IMMUNOTHERAPY AND T CELL RECEPTOR ANALYSIS IN RECURRENT TYPE 1 DIABETES

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ABSTRACT

ALAINA LEIGH GARLAND: Immunotherapy and T Cell Receptor Analysis in Recurrent Type 1 Diabetes
(Under the direction of Dr. Roland M. Tisch)

Type 1 Diabetes (T1D) is a chronic autoimmune disease characterized by the T cell-mediated destruction of insulin-producing β cells in the islets of Langerhans. For T1D patients, life-long insulin injections are necessary to help maintain normoglycemia, although constant blood glucose fluctuations lead to a variety of complications. Currently, the only way to "cure" T1D is with islet or pancreas transplantation. The aims of the studies described herein are to: i) test the hypothesis that immunomodulating therapies targeting T cells will be able to prevent recurrent autoimmunity, and ii) to understand the kinetics and specificities of the pathogenic T cells involved in recurrent autoimmunity.

Results from our first study in the non-obese diabetic (NOD) mouse model show that non-depleting αCD4 and αCD8 coreceptor antibodies extend survival of syngeneic islet grafts in diabetic recipients. We also determined that via adeno-associated virus (AAV) vector gene delivery, ectopic expression of IL-2 by β cells also extended protection of syngeneic islet grafts. Surprisingly, the combination of αCD4 and αCD8 coreceptor antibodies with AAV vector mediated gene transfer of IL-2 did not extend islet graft protection over αCD4 and αCD8 treatment alone. Taken together, our results show that these two treatments do not act synergistically, although individually these immunotherapies extend islet graft survival.

Our second study examined the T cell receptor (TCR) variable (V)β repertoire in islet grafts. In islet grafts of diabetic NOD recipients the effector/memory (eff/mem) $CD8^+$ T cell repertoire in the islet graft showed decreased entropy, and is dominated by one to four TCR Vβ chains which varied markedly by mouse. The eff/mem $CD4^+$ T cell repertoire in the islet graft was more diverse, though all NOD recipients showed an increase in frequency of TCR Vβ12-bearing T cells. Additionally, the eff/mem TCR repertoire of T cells infiltrating the islet graft was more similar to the pancreas repertoire than the TCR repertoires found in the draining renal lymph node, pancreatic lymph node, or spleen. This suggests that, in individual NOD recipients, the same specificies of effector/memory T cells may be involved in both initial and recurrent autoimmunity.

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LIST OF ABBREVIATIONS

AAV	adeno-associated virus
APC	antigen presenting cell
BCR	B cell receptor
C	constant
CDR3	complementarity determining region 3
cTEC	cortical thymic epithelial cell
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
D	diversity
DBY	dead box RNA helicase Y
DC	dendritic cell
DN	double negative (thymocytes)
DP	double positive (thymocytes)
ds	double-stranded
EAE	experimental autoimmune encephalomyelitis
ECDI	ethylene carbodiimide
EGFP	enhanced green fluorescent protein
Ex-4	exendin-4
FoxP3^{+}Treg	forkhead-box protein 3-expressing regulatory T cell
GAD65	the 65kd isoform of glutamic acid decarboxylase
GFP	green fluorescent protein
GLP-1	glucagon-like peptide 1

HEL	hen egg lysozyme
HGG	human immunoglobulin
HLA	human leukocyte antigen
Hsp60	heat shock protein 60
Idd	insulin dependent diabetes (loci)
IFIH1	interferon induced with helicase C domain 1
IGRP	islet-specific glucose-6-phosphatase catalytic subunit-related protein
IL2RA	IL-2 receptor alpha (CD25)
IU	infectious units
J	joining
MHC	major histocompatibility complex
MIP	mouse insulin promoter
MOG	myelin/oligodendrocyte glycoprotein
mTEC	medullary thymic epithelial cell
NOD	non-obese diabetic (mouse)
PAMP	pathogen-associated molecular pattern
PLN	pancreatic lymph nodes
PTPN22	protein tyrosine phosphatase non-receptor type 22
RLN	renal lymph nodes
ss	single-stranded
STZ	streptozotocin
T1D	Type 1 Diabetes

TCR	T cell receptor
Treg	regulatory T cells
V	variable
vp	viral particles
α	alpha
β	beta
γ	gamma
Δ/δ	delta

CHAPTER 1

INTRODUCTION

1.1 The Immune System

The immune system is responsible for defending the body from foreign pathogens. This system must be able to respond effectively to both intracellular (e.g. viruses) and extracellular pathogens (e.g. bacteria) that invade the body. To do this, cells of the immune system must also be able to differentiate between self and non-self proteins. This involves a complex interplay between innate and adaptive immune effector cells. Innate immunity functions largely through recognition of pathogen-associated molecular patterns (PAMPs) present on foreign invaders, as well as complement activation[1,2]. The adaptive immune response includes T lymphocytes (T cells) and B lymphocytes (B cells), and is responsible for specific recognition of antigens. Simplistically, B cells that bind cognate antigen via the B cell receptor (BCR) and that receive appropriate "co-stimulatory" signals become activated, differentiate into plasma cells, and produce large amounts of antibody[3]. T cells are a varied population, which function together to help orchestrate the antigen-specific immune response. This system involves a series of checks and balances wherein effector T cells can become activated and fight infection, and regulatory T cells (Treg) limit the course of these ongoing responses[4,5]. When the system works properly, this balance allows the immune system to protect the body from infectious disease while limiting excess damage to the tissues involved. Additionally, the body is protected from autoimmunity by central and peripheral self tolerance-inducing mechanisms[6-8].

1.2 Central and peripheral T cell tolerance

In a healthy individual, negative selection in the thymus deletes the majority of autoreactive T cells via clonal deletion[8]. T cells originate in the liver of neonatal mice, and then from the bone marrow throughout adult life. T cell progenitors migrate to the thymus, where development occurs through interactions that are both antigen-independent and –dependent[8]. Double negative (DN) thymocytes ($CD4^-$ and $CD8^-$) undergo gene rearrangement within the T cell receptor (TCR) locus. To create a diverse repertoire of TCR capable of recognizing wide varieties of antigens, different variable (V), diversity (D) and joining (J) gene segments undergo rearrangement and combine to generate TCRs with distinct specificities[9]. The TCR is a heterodimer composed of either alpha (α) and beta (β) chains (95% of T cells) or gamma (γ) and delta (δ) chains (5% of T cells). Upon functional gene rearrangement, a TCR is expressed on the surface, followed by upregulaton of both co-receptor molecules CD4 and CD8[10]. These double positive (DP) thymocytes first undergo positive selection in the cortex of the thymus by interacting with cortical thymic epithelial cells (cTECs)[10]. cTECs present peptides bound by surface major histocompatibility complex (MHC) class I and II molecules. DP thymocytes that express TCRs able to bind peptide-MHC complexes receive survival signals from cTECs and further differentiate into single positive (SP) $CD4^+$ and $CD8^+$ thymocytes[11]. DP thymocytes that express TCRs unable to bind peptide-MHC complexes fail to receive survival signals, and die from "neglect[11]." Positively selected thymocytes then migrate to the medulla of the thymus to undergo negative selection[12]. SP thymocytes interact with medullary TECs (mTECs) or dendritic cells (DC) displaying self-peptide-MHC complexes[12]. Elimination of potential autoreactive T precursors occurs when

SP thymocytes bind self-peptide-MHC with high affinity/avidity, thereby inducing apoptosis. A small population of $CD4^+$ thymocytes with moderate to high affinity/avidity develop into forkhead-box protein 3 (FoxP3)-expressing Treg ($FoxP3^+$Treg), which contribute to peripheral tolerance[13] (see below). Following positive and negative selection, surviving SP $CD4^+$ and $CD8^+$ thymocytes migrate from the thymus into the periphery.

In the periphery, T cell tolerance is maintained by a variety of mechanisms. Presentation of self-peptides on immature APCs contributes to the maintenance of tolerance. Activation of naive T cells simplistically requires two signals; signal 1 is delivered by the TCR recognizing its cognate antigen in the context of MHC molecules, and signal 2 is provided by "co-stimulatory" molecules expressed by APCs[14]. Co-stimulatory signals are delivered by B7-1 (CD80)/B7-2 (CD86) and CD40 expressed by APCs, which interact with CD28 and CD40 ligand (CD40L) on the surface of T cells, respectively[14]. In the absence of an inflammatory environment, immature APCs lack expression of the co-stimulatory molecules required to activate T cells. Additionally, the co-receptor molecules CD4 and CD8 expressed by T cells contribute to efficient T cell activation by interacting with conserved regions of MHC class II and class I molecules, respectively, expressed by APC[15]. Resultant signaling by CD4 or CD8 within the T cell in part involves phosphorylation of the src kinase Lck[15]. MHC molecules present peptide antigens to T cells and thus play a major role in T cell selection in the thymus, so these molecules are instrumental in determining the T cell repertoire. Additionally, peptide-MHC complexes on APCs control regulation and activation of T cells in the periphery.

While it was previously thought that the immune system was simply a balance between pathogenic $CD4^+$ and $CD8^+$ type 1 effectors and more "suppressive" $CD4^+$ T helper 2 (Th2) cells, it is now recognized that other Treg populations play a large role in maintaining peripheral T cell tolerance. These include IL-4 secreting $CD4^+$ Th2 cells and IL-10 secreting $CD4^+$ Tr1 cells, as well as "natural" $FoxP3^+$Treg[16]. FoxP3 is a transcription factor that regulates the expression of genes required for the phenotype and function of $FoxP3^+$Treg[17,18]. $FoxP3^+$Treg exhibit a potent suppressive capacity, and play a critical role in maintaining peripheral tolerance[19-21]. $FoxP3^+$Treg regulate effector responses in a variety of ways including: 1) secretion of suppressive cytokines (IL-10, TGFβ), 2) IL-2 consumption, 3) cytolysis via granzyme b, and 4) cell-cell contact mechanisms (galectin-1, CTLA-4, TGFβ) [22]. Dysregulation within the overall pool of Treg can contribute to the development of autoimmunity.

1.3 Type 1 diabetes (T1D)

Type 1 Diabetes (T1D) is characterized by the autoimmune attack of β cells present in the islets of Langerhans[23]. β cells are responsible for the production of insulin, which regulates glucose transport and metabolism[24]. Early β cell autoimmunity involves a response characterized by autoantibodies to islet antigens and autoreactive T cells. This chronic immune response causes the gradual destruction of β cells, ultimately resulting in an inability to manage blood glucose levels. Though T1D can be managed with daily insulin injections, lack of complete blood glucose control results in a variety of debilitating

complications including heart disease, blindness, nerve damage, and kidney damage, as well as a reduced life expectancy of 10-15 years[25]. Like many other autoimmune diseases, T1D is rapidly growing in incidence. This increase in incidence is especially pronounced in industrialized nations[26], and affects at least 1-2 million people in the United States alone. Currently, the only "cure" for T1D is through islet cell or pancreas transplantation. However, lifelong immunosuppression is needed to prevent rejection of islet grafts due by both autoimmune- and allogeneic-reactive T cells present in the recipient. This severely limits the utility of this approach, and will be discussed later in further detail.

1.4 Genetic and environmental factors of T1D

Susceptibility to T1D is influenced by both genetic and environmental factors, which combine to cause the breakdown of self-tolerance to β cells[27-32]. Though the inheritance pattern of T1D is complex, a strong genetic link has been observed. The risk of T1D development in siblings of T1D patients is 5-10% by age 20, a 15-fold higher rate than that for the general population[31]. Offspring of T1D fathers have a 12% disease rate, and those of diabetic mothers have a 6% disease rate. The incidence of diabetes in monozygotic twins is ~65%, indicating both a strong genetic linkage as well as a likely role for environmental factors. Twenty-six insulin-dependent diabetes (*Idd*) loci have been identified in humans, including 19 loci associated with immune regulation[29]. The strongest genetic association with T1D susceptibility and resistance maps to genes found in the MHC (mice) and human leukocyte antigen (HLA; humans) regions. Non-obese diabetic (NOD) mice, which spontaneously develop T1D, express specific class I and class II MHC molecules ($H2K^d/D^b$

and I-A^{g7}, respectively), which are instrumental in contributing to diabetes progression. Notably, the NOD MHC class II molecule I-A^{g7} is similar in structure to human HLA class II DQ8, and both confer a susceptibility to T1D. The P9 peptide-binding pocket in both lacks a negatively charged aspartate residue at position B57, which results in binding of peptides distinct from those bound by other class II molecules [33], thus influencing the T cell repertoire. A variety of other genes present in *idd* loci contribute to disease risk including: the insulin gene, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), protein tyrosine phosphatase non-receptor type 22 (PTPN22 gene), IL-2 receptor alpha (IL2RA/CD25), and interferon induced with helicase C domain 1 (IFIH1) genes[30,31,34].

Additionally, polymorphisms and mutations in key genes can cause dysregulation of thymic clonal deletion and contribute to the escape of autoreactive T cells into the periphery[8]. For example, autoimmune regulator protein (AIRE) is a transcription factor important for driving expression of various tissue-specific antigens (TSA) by mTECs and allowing efficient negative selection[35,36]. TSA restricted to various tissues including pancreas, salivary gland, and eye, are included in this process. Importantly, insulin is an AIRE-regulated islet protein expressed by mTECs. AIRE mutations are thought to cause a peripheral increase in autoreactive T cells specific for these tissues[35,36]. Indeed, AIRE-knockout mice develop multi-organ autoimmunity and mutations in AIRE cause multi-organ human disease, with 20% developing T1D[28].

Peripheral regulation can also be influenced by gene variants affecting the immune system as a whole. For example, both IL-2 and CD25 (the high-affinity member of the IL-2 receptor) are located within *idd* loci. FoxP3^{+}Treg constitutively express CD25, and require IL-2 for maintenance/homeostasis in the periphery. Additionally, IL-2 is involved in expression of Bcl-2, a mitochondrial protein that protects from apoptosis. Thus, mutations involving the IL-2/IL-2 receptor axis have the potential to adversely affect the preservation of peripheral tolerance. Indeed, a reduction of both CD25 and Bcl-2 expression has been correlated with reduced survival and function of intra-islet FoxP3^{+}Treg in NOD mice[37]. An imbalance between functional FoxP3^{+}Treg and pathogenic effector T cells in the islets is believed to contribute to diabetes progression[28]. Accordingly, enhancing the numbers and/or function of FoxP3^{+}Treg has been the focus of several strategies to prevent or suppress β cell autoimmunity[38].

Poorly-defined environmental factors also contribute to T1D. Factors such as microbes, milk products, sun exposure, and vitamin D have been suggested to play a role[27,28]. NOD mice monocolonized with aerobic spore-forming gram positive bacteria show a lower diabetes incidence than germ-free NOD mice[39]. Additionally, diabetes onset is inhibited by infection with a gastrointenstinal helminth[40]. There is also evidence in mouse models that viral infections trigger an autoimmune reaction through molecular mimicry of viral proteins and β cell proteins, bystander activation of autoreactive T cells, and altering of the pathogenic T effector-Treg balance[41]. So, T1D is a mulitifactoral disease resulting from a

combination of various genetic mutations in addition to various environmental factors that lead to the development of β cell autoimmunity.

1.5 T1D is a chronic inflammatory disease

T1D is a viewed as a chronic inflammatory disease. Typically, β cell autoimmunity progresses over a number of years before a sufficient amount of β cells are destroyed and clinical diabetes diagnosed. The spontaneous and chronic autoimmune disease observed in NOD mice mimics the human form of the disease, making this mouse model a highly useful tool to investigate T1D. Studies in this well-established model have shown that islet inflammation or insulitis occurs in "stages"[28]. Peri-insulitis is first observed at 3-4 weeks of age in NOD mice, and involves the infiltration of T cells, B cells, macrophages, and DC around the edge of an islet[42]. This is followed by a progressive infiltration of the immune cells into the islets (intra-insulitis). The last stage entails efficient β cell destruction and the onset of overt diabetes, which typically occurs between 12 and 35 weeks of age in 70-80% of female NOD mice[42]. Diabetes occurs when 80 to 90% of β cell mass has been destroyed and insulin levels are no longer sufficient to regulate glucose metabolism. The loss of β cell-specific tolerance is mediated by a variety of defects in the NOD mouse. As mentioned above, defects in negative selection in the thymus are thought to contribute to the increased frequency of β cell specific T cells present in the periphery. Additionally, dysregulation of the pool of Treg plays a key role in the apparent preferential differentiation of β cell-specific, pathogenic $CD4^+$ and $CD8^+$ type 1 T effectors. As alluded to earlier, dysregulation of $FoxP3^+$Treg in frequency, number, and/or function has been reported as β

cell autoimmunity progresses in NOD mice[43,44]. It is thought that the progression of diabetes is promoted by a gradual decrease in numbers and/or function of FoxP3$^+$Treg within the islets, resulting in a concomitant expansion of pathogenic type 1 effector T cells. Additionally, FoxP3$^+$Treg have been shown to be phenotypically different in the blood of diabetic patients than that of healthy controls, indicating a role for FoxP3$^+$Treg dysfunction in the human form of the disease[45].

1.6 CD4$^+$ and CD8$^+$ T cells mediate β cell destruction in T1D

Both CD4$^+$ and CD8$^+$ T cells are necessary for diabetes development in NOD mice. For example, co-transfer of CD4$^+$ and CD8$^+$ T cells from diabetic NOD mice is needed to induce diabetes in appropriate recipient mice[46], and antibody depletion of CD4$^+$ T cells prevents the onset of diabetes in NOD mice[47]. Furthermore, β-2 microglobulin knockout NOD mice, which express almost no MHC class I molecules, and thus no CD8$^+$ T cells, remain diabetes-free[48,49]. Additionally, treatment of young NOD mice with a non-depleting CD8 antibody delayed or prevented the progression of insulitis in NOD mice[50]. Although the roles of both T cell subsets in diabetes have been extensively studied, how TCR specificities for β cell autoantigens and changes in the TCR repertoire influence disease progression are not well understood. During the early stages of the diabetogenic response, it is thought that relatively few β cell autoantigens are targeted by CD4$^+$ and CD8$^+$ T cells. Therefore, the initial infiltrating TCR repertoire is believed to be limited as well. Over time, additional epitopes are exposed through progressive β cell death. This “epitope spread” and concomitant expansion of the TCR repertoire enhances β cell destruction. Part of what

initiates activation of autoreactive T cells may be a wave of β cell death that happens naturally in NOD mice at about 2 weeks of age[51,52]. β cell antigens would be available to APCs, which could then migrate to the PLN and activate T cells[52,53].

1.7 Specificity of diabetogenic T cells

Since knowledge of the β cell autoantigens recognized by pathogenic T cells may aid in better defining the disease process and the development of immunotherapies, the interest in defining these targets has been understandably high. Identified $CD4^+$ T cell epitopes including insulin B chain, proinsulin, glutamic acid decarboxylase 65 (GAD65; the 65kd β-cell specific isoform of GAD), the protein tyrosine phosphatase IA-2, and heat shock protein 60 (hsp60) have been found in NOD mice (reviewed in [33,54,55]). Important β cell autoantigens recognized by $CD8^+$ T cells include the insulin B chain and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and $CD8^+$ T cells specific for these proteins have been observed early in disease progression in NOD mice[33,54,55]. Both NOD mice and human type 1 diabetics have autoantibodies to many of these antigens, indicating a potential role for many of these autoantigens in human disease[33]. Using MHC class II tetramers, GAD65 and proinsulin-specific $CD4^+$ T cells were detected in 61% of diabetic patients assayed and only 9.5% of controls, also indicating a potential role in human diabetes[56]. Interestingly, when children with high-risk HLA genotypes were evaluated, the appearance of insulin antibodies prior to antibodies specific for GAD65 or IA-2 was associated with a more aggressive form of disease[57].

Despite knowledge that certain autoantigens are involved in T1D, it remains unclear which if any of these antigens play an important role in initiating the autoimmune process, and how each may contribute to disease progression. Several studies have implicated the involvement of GAD early in the immune response within the islets[58,59], however, NOD mice deficient in GAD65[60,61] and NOD mice tolerized to GAD65[62] both develop diabetes with the same frequency as unmanipulated NOD mice. Additionally, a study where mutating the main insulin epitope (InsB9-23) eliminated diabetes development indicates a prime role for insulin in disease onset[63]. Additionally, insulin-reactive, clonally expanded T cells have been isolated from the PLNs of diabetic patients[64]. Yet another study found that the earliest clones present in the islets of NOD mice were reactive to whole islet lysate, but not GAD65 or insulin, suggesting that other, perhaps as yet unknown epitopes play a role in disease initiation[65]. Additionally, T cells clones reactive to various islet epitopes (GAD65, IA2) were not all able to mediate insulitis or diabetes[66]. Notably, administration of whole insulin, insulin B chain peptide 9-23, GAD65, and hsp60 peptide p277, has been shown to prevent and/or suppress the diabetogenic response in pre-diabetic NOD mice[33,55]. Thus, while several of these antigens likely play a role in disease initiation and/or propagation, the mechanisms controlling these processes are as yet undefined.

1.8 TCR Vβ chain usage in T1D

Islet-infiltrating T cells have also been studied by examining the Vα and Vβ chains of the TCR. The TCR is a heterodimer consisting of disulfide-linked α and β chains, which have constant (C) and variable (V) regions. The V region is further composed of variable (Vβ),

diversity (Dβ), and joining (Jβ) segments. At least 31 Vβ genes have been identified[67]. Early studies determined that in two autoimmune diseases, experimental allergic encephalomyelitis (EAE) and collagen-induced arthritis, TCR Vβ repertoires were restricted[68-70]. Both models showed preferential use of TCR Vβ8.2 by pathogenic T cells, and disease was ameliorated when mice were treated with αTCR Vβ8 or 8.2 antibodies[68-70]. This provided rationale to explore the TCR Vβ specificity of islet-infiltrating T cells in T1D, specifically early islet-infiltrating (and perhaps disease-initiating) T cells. Several studies found skewed TCR Vβ repertoires in young NOD mice, but with little consensus on the dominant TCR Vβ chains. At 2-5 weeks of age, various studies showed that T cells found in NOD islets demonstrated preferential usage of Vβ1[71], Vβ1 and Vβ12[65],Vβ2[72], Vβ3 and Vβ7[73], Vβ11[74], and Vβ8.2[75].

Due to the limited TCR usage observed in their studies, several authors postulated that T1D is initiated by recognition of a single autoantigen. While this may indeed be the case, the great variety of TCR Vβ chain preferences observed by different groups in distinct NOD mouse colonies suggests that the initiating TCR specificities may vary considerably. There is more consensus on the repertoire as mice age, as several studies have shown a more diversified TCR Vβ repertoire as islet infiltration progresses in older, prediabetic animals[72,76,77], and in islet-reactive T cell clones[76,78,79].

Though most studies show heterogeneity in the autoreactive T cell response, a few studies have shown some degree of TCR Vβ skewing. One study found preferential use of Vβ1 and

Vβ12 in 2-4 week old NOD islets, and that both exhibited conserved motifs. Interestingly, though the Vβ1 motifs found in the islets were diluted by age 11-12 weeks, at that age the Vβ12 transcripts still showed similar junctions and CDR3 lengths[65]. Interestingly, another study noted that Vβ12 RNA levels were significantly increased in the islets of the two pre-diabetic mice examined in comparison to spleen RNA levels[80].

To determine if several prevalent TCR Vβ chains were necessary for disease onset, NOD mice were bred with a deletion of TCR Vβ5, 8, 9, 11, 12, and 12[81]. Disease developed at lower incidence; overt diabetes was observed in 12.5% of mice as compared to 33% in control mice, [81] but diabetes could still develop in some mice in the absence of these TCR Vβ chains. This indicates plasticity in the TCR repertoire, and that while certain TCR Vβ chains may accelerate diabetes, it is unlikely that any one TCR Vβ chain is essential for disease initiation. Indeed, T cells may express different TCR Vβ chains yet be specific for the same epitope. Indeed, T cell clones all specific for the same epitope, Ins9-23, were shown to express a wide variety of TCR Vβ chains[82,83]. Two different but overlapping GAD65 epitopes were shown to express different Vβ chains[84]. The p530 clones spontaneously developed, were diabetogenic, and expressed TCR Vβ4. The p524 clones were found after GAD65 peptide immunization, were protective against disease, and expressed TCR Vβ12[84]. However, T cells specific for certain β cell autoantigens appear to preferentially use particular TCR Vβ chains. For example, tetramer-binding IGRP-specific $CD8^+$ T cells sorted via flow cytometry from the islets of NOD mice predominantly express TCR Vβ 8.1/8.2, indicating a distinct preference for this TCR Vβ chain[85,86]. So, while it is not likely that one

TCR Vβ chain is essential for disease development, certain diabetogenic T cells, like those specific for IGRP, show a preference for a certain TCR Vβ chain. Thus, the immune response can be explored to a certain extent by studying the TCR Vβ repertoire. It is important to note that despite uniform MHC class I and II alleles in the NOD mouse, there is high mouse to mouse variability within the TCR repertoire. One study showed skewed TCR Vβ usage in peripheral blood of several multiple sclerosis patients, but disease progression was not affected by this skewing[87]. It is likely that the variability of human islet-infiltrating T cells is even greater from patient to patient due to the much wider range of HLA class I and II alleles possible.

1.9 Islet transplantation for the treatment of T1D

Though T1D can be managed with daily insulin injections, islet or pancreas transplantation is currently the only way to "cure" T1D. Even with insulin therapy, complications can ensue from continual blood glucose fluctuations in diabetic patients, including heart disease, kidney disease, liver disease, and blindness. Additionally, while intensive insulin therapy can delay the onset of these events, patients remain at risk for severe or fatal hypoglycemic events. In humans, islets have been isolated and transplanted via the "Edmonton protocol" [88]. Currently, islet or pancreas transplants are only performed if the patient is already receiving a kidney or liver transplant, or if insulin alone is insufficient to maintain appropriate blood glucose control. Excitingly, with the advent of the Edmonton protocol the success rate of islet transplants increased dramatically to a rate of 58%[88]. Here, success has been defined as attaining insulin independence at some point following transplantation. In

this procedure, islets are harvested from donor cadavers, purified, and injected via the portal vein into the recipient's liver. This minimally invasive technique is associated with a low morbidity rate, and allows for repeated administration of islets if necessary to achieve full insulin independence. Though this shows the promise of islet transplantation as a treatment for T1D, several factors nevertheless limit general application of the approach. These include the lack of donor cadaver pancreases (2 or more are needed for necessary islet yield), the recovery and viability of islets, toxicity of the immunosuppressive drug regime to the β cells, immune-mediated rejection of islets, and eventual failure of most grafts within 2 years. Additionally, the benefits of increased blood glucose control are likely outweighed by the cost of the lifelong systemic immunosuppression needed to prevent autoreactive and alloreactive rejection of the islet graft. Currently, an immunosuppressive regime involving sirolimus and tacrolimus is used to prevent islet graft rejection by both autoreactive[89,90] and alloreactive T cells[91]. This immunosuppression decreases the efficiency of the entire immune system, leaving patients more vulnerable to opportunistic infections. However, if "targeted" immune suppression is achieved in which the auto- or alloreactive T cells are selectively suppressed while preserving the function of the remainder of the immune system, islet grafts may become a viable option for a larger population of T1D patients. By decreasing the probability and incidence of complications resulting from severe blood glucose fluctuations, this treatment could have the potential to increase both length and quality of life.

Efforts to enhance islet transplantation such as improving islet cell processing and culturing have been ongoing. For instance, the function of islets may be augmented by cytoprotective regimes involving pre-culture with factors such as 17-β-estradiol, nicotinamide, and metal protoporphyrins[92]. Various molecules have been implicated in enhancing β cell viability and/or regeneration, including glucagon-like peptide 1 (GLP-1) and a longer-lived analog, exendin-4 (Ex-4), and used to augment therapeutic regimes[93-95]. Studies have shown that these molecules can increase the number and size of β cells, and promote β cell neogenesis from pancreatic ductal cells[96]. Further, the combination of Ex-4 and complete Freund's adjuvant, was seen to reverse new-onset diabetes in 86% of NOD mice[96]. Use of potential therapeutics may prevent effects of oxidative stress and minimize proinflammatory cytokines following transplantation, thereby improving function and survival of islet cells and possibly decreasing the number of β cells necessary for transplantation. However, the need for immunosuppression would still exist. Therefore, to make islet grafts a viable option to treat diabetes, these issues must first be addressed.

1.10 Islet grafts and recurrent autoimmune diabetes

As discussed above, autoreactive $CD4^+$ and $CD8^+$ T cells mediate β cell destruction. In islet transplantation, both autoreactive and alloreactive $CD4^+$ and $CD8^+$ T cells play important roles in the development of recurrent diabetes. However, the specificities of graft-infiltrating autoreactive T cells are relatively unknown. Though many studies have examined the TCR repertoire during the initial autoimmune phase, the specificity of T cells involved in autoimmune-mediated islet graft destruction may not mimic that seen in the endogenous

islets. Indeed, the frequency of IGRP-specific $CD8^+$ T cells is 6 times higher in the islet graft compared to the endogenous pancreas at day 7 post-transplantation, averaging 25% of the $CD8^+$ T cell infiltrate by tetramer staining[86]. At day 13, only 5% of islet-infiltrating $CD8^+$ T cells are IGRP-specific, suggesting that a more polyclonal response develops with time. However, since the specificity of the remaining 95% of T cells was not determined in this study, it is not known whether the response becomes more diverse through epitope spread and recruitment of a variety of different β cell-specific T cells, or T cells specific for other antigens dominate the immune response. Unlike the relatively slow pace of islet infiltration and destruction in the primary autoimmune response of NOD mice (13-35 weeks), syngeneic islet graft rejection takes place quickly - typically within 7-13 days of implantation, presumably through the activation of a β cell specific memory T cell population. It is possible that certain T cell clones are recruited from the pancreas to the islet graft to mediate β cell destruction; however, it is also possible that T cells of specificities not observed in the pancreas will be activated by this new source of antigen. As yet, it is not known which T cells are migrating to the graft and causing graft rejection. In order to develop rational strategies for improving the survival of "new" β cells, whether through allogeneic islet transplantation or through regeneration/expansion of HLA-matched β cells, it will be important to define the T cell specificity during this immune reaction.

Interestingly, by expressing diabetogenic (BDC2.5 or NY4.1) and non-diabetogenic (two HEL-specific clones) transgenic TCRs by $CD4^+$ T cells within the same NOD mouse, it was shown that T cell accumulation within the islets was restricted to the diabetogenic population[97].

This suggests that the majority of T cells within diabetic islets should be β cell-specific and not merely accumulating due to a bystander effect, and provides further reason to examine this population in islet grafts more thoroughly. Importantly, identification of β cell epitopes or certain TCR Vβ (and Vα) patterns may help lead to the development or refinement of β cell-specific therapies. Indeed, the use of toxin-coupled IGRP-containing MHC class I tetramers specifically deleted IGRP-specific $CD8^+$ T cells *in vivo* and delayed the onset of diabetes[85].

1.11 Antibody-based Immunotherapy in T1D

One approach to block autoimmune-mediated destruction of islet grafts is to re-establish the "balance" between pathogenic and immunoregulatory T cells. The use of monoclonal antibodies provides an approach to target specific cell populations. Antibody-based therapies to target T cells have been used with success in both mice and humans, though current methods still leave much room for improvement. Many of these therapies have focused on the TCR and co-stimulatory molecules as targets. As mentioned above, activation of T cells requires both TCR-peptide-MHC interactions, as well as co-stimulatory signals. Importantly, co-receptor molecules CD4 and CD8 interact with MHC class I and II molecules, allowing for efficient T cell activation via phosphorylation of Lck. The rationale for using antibodies to target molecules involved in T cell activation is to block complete T cell activation, which in turn has been shown to promote T cell anergy, clonal deletion, or under certain conditions Treg differentiation. For instance, Ethylene carbodiimide (ECDI)-

"fixed" peptide-pulsed APCs, which lack co-stimulatory molecule expression, efficiently induce T cell anergy[98].

One noted success story in the induction of tolerance has been the use of monoclonal antibodies specific for CD3. αCD3 antibody treatment induces remission in 64-80% of new-onset diabetic NOD mice[99]. This approach causes rapid systemic depletion of the majority of T cells by 24 hours post-administration, followed by a gradual re-appearance of T cells over a period of several weeks[100]. TGFβ1 levels are increased at 24 hours post-treatment, which in this system are required for the induction/expansion of FoxP3$^+$Treg. Interestingly, the primary sources of the elevated TGFβ1 are macrophages and immature DC[101]. Here, TGFβ1 is induced in APC following phagocytosis of T cells that have undergone apoptosis following αCD3 antibody binding. Additionally, in a myelin/oligodendrocyte glycoprotein (MOG)-induced model of experimental autoimmune encephalitis (EAE), αCD3 antibody treatment increases the frequency of FoxP3$^+$Treg in the spinal cord, decreases the disease score, and reestablishes tolerance[102]. Finally, αCD3 antibody has also been administered to recent onset T1D patients with some early clinical success[103,104].

It is well established that both CD4$^+$ and CD8$^+$ T cells play essential roles in the development of T1D, and since the 1990s various studies have shown the therapeutic effects of anti-lymphocyte serum and depleting antibodies specific for CD4 and CD8. In transplantation studies, MHC-mismatched skin grafts are protected using non-depleting αCD4 and αCD8 antibodies in combination; either alone, however, is ineffective[105]. One study showed that

using donor antigen under the "cover" of a non-depleting αCD4 antibody treatment induces tolerance to cardiac allografts[106]. Further, non-depleting αCD4 and αCD8 antibodies have been used to induce tolerance to human immunoglobulin (HGG) as well as bone marrow and skin grafts mismatched at multiple transplant antigens[107]. Tolerance to HGG is induced using αCD4 alone, but skin and bone marrow graft tolerance is only achieved with the combination of αCD4 and αCD8 antibodies. Interestingly, HGG tolerance is only maintained through repeated injection of the protein, whereas bone marrow and skin grafts provide a constant source of donor antigen, indicating that antigen persistence likely plays a role in long-term tolerance induced by this strategy[107]. Further studies demonstrated that a short course of αCD4 antibody alone induces tolerance to MHC-mismatched heart allografts, and that this tolerance is allograft-specific. For example, second grafts of donor-matched MHC hearts or skin are accepted, while third-party MHC skin grafts are rejected[108]. Additionally, long term survival of both skin and cardiac grafts has been reported when recipient animals are pretreated with a donor-specific transfusion "under the cover" of αCD4 antibody[106,109,110]. Furthermore studies showed that antibody-mediated co-receptor blockade-induced transplant tolerance is dominant and "infectious," and mediated by $CD4^+$ T cells[111-113]. It was further determined that non-depleting αCD4 antibodies promote conversion of naïve $CD4^+$ T cells to $Foxp3^+$ Treg. For instance, when female TCR transgenic mice specific for the male peptide dead box RNAhelicaseY (DBY) are tolerized to male skin grafts using anti-CD4 antibodies, the previously Treg-deficient recipient mice are found to have $CD4^+CD25^+FoxP3^+$ cells within spleens and skin grafts[114]. Interestingly, tolerance induction to a model antigen, equine immune globulin, was demonstrated in non-human

primates using a non-depleting αCD4 antibody, raising hopes for potential use in the clinic[115]. However, the success of this treatment regime in preventing allograft rejection may involve different mechanisms than those involved in autoimmune rejection of syngeneic islet grafts.

Allogeneic responses are typically directed at MHC molecules, and involve the activation of a naïve population of allogeneic T cells. However, diabetic recipients of islet grafts have established pathogenic β cell-specific effector and memory T cells. Thus, considering that the result of αCD4 and αCD8 non-depleting antibody binding to naïve, memory, effector, or regulatory T cells may be very different, it is difficult to predict the exact nature of the effect on the immune response in autoimmune diabetes. Nevertheless, due to the success of these antibodies in establishing transplantation tolerance in various allograft models, non-depleting αCD4 and αCD8 antibodies may be effective in tolerizing autoreactive T cells in the context of islet transplantation.

1.12 Systemic Adeno-associated Virus (AAV) Immunotherapy in T1D

AAV received its name because it is often found in cells also infected with adenovirus. Unlike adenovirus, however, AAV is nonimmunogenic in mice, can enter non-dividing cells, and either integrates into one specific area on the genome or remains as an episomal plasmid. Recombinant AAV vectors provide a clinically amenable approach for *in vivo* gene delivery[116,117]. Importantly, AAV vectors: i) cannot replicate without a helper virus, ii) stably express transgenes for long periods of time *in vivo*, and iii) infect a wide variety of tissues

depending on the serotype of capsid protein used to package the recombinant[117,118]. Various AAV vector-based therapies have been used in both streptozotocin (STZ)-induced and spontaneous diabetes models. Earlier studies showed the potential of this approach by demonstrating that various AAV serotypes successfully transduce both human and mouse islets. Notably, long-term expression of a green fluorescent protein (GFP) transgene was observed in AAV-GFP vector transduced islets implanted into non-autoimmune STZ-induced diabetic Balb/c mice[119]. AAV vectors have been widely used in diabetes prevention and treatment studies, and transgenes expressing β cell autoantigens, immunoregulatory cytokines and soluble factors have been employed. In NOD mice, an AAV1 vector expressing a GAD500-585 transgene prevents diabetes, possibly due to the induction of GAD65-specific FoxP3^{+}Treg and Th2 cells[120,121]. An AAV2 vector expressing α-1 antitrypsin, a glycoprotein that inhibits neutrophil elastase and proteinase 3, reduces diabetes incidence at 32 weeks from 70% to 30% when injected into NOD mice at 4 weeks of age[122]. Systemic expression of AAV vector encoded heme oxygenase-1, a stress-response enzyme with immunoregulatory capacity, results in a delay in diabetes onset as well as suppression of DC activation and Th1 effector cell reactivity[123]. Additionally, an AAV2 vector expressing IL-10, but not IL-4, prevents diabetes, reduces insulitis, and preserves insulin production in NOD mice[124]. A later paper further demonstrated that diabetes prevention by AAV-IL10 vector treatment is accompanied by an increase in the percentage of CD4^{+}CD25^{+} Treg[125]. Additionally, this effect is dose-dependent, with the highest vector dose ($1x10^{9}$ infectious units (IU)) preventing diabetes in 12 week-old “prediabetic” NOD mice. An additional study showed that systemic expression of AAV-IL10 vector administration prolongs the survival of

syngeneic islet grafts in NOD mice[126]. Though initial success with AAV vectors is exciting, there are drawbacks to this method of gene therapy. AAV vector gene therapy initially employed single-stranded (ss) DNA vectors. The kinetics of transgene expression by ssAAV vectors, however, is limited by the conversion of ssAAV to double stranded (ds) AAV forms. The engineering of dsAAV vectors has markedly enhanced the application of the approach[127,128]. By mutating the inverted terminal repeat, AAV vectors package the self-complementary dsAAV genome, resulting in rapid and more robust transgene expression[127]. Consequently, the dose of dsAAV vector relative to ssAAV recombinants can be reduced to minimize the likelihood of a vector-specific immune response. One drawback to dsAAV vectors, however, is that the size of transgene is limited to ~2.3 kb to ensure efficient packaging of the vector.

1.13 *In vivo*-targeted AAV Immunotherapy in T1D

Systemic and long-term expression of an immunomodulatory molecule may affect the "normal" function of the immune system. AAV vector transgene expression localized in a cell- or tissue-specific manner circumvents this problem. In the case of T1D, one way to target transgene expression to the β cells has been the use of the mouse insulin promoter (MIP). AAV vectors have been engineered in which transgene expression is driven by the MIP, and in turn targeted to β cells[129]. Glucose-dependent transgene expression has been demonstrated in both β cell lines *in vitro* and islets *in vivo* following transduction with AAV vectors containing MIP. Notably, 4 week-old NOD mice given a dsAAV8 vector encoding a

MIP driven IL-4 transgene show a delay in diabetes onset relative to dsAAV8 MIP-GFP control-treated mice and untreated mice[130]. The protective effect is believed to be due to an increased frequency of FoxP3^{+}Treg. Our group has also shown that a dsAAV8 vector encoding a MIP-driven IL-2 transgene prevents diabetes in NOD mice at a late preclinical stage (Johnson, M. and Tisch, R. unpublished data). Though using MIP to target transgene expression to β cells should localize transgene effects to the islets, AAV can still integrate into a variety of cell types, raising the possibility of a virus-specific response. Additionally, high amounts of virus may be needed in order to achieve high transduction rates of β cells, which could be cause for concern in clinical trials

1.14 *Ex vivo*-targeted AAV Immunotherapy in T1D

Ex vivo islet transduction by AAV vectors has several benefits over direct injection into a patient. For one, exposure of non-islet cells to the AAV vector is limited, and transgene expression is directly targeted to the islets. This organ-specific expression should be beneficial in that off-target effects of transgene expression should be minimal. Furthermore, *ex vivo* manipulation of islets requires reduced doses of AAV vector. Early work has shown the potential for *ex vivo* islet transduction prior to islet transplantation. Both AAV2 and AAV5 vectors efficiently transduce both β cells and other islet-resident cell types in vitro, and do not interfere with insulin production upon transplantation into mice[131]. Also, dsAAV vectors have been shown to efficiently transduce both mouse and human islets using recombinants packaged with serotype 2, 6, and 8 capsid proteins[119]. EGFP expression in AAV vector transduced islet grafts is long-term, being detected at least 6

months post-implantation[119]. Though still in early stages, these results show the potential for using AAV vectors to express various immunotherapeutic transgenes in islets.

1.15 The role of IL-2 in T1D

IL-2 is a cytokine with pleotropic effects on T cells, including: stimulation of proliferation, induction of activation-induced cell death, and the generation, expansion, and maintenance of FoxP3$^+$Treg. The IL-2 gene is associated with diabetes susceptibility in NOD mice, being mapped to *idd3* [132,133]. FoxP3$^+$Treg constitutively express CD25, the high-affinity α chain of the IL-2 receptor, and IL-2 has been shown to be important for FoxP3$^+$Treg survival. As discussed earlier the lack of proper FoxP3$^+$Treg function and survival in older NOD mice has been linked to the progression of β cell autoimmunity[43,44]. Also, NOD mice exhibit a defect in IL-2 production, and introgression of the *idd3* locus from C57BL/6 (B6) mice into the NOD genome markedly reduces the frequency of diabetes[32]. Performing transplants into NOD mice congenic for the B6 *idd3* allele or injection of IL-2 in combination with costimulation blockade (e.g. αCD40L) improves islet allograft survival[134], further highlighting the importance of IL-2 in the NOD mouse. A combination of IL-2 and the immunosuppressant sirolimus, which inhibits IL-2-induced T cell proliferation but not apoptosis, prevents diabetes as well as protects syngeneic islet grafts from autoimmune destruction. This occurs through induction of a shift from pathogenic Th1 to Th2 (IL4$^+$IL10$^+$) and Th3 (TGFβ1$^+$)-type cells within the graft[135]. Intramuscular injection of an AAV1 vector encoding IL-2 driven by an inducible tetracycline promoter prevents diabetes in NOD mice at a late preclinical

stage[136]. Protection correlates with an increase in the frequency of FoxP3$^+$Treg residing in the pancreas.

1.16 Our hypothesis and goals

Islet grafts have the potential to greatly improve both the quality and duration of life for T1D patients. Since the success achieved via the Edmonton protocol, though substantial, still entails long-term administration of immunosuppressive drugs, we chose to assess an antibody-based method of tolerance induction for syngeneic islet grafts. αCD4 and αCD8 non-depleting antibodies have shown efficacy in preventing allograft rejection, and may be useful in the protection of islet grafts from autoimmune rejection as well. Additionally, these antibodies may provide a method to selectively tolerize autoreactive T cells while preserving the function of the remainder of nonautoreactive T cells. Accordingly, we tested the ability of αCD4 and αCD8 antibodies alone and in combination to prevent rejection of syngeneic islet grafts in NOD mice. Because it is a possibility that the lack/dysregulation of IL-2 in our model may inhibit the development or expansion of a highly functional population of FoxP3$^+$Treg observed in other models using the non-depleting αCD4 and αCD8 antibodies, we chose to augment our approach with the expression of IL-2 by β cells. Using an AAV8 vector encoding a MIP-driven IL-2 transgene, we examined whether IL-2 expression by β cells could delay or prevent graft rejection both alone and in combination with αCD4 and αCD8 antibody therapy. Lastly, recurrent autoimmunity is less well-characterized than the primary immune response in T1D, so further understanding of the autoimmune response to “neo-β cells” may provide valuable information and insight into

developing methods to protect islet grafts. If the TCR repertoire in the islets is altered in recurrent autoimmunity, knowledge of the TCR specificities may aid in developing more effective immunotherapies. Therefore, we investigated the TCR specificity of various subsets of $CD4^+$ and $CD8^+$ T cells in rejecting syngeneic grafts. We hypothesize that a subset of T cells found in the pancreas, possibly memory cells, migrate to the islet graft, and cause destruction of the β cells. By examining the TCR Vβ specificities of a variety of populations, including $CD4^+$ and $CD8^+$ naïve and effector/memory T cells, insight into the dynamics and specificities of recurrent autoimmunity have been gained.

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CHAPTER 2

BLOCKING AUTOIMMUNE RECOGNITION OF ISLET GRAFTS VIA T CELL CORECEPTOR ANTIBODY BINDING AND ADENO-ASSOCIATED VIRUS VECTOR MEDIATED GENE TRANSFER

2.1 Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin-producing β cells within the pancreas. Islet or pancreas transplantation is currently the only way to "cure" T1D. Though transplantation has the potential to achieve long-term, stable blood glucose control and avoid diabetes-associated health complications, it also requires lifelong systemic immunosuppression. Thus, we assessed the ability of non-depleting anti-(α)CD4 and αCD8 antibodies to prevent recurrent autoimmunity and enhance islet graft survival. Whereas isotype control antibodies provided no protection to syngeneic islet grafts, the use of αCD4 or αCD8 antibody alone, or in combination extended graft survival significantly. Additionally, we assessed the ability of an adeno-associated virus (AAV) vector expressing IL-2 via the mouse insulin promoter (MIP) to prolong islet graft survival. Double-stranded AAV-MIP-IL2 serotype 8 (dsAAV8-MIP-IL2) transduced islet grafts exhibited enhanced survival whereas dsAAV-MIP-IL2 serotype 1 transduced islet grafts did not. We also combined dsAAV8-MIP-IL2 administration with non-depleting αCD4/CD8 treatment, and found that this combination provided islet protection similar to that of αCD4/CD8 treatment alone. These findings demonstrate that: 1) αCD4/CD8 combination treatment extends graft survival to create a window for additional interventions, and 2) recombinant AAV vectors can be readily used to genetically modify β cells *in vitro*, and enhance islet graft survival.

Type 1 Diabetes (T1D) is an autoimmune disease characterized by the T-cell mediated destruction of the insulin-producing β cells present in the islets[1,2]. T1D can be managed with daily insulin injections, but effects of continual blood glucose fluctuations can result in

long-term complications affecting eyes, kidney, limbs and other organs, and resulting in early death[3]. The only way to provide "normal" blood glucose control in T1D patients is with whole pancreas or islet transplantation, which can reduce or eliminate the need for insulin injections[4-6]. However, islet graft protection against both autoreactive and alloreactive T cells is dependent on continuous administration of immunosuppressive drugs. The drugs used systemically affect the immune system, opening the patient up to a host of opportunistic infections. Thus, the benefit of increased blood glucose control is typically outweighed by the detriment of lifelong systemic immunosuppression required to prevent graft rejection. Consequently there is a need for immunotherapies that selectively block autoimmune and allogeneic recognition of islet grafts while permitting normal immune function. Optimally, an immunotherapy would target pathogenic effector T cells and/or increase β cell-specific regulatory T cells (Treg) while maintaining normal immune function.

$CD4^+$ and $CD8^+$ T cells play important roles in the development of T1D. For instance, nonobese diabetic (NOD) mice, a spontaneous model of T1D, remain diabetes-free when $CD8^+$ T cells are depleted[7]. Additionally, major histocompatibility complex (MHC) class I is required for β cell destruction. In β2 microglobulin-deficient NOD mice, $CD4^+$ T cells fail to infiltrate the islet and diabetes onset does not occur[8-11]. Furthermore, NOD mice lacking expression of MHC class II and therefore devoid of $CD4^+$ T cells also remain protected from diabetes[12]. Only in certain transgenic NOD mouse models involving β cell-specific T cell receptors (TCRs) are $CD4^+$ or $CD8^+$ T cells alone capable of causing, transferring, or accelerating T1D, often in young or immunocompromised NOD mice[13-15]. As is the case with spontaneous diabetes in NOD mice, both $CD4^+$ and $CD8^+$ T cells play key roles in

autoimmune-mediated destruction of islet grafts. Wang *et al.* found that islet destruction was dependent on $CD4^+$ but not $CD8^+$ effector T cells[16]. However, others found that in the absence of MHC class I expression, syngeneic islet grafts were protected from rejection[17,18]. This would indicate that $CD8^+$ T cells are also involved in mediating islet destruction. In addition, β cell destruction is associated with an accumulation of both $CD4^+$ and $CD8^+$ T cells capable of secreting IFNγ within the newly-engrafted islets[19]. Taken together, these results demonstrate that both $CD4^+$ and $CD8^+$ T cells likely play important, though not fully delineated, roles in syngeneic islet graft rejection.

A number of approaches have been studied to target pathogenic T cells in autoimmune and/or allograft settings. The most straightforward has been the administration of antibodies specific for various proteins expressed by T cells, such as the CD3 molecule. While there has been some success in achieving long-term remission in NOD mice[20,21], clinical trial results in humans have been less striking. While initial studies administering αCD3 antibody showed some improvement in β cell function for up to two years[22,23], more recent studies have shown that the protective effect is limited in the majority of patients, and that a second dose of αCD3 antibody and/or higher dosage treatments can be detrimental rather than extend protection[24-26]. Additionally, the majority of these antibodies deplete T cells resulting in immunosuppression, and often result in no long-term tissue-specific tolerance.

On the other hand, non-depleting antibodies specific for the CD4 and CD8 co-receptor molecules have proven to be highly effective at inducing tissue/antigen-specific tolerance without systemic depletion of T cells[27]. The best characterized of these

nondepleting αCD4 and αCD8 antibodies are YTS177 and YTS105, respectively. YTS177 and YTS105 are rat IgG2a antibodies which are much less efficient at binding murine Fc receptors and fixing murine complement than other isotypes (i.e. rat IgG2b) [28]. Importantly, these two monoclonal antibodies have not been observed to deplete murine T cells *in vivo* upon binding in transplant models[29-31]. YTS177 and YTS105 have been successfully used to induce long-term tolerance in murine skin and cardiac allograft models[29-33]. Protection established by YTS177.9 and YTS105.18 is in part due to induction of alloantigen-specific FoxP3-expressing Treg (FoxP3$^+$Treg) [33-35]. Our group has also recently found that YTS177 and YTS105 induce long-term remission in recent-onset diabetic NOD mice ([36] and Yi and Tisch, unpublished).

It is well established that IL-2 is a critical growth factor for T cells, and is necessary to drive T cell-mediated proinflammatory responses. Because of its essential role in regulating multiple aspects of immune responses, IL-2 is tightly regulated[37]. Notably, IL-2 has also been shown to play a key role in peripheral maintenance of FoxP3-expressing Treg (FoxP3$^+$Treg)[38,39]. FoxP3$^+$Treg constitutively express the high affinity alpha chain (CD25) of the IL-2 receptor, and can act as a "sink" for IL-2 to downregulate pathogenic effector responses. In combination with TGFβ, IL-2 is necessary to up-regulate FoxP3-expression and convert naïve "conventional" CD4$^+$ T cells into "adaptive" FoxP3$^+$Treg. Notably, dysregulation of IL-2 expression has been shown to contribute to T1D in NOD mice, leading to a decrease in the function and frequency of FoxP3$^+$Treg[40,41]. The IL-2/CD25 pathway has also been linked to T1D in humans[41]. Interestingly, several recent studies have shown that IL-2 can be therapeutic in treating T1D in NOD mice. Low-dose IL-2 promotes survival of

FoxP3$^+$Treg in the islets, and prevents diabetes onset[40]. Additionally, a brief pulse of systemic IL-2 delivered by intra-muscular injection of an adeno-associated virus (AAV) vector prevents diabetes in NOD mice via an increase in islet-resident FoxP3$^+$Treg [42]. Short-term IL-2 administration at diabetes onset can also induce remission in diabetic NOD mice and result in long-lasting protection through modification of pancreatic FoxP3$^+$Treg[43]. The above approaches use systemic IL-2 as a therapeutic, which, though effective in these cases, might become problematic when applied to humans. Indeed, high dose IL-2 therapy is also used for treatment of various cancers, and treatment of mice and human patients with high-dose IL-2 can expand effector cells and result in an anti-tumor response[44-46]. Thus, a targeted approach for administration of IL-2 may make clinical application of this therapeutic more amenable. Since T1D is associated specifically with pancreatic Treg dysfunction[40], targeting IL-2 transgene expression to the pancreas should not only promote Treg induction/expansion, but also decrease the possibility of adverse effects associated with systemic IL-2 production.

Since non-depleting YTS177 and YTS105 antibodies have been shown to prevent rejection of allogeneic grafts in a variety of model systems, we investigated whether this approach also induced tolerance to syngeneic islet grafts in diabetic NOD female mice. We hypothesized that the combination of αCD4 and αCD8 antibody treatment would result in syngeneic islet graft protection through a combination of FoxP3$^+$Treg induction/expansion, as well as blocking the function of pathogenic CD4$^+$ and CD8$^+$ T cells. Additionally, we assessed the ability of an AAV vector encoding an IL-2 transgene driven by the mouse insulin promoter (MIP) (dsAAV8-MIP-IL2) to protect islet grafts. Since IL-2 can promote

FoxP3^{+}Treg survival and protect mice from diabetes onset, we hypothesized that production of IL-2 in the microenvironment of the islet graft would promote local (and potentially β cell-specific) FoxP3^{+}Treg survival and thus extend graft survival. Finally, we assessed whether the combination of dsAAV8-MIP-IL2 and YTS177 and YTS105 antibody treatment enhances protection of islet grafts.

2.2 Results

Non-depleting αCD4 (YTS177) and αCD8 (YTS105) antibodies enhance syngeneic islet graft survival.

We assessed the capacity of YTS177 and YTS105 to block autoimmune-mediated destruction of syngeneic islet grafts. Diabetic NOD female mice (e.g. blood glucose levels >250 mg/ml) were used as islet graft recipients. These animals received 500 NOD.*scid* islets under the kidney capsule. Transplantation was carried out after 2-3 weeks of diabetes onset to ensure that only minimal endogenous β cell mass was present. For instance, we have found that administration of YTS177 and YTS105 within 10 days of diabetes onset reverses diabetes. To make certain that the isolated islets lacked infiltrating lymphocytes, NOD.*scid* mice were used as syngeneic donors. Graft recipient mice received intraperitoneal (i.p.) injections of 600 ug of YTS177 and YTS105 either alone or in combination, or 2A3, an isotype control antibody, on days 0, 2, and 4 post-graft implantation. Administration of 2A3 resulted in a minor delay in the onset of recurrent diabetes relative to untreated NOD recipients; islet graft median survival time was extended from 10.5 days to 14.5 days ($p<0.01$) (Figure 2.1). In contrast, YTS105 or YTS177 alone significantly delayed the onset of recurrent diabetes to a similar extent (Figure 2.1). Furthermore, as expected the combination of YTS105 and YTS177 delayed the onset of recurrent diabetes; however efficacy was not significantly different compared to that seen with either antibody alone (Figure 2.1). These results demonstrate that administration of YTS105 and YTS177 alone enhances syngeneic islet graft survival in diabetic NOD recipients, and that co-injection of the αCD4 and αCD8 antibodies does not markedly enhance efficacy.

Rejected islet grafts from YTS177 and YTS105-treated NOD recipients have an increased frequency of graft infiltrating FoxP3$^+$Treg.

The development of recurrent diabetes in YTS177 and YTS105-treated NOD recipients could be attributed to autoimmune-mediated destruction of the islet graft or a failure of islet grafts to secrete adequate levels of insulin. To distinguish between these two possibilities, islet grafts were harvested from YTS177 and YTS105-treated NOD recipients at the time of recurrent diabetes onset, and immunofluorescence analysis carried out. Islet grafts exhibited minimal insulin staining consistent with graft failure (Figure 2.2A). In addition, islet grafts were heavily infiltrated with Thy1.2$^+$CD4$^+$ and Thy1.2$^+$CD4$^-$ (e.g. CD8$^+$) T cells (Figure 2.2A). This result demonstrates that recurrent diabetes detected in YTS177 and YTS105-treated NOD recipients is likely due to autoimmune-mediated islet graft destruction.

Flow cytometric analyses were carried out to characterize the nature of the T cell infiltrates found in the rejected islet grafts from YTS177 and YTS105- and 2A3-treated NOD recipients. A moderate but significant increase in the percentage of FoxP3$^+$CD4$^+$ T cells was detected in islet grafts from NOD recipients treated with YTS177 and YTS105 (18.5$\pm$ 1.9) versus 2A3 (12$\pm$1.7) (Figure 2.2B). On the other hand, the average frequency of CD4$^+$ and CD8$^+$ T cells infiltrating the islet grafts of YTS177 and YTS105-treated recipients was similar to that of the control group. Furthermore, an equivalent frequency of islet graft infiltrating, IFNγ-secreting CD8$^+$ and CD4$^+$ T cells was observed for the respective groups (Figure 2.2C), and no marked difference was detected in the effector T cell to FoxP3$^+$Treg ratio in the

grafts from NOD recipients treated with YTS177 and YTS105, and 2A3 (Figure 2.2D). Finally, no differences in FoxP3$^+$Treg and CD4$^+$ and CD8$^+$ T effectors were detected in the renal lymph nodes (RLN) draining the islet graft or the nondraining RLN of the respective two groups of NOD recipients (Figure 2.2). These results indicate that the delayed onset of recurrent diabetes observed in YTS177 and YTS105-treated NOD recipients correlates with an increase in FoxP3$^+$Treg infiltrating the islet grafts relative to the control group, even after graft failure.

Characterization of T cells infiltrating the islet grafts shortly after YTS177 and YTS105 treatment.

Since the above islet grafts were examined after failure, protection mediated by YTS177 and YTS105 treatment may have been obscured by effector T cell responses. Therefore islet grafts were examined at 14 days post-implantation, a time point when all YTS177 and YTS105-treated NOD recipients remained free of recurrent diabetes, and islet grafts were in the process of being rejected in the 2A3-treated control group. No significant difference in the frequency of IFNγ-secreting CD4$^+$ and CD8$^+$ T effectors (Figure 2.3B) was seen in the infiltrating grafts or draining RLN of the two respective groups. In contrast to observations made in the rejected grafts, a trend towards a reduced frequency of FoxP3$^+$CD4$^+$ T cells was seen in the islet grafts of NOD recipients treated with YTS177 and YTS105 versus 2A3 (Figure 2.3A). Furthermore, the ratio between effector T cells and FoxP3$^+$Treg was increased in YTS177 and YTS105-treated NOD recipients, with the difference between effector CD4$^+$ T cells and FoxP3$^+$Treg reaching statistical significance (Figure 2.3C). No significant differences

were detected in the RLN of YTS177 and YTS105- and 2A3-treated recipients for the different T cell subsets (Figure 2.3). These results suggest that early islet graft infiltration by $CD4^+$ and $CD8^+$ T cells able to secrete IFNγ following stimulation is not significantly affected by YTS177 and YTS105 treatment, although trafficking of $FoxP3^+$Treg is delayed.

Ectopic expression of IL-2 by β cells prolongs syngeneic islet graft survival.

Our laboratory has reported that β cell autoimmunity is effectively suppressed in NOD mice at a late preclinical stage of T1D by an intramuscular injection of AAV vector encoding IL-2[42]. With this in mind, we assessed the therapeutic efficacy of targeting IL-2 expression to β cells in islet grafts using dsAAV-MIP-IL2. Notably, IL-2 expression is driven by MIP to selectively direct expression to β cells. Islet specificity of the AAV-MIP vector was confirmed in the laboratory. Since untreated syngeneic islet grafts are typically rejected within 10-14 days (Figure 2.1), rapid transgene expression is critical for prolonging islet graft survival. With this in mind, we employed dsAAV vectors which exhibit earlier and more robust transgene expression than conventional single stranded (ss) AAV vectors.

Initially, efforts focused on establishing the optimal conditions to transduce islets *in vitro* with dsAAV vectors. Here a dsAAV-MIP vector encoding enhanced green fluorescent protein (GFP) and packaged with serotype 8 capsid was utilized. Islets isolated from NOD.*scid* donor mice were incubated with $1x10^{11}$ viral particles (v.p.) of dsAAV8-MIP-GFP, and GFP expression examined via immunofluorescence microscopy over time. Robust GFP expression in islets was readily detected within 3 days of transduction (Figure 2.4A, B). To determine the time of incubation needed for efficient transduction, islets were cultured

with various viral doses. dsAAV8-MIP-GFP was added to islets, washed off with PBS after 1, 12, or 96 hours, and islets were returned to culture. GFP expression was assessed after 4 days. Strikingly, GFP expression was detected in islet cultures transduced with dsAAV8-MIP-GFP for only 1 hour (Figure 2.4C). This is not unexpected, as a report by Bartlett *et al.* showed that AAV particles enter the cells through receptor-mediated endocytosis within 30 minutes postinfection[47]. As expected, increased GFP expression was detected with longer transduction times although the effect was only moderate (Figure 2.4C, D). For instance, an approximate 3-fold increase in fluorescence was seen in islets incubated with dsAAV8-MIP-GFP for 96 versus 1 hour(s) (Figure 2.4D).

After optimizing *in vitro* transduction conditions using dsAAV8-MIP-GFP, the function of dsAAV8-MIP-IL2 was tested. Vector dose-dependent IL-2 secretion was detected for islets transduced *in vitro* with dsAAV8-MIP-IL2 (Figure 2.4E). Next, NOD.*scid* islets were transduced with two different doses of dsAAV8-MIP-IL2 v.p. for 2 hours, and then implanted under the kidney capsule of diabetic NOD.*scid* recipients, which had been previously treated with streptozotocin (STZ) to destroy endogenous β cells. Euglycemia was achieved in all of the NOD.*scid* recipients after islet transplantation, indicating that transduction with dsAAV8-MIP-IL2 had no adverse effects on β cell function. Islet grafts were harvested at day 10 post-implantation, cultured for 2 days, and IL-2 secretion measured via ELISA. As demonstrated in Figure 2.4F, IL-2 secretion by transduced islets was readily detected. Furthermore, levels of IL-2 expression correlated with the dose of dsAAV8-MIP-IL2 used to transduce the islets (Figure 2.4F). These results demonstrate that β cells transduced with dsAAV8-MIP-IL2 are functional *in vivo* and express IL-2.

***In vitro* transduction of islets with dsAAV8-MIP-IL2 extends graft survival.**

We then tested whether dsAAV8-MIP-IL2 enhanced islet graft survival in diabetic NOD female mice. Included in the study was dsAAV-MIP-IL2 packaged with serotype 1 capsid. AAV1 vector has been reported to transduce islets *in vitro* with increased efficiency compared to AAV8 vector[48]. Indeed, we detected a 5-fold increase in the level of IL-2 secretion by β cells transduced *in vitro* with an equivalent number of v.p. of dsAAV1-MIP-IL2 versus dsAAV8-MIP-IL2 on day 7 in culture (data not shown). NOD.*scid* islets were transduced *in vitro* with $1.5x10^{10}$ v.p. of dsAAV1-MIP-IL2 or dsAAV8-MIP-IL2, implanted into diabetic NOD mice, and blood glucose levels monitored. Unmanipulated islets were transplanted into NOD diabetic mice as a control. Recipients of the dsAAV8-MIP-IL2 transduced islets exhibited a significant delay in the onset of recurrent diabetes (median survival of 23 days) relative to the control group (median survival of 10.5 days; Figure 2.5). In contrast, there was no significant difference in the time of onset or frequency of recurrent diabetes in NOD mice receiving islets transduced with dsAAV1-MIP-IL2 (Figure 2.5). These findings indicate that ectopic expression of IL-2 by β cells following dsAAV8-MIP-IL2 transduction enhances islet graft survival.

Co-administration of dsAAV8-MIP-IL2 does not increase islet graft survival above αCD4 and αCD8 antibody treatment alone.

Although YTS177 and YTS105 treatment of NOD recipients, and dsAAV8-MIP-IL2 transduction of β cells *in vitro* increased islet graft survival, long-term protection (e.g. >100 days) was not achieved by either approach. Therefore, whether the combination of the two

approaches would have an additive or synergistic effect on islet graft survival was tested. Diabetic NOD female mice received 500 NOD.*scid* islets that were transduced *in vitro* with $1.5x10^{10}$ v.p. dsAAV8-MIP-IL2 or mock transduced. Islet graft recipients were then treated with 600 ug of YTS177 and YTS105 or 2A3 on days 0, 2, and 4 post-graft implantation. As expected, the onset of recurrent diabetes was markedly delayed in NOD recipients treated with YTS177 and YTS105 and receiving dsAAV8-MIP-IL2 transduced islets relative to the control group (e.g. NOD mice treated with 2A3 and receiving unmanipulated islets) (Figure 2.6). However, no significant difference in the time of recurrent diabetes onset was observed between the YTS177 and YTS105 treatment group versus the group of NOD mice receiving the combinatorial YTS177 and YTS105 and dsAAV8-MIP-IL2 therapy (Figure 2.6). Consistent with results from earlier islet graft experiments (Figure 2.2), the frequency of $FoxP3^+$Treg was increased in rejected grafts from NOD recipients treated with YTS177 and YTS105 alone or receiving YTS177 and YTS105 plus AAV8-MIP-IL2 transduced islets relative to the control group (Figure 2.7A). However, the frequency of islet graft infiltrating $FoxP3^+$Treg between the two respective treatment groups was not significantly different (Figure 2.7A). No significant difference in the frequency of IFNγ-secreting $CD4^+$ and $CD8^+$ T effectors (Figure 2.7B) was seen in the infiltrating grafts or draining RLN of the two respective groups. Furthermore, the ratio between effector T cells and $FoxP3^+$Treg showed a trend towards an increase in 2A3 (isotype)-treated NOD recipients in comparison to the two treated groups, likely because isotype animals had a lower percentage of $FoxP3^+$Treg in the grafts. (Figure 2.7C). These results demonstrate that despite the capacity of YTS177 and

YTS105 and ectopic expression of IL-2 by β cells to increase islet graft survival individually, the combination of the two approaches does not further enhance protection.

2.3 Discussion

Lifelong immunosuppression is necessary to prevent islet graft rejection in T1D patients. Hence, there is an urgent need for immunotherapies that selectively block autoimmune (and allogeneic) recognition of islet grafts while allowing normal immune function. The ideal immunotherapy would selectively tolerize pathogenic effector T cells, and concomitantly enhance the β cell specific Treg pool. Since non-depleting YTS177 and YTS105 treatment prevents rejection of allogeneic grafts in an alloantigen-specific manner, we investigated whether this approach also induced T cell tolerance to syngeneic islet grafts in diabetic NOD female mice. YTS177 and YTS105 treatment resulted in a significant delay in autoimmune-mediated rejection of islet grafts, which in turn correlated with an increased frequency of FoxP3^{+}Treg found in the grafts relative to 2A3 treated recipients. Additionally, transduction of islets with dsAAV8-MIP-IL2 alone delayed islet graft rejection. Surprisingly, the combination of dsAAV8-MIP-IL2 and YTS177 and YTS105 treatment failed to improve islet graft survival beyond what was observed for YTS177 and YTS105 alone.

Autoimmune-mediated destruction of the islet grafts was delayed by administration of either YTS177 or YTS105, and efficacy was not significantly improved by co-administering the αCD4 and αCD8 antibodies under the conditions employed (Figure 2.1). These results further implicate that similar to the autoimmune attack on endogenous β cells, both β cell-specific CD4^{+} and CD8^{+} T cells are required for efficient destruction of islet grafts. Suppressing the diabetogenicity of one T cell subset alone is sufficient to effectively delay islet graft rejection with a similar efficacy seen when targeting both CD4^{+} and CD8^{+} subsets.

An increased frequency of FoxP3$^+$Treg was detected in graft infiltrates at the time of rejection, but not at day 14 post-islet implantation in YTS177 and YTS105-treated NOD recipients (Figures 2.2, 2.3). This observation suggests that while FoxP3$^+$Treg may contribute to protection at later stages of graft infiltration, protection seen 14 days post-islet graft implantation is likely independent of FoxP3$^+$Treg. Accordingly, early events in islet graft protection may be attributed to direct suppression of established effector and/or memory T cells by YTS177 and YTS105 binding. Numerous reports have shown that antibody-mediated CD4 and CD8 blockade induces a hypo-responsive phenotype in naïve T cells by interfering: i) with TCR-MHC interactions and/or ii) co-receptor molecule-mediated activation of the src kinase lck that is required for efficient T cell activation[49]. Notably, Phillips *et al.* demonstrated that effector CD4$^+$ T cells are also suppressed upon YTS177 treatment *in vivo*[49]. YTS177 binding was found to reduce proliferation and IFNγ production by the effector CD4$^+$ T cells. In contrast to this study, we found that upon stimulation, the frequency of graft infiltrating IFNγ-secreting CD4$^+$ and CD8$^+$ T cells was similar between recipients treated with YTS177 and YTS105, and 2A3 by 14 days post-implantation (Figure 2.3). This data would suggest that the activity of effector T cells within the graft was minimally influenced by YTS177 and YTS105 binding. An important caveat with this interpretation, however, is that graft-infiltrating T cells were given a strong *in vitro* stimulus, namely PMA/ionomycin, to detect intracellular IFNγ. PMA/ionomycin stimulation may "over-ride" the tolerogenic effect of YTS177 and YTS105 binding. Consequently these results may not accurately reflect the *in vivo* status of graft-infiltrating T cells. In addition, CD8$^+$ T cells may express IFNγ but still lack cytolytic activity, as other molecules and pathways

including Fas/FasL, perforin, and granzymes are involved in $CD8^+$ T cell-mediated destruction of beta cells[50]. This, in turn would be expected to delay β cell destruction in the islet grafts. Early islet graft protection by YTS177 and YTS105 may also be due to a reduced efficiency in trafficking of β cell-specific effector and memory T cells into the graft. Studies have shown that the trafficking properties of T cells are altered after crosslinking of CD4 by antibody or natural ligands such as HIV gp120, in the absence of TCR crosslinking[51,52]. The latter is attributed to membrane reorganization of adhesion molecules and chemokine receptors, in addition to desensitization of chemokine receptor signaling. Although similar frequencies were observed, a reduced number of effector and memory T cells due to inefficient trafficking would be expected to delay islet graft destruction in YTS177 and YTS105 treated recipients. Unfortunately, an assessment of the number of islet graft-infiltrating T cells has proven to be problematic. The number of T cells obtained from islet grafts has been highly variable among individual mice, in part due to the amount of grafted tissue that can be harvested. So why is the inhibitory effect of YTS177 and YTS105 transient? Under the conditions used in our study, YTS177 and YTS105 binding of T cells persists *in vivo* for approximately 30 days. We propose that once YTS177 and YTS105 are cleared *in vivo,* the inhibitory effect of the antibodies is alleviated thereby permitting effector and memory $CD4^+$ and $CD8^+$ T cells to mediate islet graft destruction.

Waldmann and others have shown that in various allograft models YTS177 treatment results in an increase in alloantigen-specific $FoxP3^+$Treg[33-35]. In addition, *in vitro* experiments have shown that YTS177 binding to naïve conventional T cells in the presence of antigen and TGFβ1 significantly enhances up-regulation of FoxP3 expression[34]. Albeit

modest, an increased frequency of FoxP3$^+$Treg was in fact observed in failed grafts of NOD recipients treated with YTS177 and YTS105 versus 2A3 (Figures 2.2B, 2.7A). The increase in FoxP3$^+$Treg was insufficient to mediate long-term protection, but may still contribute to the delay in islet graft rejection by regulating the expansion and/or activity of pathogenic T effectors in the graft. Accordingly, additional injections of YTS177 and YTS105 over time may "hold in check" the pool of pathogenic effector and memory T cells and permit induction and/or expansion of sufficient numbers of FoxP3$^+$Treg needed to mediate long-term islet graft survival. It is worthwhile to note that in the few NOD recipients remaining free of recurrent diabetes (Figure 2.1), increased FoxP3$^+$Treg were detected in the grafts in these recipients, further suggesting a protective role for FoxP3$^+$Treg. Our data also indicates that the graft itself is the critical site for the protective events induced by YTS177 and YTS105. Increased FoxP3$^+$Treg were found in the islet graft but not the draining RLN in YTS177 and YTS105 treated NOD recipients (Figures 2.2B, 2.7A). Similarly, no difference in the frequency of IFNγ$^+$ effector T cells was seen between the RLN draining the islet graft and the nondraining RLN, which reflects the T cell *milieu* under steady conditions (Figures 2.2, 2.7). The draining RLN would be expected to be a key site for activation, expansion and/or differentiation of effector T cells and FoxP3$^+$Treg. Our results, however, suggest that the majority of islet infiltrating effector T cells and FoxP3$^+$Treg are "recruited" from pools previously established during the autoimmune attack on endogenous β cells.

Ectopic expression of IL-2 by β cells has the potential to create a microenvironment within the graft to promote FoxP3$^+$Treg survival and expansion, and enhance graft survival. Expression of IL-2 limited to the islet graft would also minimize nonspecific activation of T

and natural killer cells for instance, typically associated with elevated systemic levels of IL-2. Islet graft survival was significantly increased following *in vitro* transduction of islets with dsAAV8-MIP-IL2 (Figure 2.5). Notably, serum levels of IL-2 were not increased in the NOD recipients (data not shown) indicating that ectopic IL-2 production was indeed limited to the islet graft site. Currently, it is not clear whether the level of IL-2 expressed by transduced β cells is optimal for effective graft protection *in vivo*. For instance, an increased dose of dsAAV8-MIP-IL2 and in turn elevated levels of ectopic IL-2 expression may be needed to expand the appropriate number of graft infiltrating FoxP3^{+}Treg for efficient protection. Increased levels of IL-2 in the graft may also elicit efficient activated induced cell death (AICD) in islet graft infiltrating effector T cells. It is well established that IL-2 is a potent inducer of apoptosis in replicating T cells. A reduced effector T cell pool due to AICD would allow for fewer FoxP3^{+}Treg to mediate efficient suppression and long-term islet graft survival. An alternative possibility is that the level of IL-2 produced by transduced β cells is too high and permits expansion of effector T cells that over time leads to islet graft destruction. This scenario is suggested by results obtained with islets transduced with dsAAV1-MIP-IL2. Islet graft survival was markedly reduced following transduction with the same dose of dsAAV1-MIP-IL2 versus dsAAV8-MIP-IL2 (Figure 2.5), despite a 5-fold increase in IL-2 secretion *in vitro* by dsAAV1-MIP-IL2-transduced islets. The rejected islet grafts transduced with dsAAV1-MIP-IL2 were infiltrated, ruling out the possibility that the level of ectopic IL-2 expression was cytotoxic to the β cells. For instance, β cells are highly sensitive to stress resulting from high levels of protein expression and/or inappropriate protein folding. Additionally, in dsAAV1-MIP-IL-2-treated mice, significantly higher percentages of

$CD4^+$ and $CD8^+$ T cells secreted IFNγ when stimulated in vitro as compared to both isotype controls and dsAAV8-MIP-IL-2 (data not shown). This could be explained by higher levels of IL-2 causing the preferential expansion of effector T cells. Alternatively, AAV serotype 1 is more immunogenic than AAV serotype 8 due to its ability to transduce dendritic cells, which may play a role in mounting an immune response to the virus[53]. Constitutive expression of the high affinity IL-2 receptor provides $FoxP3^+$Treg an advantage over naïve and effector T cells when IL-2 levels are limiting. Therefore reduced IL-2 expression by β cells may in fact favor $FoxP3^+$Treg expansion and survival in the islet grafts. Further work is required to determine the optimal dose of dsAAV-MIP-IL2 for transduction in order to achieve the appropriate expansion of $FoxP3^+$Treg and reduction of effector T cells in the graft site.

Surprisingly, YTS177 and YTS105 treatment coupled with islet grafts transduced with dsAAV8-MIP-IL2 failed to significantly enhance islet graft survival relative to that seen with YTS177 and YTS105 injection alone. This result may be due to the effects of YTS177 binding on $FoxP3^+$Treg. Preliminary data suggests that YTS177 binding inhibits *in vivo* proliferation of $FoxP3^+$Treg elicited by ectopic IL-2 expression by endogenous β cells (Johnson and Tisch, unpublished data). Therefore, similar to naïve T cells, antibody binding of CD4 may induce a hypo-responsive phenotype in $FoxP3^+$Treg, and therefore "neutralize" the effect of ectopic IL-2 expression on $FoxP3^+$Treg in the graft site. This scenario may also explain why the frequency of $FoxP3^+$Treg was reduced in islet grafts of YTS177 and YTS105-treated NOD recipients 14 days post-implantation (Figure 2.3). This finding also suggests that molecules targeting cell types other than T cells (e.g. APC) may prove to be more effective in synergizing with YTS177 and YTS105.

In conclusion, we have shown that YTS177 and YTS105 treatment and AAV8-MIP-IL-2 transduction of β cells *in vitro* extend protection of syngeneic islet grafts in diabetic NOD mice. However, neither approach provided long-term protection, either alone or in combination. Nevertheless, further examination of mechanisms associated with protection induced by the respective approaches and better definition of treatment parameters may lead to improved efficacy required to effectively block autoimmune- and possibly allogeneic-mediated destruction of islet grafts.

2.4 Materials and Methods

Mice

NOD/LtJ and NOD.CB17.Prkdc*scid*/J (NOD.*scid*) mice were bred and housed under pathogen-free conditions in an American Association for Laboratory-accredited animal facility. NOD mice were considered to be diabetic after two successive days of ≥250mg/dl blood glucose as measured by a Freestyle *Lite* blood glucose monitor and strips (Abbott Diabetes Care Inc.). All procedures were reviewed and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Flow cytometry and Abs

Single cell suspensions were prepared from the RLN and islet grafts, and filtered with a 70-mM strainer (Fisher Scientific). PBL to examine peripheral FoxP3 T cell levels after IL-2 grafts were acquired via submandibular puncture with lancets (Golden Rod). PBL were RBC-lysed with TAC buffer. Total cells were stained with a panel of fluorochrome-conjugated monoclonal antibodies including: αCD3 (2C11), αCD4 (L3T4), αCD8 (Ly-2), αCD25 (PC61.5), αCD44 (IM7), αCD62L (MEL14), and αFoxP3 (FJK.16 kit) (eBioscience). Data were acquired on a Cyan flow cytometer (DakoCytomation), and analyzed using Summit software (DakoCytomation).

Intracellular cytokine staining was performed on single cell suspensions of RLN and islet grafts. Briefly, lymphocytes were stimulated with 10 ng/mL PMA (Sigma-Aldrich) and 150 ng/mL ionomycin (Sigma-Aldrich) in complete RPMI 1640 medium for 5 h at 37^0C. 10

mg/mL of Brefeldin A (Sigma-Aldrich) was also added for the 5 hour incubation. Cells were stained for surface molecules, fixed and permeabilized with cytokfix/cytoperm reagents (BD Biosciences), and stained for intracellular IFNγ (XMG1.2) (eBioscience).

Pancreatic islet isolation and islet culture

Pancreases were perfused with 0.2mg/ml Collagenase P (Roche) and digested for 20 minutes at 37^0C. Islets were purified via Ficoll (Sigma-Aldrich) gradient and handpicked. For transplantation, islets were washed twice with PBS, and collected in Silastic laboratory tubing (Dow Corning) for implantation. For culture, islets were washed twice with PBS in an eppendorf tube. Excess liquid was removed, and $1.5x10^{10}$ v.p. in PBS were added directly to the islets. After 5 minutes, 175ul RPMI (RPMI 1640 medium (Gibco) containing 10% heat-inactivated FBS, 100 U/mL penicillin/streptomycin (Gibco), and 50 mM 2-ME (Sigma-Aldrich)) was added to the islets. Islets were cultured at 37^0C, and RPMI was added in a stepwise fashion – 250ul added after 30 min, and 500 ul added at 1 hour. Islets were cultured for a total of 2 hours. Islets were then transferred to an eppendorf tube using a P1000 pipette tip, and washed gently twice with PBS. For further culture, islets were added to low-cluster culture plates. For transplantation, islets were then gently pulled into laboratory tubing as above.

Islet transplantation

Diabetic NOD female mice received 5 units of insulin daily prior to transplantation. Five hundred syngeneic (NOD.*scid*) islets were transplanted under the renal capsule of the left

kidney. Blood glucose values were monitored daily, biweekly, or weekly post-transplantation.

YTS 105.18 (αCD8) and YTS 177.9 (αCD4) purification

YTS105.18 and YTS177.9 were produced as ascites in nude mice. The antibody was then purified by negative selection using Melon Gel IgG purification kit (ThermoScientific). Antibody yield was quantified using an αrat IgG ELISA. A rat IgG2a antibody (2A3, BioXCell) was used as an isotype control.

Immunotherapy in NOD islet graft recipients

Mice were given intraperitonael injections of 600 ug purified YTS105 and 600 ug purified YTS177 on day 0, 2, and 4 following transplantation. Transplants were performed only after two successive blood glucose readings of over 500 ug/dl were observed in the diabetic mice. This typically occurred over two weeks after the onset of diabetes. Mice were maintained on 5 units of insulin daily prior to transplantation.

AAV vector packaging

To package dsAAV vector, HEK 293 cells were transfected via calcium phosphate with adeno-helper-encoding plasmid DNA (pXX6-80), AAV8 or 1-encoding plasmid DNA (pXR-1), and the transgene encoding plasmid DNA. Nuclear fractions were harvested and virus

purified with an iodixonal (Sigma-Aldrich) gradient. The virus-containing fractions and titer were determined by Southern dot blot.

ELISA

Serum was collected, diluted 1:2 in RPMI with 10% FBS, and levels of IL-2 at varying times post-transplantation were measured. The anti–IL-2 Ab set (JES6-1 and JES6-5; eBioscience) was used at 2 μg/ml on a high-binding ELISA plate (Costar).

Statistical analyses

Data were analyzed using Prism 4.0 (GraphPad, San Diego, CA). Where appropriate, data were evaluated via Student's paired t test, one way ANOVA, or two way ANOVA. The Log-rank (Mantel-Cox) Test was used to determine the significance in difference in diabetes incidence between treated mice groups. In all analyses, the significance level was 0.05.

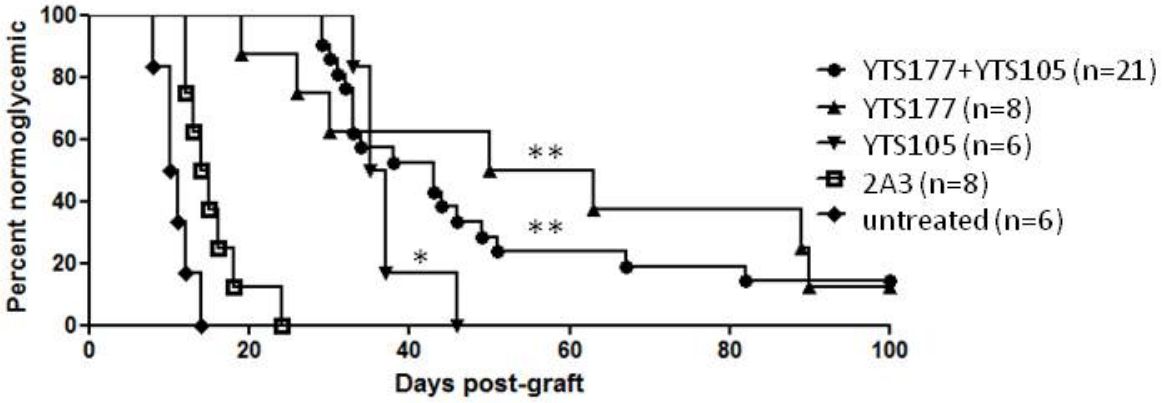

Figure 2.1. Nondepleting YTS177 and YTS105 protect syngeneic islet grafts
Diabetic NOD mice were transplanted with 500 NOD.*scid* islets on day 0. Mice were treated with 600ug of YTS177, YTS105, and isotype antibody (2A3) alone or 600ug of both YTS177 and YTS105 on days 0, 2, and 4 post-implantation. *p<0.0005, **p<0.0001, statistical significance was determined by Log-rank (Mantel-Cox) Test versus isotype control for all groups.

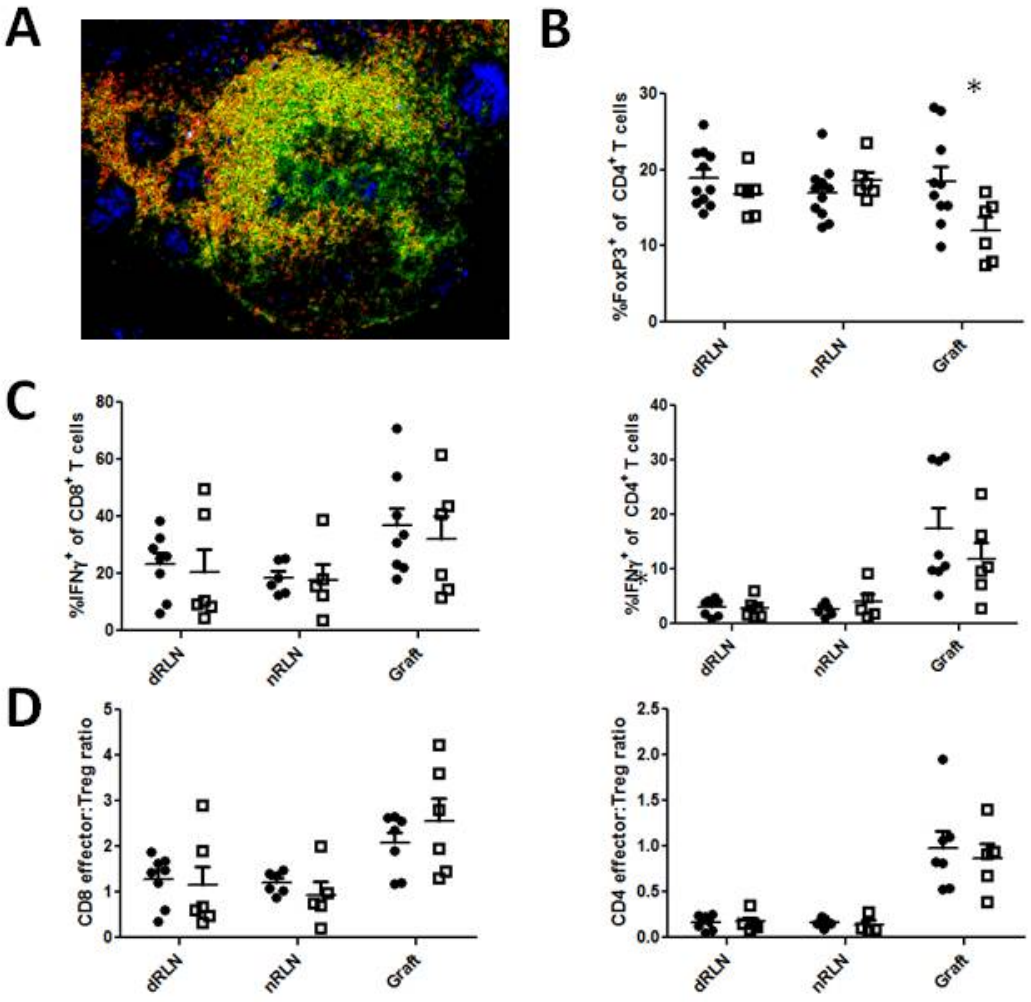

Figure 2.2. Analysis of T cells from YTS177 and YTS105- and 2A3-treated NOD recipients following graft failure (long-term).

Tissues were analyzed from islet grafted NOD recipients treated with YTS177 and YTS105 (black circles) and 2A3 (open squares). A.) A representative immunofluorescence image of a rejected YTS177 and YTS105-treated graft at 30 days post-implantation. (Blue – insulin, green – Thy1.2, and red – CD4). B.) FoxP3$^+$Treg frequency was determined by gating on live Thy1.2$^+$, CD4$^+$, and FoxP3$^+$ cells via flow cytometry. C.) The frequency of CD4$^+$ and CD8$^+$ T cells staining for IFNγ was determined in the the respective tissues following PMA and ionomycin stimulation. D.) The ratio of effector T cells to FoxP3$^+$Treg was determined by dividing the percent of IFNγ$^+$CD8$^+$Thy1.2$^+$ or IFNγ$^+$CD4$^+$Thy1.2$^+$ by the percent of FoxP3$^+$CD4$^+$Thy1.2$^+$; *p<0.05, statistical significance was determine using unpaired two-way ANOVA.

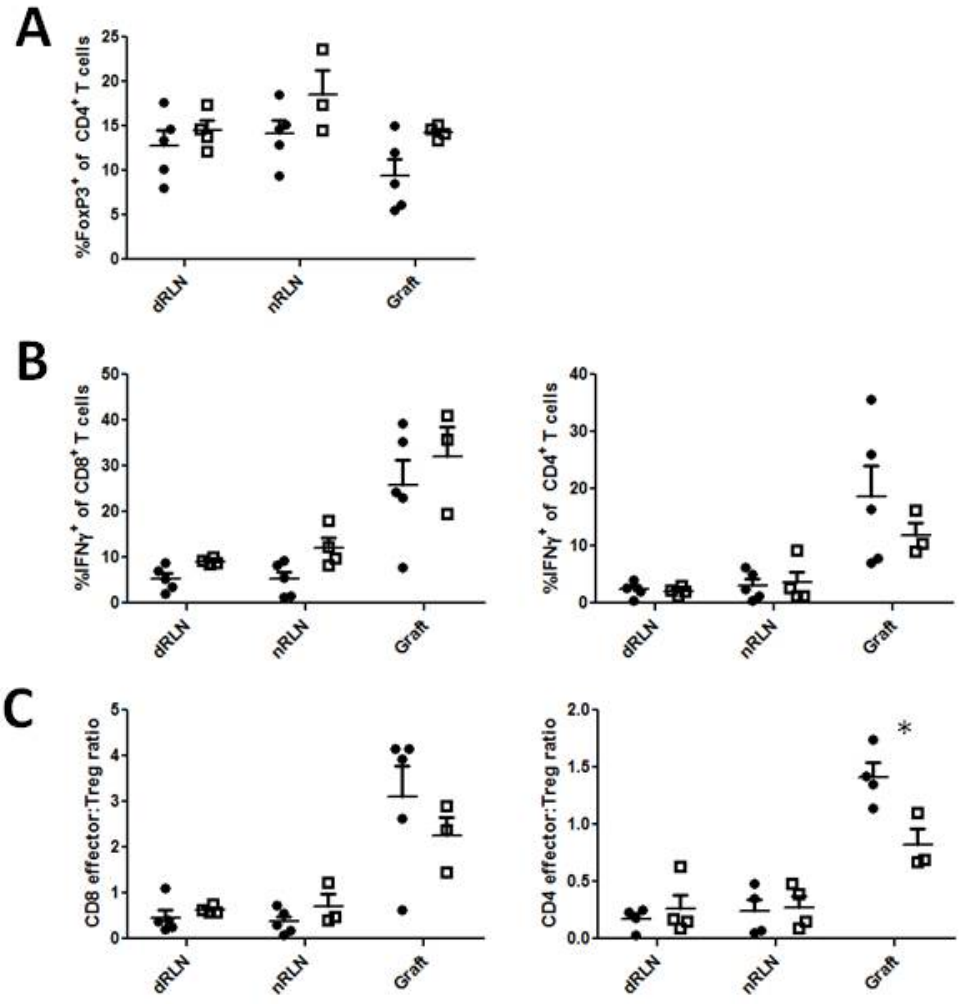

Figure 2.3. Analysis of T cells in YTS177 and YTS105- and 2A3-treated NOD recipients at day 14 (short-term) post-islet graft implantation.

Tissues were harvested on day 14-post islet graft implantation from NOD recipients treated with YTS177 and YTS105 (black circles) and 2A3 (open squares), and analyzed via flow cytometry. A.) FoxP3$^+$Treg frequency was determined by gating on live Thy1.2$^+$, CD4$^+$, and FoxP3$^+$ cells. B.) The frequency of CD4$^+$ and CD8$^+$ T cells staining for IFNγ was determined in the the respective tissues following PMA and ionomycin stimulation. C.) The ratio of effector T cells to FoxP3$^+$Treg was determined by dividing the percent of IFNγ$^+$CD8$^+$Thy1.2$^+$ or IFNγ$^+$CD4$^+$Thy1.2$^+$ by the percent of FoxP3$^+$CD4$^+$Thy1.2$^+$; *p<0.01, statistical significance was determine using two way ANOVA.

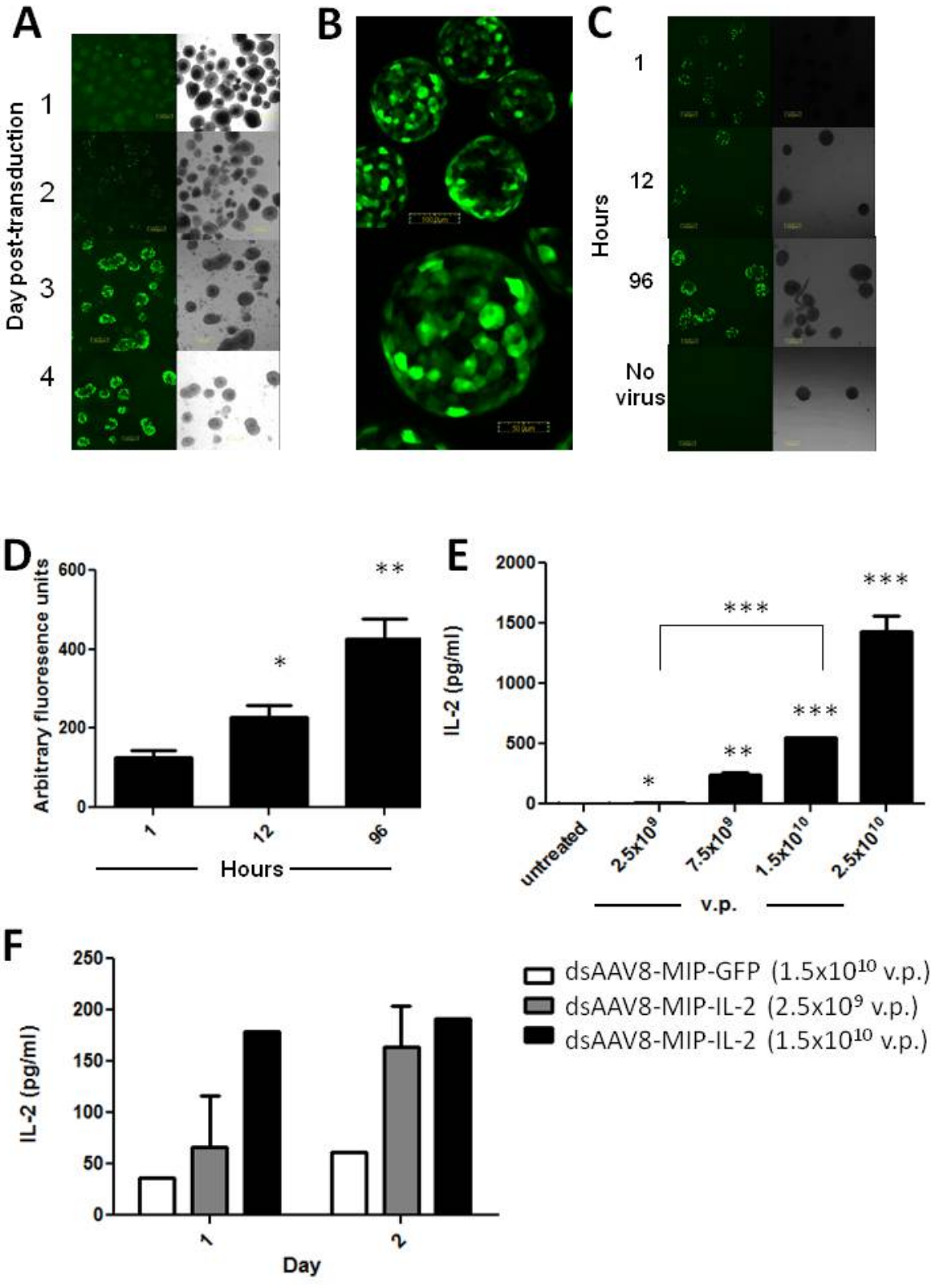
A
Day post-transduction
1
2
3
4
B
C
Hours
1
12
96
No virus
D
Arbitrary fluoresence units
600
400
200
0
*
**
1
12
96
Hours
E
IL-2 (pg/ml)
2000
1500
1000
500
0
*
**
***
***
***
untreated
2.5x10^9
7.5x10^9
1.5x10^10
2.5x10^10
v.p.
F
IL-2 (pg/ml)
250
200
150
100
50
0
1
2
Day
dsAAV8-MIP-GFP (1.5x10^10 v.p.)
dsAAV8-MIP-IL-2 (2.5x10^9 v.p.)
dsAAV8-MIP-IL-2 (1.5x10^10 v.p.)

Figure 2.4. AAV vector islet transduction *in vitro*.
For *in vitro* transduction, isolated islets were cultured and transduced with dsAAV. A.) Islets were transduced *in vitro* with dsAAV8-MIP-GFP at $1x10^{11}$ v.p. per 100 islets, and GFP expression demonstrated by confocal microscopy; scale bar 200um. B.) Islets transduced with dsAAV-MIP-GFP day 4 post-transduction; scale bar 100um (top) and 50um (bottom). C. Islets were cultured with dsAAV-MIP-GFP for varying times, washed with PBS, and cultured for 4 days total. GFP expression is shown by confocal microscopy with scale bar 200um. D. GFP expression of islets shown in (C) was quantified by image J; *p<0.005, **p<0.0001, statistical significance was determined with unpaired Student's t test relative to the 1hr time-point. E.) Islets were transduced *in vitro* with dsAAV8-MIP-IL2, and supernatant IL-2 levels on day 7 post-transduction measured by ELISA; *p<0.05, **p<0.01 ***p<0.001, statistical significance was determined using unpaired Student's t test. F.) NOD.*scid* islets were transduced with dsAAV8-MIP-IL2 or dsAAV8-MIP-GFP for 2 hours, and then implanted under the kidney capsule of STZ-diabetic NOD.*scid* recipients. Grafts were harvested at day 10, cultured, and supernatants measured for IL-2 production.

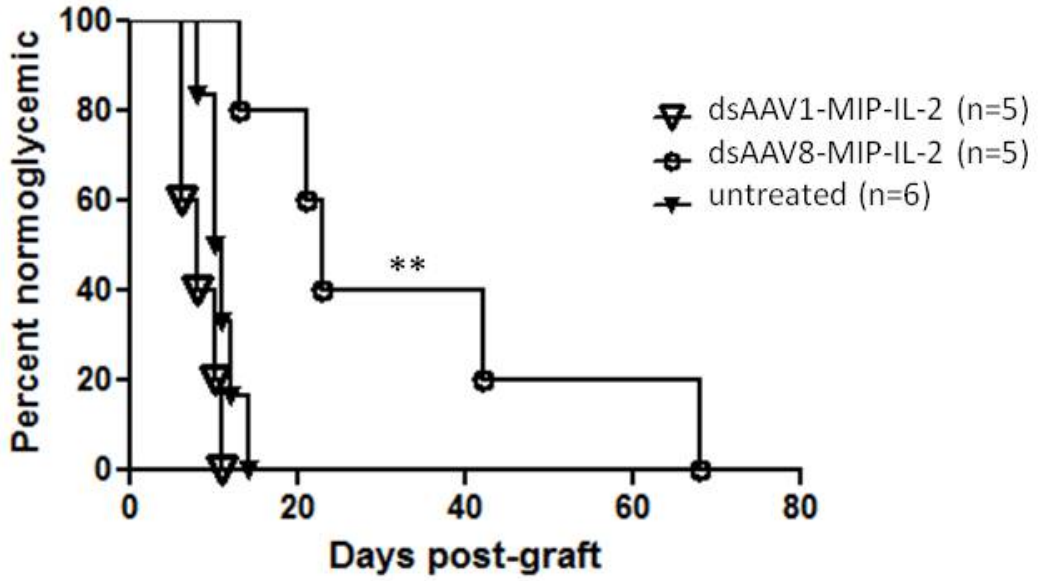

Figure 2.5. Transduction of islets with dsAAV8-MIP-IL2 extends graft survival. NOD.*scid* islets were isolated and cultured with $1.5x10^{10}$ v.p. of either dsAAV1- or dsAAV8-MIP-IL2 for 2 hours before transplantation, or left untreated. Diabetic NOD female mice were then engrafted with 500 islets at day 0. **p<0.005, AAV8-MIP-IL2 versus untreated and AAV1-MIP-IL2-transduced islets; statistical significance was determined by Log-rank (Mantel-Cox) Test.

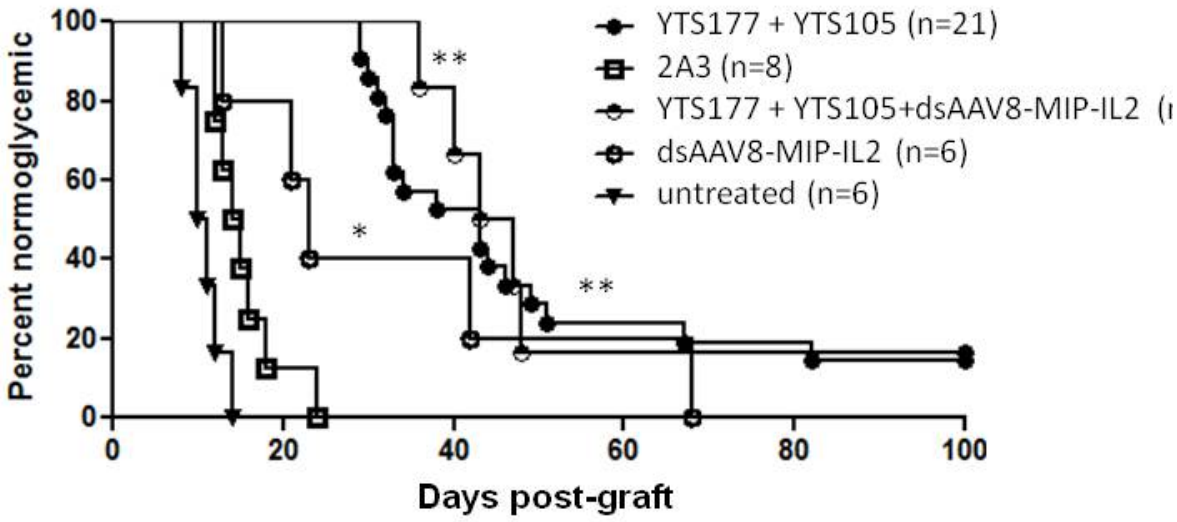

Figure 2.6. dsAAV8-MIP-IL2 plus YTS177 and YTS105 treatment does not significantly extend islet graft protection compared to YTS177 and YTS105 treatment alone.
Diabetic NOD female mice were transplanted with either 500 non-transduced or dsAAV8-MIP-IL2 transduced ($1.5x10^{10}$ v.p.) NOD.*scid* islets on day 0. Some mice received 600ug of either 2A3 or 600ug both YTS177 and YTS105 on days 0, 2, and 4 post-graft. *p<0.005, **p<0.001 versus isotype antibody control; statistical significance was determined by Log-rank (Mantel-Cox) Test.

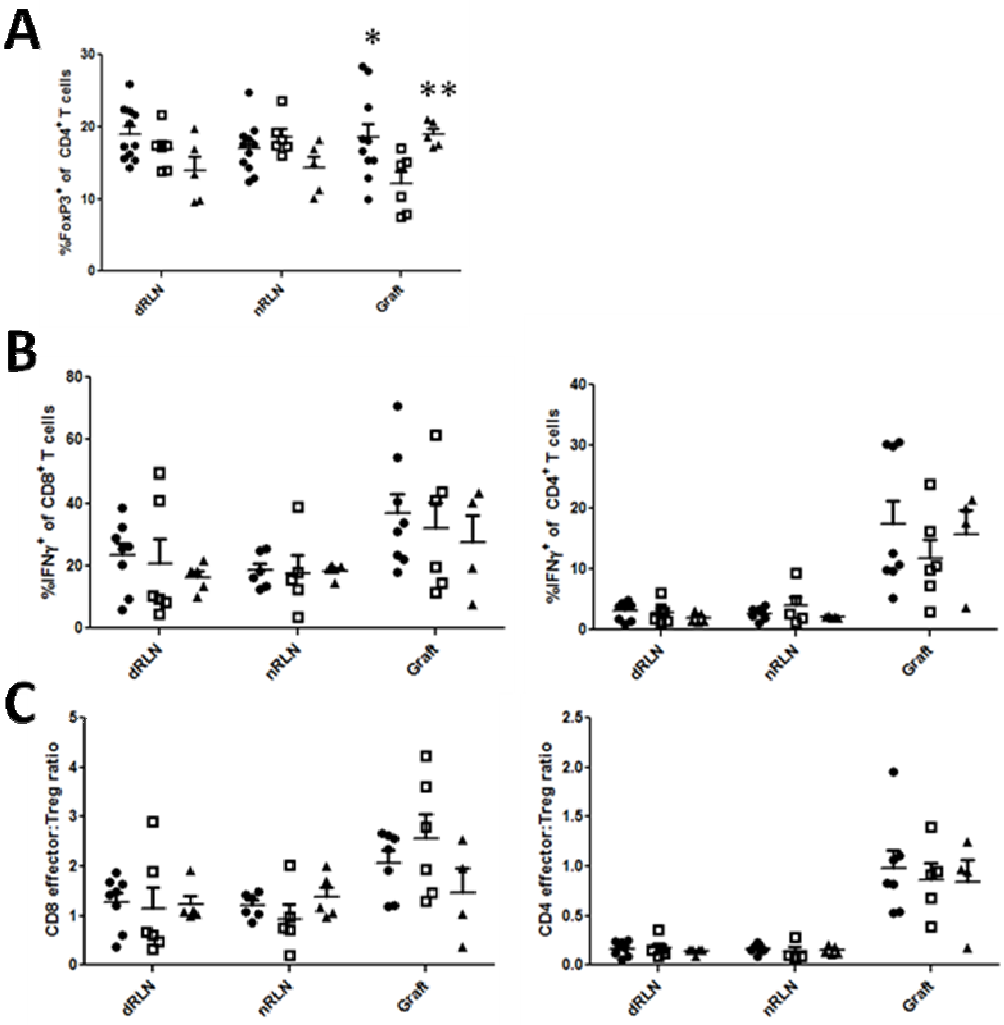

Figure 2.7. Analysis of T cells in YTS177 and YTS105 plus dsAAV8-MIP-IL2 treated NOD recipients following graft rejection.

Tissues were analyzed via flow cytometry following graft rejection in NOD recipients treated with YTS177 and YTS105 (black circles), 2A3 (open squares), and YTS177 and YTS105 + dsAAV8-MIP-IL2 mice (black triangles). A.) $FoxP3^+$Treg frequency was determined by gating on live $Thy1.2^+$, $CD4^+$, and $FoxP3^+$. B.) The frequency of $CD4^+$ and $CD8^+$ T cells staining for IFNγ was determined in the the respective tissues following PMA and ionomycin stimulation. C.) The ratio of effector T cells to $FoxP3^+$Treg was determined by dividing the percent of $IFN\gamma^+CD8^+Thy1.2^+$ or $IFN\gamma^+CD4^+Thy1.2^+$ by the percent of $FoxP3^+CD4^+Thy1.2^+$. *$p<0.01$, statistical significance was determine using two way ANOVA.

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CHAPTER 3

T CELL RECEPTOR VARIABLE β CHAIN DIVERSITY IN RECURRENT AUTOIMMUNE DIABETES

3.1 Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the T cell-mediated destruction of insulin-producing β cells within the pancreatic islets. Currently, islet or pancreas transplantation provides the only "cure" for T1D. However, the use of this procedure is limited due to both allo- and auto-reactivity against the graft. The T cell receptor (TCR) repertoire involved in recurrent autoimmunity is at present poorly defined. Understanding the repertoire and specificities of the T cells involved in the recurrent autoimmune response may aid in the development of therapies to prevent islet graft rejection. Thus, the TCR variable β (Vβ) chain repertoires in rejecting syngeneic islet grafts in diabetic NOD mice were assessed. In most NOD recipients, the effector/memory $CD8^+$ T cell repertoire in the islet graft showed decreased entropy, and was dominated by one to four TCR Vβ chains. However, specific TCR Vβ chain usage varied markedly from recipient to recipient. In contrast, the effector/memory $CD4^+$ T cell repertoire in the islet graft was more diverse, though strikingly, all NOD recipients showed an increase in the percentage of TCR Vβ12-bearing T cells in the islet graft and pancreas. Importantly, these T cells were shown to be proliferating preferentially in the islet graft and pancreas. Interestingly, the naïve T cell repertoire in all organs was similar, even in the pancreas and islet grafts. Additionally, the TCR repertoire of effector/memory T cells infiltrating the islet graft exhibited greater similarity to the repertoire found in the pancreas than that found in the draining renal lymph node, pancreatic lymph node, or spleen. This suggests that the same specificities of effector/memory T cells drive both initial and recurrent autoimmune responses in individual recipient basis.

Type 1 Diabetes (T1D) is an autoimmune disease mediated by T cells specific for the insulin-producing β cells present in the islets of Langerhan's of the pancreas[1]. In humans and the NOD mouse, a model for T1D, autoimmunity is typically viewed as a chronic inflammatory response leading to gradual β cell destruction. Once 80-90% of β cell mass has been destroyed, hyperglycemic blood levels are achieved and overt diabetes is established. In order to replace β cells and restore euglycemia, islet transplants can be performed. In the clinic, this procedure is limited by several factors, including both allo- and auto-reactivity against the transplanted tissue[2-5].

It is well established that both autoreactive $CD4^+$ and $CD8^+$ T cells play essential roles in driving β cell destruction in T1D[6-11]. Concerted efforts have been made to identify the β cell autoantigens and corresponding epitopes targeted by T cells. $CD4^+$ T cells responsive to antigens including insulin B chain, proinsulin, glutamic acid decarboxylase 65 (GAD65), the protein tyrosine phosphatase IA-2, and heat shock protein 60 (hsp60) have been found in NOD mice (reviewed in [12-14]). Important β cell autoantigens recognized by $CD8^+$ T cells include the insulin B chain and islet-specific glucose-6-phosphatase catalytic subunit-related protein ($IGRP_{206-214}$). $CD8^+$ T cells specific for $IGRP_{206-214}$ have been observed in islets of young, prediabetic, and diabetic NOD mice[12-16].

$IGRP_{206-214}$-specific $CD8^+$ T cells play a role in recurrent autoimmunity as well. Wong *et al.* determined that at day 7 post-implantation of a syngeneic islet graft, ~25% of graft-infiltrating $CD8^+$ T cells were $IGRP_{206-214}$-specific, though this number decreased to ~5% by

day 13[15]. Graft rejection was delayed by depleting $IGRP_{206-214}$-specific $CD8^+$ T cells, further highlighting the importance of this antigen in recurrent autoimmunity in NOD mice[15]. Notably, the rapid kinetics of syngeneic islet graft rejection, generally seen within 10-14 days post-implantation, suggests that pre-existing $IGRP_{206-214}$-specific memory T cells are activated, and contribute to destruction of the implanted β cells. While this study indicated a key role for $IGRP_{206-214}$-specific $CD8^+$ T cells in graft destruction, the identity of other β cell autoantigens mediating the rejection process remains ill-defined.

Since relatively few β cell autoantigens have been identified, T cell receptor (TCR) variable (V)α and Vβ chain usage has been used to gain insight into the overall repertoire of islet-infiltrating T cells. The TCR is a heterodimer consisting of α and β chains, which have constant (C) and variable (V) regions. The V region is further composed of variable (Vβ), diversity (Dβ), and joining (Jβ) segments. At least 31 Vβ genes have been identified[17]. Restricted TCR repertoires have been observed in several autoimmune diseases, including experimental allergic encephalomyelitis (EAE) and collagen-induced arthritis[18-20]. In these models, pathogenic T cells preferentially expressed TCR Vβ8.2, and disease was ameliorated following treatment with an αTCR Vβ8.2 antibody[18-20]. Though most studies of pre-diabetic NOD mice showed no significant TCR repertoire skewing[21-23], one study found that TCR Vβ12 expression was increased 2-fold in the pancreas compared to spleen[24], indicating a possible role for this TCR Vβ chain. However, many of these studies used bulk populations of T cells and did not distinguish between $CD4^+$ and $CD8^+$ T cells or naïve and

effector/memory (eff/mem) T cells. Therefore, possible selective TCR usage by these respective T cell subsets would be obscured.

Understanding the basis for recurrent autoimmunity, in particularly the TCR repertoire of pathogenic T effectors, may provide important insight for the development of rational strategies to establish islet graft tolerance. With this in mind, TCR Vβ usage by naïve and eff/mem $CD4^+$ and $CD8^+$ T cells in day 10 syngeneic islet grafts was studied in order to better understand the dynamics and specificities of recurrent autoimmunity.

3.2 Results

TCR Vβ repertoires of islet graft-infiltrating T cells can be evaluated by a novel flow cytometry technique.

Typically, the TCR Vβ repertoire of islet-infiltrating T cells has been studied by RT-PCR from RNA isolated from bulk T cell preparations[21-23] or flow cytometric-sorted MHC tetramer-binding T cells[15,16]. The former approach fails to address potential TCR repertoire differences among distinct T cell subsets, whereas the latter strategy provides information only for a given set of clonotypes. Accordingly, we developed a flow cytometry technique allowing characterization of the TCR Vβ repertoire by multiple T cell subsets. Flow cytometry has the advantage of identifying TCR Vβ usage by several T cell subsets so that broader insight can be gained into how the TCR repertoire evolves during the autoimmune process. In addition the strategy permits analyses of relatively few T cells, which is a key issue when examining the small numbers of islet graft-infiltrating T cells (typically between 20,000 and 100,000 T cells).

Cells were stained with αCD90.2 (Thy1.2) to identify T cells, and αCD8 and αCD4 to identify cytotoxic and helper T cells, respectively. Further, cells were stained with antibodies specific for CD44, a marker for eff/mem T cells, and CD62L, a marker for naïve T cells; $CD44^{hi}$ T cells were defined as eff/mem, whereas $CD62L^{hi}$ T cells were defined as naïve T cells. Although CD44 did not permit distinction between effector versus memory T cells, activated versus naïve T cells, however, could be readily distinguished. Three different Vβ staining panels were used to determine the T cell TCR Vβ repertoire. Using this approach up to 6

different TCR Vβ chains could be detected per well in addition to the markers required to define T cell subsets (Figure 3.1A, B). This minimized sample division, and provided enough cells for the analysis to have statistical power. A schematic representation of the TCR Vβ flow cytometric data is provided in Figure 3.1B. Here, TCR Vβ2-expressing T cells would stain positive for FITC-only, TCR Vβ4-expressing T cells would stain positive for both FITC and PE, and TCR Vβ13-expressing T cells would stain positive for PE alone (Figure 3.1A,B). Adding a biotinylated antibody to incorporate a third color (e.g. streptavidin PECy7 (SAV-PECy7)) permits identification of three additional TCR Vβ chains. For instance, TCR Vβ5.1/2 -expressing T cells would stain positive for SAV-PECy7 only, TCR Vβ11-expressing T cells would stain positive for both SAV-PECy7 and PE, and TCR Vβ8.1/2-expressing T cells would stain positive for SAV-PECy7 and FITC (Figure 3.1A,B). In this way, 6 TCR Vβ chains can be examined using only three colors. A representative flow cytometric plot is shown in Figure 3.1C. A similar approach has been used to characterize T cells in human peripheral blood[25], but application of this technique to characterize TCR Vβ repertoires within islet grafts is novel.

Eff/mem CD8$^+$ and CD4$^+$ T cells infiltrating an islet graft exhibit restricted TCR Vβ chain usage.

TCR Vβ usage by T cell subsets in the islet graft, pancreas, renal lymph node (RLN), pancreatic lymph node (PLN), and spleen was studied in individual NOD recipients. Diabetic female NOD mice (blood glucose levels >250 mg/ml) were implanted with 500 syngeneic NOD.*scid* islets under the kidney capsule. Function of an implanted islet graft was confirmed

upon return of the NOD recipient to normoglycemia. Ten days post-implantation, naïve and eff/mem $CD4^+$ and $CD8^+$ T cells were examined via flow cytometry. The composition of the TCR Vβ repertoire of naïve $CD4^+$ and $CD8^+$ T cells was essentially identical between the respective organs. Interestingly, naïve T cells present in the islet graft, which accounted for between 10%-50% of T cells, exhibited similar TCR Vβ usage to naïve T cells seen in the periphery e.g. spleen (Figure 3.2A, B).

In the pool of eff/mem versus naïve $CD4^+$ T cells, a trend towards more selective TCR Vβ usage was observed, particularly in the pancreas and islet grafts of recipient animals (Figure 3.2A). Strikingly, TCR Vβ12 usage by eff/mem $CD4^+$ T cells was markedly increased in the islet graft and pancreas compared to naïve $CD4^+$ T cells in those tissues, in addition to eff/mem $CD4^+$ T cells found in the spleen, PLN and RLN (Figure 3.2A). In several NOD recipients >15% of eff/mem $CD4^+$ T cells expressed TCR Vβ12 (Figure 3.2 A). This skewing towards TCR Vβ12 usage by eff/mem $CD4^+$ T cells in the islet graft (and less so in the pancreas) was more evident when TCR Vβ usage within the respective tissues was normalized to the TCR repertoire used by naïve $CD4^+$ T cells residing in the spleen (Figure 3.3A). Importantly, TCR Vβ usage was statistically higher in the graft and pancreas than spleen and PLN (Figure 3.3C). Strikingly, this trend was also mirrored in the RLN, though to a lesser degree (Figure 3.3C). This suggests T cell proliferation in the RLN prior to migration into the graft.

TCR Vβ usage among eff/mem $CD8^+$ T cells was similar in the spleen, RLN, and PLN (Figure 3.2B and 3.3B). However, preferential TCR Vβ usage was detected in both the islet graft and pancreas of NOD recipients (Figure 3.2B and Figure 3.3B). In most NOD recipient mice, the eff/mem $CD8^+$ T cells infiltrating the islet graft preferentially used one to four TCR Vβ chains (Figure 3.2B) Interestingly, preferential TCR Vβ usage varied markedly between the islet grafts of individual recipients (Figure 3.2B,C). For example, the islet graft TCR Vβ repertoire of mouse #1 was dominated by TCR Vβ8.1/2 (29%), Vβ10 (29%), and Vβ6 (8%), whereas the TCR Vβ repertoire of mouse #2 was largely composed of TCR Vβ4 (21%), Vβ5.1/2 (19%), Vβ2 (11%), and Vβ8.1/2 (10%) (Figures 3.2C). Generally, the dominant TCR Vβ chains found in the graft-infiltrating repertoire were also prominent in the pancreas (Figure 3.2B, C); the TCR Vβ repertoire of mouse #1 was dominated by TCR Vβ8.1/2 (25%), Vβ10 (9%), and Vβ6 (19%). On the other hand, the TCR Vβ repertoire of mouse #2 exhibited preferential usage of TCR Vβ4 (24%), TCR Vβ5.1/2 (14%), and TCR Vβ8.1/8.2(14%), though not TCR Vβ2 (3%) (Figure 3.2C). Relative to eff/mem $CD4^+$ T cells, TCR usage by eff/mem $CD8^+$ T cells in the islet graft and pancreas was overall more diverse and variable (Figure 3.2 B,C). This data suggests that preferential TCR Vβ usage is detected for eff/mem T cells found infiltrating an islet graft relative to other tissues and to naïve T cells.

The TCR Vβ repertoire of eff/mem $CD8^+$ T cells is less diverse in the islet graft compared to the RLN, PLN, and spleen.

To more accurately assess the level of diversity among TCR Vβ chains used by naïve and eff/mem T cells in the respective tissues, Shannon entropy was calculated. The posterior

distribution of the Shannon entropy in a given population represents the abundance and diversity for a given TCR Vβ chain[16]. Hence, if a pool of T cells express few (e.g. less diverse) TCR Vβ chains, the entropy will correspondingly be lower: more dominance corresponds to less diversity. As expected, the entropy of the naïve $CD4^+$ and $CD8^+$ T cells was nearly identical for all tissues (Figure 3.4A, C), indicating a similar level of TCR Vβ diversity. In contrast, the entropy for TCR Vβ chains of eff/mem $CD8^+$ T cells was reduced in the pancreas, and significantly lower in the islet graft compared to spleen, RLN and PLN (Figure 3.4D). On the other hand, entropy of TCR Vβ chains used by eff/mem $CD4^+$ T cells was similar in all tissues, although a slight trend towards reduced entropy in islet grafts was detected (Figure 3.4B). This data demonstrates that the level of TCR Vβ chain diversity is significantly reduced in the islet grafts and to a lesser extent the pancreas for eff/mem $CD8^+$ T cells.

The TCR Vβ chain repertoires of the islet graft and pancreas are similar.

Within individual NOD recipients, TCR Vβ usage by eff/mem $CD4^+$ and $CD8^+$ T cells appeared to be similar between the islet graft and pancreas (Figure 3.2). The Kullback-Liebler divergence test was employed to directly determine the level of similarity of TCR Vβ chain usage among naïve and eff/mem $CD4^+$ and $CD8^+$ T cells in the respective tissues. This test measures the variance between two populations, and allows the comparison of divergence. Hence, if two populations are similar, their divergence will be low. As expected, TCR Vβ usage among naïve T cells was similar among the respective tissues, with a divergence index of approximately 0.0 (Figure 3.5A). Notably, TCR Vβ usage for eff/mem $CD4^+$ and $CD8^+$ T

cells was also more similar between the islet graft and pancreas. For instance, the average level of divergence detected for eff/mem $CD8^+$ T cells in the islet graft versus pancreas (0.109±.048) was reduced relative to that seen when islet grafts were compared to the spleen (0.181±.03), PLN (0.215±.028), and significantly reduced when compared to RLN (0.243±.026) (Figure 3.5D). Similar results were obtained for eff/mem $CD4^+$ T cells found in the islet graft, where the islet-pancreas repertoire divergence was significantly less than the islet-spleen or islet-PLN divergence (Figure 3.5B). Together these findings indicate that a strong trend towards islet graft versus pancreas repertoire similarity is apparent in eff/mem $CD4^+$ and $CD8^+$ T cells. These results suggest that the specificity of T cells that mediate islet destruction in the initial response in the endogenous pancreas are the same as those that mediate the recurrent response seen in the islet graft.

Islet graft infiltrating eff/mem $CD4^+$ and $CD8^+$ T cells are proliferating.

Preferential usage of specific TCR Vβ chains by eff/mem T cells could be explained by selective expansion/proliferation within the islet graft. Accordingly, to determine if the dominant TCR Vβ chain populations were actively expanding, Ki67, a marker for ongoing cellular division, was examined via flow cytometry. An increased frequency of islet graft infiltrating eff/mem $CD4^+$ and $CD8^+$ T cells expressing prevalent TCR Vβs were Ki67-positive (Figure 3.6A-D). Strikingly, TCR Vβ12-bearing $CD4^+$ T cells in the islet graft and pancreas were proliferating in all graft recipients examined (Figure3.6 A, B). The TCR Vβ12 population in the graft proliferated significantly more than the populations in the spleen ($p<0.01$) and PLN ($p<0.001$). Additionally, TCR Vβ12 population in the pancreas proliferated significantly

more than the population in the PLN ($p<0.05$). Likely due to the variability observed in proliferation and to the low number of mice used, no significance was observed between the RLN and PLN or spleen, though there was a trend of slightly higher proliferation in the RLN. Together these results demonstrate that dominant TCR Vβ usage corresponds with eff/mem T cells actively proliferating within the islet grafts.

A similar frequency of IGRP$_{206\text{-}214}$-specific CD8$^+$ T cells is detected in the islet graft and pancreas of individual NOD recipients.

Studies by our group and others[15,16] have shown that up to 85% of IGRP$_{206\text{-}214}$-specific CD8$^+$ T cells use TCR Vβ8.1/2. In view of the relatively high prevalence of TCR Vβ8.1/2 usage by eff/mem CD8$^+$ T cells infiltrating an islet graft and pancreas (Figure 3.2B), the frequency of IGRP-specific eff/mem CD8$^+$ T cells within these tissues was measured in 6 NOD graft recipients using IGRP$_{206\text{-}214}$-H2K^d tetramers. Interestingly, for a given NOD recipient similar frequencies of IGRP$_{206\text{-}214}$-specific eff/mem CD8$^+$ T cells were detected in the islet graft and pancreas (Figure 3.7A). For example, in one NOD recipient, IGRP$_{206\text{-}214}$-specific CD8$^+$ T cells made up ~26% and 20% of the eff/mem CD8$^+$ T cells infiltrating the islet graft, and pancreas, respectively (Figure 3.7A). Furthermore, >85% of IGRP$_{206\text{-}214}$-specific eff/mem CD8$^+$ T cells in the islet graft and pancreas expressed TCR Vβ8.1/2 (Figure 3.7B). These results demonstrate that similar β cell-specific eff/mem CD8$^+$ T cells target the islet graft and pancreas in a given NOD recipient.

3.3 Discussion

In order to develop effective immunotherapies to block autoimmune-mediated islet graft destruction, the nature of the recurrent autoimmune response needs to be better defined. With this in mind, a multi-parameter flow cytometry method was established and applied to examine the TCR Vβ repertoires found in the islet graft, pancreas and respective draining lymph nodes in diabetic NOD recipients. This approach allows for definition of TCR Vβ chain usage by distinct T cell lineages (e.g. $CD4^+$, $CD8^+$) varying in activation and proliferative status from multiple tissues. Resolution of TCR Vβ usage by T cells under these different parameters has provided a more accurate account of events driving recurrent autoimmunity. For instance, the surprisingly high frequency of naïve T cells infiltrating an islet graft (up to 50%) and other tissues would clearly affect the interpretation of the results, if T cells were studied independent of activation status. Selective analysis of eff/mem T cells provides insight into TCR Vβ usage by clonotypes directly mediating β cell destruction. Three key observations were made in this study. First, eff/mem T cells infiltrating an islet graft exhibit skewed TCR Vβ usage. Secondly, TCR Vβ usage by islet graft infiltrating eff/mem $CD8^+$ versus $CD4^+$ T cells is more diverse and variable among NOD recipients. Finally, similar TCR Vβ usage is seen by eff/mem T cells found infiltrating the islet graft and pancreas of a given NOD recipient.

The majority of eff/mem $CD8^+$ T cells infiltrating an islet graft typically expressed one of 1 to 4 dominant TCR Vβ chains for given NOD recipient (Figure 3.2B). Accordingly, the repertoire of eff/mem $CD8^+$ T cells infiltrating the islet graft exhibited reduced entropy (diversity)

compared to eff/mem $CD8^+$ T cells residing in the RLN, PLN and spleen, again attributed to the dominance of few TCR Vβ chains (Figure 3.4D). Notably, TCR Vβ usage by islet infiltrating eff/mem $CD8^+$ T cells varied markedly among the recipients (Figure 3.2B, C, 3.3B). TCR Vβ8.1/2 was expressed prominently by islet infiltrating eff/mem $CD8^+$ T cells in a number of NOD recipients (Figure 3.2B, 3.3B). However, several other TCR Vβ chains were highly expressed by islet graft eff/mem $CD8^+$ T cells, including TCR Vβ2 (mouse 12, 19%), TCR Vβ4 (mouse 2, 21.4%), TCR Vβ5 (mouse 2, 19.5%), TCR Vβ6 (mouse 3, 20.7%), TCR Vβ10 (mice 1 and 10, 29.3 and 26.9%), TCR Vβ1 (mouse 8, 25.16%), and TCR Vβ13 (mouse 4, 26.1%). These findings indicate that a broad TCR repertoire is utilized by eff/mem $CD8^+$ T cells to mediate recurrent autoimmunity, but in a given recipient extensive clonal selection occurs within the islet graft. Usage of particular TCR Vβ chains may reflect increased TCR affinity for the corresponding peptide-MHC complex (pMHC), and/or an increased frequency of the respective pMHC found within the islet graft. Interestingly, a high frequency of eff/mem $CD8^+$ T cells infiltrating an islet graft were found to be actively proliferating based on Ki67-staining, arguing for clonal expansion of those T cells. Preliminary data shows that the TCR Vβ repertoire of eff/mem $CD8^+$ T cells infiltrating an islet graft is more diverse and less skewed at day 5 versus 10 post-implantation, further indicating clonal selection ongoing in the islet graft.

Interestingly, TCR Vβ usage by eff/mem $CD4^+$ versus $CD8^+$ T cells found in islet grafts and the pancreas was less skewed (Figure 3.2A, C, 3.3A), reflected by a minimal (if any) decrease in entropy compared to other tissues (Figure 3.4B). Strikingly, however, TCR Vβ12 usage was

markedly increased by eff/mem $CD4^+$ T cells infiltrating the islet graft (Figure 3.3A, C) and less so in the pancreas (Figure 3.3A, C), in all NOD recipients examined. Baker *et al.* also showed preferential usage of TCR Vβ12 (as well as TCR Vβ1) in the islets of 2-3 week-old NOD mice[26]. Interestingly, the length of the TCR complementary determining region 3 (CDR3) and motif of these T cells was conserved over time, suggesting a key role in mediating β cell destruction[26]. A two-fold increase in TCR Vβ12 RNA expression has also been reported in the pancreas versus spleen of pre-diabetic NOD female mice[24]. Furthermore, >60% of pancreas infiltrating $CD4^+$ T cells specific for the BDC mimetic peptide (mBDC) expressed TCR Vβ12 in pre-diabetic 16 week-old NOD mice[27]. On the other hand, only 10% of mBDC-specific $CD4^+$ T cells in the periphery expressed TCR Vβ12 chain. Since mBDC-specific $CD4^+$ T cells in general are highly pathogenic[28-30], and a high percentage of these clones express TCR Vβ12, it is tempting to speculate that the eff/mem $CD4^+$ T cells expressing TCR Vβ12 found infiltrating the islet grafts also play a critical role driving recurrent autoimmunity.

Our results indicate that the TCR Vβ repertoire of eff/mem T cells observed in the islet graft is more similar to the repertoire of eff/mem T cells residing in the pancreas rather than the PLN, RLN, and spleen (Figure 3.4B, D). This observation supports a scenario in which β cell-specific T cells mediating recurrent autoimmunity have been recruited from the pancreas, rather than being selected from clonotypes found in the periphery. In support of this hypothesis, similar frequencies of $IGRP_{206\text{-}214}$-specific $CD8^+$ T cells were detected in the islet graft and pancreas for a given NOD recipient (Figure 3.7A). $IGRP_{206\text{-}214}$-specific $CD8^+$ T cells

are believed to play a major role in mediating spontaneous diabetes in NOD mice[12,16,31]. Accordingly, IGRP$_{206-214}$-specific CD8$^+$ T cells may play an equally important role in recurrent autoimmunity. Noteworthy is that ~80% of IGRP$_{206-214}$-specific CD8$^+$ T cells sorted from NOD islet grafts expressed TCR Vβ8.1[15], consistent with our results for IGRP$_{206-214}$-specific eff/mem CD8$^+$ T cells infiltrating islet grafts (Figure 3.7B). Therefore, one interesting possibility is that prevalent usage of TCR Vβ8.1/2 by eff/mem CD8$^+$ T cells in the islet graft maybe representative of expansion of IGRP$_{206-214}$-specific CD8$^+$ T cells.

Whether the diversity seen in TCR Vβ usage by eff/mem T cells among individual NOD recipients is due to targeting of: i) the same autoantigen by distinct clones, and/or ii) multiple autoantigens and/or epitopes is unclear. For example, 6 insulin-specific T cell clones recognizing the same epitope (9-23) expressed at least 4 different TCR Vβ chains, though these clones preferentially expressed TCR Vα13[32]. To further examine this issue an extensive panel of β cell antigen-specific MHC class I and II tetramers can be employed to determine how antigen/peptide specificity correlates with TCR Vβ usage profiles. For instance, the diversity of antigen-specificity of eff/mem CD4$^+$ T cells expressing TCR Vβ12 would be highly amenable for such a study.

A somewhat puzzling observation was the relatively high frequency of naïve CD4$^+$ and CD8$^+$ T cells found infiltrating an islet graft. Preliminary results show that at day 5 post-implantation over 90% of T cells in the islet graft exhibited an eff/mem phenotype. However, at day 10 post-implantation, the frequency of eff/mem decreases to between 50

and 90% of islet infiltrating T cells, indicating an influx of naïve T cells into the islet graft. The latter may be due to the inflammatory environment established in the islet graft, as naïve T cells respond to chemokines and other proinflammatory cues.

In conclusion, for a given recipient eff/mem $CD8^+$ T cells use only few Vβ chains resulting in reduced TCR diversity in the islet graft compared to that detected in the RLN, PLN, and spleen. However, $CD8^+$ TCR Vβ usage varies considerably between NOD recipients, raising the possibility that certain antigens are more important in different recipients. These results are supported by data showing that $IGRP_{206\text{-}214}$-specific $CD8^+$ T cells compose a large portion of the response in some islet graft recipients, but not others. The TCR Vβ repertoire of eff/mem $CD4^+$ T cells infiltrating the islet graft generally shows more heterogeneity. However, every NOD recipient examined showed increased TCR Vβ12 usage by islet graft eff/mem $CD4^+$ T cells. Additionally, both eff/mem $CD4^+$ and $CD8^+$ T cells exhibited TCR Vβ repertoires that were similar between the islet graft and pancreas. Since the TCR Vβ repertoire of eff/mem $CD8^+$ versus $CD4^+$ T cells is more broad, it is possible that multiple and distinct antigens are targeted by $CD8^+$ T cells during islet graft destruction among recipient animals. On the other hand, $CD4^+$ T cells with specificity for a limited set of antigens are necessary to drive recurrent autoimmunity, and thus conserved among NOD recipients. Further examination of T cell specificities involved in recurrent autoimmunity may lead to improved therapies for the prevention of islet graft rejection.

3.4 Materials and Methods

Mice

NOD/LtJ and NOD.CB17.Prkdc*scid*/J (NOD.*scid*) mice were bred and housed under pathogen-free conditions in an American Association for Laboratory-accredited animal facility. NOD mice were considered to be diabetic after two successive days of ≥250mg/dl blood glucose as measured by a Freestyle *Lite* blood glucose monitor and strips (Abbott Diabetes Care Inc.). All procedures were reviewed and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Islet transplantation

Diabetic NOD female mice received 5 units of insulin daily prior to transplantation. Five hundred syngeneic (NOD.*scid*) islets were transplanted under the renal capsule of the left kidney. Blood glucose values were monitored daily, biweekly, or weekly post-transplantation.

Flow Cytometry

Spleen, PLN, RLN, and pancreas single-cell suspensions were prepared by grinding tissue between frosted slides in RPMI complete containing 100nM Dasatinib. When required, red cells were lysed with RBC lysis buffer. Islet grafts were excised from the kidney and gently grinded to release infiltrating cell under the capsule and minimize kidney cell contamination. Cells were washed with FACS buffer (PBS plus 0.5% BSA), filtered and blocked with αCD16/32 (2.4G2). Cells were always kept in media containing 100nM

Dasatinib. Samples were then split into three wells and stained. Thy1.1^{+} spleen cells (1X10^{6}) were added to wells containing cells from grafts and PLN/RLN prior to addition of antibodies as a staining internal control. Cells were stained with antibodies specific for CD90.2 (53-2.1), CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14) (BD Biosciences), Thy1.1 (OX-7) (BioLegend), CD4^{+} (RM4-5) (Invitrogen) and three different anti-TCR Vβ panels. **Panel A:** αTCR Vβ2-biotin (B20.6), αTCR Vβ3-PE (KJ25), αTCR Vβ4-biotin (KT4), αTCR Vβ4-FITC, αTCR Vβ6-biotin (RRA-7), αTCR Vβ6-PE and αTCR Vβ9-FITC (MR10-2); **Panel B:** αTCR Vβ5.1/2-biotin (MR9-4), αTCR Vβ7-PE (TR310), αTCR Vβ8.1/2-FITC (MR5-2), αTCR Vβ 8.1/2-biotin and αTCR Vβ8.3-FITC (1B3.3); **Panel C:** αTCR Vβ10[b]-FITC (B21.5), αTCR Vβ 10[b]-PE, αTCR Vβ11-PE (RR3-15), αTCR Vβ11-biotin, αTCR Vβ12-biotin (MR11-1), αTCR Vβ13-PE (MR12-4) (Biolegend) and αTCR Vβ14-FITC (14-2). All αTCR Vβ antibodies were purchased from BD Biosciences unless noted. Binding of biotin-labeled antibodies was determined with streptavidin Alexa 594 (Invitrogen). Cells were washed twice with PBS and stained with LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (Invitrogen) to exclude dead cells. To stain for FoxP3 (FJK-16s) or Ki-67 (B56) samples were washed, fixed and permeabilized with eBiosciences Fix/Perm kit following manufacturer's indications. For tetramer analysis, IGRP-H2K^{d} tetramers were prepared as previously described[15]. Cells were first stained in 100uL containing IGRP-H2K^{d} for 40 minutes at room temperature, and then placed on ice and incubated for 20 minutes with 100uL of a 2x cocktail of antibodies specific for T cell markers and TCR Vβ chains. Data was acquired at the University of North Carolina Flow Cytometry Facility using a 6 laser, 18 parameter LSRII flow cytometer (BD Biosciences). Analysis was performed with FlowJo software (Tree Star Inc.).

Diversity Analysis

As described by Vincent *et al*[16], "the Shannon entropy of a T cell population is determined by two parameters: 1) the number of different T cell clones that are present, and 2) the frequency of each individual clone. Entropy is greatest when there are many different T cell clones and when there are few clones that are highly represented in the population (i.e. few "dominant" clones). If *S* is the total number of unique clonotypes in the pool, and p_i is the proportion of the pool represented by clonotype *i*, the Shannon entropy *H* is defined as:"

$$H = -\sum_{i=1}^{S} p_i \log p_i$$

The Kullback-Leibler diversity index was adapted from Muller et al.[33] and is a measure of divergence.

A

TCRVβ chain	antibodies conjugated to:
TCRVβ 2	FITC
TCRVβ 4	FITC + PE
TCRVβ 8.1/8.2	Biotin + FITC
TCRVβ 13	PE
TCRVβ 11	Biotin + PE
TCRVβ 5.1/5.2	Biotin

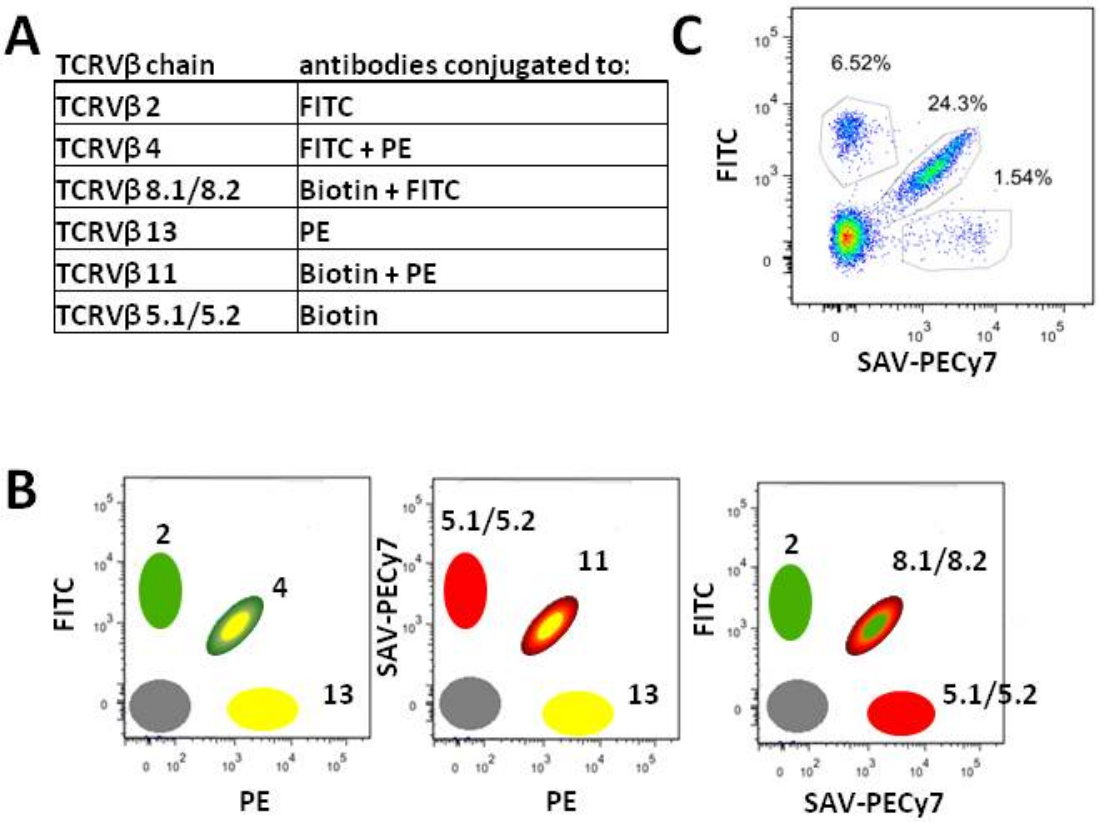
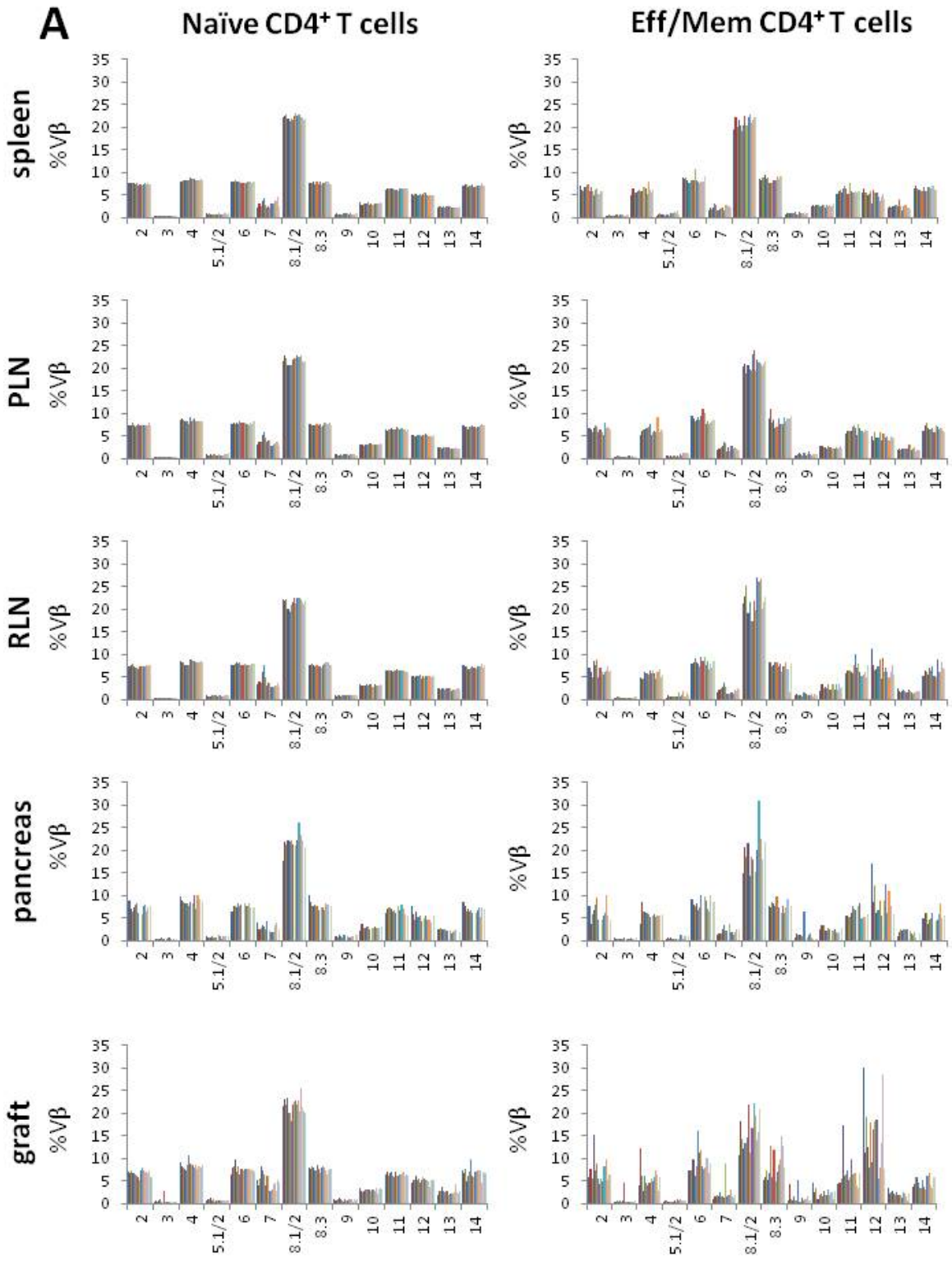
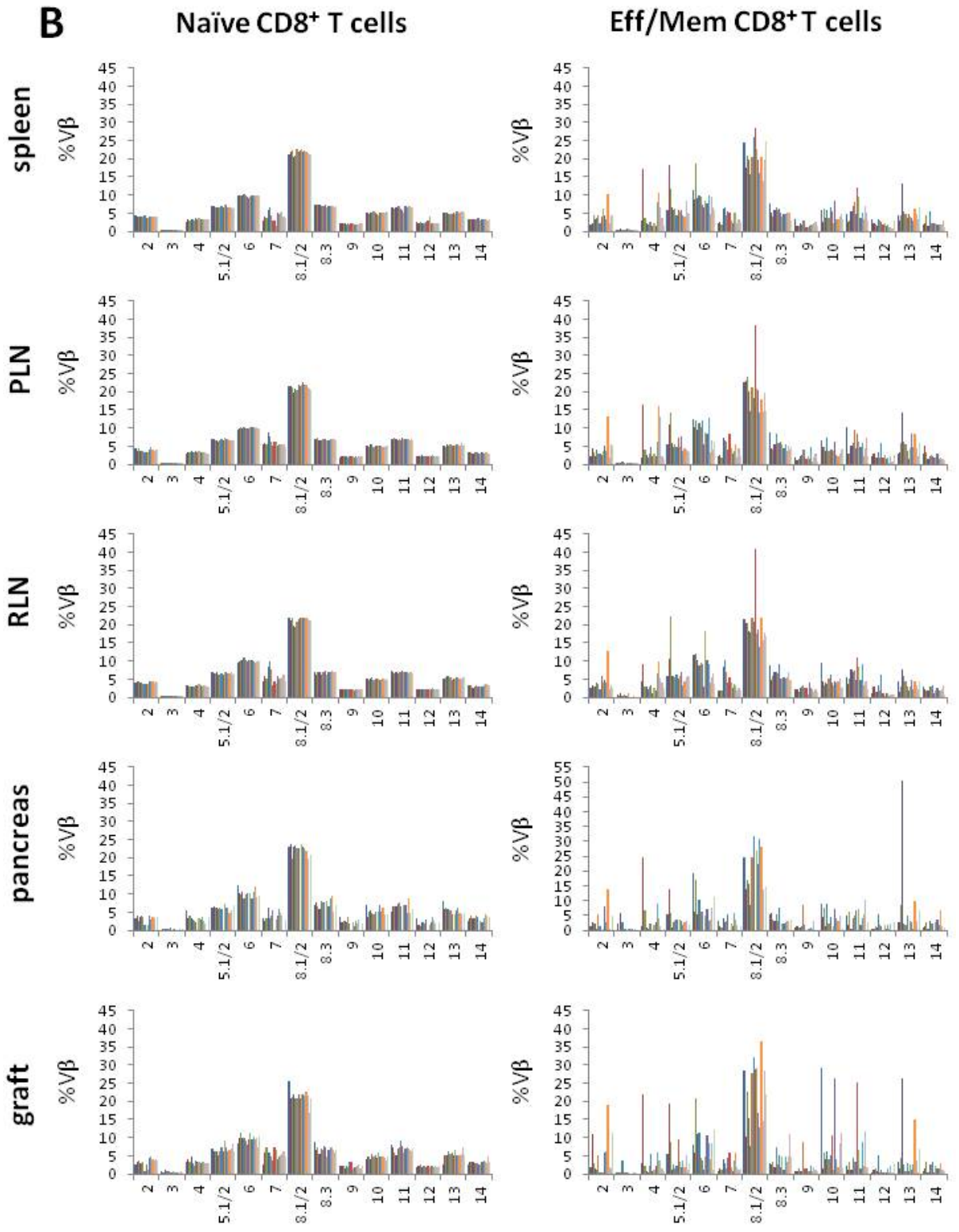


Figure 3.1. Multiple TCRs can be examined in a pool of T cells.
A.) A representative antibody set for examining 6 different TCR Vβ chains within one sample using three channels. B.) Schematic representation of the flow cytometry scatter plots of cells stained with the antibody set in Figure A. Streptavidin(SAV)-PE-Cy7 was used to detect biotinylated (bio) antibodies. For example, αTCR Vβ8.1/8.2-FITC and αTCR Vβ8.1/8.2-bio are used to stain the cells, so cells positive for TCR Vβ 8.1/8.2 will be detected in both the FITC and PE-Cy7 channels and appear double-positive in the FITC-PE-Cy7 schematic plot. C.) Actual flow cytometry scatter plot showing $CD8^+$ T cells stained with antibody set in A. As in the schematic presented in (B), there is a TCR $V\beta2^+$ $FITC^+$ population, a TCR $V\beta5.1/5.1^+$ $PECy7^+$ population, and TCR $V\beta8.1/8.2^+$ $FITC^+$ and $PECy7^+$ population.


A
Naïve CD4+ T cells
Eff/Mem CD4+ T cells
spleen
PLN
RLN
pancreas
graft
%Vβ
0
5
10
15
20
25
30
35
2
3
4
5.1/2
6
7
8.1/2
8.3
9
10
11
12
13
14


B
Naïve CD8+ T cells
Eff/Mem CD8+ T cells
spleen
PLN
RLN
pancreas
graft
%Vβ
2
3
4
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6
7
8.1/2
8.3
9
10
11
12
13
14

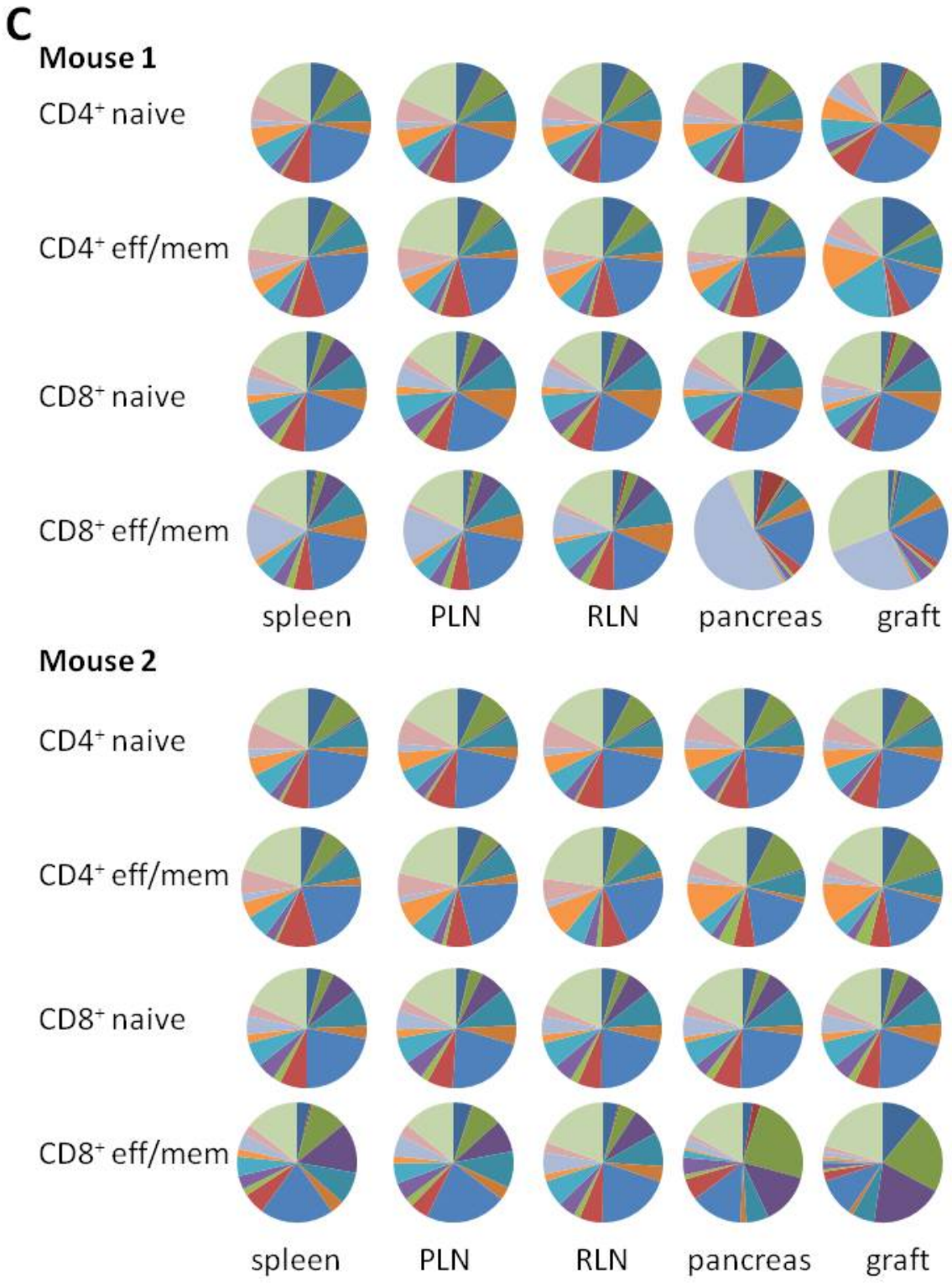
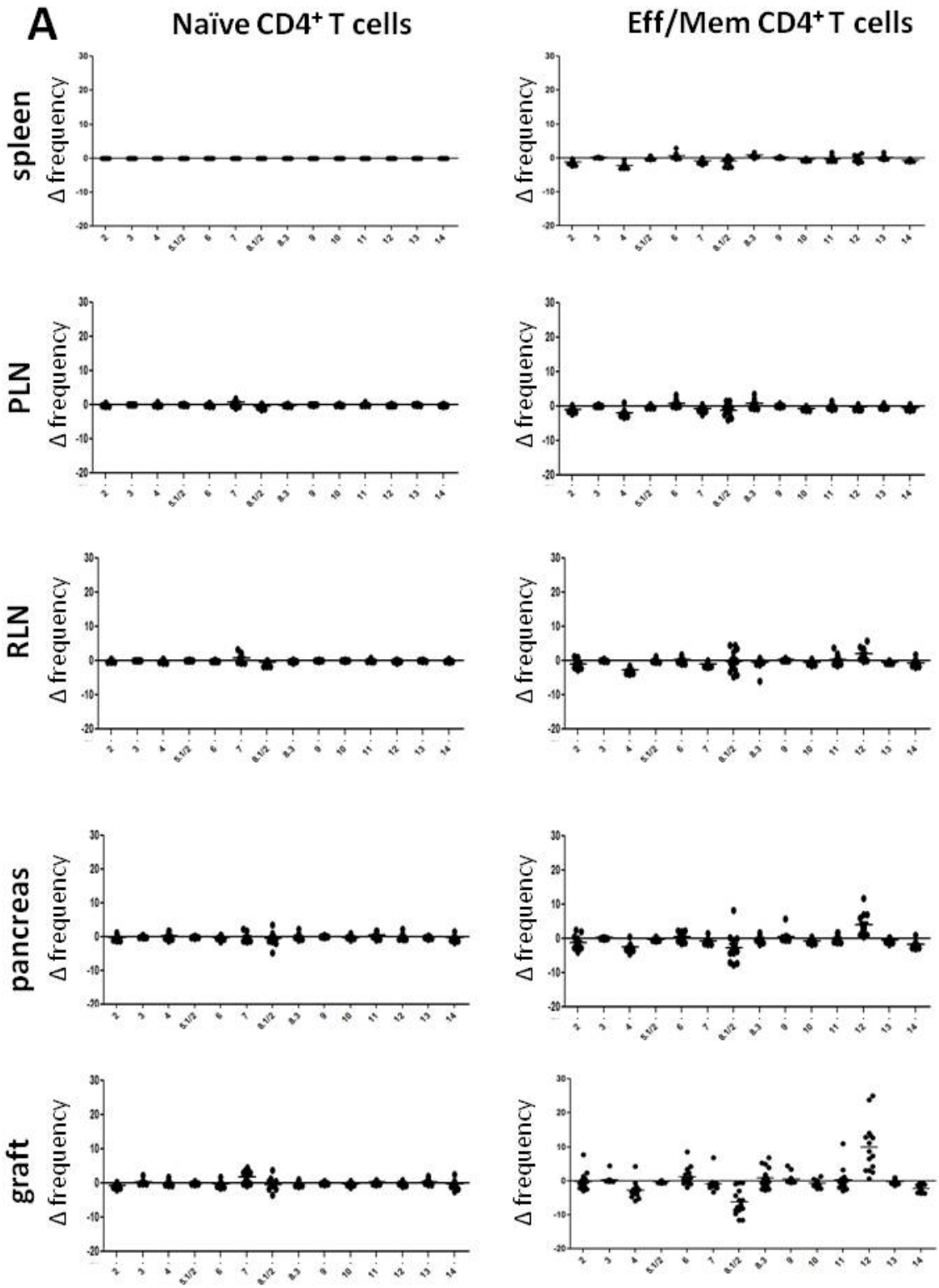
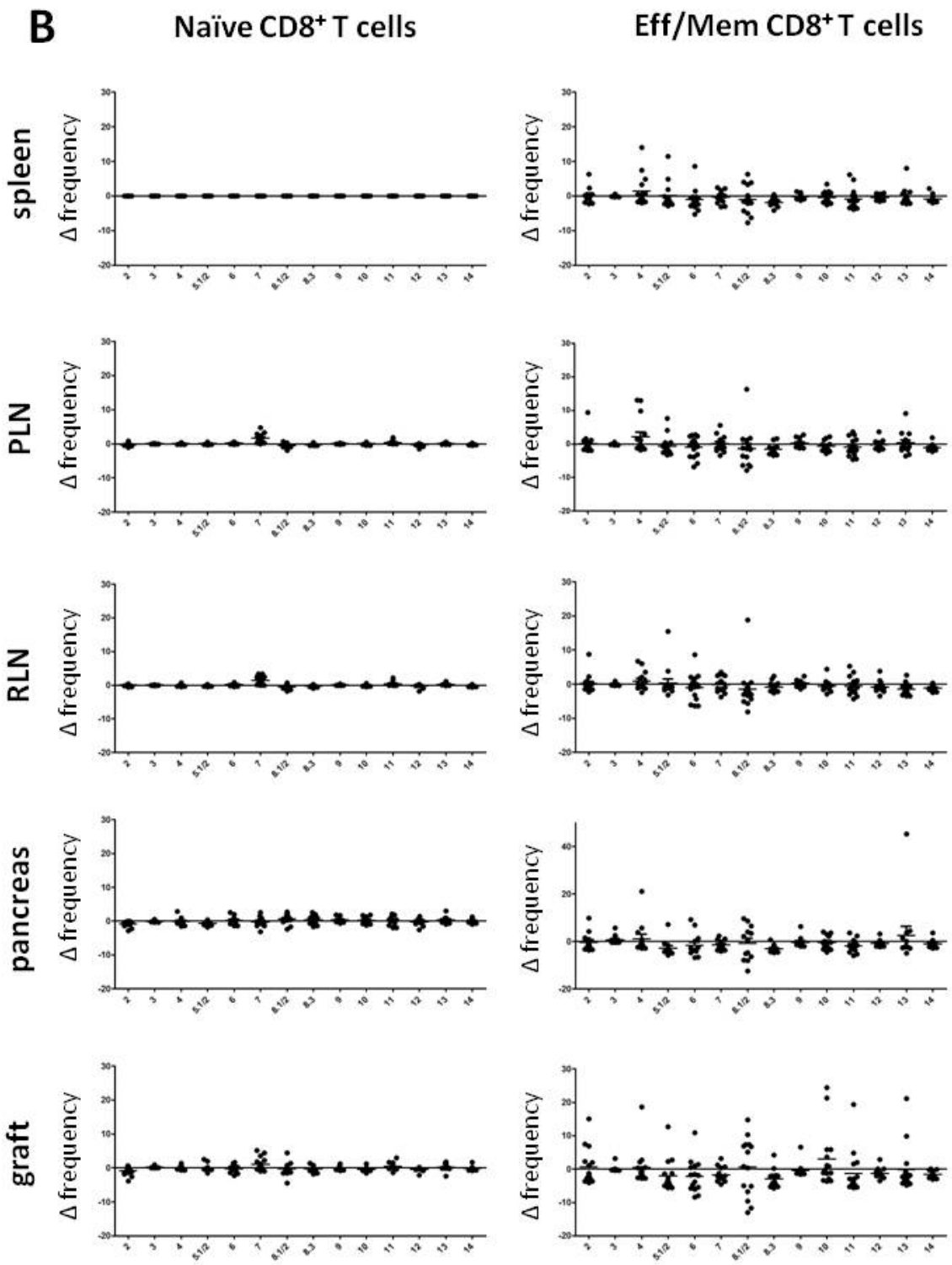

C
Mouse 1
CD4+ naive
CD4+ eff/mem
CD8+ naive
CD8+ eff/mem
spleen
PLN
RLN
pancreas
graft
Mouse 2
CD4+ naive
CD4+ eff/mem
CD8+ naive
CD8+ eff/mem
spleen
PLN
RLN
pancreas
graft

Figure 3.2. Analyses of TCR Vβ chain diversity in various tissues of islet graft NOD recipients.

Tissues from NOD recipients were harvested on day 10-post islet graft implantation, and the percentage of T cells expressing specific TCR Vb chains (A, B) assessed via flow cytometry. A.) Percentage of naïve and eff/mem $CD4^+$ (A) and $CD8^+$ (B) T cells expressing specific TCR Vb chains in spleen, PLN, RLN, pancreas, and islet graft. Individual mice are represented by the same bar color in all panels ($n \geq 13$). (C) Representative data from two individual mice.


A
Naïve CD4+ T cells
Eff/Mem CD4+ T cells
spleen
PLN
RLN
pancreas
graft
Δ frequency


B
Naïve CD8+ T cells
Eff/Mem CD8+ T cells
spleen
PLN
RLN
pancreas
graft
Δ frequency

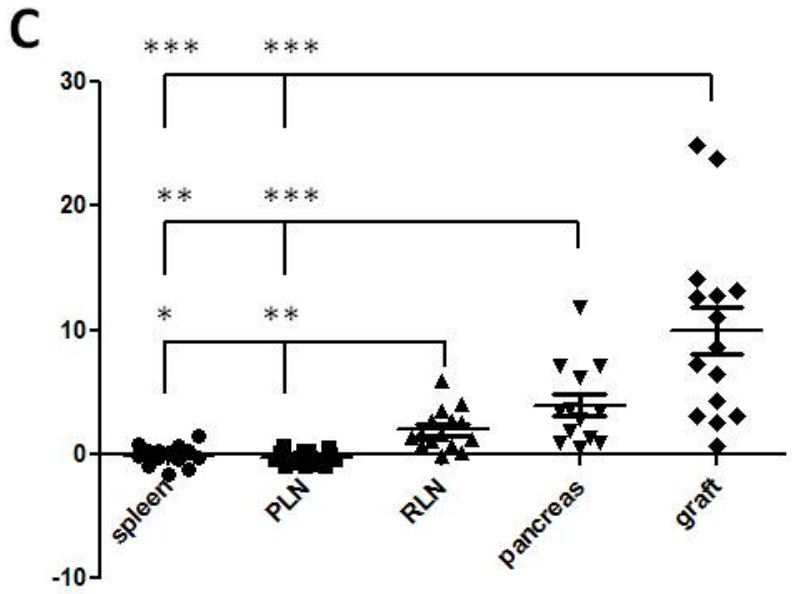


Figure 3.3. Vb12 usage is increased by eff/mem CD4$^+$ T cells in the islet graft and pancreas. TCR Vb usage by eff/mem T cells normalized to the repertoire of naïve splenic T cells in individual recipients (% Vb in islet grafts/pancreas minus % Vb in spleen) (A-C)). CD4$^+$ (A) and CD8$^+$ (B) naïve and eff/mem T cells in the spleen, PLN, RLN, pancreas, and islet grafts of NOD recipients (n>13). Data was normalized to naïve T cells in spleen of the respective recipients C.) % Vb12 in islet graft, pancreas, RLN, and PLN minus % Vb12 in spleen (n>13). *** p<0.001, **p<0.01, *p<0.05, statistical significance was determine using one-way ANOVA (Kruskal-Wallis test with two-sided Dunn's post-test).

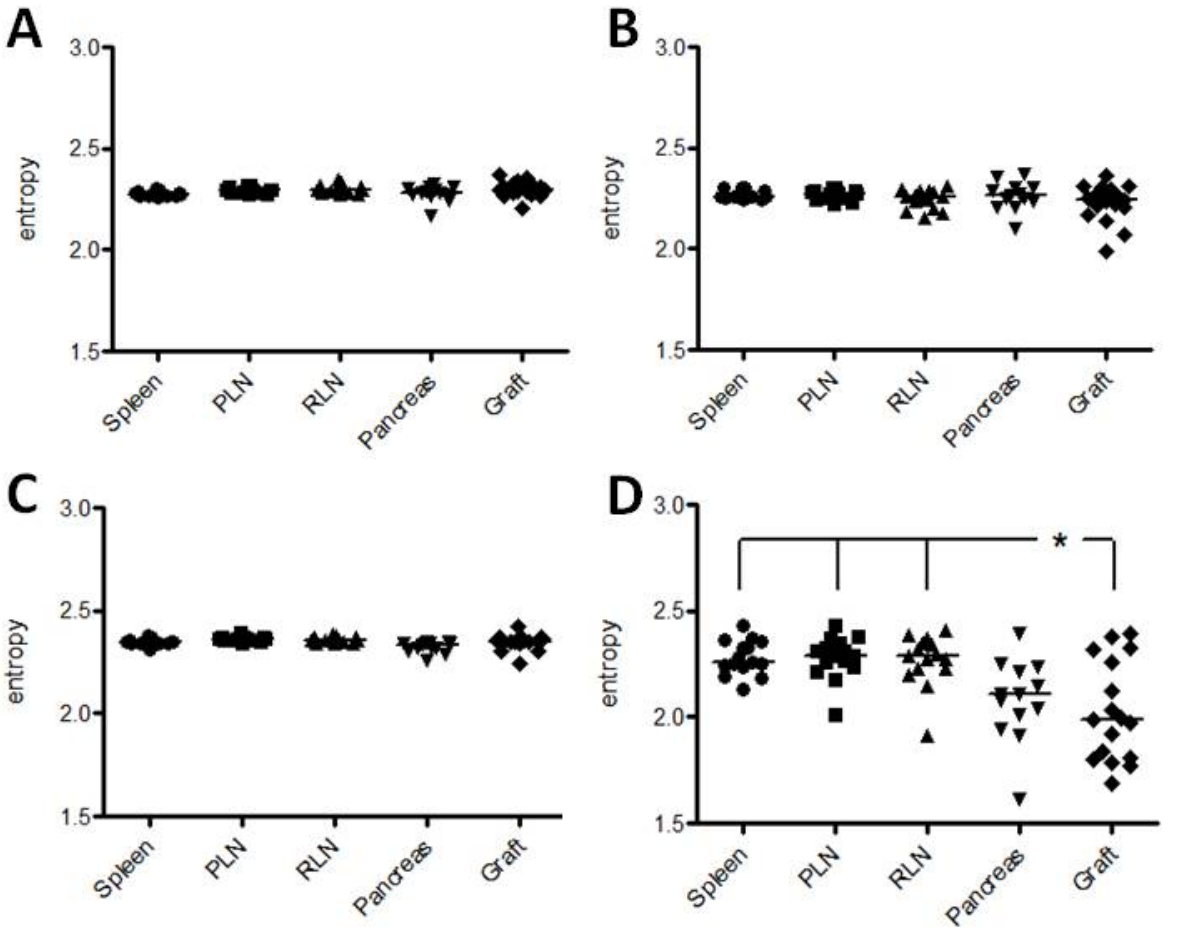


Figure 3.4. Shannon Entropy is decreased in eff/mem CD8$^+$ T cells infiltrating the islet graft.

Diversity of Vb chain usage in different tissues of NOD recipients ($n \geq 13$) was assessed using Shannon entropy. Naïve (A,C) and eff/mem (B,D) T cells expressing CD4$^+$ (A,B) or CD8$^+$(C,D) . *$p<0.05$, statistical significance was determine using one-way ANOVA (Kruskal-Wallis test with two-sided Dunn's post-test).

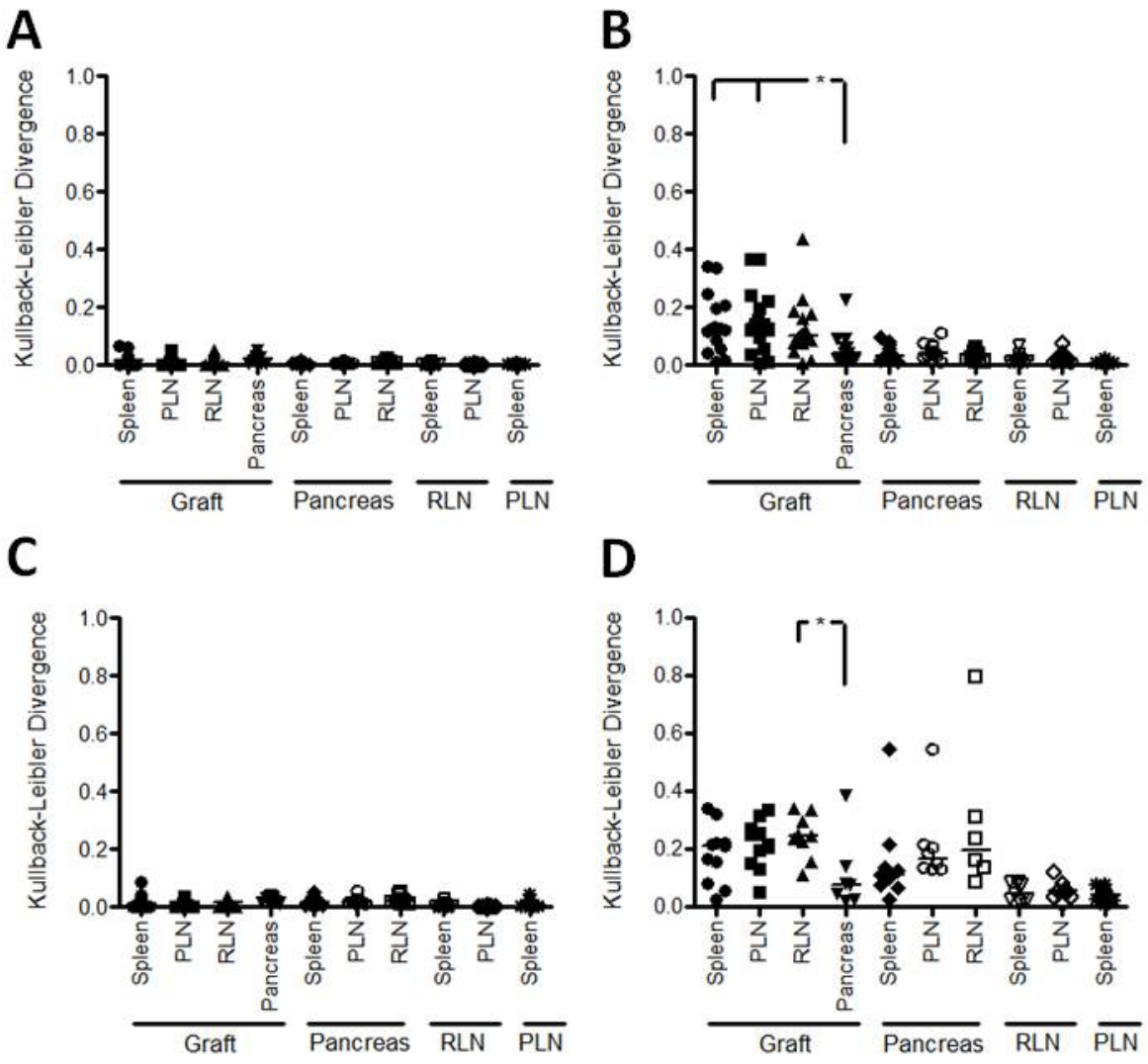
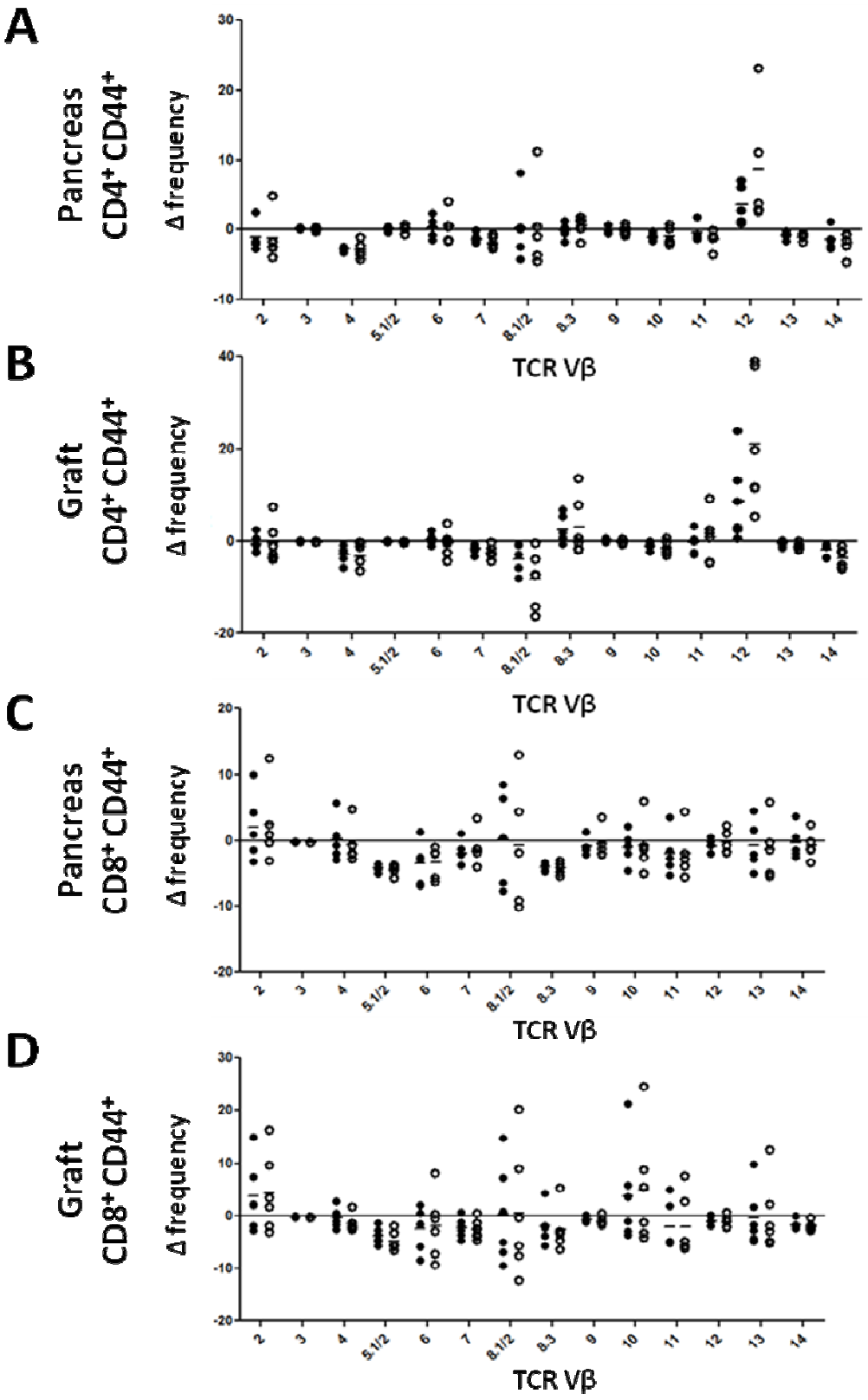


Figure 3.5. The eff/mem TCR Vb repertoire in the islet graft is more similar to the repertoire in the pancreas than the PLN, RLN, or spleen.
Divergence of Vb chain usage in islet grafts of NOD recipients (n>6) was assessed via the Kullback-Leibler divergence test. Naïve (A,C) and eff/mem (B,D) expressing $CD4^+$ (A,B) or $CD8^+$ (C,D) T cells. The X axis represents comparisons for among the respective tissues. *p<0.05, statistical significance was determine using one-way ANOVA (Kruskal-Wallis test with two-sided Dunn's post-test).


A
Pancreas
CD4+ CD44+
Δ frequency
B
Graft
CD4+ CD44+
Δ frequency
TCR Vβ
C
Pancreas
CD8+ CD44+
Δ frequency
TCR Vβ
D
Graft
CD8+ CD44+
Δ frequency
TCR Vβ
2
3
4
5.1/2
6
7
8.1/2
8.3
9
10
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12
13
14

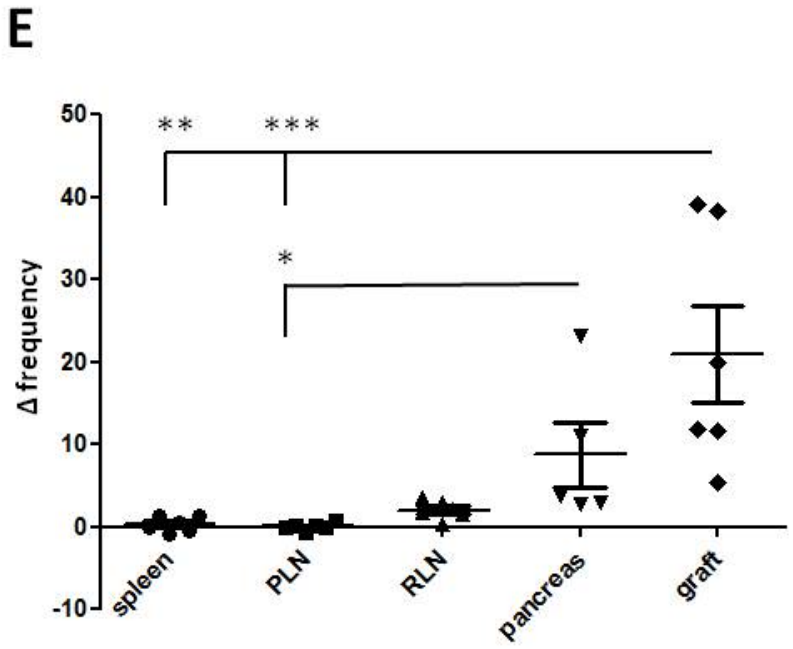

Figure 3.6 TCR Vβ12 proliferation is preferentially increased in pancreas and graft.
Data was normalized to naïve T cells in spleen of the NOD recipients. $CD4^+$ (A,B) and $CD8^+$ (C,D) eff/mem T cells in the pancreas (A,C) and islet graft (B,D) of NOD recipients ($n \geq 13$). Closed circles represent change in %TCR Vb from naïve spleen population. Open circles represent change in %TCR Vb $KI67^+$ population from naïve spleen TCR Vb $KI67^+$ population. Changes in $CD4^+$ Vb12 proliferation in the islet graft, pancreas, RLN, and PLN were examined (E) (n=6). *** $p<0.001$, ** $p<0.01$, * $p<0.05$, statistical significance was determine using one-way ANOVA (Kruskal-Wallis test with two-sided Dunn's post-test).

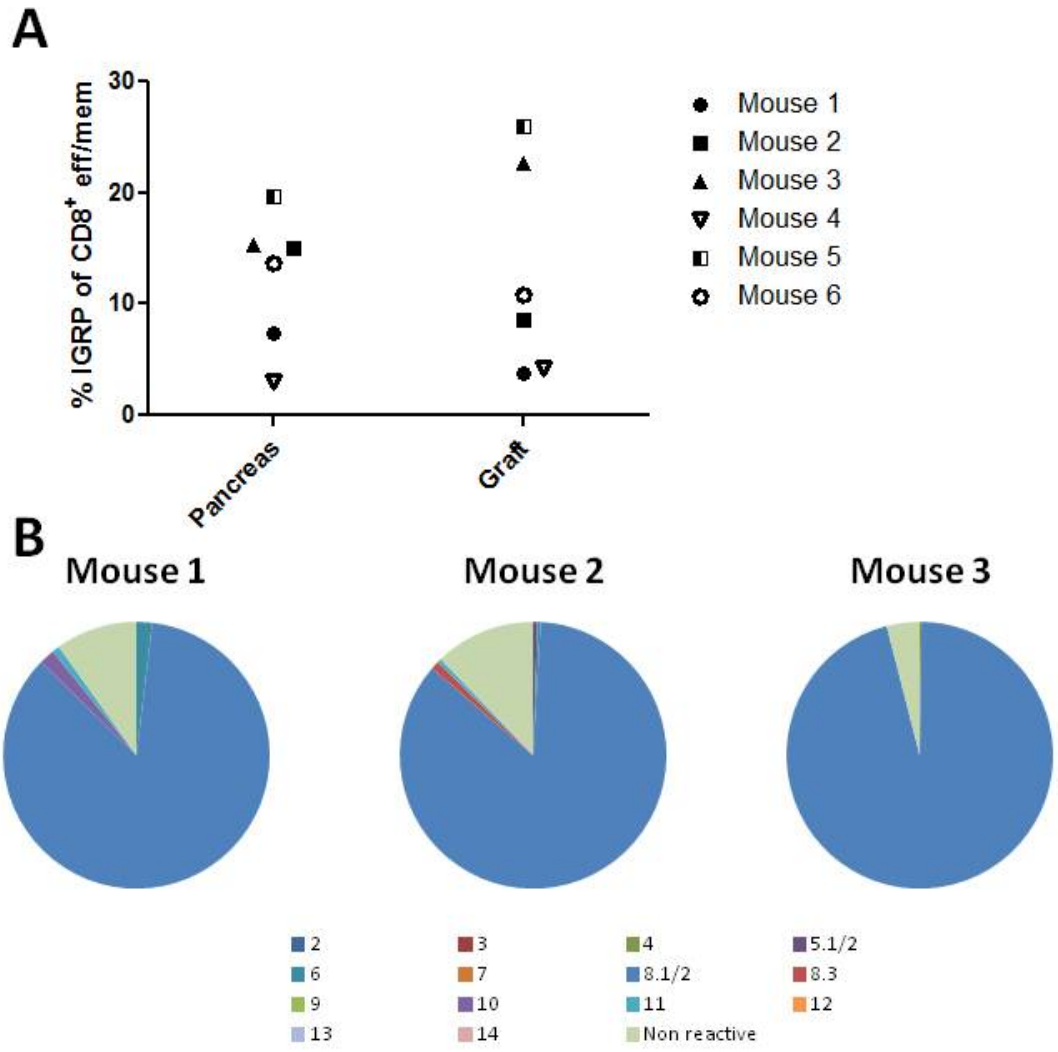

Figure 3.7. Corresponding frequencies of IGRP-specific CD8$^+$ T cells are detected in the islet graft and pancreas of individual NOD recipients.
The frequency of IGRP-H2K^d tetramer binding eff/mem CD8$^+$ T cells was measured via flow cytometry in the pancreas and islet graft. A.) Similar frequencies of IGRP-specific CD8$^+$ T cells are detected in the islet graft and pancreas of individual NOD recipients B.) IGRP-specific CD8$^+$ T cells in the islet graft predominantly express TCR Vb8.1/8.2, with frequencies of 85.4% (mouse 1), 85.4% (mouse 2), and 95.8% (mouse 3).

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CHAPTER 4

FUTURE PERSPECTIVES

4.1. Blocking autoimmune-mediated destruction of islet grafts in diabetic recipients.

Here, we showed that co-administration of the non-depleting anti- (α)CD4 and αCD8 antibodies YTS177 and YTS105, respectively, delayed recurrent autoimmunity in diabetic NOD mice receiving syngeneic islet grafts. In addition, adeno-associated virus (AAV) vector expressing IL-2 via the mouse insulin promoter (MIP) (AAV8-MIP-IL2) was also seen to delay islet graft rejection. Surprisingly, however, the combination of YTS177, YTS105 and AAV8-MIP-IL2 failed to significantly enhance islet graft survival relative to YTS177 and YTS105 treatment alone.

Our findings with YTS177 and YTS105 treatment are in contrast to studies demonstrating induction of long-term allogeneic T cell tolerance in a variety of allograft models by these antibodies[1,2]. One important difference between the respective models is the nature of the pathogenic T effectors. Allogeneic T cells are primarily found in a naïve state at the time of YTS antibody treatment. On the other hand, autoimmune-mediated destruction of syngeneic islet grafts in diabetic NOD mice is likely to be mediated primarily by β cell-specific memory T cells. The latter is supported by our findings made in Chapter 3. We believe that the apparent difference in efficacy of YTS177 and YTS105 treatment to block recurrent autoimmune versus allogeneic mediated graft destruction is attributed to the antibodies having distinct effects on naïve and memory T cells. Studies have shown for instance that YTS177 and YTS105 treatment induces tolerance in skin, cardiac, and bone marrow allograft models, in which the alloreactive T cells are presumably exhibit a naïve phenotype (reviewed in[1-3]). Here, it is believed that antibody binding to the co-receptor molecules establishes a hypo-responsive phenotype, thereby blocking efficient T cell

activation upon antigen recognition[1-3]. Additionally, *in vitro* experiments have demonstrated that YTS177 binding to conventional naïve $CD4^+$ T cells in the presence of antigen and TGFβ resulted in increased FoxP3 expression and conversion into adaptive $FoxP3^+$Tregs[4]. On the other hand, YTS177 and YTS105 binding may have distinct effects on memory T cells. Noteworthy is that the requirements for efficient activation and subsequent proliferation of memory T cells are less stringent than those needed for activation of naïve T cells[5-7]. Indeed, recent work carried out by our group has shown that in an Lymphocytic Choriomeningitis Virus (LCMV) infection model, YTS177 and YTS105 treatment dampened, but did not completely abrogate the response of memory $CD8^+$ T cells, suggesting that memory T cells are more resistant to the effects of the YTS antibodies than naïve T cells (Diz and Tisch, unpublished results). Further definition of the biochemical and molecular events induced by antibody binding of the co-receptor molecules in different subsets of T cells (e.g. naïve, memory) would provide needed insight into the parameters that determine the efficacy of this strategy in different contexts of T cell-mediated pathology.

Although YTS177 and YTS105 treatment failed to induce long-term protection, islet graft survival was enhanced relative to control groups. The mechanism by which the YTS antibodies mediate this transient protective effect needs to be further explored. YTS177 and YTS105 binding did not block the migration of T cells into the islet graft. In addition, equivalent IFNγ secretion by islet graft infiltrating T cells was detected in YTS177 and YTS105-treated and control NOD recipients suggesting that YTS antibody binding had no marked effect on the pathogenic T cells. As discussed earlier, however, IFNγ production was

assayed following *in vitro* PMA/ionomycin stimulation of the T cells, which in turn may "over-ride" an inhibitory effect induced by YTS antibody binding. It is also possible that YTS antibody binding blocks other T effector mechanisms independent of IFNγ secretion. For instance secretion of TNFα and/or IL-1, cytokines known to have cytotoxic effects on β cells, may be down-regulated by YTS antibody binding. Similarly, $CD8^+$ T cell-mediated lysis of β cells may be reduced via inhibition of perforin and/or granzyme b release/production. Intracellular staining and/or qRT-PCR analyses for these different effector molecules in islet graft-infiltrating $CD4^+$ and $CD8^+$ T cells would be a first step to address the latter possibilities.

Additionally, we showed that an adeno-associated virus (AAV) vector expressing IL-2 via the mouse insulin promoter (MIP) was able to significantly prolong islet graft survival, though long-term protection was not established. Importantly, this work provides evidence that recombinant AAV vectors can be used to efficiently transduce β cells *in vitro* as a means to enhance islet graft survival. To improve this approach, a combination of AAV vectors producing IL-2 and TGFβ, which together induce $FoxP3^+$Treg formation, could be employed. Additionally, defining the optimal dose of AAV-MIP-IL2 and corresponding ectopic levels of IL-2 expressed by the transduced β cells is likely to improve the efficacy of this therapy. Further, expression of molecules that enhance β cell viability and/or regeneration, including glucagon-like peptide 1 (GLP-1) and exendin-4 (Ex-4)[8-10], may enhance survival of the islet graft.

4.2 Characterization of the TCR Vβ repertoire in rejecting syngeneic islet grafts.

A significant effort has been made to delineate the specificities and TCR repertoires of diabetogenic T cells. Determining the key autoantigens and corresponding TCR specificities involved in recurrent diabetes may aid in developing approaches to block autoimmune recognition of an islet graft transplanted in T1D patients. Our results show that the $CD8^+$ T cell response in syngeneic islet graft rejection is highly skewed but variable among NOD recipients. These results suggest that particular $CD8^+$ T cell clones are expanded within the graft the site. Currently, whether differences in TCR Vβ usage by islet graft infiltrating $CD8^+$ T cells is reflective of distinct β cell autoantigen/epitope specificities being targeted among individual NOD recipients is unclear. To address this issue, a panel of β cell antigen-specific MHC class I tetramers can be employed to determine the frequency of particular β cell-specific $CD8^+$ T cells found residing in the islet graft and pancreas. Using tetramer staining in conjunction with the TCR Vβ staining protocol developed in Chapter 3, would be an approach to determine if tetramer-specific T cells (specific for β cell antigens) preferentially use certain TCR Vβ chains. Additionally, using a panel of class I tetramers could also determine whether the similarities in TCR Vβ repertoire hold true for β cell autoantigens as well. If so, we would expect that certain tetramer-specific T cell populations would appear more prominently in both the pancreas and the islet graft of some animals but not others, as in the case for IGRP-specific $CD8^+$ T cells. Since a small expansion of the most prominent $CD8^+$ T cell TCR Vβ chains found in the islet graft and pancreas can also be detected in the periphery, it would be interesting to examine the TCR Vβ population in the blood to determine if changes in TCR Vβ usage correlate with the progression of islet graft rejection.

Strikingly, TCR Vβ12 usage was increased in the islet graft-infiltrating $CD4^+$ T cells in all NOD recipients examined. Determining the β cell autoantigen specificity of this population is of obvious interest. As a first step to better define the clonality of these T cells, TCR Vβ12 expressing $CD4^+$ T cells can be sorted and single cells analyzed for TCR CDR3β sequences. If relatively few clones are present based on TCR CDR3β usage, this would suggest that antigen recognition by TCR Vβ12 expressing $CD4^+$ T cells is limited to a few (single?) epitopes. If, however, several clones are detected, it is likely that multiple autoantigenic epitopes are targeted and drive the expansion of TCR Vβ12 in the failed islet grafts. Additionally, MHC class II multimers can be used to directly identify the antigen specificity of the TCR Vβ12 $CD4^+$ T cells. It is notable that $CD4^+$ T cells specific for hsp60 p277[11], GADp524[12], and BDC[13] (chromogranin A[14]) have been reported to use TCR Vβ12. Finally, to determine the relative role of Vβ12 $CD4^+$ T cells in recurrent autoimmunity, NOD recipients can be treated with αVβ12 antibody and tested for prolonged islet graft survival.

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