.M., Harbertson, J., Tan, J.T., McBreen, M.E., Surh, C.D., Bradley, L.M. Interleukin 7 regulates the survival and generation of memory CD4 cells. J Exp Med 2003;198(12):1797-806.

132. Dooms, H., Wolslegel, K., Lin, P., Abbas, A.K. Interleukin-2 enhances CD4+ T cell memory by promoting the generation of IL-7R alpha-expressing cells. J Exp Med 2007;204(3):547-57.

133. Fife, B.T., Guleria, I., Bupp G, M., Eagar, T.N., Tang, Q., Bour-Jordan, H., Yagita, H., Azuma, M., Sayegh, M.H., Bluestone, J.A. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. J Exp Med 2006;203(12):2737-47.

134. Surh, C.D., Sprent, J. Homeostasis of naive and memory T cells. Immunity 2008;29(6):848-62.

135. You, S., Belghith, M., Cobbold, S., Alyanakian, M.A., Gouarin, C., Barriot, S., Garcia, C., Waldmann, H., Bach, J.F., Chatenoud, L. Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. Diabetes 2005;54(5):1415-22.

136. Lepault, F., Gagnerault, M.C., Faveeuw, C., Bazin, H., Boitard, C. Lack of L-selectin expression by cells transferring diabetes in NOD mice: insights into the mechanisms involved in diabetes prevention by Mel-14 antibody treatment. Eur J Immunol 1995;25(6):1502-7.

137. Flynn, J.C., McInerney, M.F. High density insulin receptor-positive diabetogenic T lymphocytes in nonobese diabetic mice are memory cells. Immunopharmacol Immunotoxicol 2000;22(2):387-400.

138. Ansari, M.J., Salama, A.D., Chitnis, T., Smith, R.N., Yagita, H., Akiba, H., Yamazaki, T., Azuma, M., Iwai, H., Khoury, S.J., Auchincloss, H.Jr., Sayegh, M.H. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. J Exp Med 2003;198(1):63-9.

139. Liu, X., Leung, S., Wang, C., Tan, Z., Wang, J., Guo, T.B., Fang, L., Zhao, Y., Wan, B., Qin, X., Lu, L., Li, R., Pan, H., Song, M., Liu, A., Hong, J., Lu, H., Zhang, J.Z. Crucial role of interleukin-7 in T helper type 17 survival and expansion in autoimmune disease. Nat Med 2010;16(2):191-7.

140. Keir, M.E., Liang, S.C., Guleria, I., Latchman, Y.E., Qipo, A., Albacker, L.A., Koulmanda, M., Freeman, G.J., Sayegh, M.H., Sharpe, A.H. Tissue expression of PD-L1 mediates peripheral T cell tolerance. J Exp Med 2006;203(4):883-95.

141. Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G.J., Ahmed, R. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 2006;439(7077):682-7.

142. Bending, D., Pena DL, H., Veldhoen, M., Phillips, M. J, Uyttenhove, C., Stockinger, B., Cooke, A. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. J Clin Invest 2009.

143. Pellegrini, M., Calzascia, T., Elford, A.R., Shahinian, A., Lin, A.E., Dissanayake, D., Dhanji, S., Nguyen, L.T., Gronski, M.A., Morre, M., Assouline, B., Lahl, K., Sparwasser, T., Ohashi, P.S., Mak, T.W. Adjuvant IL-7 antagonizes multiple cellular and molecular inhibitory networks to enhance immunotherapies. Nat Med 2009;15(5):528-36.

144. Herold, C. K, Hagopian, W., Auger, A. J, Poumian-Ruiz, E., Taylor, L., Donaldson, D., Gitelman, E. S, Harlan, M. D, Xu, D., Zivin, A. R, Bluestone, A. J. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002;346(22):1692-8.

145. Keymeulen, B., Vandemeulebroucke, E., Ziegler, G. A, Mathieu, C., Kaufman, L., Hale, G., Gorus, F., Goldman, M., Walter, M., Candon, S., Schandene, L., Crenier, L., Block D, C., Seigneurin, M. J, Pauw D, P., Pierard, D., Weets, I., Rebello, P., Bird, P., Berrie, E., Frewin, M., Waldmann, H., Bach, F. J, Pipeleers, D., Chatenoud, L. Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005;352(25):2598-608.

146. O'Connor, P., Comi, G., Montalban, X., Antel, J., Radue, W. E, Vera d, A., Pohlmann, H., Kappos, L. Oral fingolimod (FTY720) in multiple sclerosis: two-year results of a phase II extension study. Neurology 2009;72(1):73-9.

147. Kelly, W.D., Lillehei, R.C., Merkel, F.K., Idezuki, Y., Goetz, F.C. Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. Surgery 1967;61(6):827-37.

148. Gruessner, R.W., Sutherland, D.E., Gruessner, A.C. Mortality assessment for pancreas transplants. Am J Transplant 2004;4(12):2018-26.

149. Ryan, E.A., Paty, B.W., Senior, P.A., Bigam, D., Alfadhli, E., Kneteman, N.M., Lakey, J.R., Shapiro, A.M. Five-year follow-up after clinical islet transplantation. Diabetes 2005;54(7):2060-9.

150. McCall, M.D., Toso, C., Baetge, E.E., Shapiro, A.M. Are stem cells a cure for diabetes? Clin Sci (Lond) 2010;118(2):87-97.

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