

# **INTERLEUKIN-2 BASED THERAPY FOR THE TREATMENT OF TYPE I DIABETES**

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## **ABSTRACT**

### **MARK CHRISTOPHER JOHNSON: INTERLEUKIN-2 BASED THERAPY FOR THE TREATMENT OF TYPE I DIABETES**

(Under the direction of Dr. Roland M. Tisch)

Type I diabetes (T1D) is an autoimmune disease characterized by the destruction of the insulin producing  $\beta$  cells. Although multiple cell types contribute, the main mediators of  $\beta$  cell destruction are pathogenic Th1 effectors (Teff). Preferential differentiation and expansion of pathogenic Teff is partly attributed to dysregulation of FoxP3<sup>+</sup> regulatory T cells (FoxP3<sup>+</sup> Treg). Consequently, current strategies for treating T1D have focused on re-establishing the balance between Teff and FoxP3<sup>+</sup> Treg. The aims of the studies described are to: i) analyze the temporal effect of IL-2 on FoxP3<sup>+</sup> Treg and disease incidence, ii) to test whether  $\beta$  cell-specific IL-2 secretion prevents T1D by expanding islet resident FoxP3<sup>+</sup> Treg, and iii) to investigate the synergistic ability of T cell immunotherapies to induce remission in non obese diabetic (NOD) mice.

Our first study demonstrates that NOD mice congenic for a C57BL/6-derived disease-resistant *I2* allele (NOD.idd3) have a reduced incidence of T1D compared to NOD mice. Diabetes protection in NOD.idd3 mice was attributed to elevated systemic IL-2 levels over time that maintained more suppressive FoxP3<sup>+</sup>CD62L<sup>+</sup> Treg in the islets and draining pancreatic lymph nodes (PLN). Therefore, our findings underscore the

relationship between systemic IL-2 expression, FoxP3<sup>+</sup> Treg function *in vivo* and disease incidence.

Our second study investigated the ability of islet-localized IL-2 to prevent diabetes in NOD mice. We found that vaccination with a recombinant adeno-associated viral vector (rAAV) expressing IL-2 under control of the mouse insulin promoter (AAV8mIP-IL2) prevented diabetes. Protection correlated with increased number and function of islet FoxP3<sup>+</sup> Treg. Importantly, the effects of AAV8mIP-IL2 vaccination were islet specific. This shows that IL-2 expression driven by AAV8mIP-IL2 protected NOD mice, highlighting the potential of targeted immunotherapeutic treatment.

Our third study examined the ability of AAV8mIP-IL2 to work synergistically with other immunotherapies to induce remission. Co-administration of AAV8mIP-IL2 to diabetic NOD mice receiving nondepleting antibodies specific for the T cell co-receptors, CD4 and CD8, induced long-term remission. Notably, IL-2 levels in long-term remission NOD mice were elevated and maintained throughout treatment. Collectively, this shows that islet-localized IL-2 driven by rAAV vectors provides a viable immunotherapeutic approach for treating human patients.

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## LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AICD	Activated induced cell death
AIRE	Autoimmune regulatory
APC	Antigen-presenting cell
Bcl-2	B cell lymphoma 2
BCR	B cell receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
DC	Dendritic cell
DP	Double-positive
DS	Double-stranded
EAE	Experimental autoimmune encephalomyelitis
EGFP	Enhanced green fluorescent protein
FoxP3	Forkhead box protein 3
GAD	Glutamic acid decarboxylase
GITR	Glucocorticoid-induced TNFR-related protein
HLA	Human leukocyte antigen
IA2	Tyrosine phosphatase-related islet antigen 2
IBD	Inflammatory bowel disease
ICOS	Inducible T cell co-stimulator
Idd	Insulin dependent diabetes

IFN $\gamma$	Interferon gamma
IGRP	Islet-specific glucose-6-phosphatase
IL	Interleukin
I.M.	Intramuscular
I.P.	Intraperitoneal
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
LFA-1	Lymphocyte function-associated antigen 1
MHC	Major histocompatibility complex
MIP	Mouse insulin promoter
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cell
NK	Natural Killer
NOD	Non-obese diabetic mouse
PAMP	Patter-associated molecular pattern
PLN	Pancreatic lymph node
PTPN22	Protein tyrosine phosphatase non-receptor type 22
rAAV	Recombinant Adeno-associated virus
SS	Single-stranded
SP	Single positive
STAT	Signal transducer and activator of transcription
T1D	Type I diabetes
T2D	Type II diabetes
TCR	T cell receptor
Teff	T effectors

TET-ON	Tetracycline on
TGF $\beta$	Transforming growth factor beta
TLR	Toll-like receptors
TNF $\alpha$	Tumor necrosis factor alpha
TRA	Tissue restricted antigen
Treg	Regulatory T cell
$\alpha$	Alpha
$\beta$	Beta
$\gamma$	Gamma
$\alpha/\beta$	Alpha/Beta
$\gamma/\delta$	Gamma/Delta

## **CHAPTER 1**

### **INTRODUCTION**

#### **1.1 Diabetes Mellitus**

Diabetes mellitus is a collection of metabolic diseases characterized by the body's inability to properly produce and/or respond to insulin, resulting in high systemic blood glucose levels (1). The short peptide insulin is made by a specific cell type in the pancreatic islets of Langerhans, known as  $\beta$  cells, in response to rising systemic glucose levels (2). After its release, insulin induces glucose uptake by myocytes and adipocytes, thus reducing circulating glucose levels. If left unchecked at high levels, glucose can result in a multitude of complications, thereby underscoring the importance of insulin.

Diabetes mellitus can be further subdivided into distinct forms of disease. In addition to gestational diabetes and other situation specific forms which make up only a minor portion of all diabetes patients, the two major forms of diabetes are known appropriately enough as type I and type II diabetes ([www.diabetes.org](http://www.diabetes.org)). Type II diabetes (T2D), formerly known as non-insulin dependent diabetes, comprises 90-95% of the total diabetic population ([www.diabetes.org](http://www.diabetes.org)). It is initially characterized by the body's reduced capacity to respond to secreted insulin, which can be further complicated by inhibited synthesis of insulin from the pancreas over time, and is commonly associated with obesity as a triggering factor (1).



On the other hand, type I diabetes (T1D), formerly known as juvenile diabetes, afflicts 5-10% of the total diabetic population and is found more commonly in industrialized nations ([www.diabetes.org](http://www.diabetes.org)). Unlike T2D in which insulin is still secreted, T1D is classified as an autoimmune disease characterized by the destruction of the insulin producing  $\beta$  cells, resulting in a deficiency in insulin (3). As a result, patients are maintained on life-long insulin therapy, given as daily injections, in order to regulate blood glucose levels. Despite insulin therapy, type I diabetics are susceptible to several complications, including increased risk for heart disease, high blood pressure, blindness, kidney disease, nerve damage, limb amputation and significantly shortened life expectancy (4).

## **1.2 Islet Transplantation for the Treatment of T1D**

Currently, islet transplantation is the only approach offering a potential cure for T1D. Unfortunately, islet transplantation is hindered by a number of obstacles. First, the availability of donor islets is limited. As few as 7,000 donors in the United States each year are available (National Diabetes Information Clearinghouse), with only half being suitable for actual transplantation. Consequently, multiple donors are required for a single diabetic recipient. In addition, islet transplantation recipients are maintained on immune suppressive drugs for the remainder of their life in order to prevent rejection by both allogeneic and autoreactive T cells. Drugs used include daclizumab, sirolimus and tacrolimus, which can result in fatigue, high cholesterol, anemia, and hypertension,

as well as increased susceptibility to opportunistic infection and cancer (5-7). In spite of the widespread immune suppression, recent studies have shown that only 10% of islet transplant recipients remain insulin independent at a 5 year follow-up, even though the majority of patients experienced a decreased need for insulin and/or greater glucose stability (8). Current research is investigating the potential of encapsulating transplanted islets, preventing exposure to, and destruction by, autoimmune and allogeneic mediated events, which may reduce the need for immunosuppressive drugs (9, 10). Although the clinical results have been promising, the long-term burden on recipients and lack of available material for transplantation has hindered the progress of islet transplantation as a viable option in the short term, necessitating the investigation of alternative approaches.

### **1.3 Protective Immunity**

In simplistic terms, the immune system is responsible for the protection against foreign pathogens. Immunity to pathogens is achieved via two complementary arms of the immune system, namely innate and adaptive immunity. The innate immune system responds rapidly, but non-specifically, to extracellular and intracellular pathogens by recognizing common pattern associated molecular patterns (PAMPs) found on bacteria and/or viruses, as well as through activation of the complement system (11, 12). This leads to the recruitment of granulocytes and phagocytes, as well as secretion of various pro-inflammatory molecules, that temper the infectious agent. On the other hand, the

adaptive immune response, comprised primarily of B lymphocytes (B cells) and T lymphocytes (T cells), takes longer to develop and does so in an antigen-specific manner. B cells differentiate into antibody secreting plasma cells after recognition of antigen by the B cell receptor (BCR), in addition to “co-stimulatory” signaling (13). T cells are comprised of various subsets, including cytotoxic T cells, T helper cells and regulatory T cells (Treg). During a normal immune response, all three subsets function in concert to control the infection, with cytotoxic and T helper cells contributing to elimination of the pathogen, while Treg dampen the immune response after clearance to minimize potential damage to surrounding healthy tissue (14-16).

#### **1.4 Central and Peripheral Self-Tolerance**

A key property of the immune system is the capacity to distinguish between foreign and self antigens by both central and peripheral mechanisms. The latter is partly achieved by establishing “self-tolerance”. Very early in life, T cell progenitors migrate from the liver, and later the bone marrow, into the thymus. These progenitors enter the thymus near the cortico-medullary junction and begin the process of forming a hetero-dimeric  $\alpha/\beta$  or  $\gamma/\delta$  T-cell receptor (TCR) (17). This occurs through the rearrangement of the variable (V), diversity (D), and joining (J) gene segments to form functional TCRs with varying specificities (18). After TCR expression, double-positive (DP) thymocytes randomly traffic through the cortex, interacting with cortical thymic epithelial cells (cTECs), which present complexes of self-peptide bound by major histocompatibility complex (MHC)

molecules. TCR binding of these complexes with intermediate affinity and/or avidity (19, 20), delivers signals for DP thymocyte survival and subsequent lineage commitment into either a single-positive (SP) CD4<sup>+</sup> or CD8<sup>+</sup> T cell. Following the latter positive selection events, SP thymocytes then traffic into the medulla (17).

In the thymic medulla, SP thymocytes dwell on average 4-5 days undergoing a process known as negative selection (21, 22). Also known as clonal deletion, negative selection is the process by which SP thymocytes with a high affinity and/or avidity for self-peptide are eliminated, thereby purging T cells with an autoimmune potential (17). This process is mediated by presentation of self-peptides by medullary thymic epithelial cells (mTECs) and thymic dendritic cells (DCs) (23). In turn, the key thymic DCs driving negative selection consist of two conventional DC (cDC) subsets, namely CD11b<sup>+</sup>CD8α<sup>-</sup> /<sup>low</sup> migratory and CD11b<sup>-</sup>CD8α<sup>+</sup> intrathymic cDCs (24, 25).

Additionally, the negative selection process is mediated by the controlled expression of several tissue-restricted antigens (TRA) by the autoimmune regulator (AIRE) transcription factor (26, 27). While AIRE only allows for the expression of TRA on a small subset of mTECs at a time within a finite window, the overall process is thought to be enhanced through transfer of TRA to both thymic resident cDCs, as well as rapid turnover of the mTEC pools every 1 to 2 weeks, thereby increasing the total expression of different TRA (28-31). Notably, both human and mouse studies have shown that defects in AIRE expression lead to systemic autoimmunity (32-35).

Of interest, Treg develop during the process of negative selection from late DP to SP CD4<sup>+</sup> high avidity and/or affinity precursors that do not undergo apoptosis (14, 15, 36). It is noteworthy that mTECs and each thymic DC subset are effective in promoting immature thymocyte differentiation into Treg *in vitro*, suggesting that Treg development is T cell intrinsic, rather than one dependent on a specific antigen presenting cell (APC) interaction (37). Despite this, the interaction of other T cell expressed molecules including CD28, CD40L, and LFA-1 with appropriate binding partners on mTECs and APCs are known to contribute to Treg differentiation (38). The development of “natural” Treg from thymocytes coincides with the expression of the Forkhead box protein 3 (FoxP3), which is generally thought to be a master regulator for FoxP3<sup>+</sup> Treg function (39, 40).

After maturation, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells egress from the thymus into the periphery. While the majority of self-reactive high affinity/avidity thymocytes are deleted during negative selection, high affinity/avidity thymocytes specific for TRA not presented by mTEC and low affinity and/or avidity self-reactive thymocytes do persist (31, 41). Therefore, a number of mechanisms exist to maintain self-tolerance in the periphery. Peripheral tolerance is in part established by the action of tolergenic APC. During an active infection, APC maturation is triggered through binding of microbial products to different innate immune sensors, most notably Toll-like receptors (TLR) (12). TLR ligation signaling results in the up-regulation of the co-stimulatory molecules CD80/CD86 and CD40 on APC, as well as increased MHC class II and MHC class I expression, required for efficient T cell activation (42). In the absence of inflammation, however, the constitutive uptake and presentation of antigen to T cells by APC occurs

without co-stimulation, leading to tolerance induction in naïve T cells (43, 44).

Depending on the strength of signal, T cells may become nonresponsive to subsequent antigen stimulation, a state referred to as anergy, or with stronger signals T cells may be driven to undergo apoptosis. In addition, self-reactive T cells can be eliminated by apoptosis through continued recognition of self MHC complexes (45) involving a process mediated via Bim-dependent and/or Fas receptor engagement by FasL (46, 47).

### **1.5 Treg Play a Key Role in Peripheral Self-Tolerance**

In addition to FoxP3<sup>+</sup> Treg, which develop in the thymus, other Treg subsets differentiate in the periphery from CD4<sup>+</sup>CD25<sup>-</sup> naïve T cells, such as Tr1 and Th3 cells (48, 49). Tr1 cells, which require TGFβ, IL-10 and IL-27 for differentiation, are classically defined by the secretion of IL-10 (50, 51). Importantly, studies have shown that antibody mediated blockade of IL-10 negates the suppressive phenotype of Tr1 cells (49). On the other hand, TGFβ1 is required for differentiation and the suppressor function of Th3 cells (48, 52). Collectively, Tr1 and Th3 function with FoxP3<sup>+</sup> Treg to maintain peripheral tolerance and may benefit from the presence of FoxP3<sup>+</sup> Treg for development, in a process known as “bystander suppression”. Furthermore, the combination of TGFβ1 and IL-2 in the periphery can induce the differentiation of antigen-specific adaptive FoxP3<sup>+</sup> Treg (53). Adaptive FoxP3<sup>+</sup> Treg have been shown to play a role in maintaining peripheral tolerance, specifically in organs with ongoing inflammation, such as the gut.

Early experiments highlighted the importance of FoxP3<sup>+</sup>Treg in maintaining peripheral tolerance. Depletion of CD4<sup>+</sup>CD25<sup>+</sup>T cells, which compromise the majority of FoxP3<sup>+</sup>Treg in non-autoimmune prone mice, resulted in systemic autoimmunity and inflammatory bowel disease (IBD) (54, 55). Furthermore, mutations in the human *FOXP3* gene results in immunodysregulation polyendocrinopathy enteropathy X-linked syndrome, with up to 90% of patients developing T1D, among other diseases, within a few years of birth (56, 57). This human phenotype was later recapitulated in the scurfy mouse model, which express a naturally occurring loss-of-function mutation within *foxp3* (58). FoxP3<sup>+</sup>Treg suppression is mediated by both contact dependent and independent pathways. While no one mechanism is thought to be universally required for FoxP3<sup>+</sup>Treg mediated suppression, the combination of different mechanisms appears to contribute to the overall effectiveness of these Treg.

### **1.6 Modes of FoxP3<sup>+</sup>Treg-mediated Suppression**

In the steady state, FoxP3<sup>+</sup>Treg express high levels of surface CD25, GITR, OX40, CD62L, CTLA-4 and LFA-1, but the relative contribution of each molecule to FoxP3<sup>+</sup>Treg function has been debated. Administration of OX40 agonists in studies investigating experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis, either reduced or increased disease severity depending on the timing of administration (59). Furthermore, OX40 expression was shown to be dispensable for FoxP3<sup>+</sup>Treg mediated suppression, but co-stimulation of OX40 reduced suppressor

activity, suggesting that OX40 is a negative regulator of FoxP3<sup>+</sup> Treg (60). In addition, studies have shown that stimulation of GITR through the use of an agonist antibody abrogates the protective effect of CD4<sup>+</sup>CD25<sup>+</sup> Treg in various settings, including allograft rejection, diabetes, and gastritis, implying that GITR engagement blocks the suppressor function of FoxP3<sup>+</sup> Treg (61-63) .

CD62L plays a role in the migration of both FoxP3<sup>+</sup> Treg and conventional T cells *in vivo* to secondary lymphoid organs (64). Interestingly, FoxP3<sup>+</sup> Treg which express high levels of CD62L have been shown to be more suppressive in T1D mouse models (65, 66) and other diseases (67). Additionally, adoptive transfer of CD62L<sup>HI</sup> FoxP3<sup>+</sup> Treg from the spleen or draining pancreatic lymph nodes (PLN) resulted in enhanced suppression of  $\beta$  cell autoimmunity compared to CD62L<sup>LO</sup> FoxP3<sup>+</sup> Treg (66). Interestingly, augmented IL-2 expression was also shown to directly correlate with increased expression of CD62L on organ resident FoxP3<sup>+</sup> Treg (65), including the islets (M.C.J. and R.T., manuscript in preparation).

Reports also suggest that both CTLA-4 and LFA-1 expression enhance the suppressor function of FoxP3<sup>+</sup> Treg. Deletion or blockade of CTLA-4, which is known to interact with both CD80 and CD86 on APC (68), reduces FoxP3<sup>+</sup> Treg-mediated suppression both *in vitro* and *in vivo* (69, 70). Importantly, elimination of CTLA-4 expression specifically by FoxP3<sup>+</sup> Treg resulted in a phenotype similar to FoxP3-deficient mice (71). Furthermore, CTLA-4 and LFA-1 expression are thought to be intimately linked, as LFA-1-mediated cell adhesion and clustering was shown to be increased upon



CTLA-4 up-regulation (72). Of note, one recent study has shown that CTLA-4 and LFA-1 on FoxP3<sup>+</sup> Treg function collectively to both sequester APC away from responding T cells and cause down-regulation of the co-stimulatory molecules CD80 and CD86 (73). Lastly, CTLA-4 is thought to further alter APC function by down-regulating pro-inflammatory and/or increasing immune suppressive cytokine secretion (74, 75).

Recent studies have also shown that FoxP3<sup>+</sup> Treg express high endogenous levels of CD39 and CD73 (76), both members of ectonucleoside triphosphate diphosphohydrolase family (77, 78). CD39 hydrolyzes ATP, a classic immune “danger signal” into ADP/AMP, while CD73 further breaks down AMP into adenosine (77, 78). Combined, these two receptors serve to protect FoxP3<sup>+</sup> Treg from ATP-induced death, as well as dampen ATP driven maturation of APCs (76). Furthermore, FoxP3<sup>+</sup> Treg have been shown to lyse APCs or responding T cells through granzyme and perforin-dependent mechanisms (79, 80), in addition to secreting soluble immune modulating factors including IL-10, TGFβ, galectin-1 and/or the recently identified IL-35 (81-85). Notably, FoxP3<sup>+</sup> Treg are known to actively deprive responding T cells of pro-survival cytokines, by acting as sinks for IL-2 (86). This function is directly due to constitutively high levels of CD25 expression by FoxP3<sup>+</sup> Treg (87). In sum, these studies highlight the importance of FoxP3<sup>+</sup> Treg in maintaining peripheral tolerance.

## 1.7 Genetic and Environmental Factors Influencing T1D Susceptibility

T1D is a multi-factorial disease, with both genetic and environmental factors contributing to disease susceptibility (88-92). Although ~85% of T1D occurs in the absence of family history, there remains a strong genetic component (93). Among immediate relatives, the highest risk exists among siblings of diabetic patients, which show a 15-fold increase compared to siblings of non-diabetic individuals (93). In addition, children of diabetic fathers and mothers have approximately a 12% and 6% chance of developing disease, respectively, before the age of 20 years (94). Interestingly, the incidence for the second twin developing diabetes decreases significantly with age of diagnosis in the first twin (95). Furthermore, studies have shown that the incidence of both monozygotic twins developing diabetes is >50%, which is reduced 5-fold in dizygotic twins (93).

Currently, over 50 insulin dependent diabetes (*Id*) loci have been identified in humans (90). Of interest, several genes located within these identified regions are directly related to Treg development and/or function, including CD25, CD122, IL-2, IL-10 and CTLA-4, indicating a direct link between Treg and genetic risk for T1D (90, 91)(t1dbase.org). Other known susceptibility genes include insulin, protein tyrosine phosphatase non-receptor type 22 (PTPN22), and AIRE (90, 91) (t1dbase.org). Noteworthy is that AIRE has been shown to regulate in thymic mTECs the expression of insulin, which is believed to be a key autoantigen driving  $\beta$  cell destruction in human T1D (96, 97).

The strongest genetic association with disease susceptibility is linked to the human leukocyte antigen (HLA) region, which contains genes encoding the HLA class I and II molecules. Notably, up to 90% of young type 1 diabetic patients express one or both of the HLA class II susceptibility alleles DR3 and/or DR4 (98). This has prompted increased screening of HLA, in addition to autoantibody analysis, in at risk patients (99, 100). In NOD mice, the expression of the MHC class I H2K<sup>d</sup>/D<sup>b</sup> and MHC class II IA<sup>g7</sup> have also been shown to directly influence disease outcome. Specifically, the role of IA<sup>g7</sup> in diabetes is thought to be due to its unique structural properties, allowing increased binding of a unique repertoire of low affinity peptides (101).

The variable development of T1D in monozygotic twins suggests an environmental component for disease outcome. Noteworthy is that enteroviral infections have been strongly correlated to the emergence of autoantibodies in young at risk individuals (102). Enteroviruses, specifically coxsackie B virus, have been shown to infect  $\beta$  cells *in vitro*, resulting in cell death (103). Furthermore, enterovirus DNA has been detected in the islets of diabetic patients, suggesting that infection may act as an initiating trigger (104). In addition, rotaviruses have been associated with the initiation of disease, as viral proteins are known to mimic the T1D autoantigen glutamic acid decarboxylase (GAD) (105).

Beyond viral initiation, other environmental factors have been debated as potential triggers of T1D. Limited evidence suggests the vitamin D deficiency in infants may increase the risk of developing T1D during infancy (106). Furthermore, the timing

of, and total exposure to, bovine derived milk proteins may influence the development of autoantibodies against bovine proteins (89). Moreover, gluten and other similar proteins have been implicated as driving antigens in T1D as well (107). Lastly, one study has shown that helminth infection of the gastrointestinal track may inhibit diabetes development, possibly through skewing of the immune response away from a destructive type I response (see below), towards a protective type 2 response characterized by IL-4 secreting Treg (108). As a whole, these studies suggest that predicting the emergence of T1D is complicated and involves the collective influence of genetic and environmental factors.

### **1.8 The Non-obese Diabetic (NOD) Mouse Model of T1D**

In human patients, the progressive destruction of  $\beta$  cells typically occurs over a number of years, resulting in the destruction of 80-90% of  $\beta$  cell mass at the time of clinical onset (109). Although a variety of risk factors have been identified, one of the earliest and most reliable markers is the presence of serum autoantibodies, specifically against GAD, protein tyrosine phosphatase (IA2) and/or insulin (92). Importantly, studies have shown that the presence of autoantibodies against all three correlates with the development of T1D in 90% of patients, with risk decreasing in association with number of different autoantibodies present (110). However, understanding the earlier stages of disease has been complicated.

The NOD mouse model has been extremely useful in the study T1D, and closely mimics the disease process in humans (101). Diabetes develops spontaneously in 80-90% of females and 20-30% of males by 35 weeks of age, suggesting a sex related component in disease progression (111). Based on studies in NOD mice, T1D is understood to progress through a number of stages of islet inflammation or “insulitis”. The first stage, known as peri-insulitis occurs between 3 and 4 weeks of age, and is marked by the surrounding of islets by mononuclear infiltrates (101). As NOD mice age, disease progresses to the next stage known as intra-insulitis. Occurring on average at 6-10 weeks of age, intra-insulitis is noted by infiltration of the islets by mononuclear cells, resulting in  $\beta$  cell destruction. This process continues until a sufficient number of  $\beta$  cells have been destroyed leading to the onset of clinical diabetes, typically seen between 12-35 weeks in female NOD mice. The composition of the islet infiltrating population is composed of  $CD4^+$  and  $CD8^+$  T cells, NK cells, B cells, and APCs, including DC and macrophages (111). While all cells are thought to contribute, the major mediators of  $\beta$  cell destruction are T cells (112).

### **1.9 $CD4^+$ and $CD8^+$ T cells are the Primary Mediators of T1D**

Previous work indicates that both  $CD4^+$  and  $CD8^+$  T cells are required for the development and progression of T1D (113-115). The majority of pathogenic  $\beta$  cell-specific T cells exhibit a type I effector phenotype, characterized by the secretion of interferon-gamma (IFN $\gamma$ ) and tumor necrosis factor-alpha (TNF $\alpha$ ) (116). In addition, IL-

17 secreting Th17 cells may also be involved in this process. NOD.*scid* mice injected with diabetogenic BDC CD4<sup>+</sup> T cells skewed *in vitro* towards a Th17 phenotype developed T1D, although the majority of transferred cells converted to IFN $\gamma$  secreting type I effectors after transfer (117). Furthermore, studies show that the frequency of Th17 cells in the infiltrating islet population is low, suggesting that Th17 cells are not the main mediators of  $\beta$  cell destruction (118). Interestingly, antibody mediated neutralization of IL-17 may result in an enhanced Treg frequency through down regulation of the Th17 population (119). This result is not surprising given the mutual requirement for TGF $\beta$  in both FoxP3<sup>+</sup> Treg and Th17 cell development (120) and warrants further investigation.

Given the importance of MHC class II in disease, early studies focused on CD4<sup>+</sup> T cells. Adoptive transfer of diabetogenic BDC2.5 CD4<sup>+</sup> T cell clones was sufficient to induce diabetes in appropriate recipients (121). In addition, blockade using a CD4-specific monoclonal antibody prevented diabetes in NOD mice (122). CD8<sup>+</sup> T cells also play a key role in disease, as NOD mice either lacking MHC class I expression (123-125) or treated with anti-CD8 monoclonal antibodies (126) fail to develop diabetes.

T1D is marked by CD4<sup>+</sup> and CD8<sup>+</sup> T cells targeting multiple  $\beta$  cell autoantigens (127).  $\beta$  cell autoantigens recognized by CD4<sup>+</sup> T cell include the 65 kDa isoform of GAD (GAD65), IA-2, insulin B chain, proinsulin and heat shock protein 60 (128, 129).  $\beta$  cell autoantigens recognized by CD8<sup>+</sup> T cells include islet-specific glucose-6-phosphatase, insulin-B chain, and dystrophin myotonia kinase (128, 129). Proinsulin has been

suggested by some as the initiating autoantigen in mouse models, as thymic deletion of the second proinsulin isoform resulted in accelerated diabetes in NOD mice (130), while thymus specific transgene expression of proinsulin was found to be protective (131). However, NOD mice tolerized with proinsulin had reduced incidence (132), but were not completely protected from diabetes, and the role of proinsulin in human patients is not as clear (133, 134). Despite this, the  $\beta$  cell specificity of diabetogenic T cells is known to increase with time, in a process known as epitope spreading (135, 136). Therefore, the potential role of other autoantigens in disease progression has been investigated.

IA-2 is known to be required for normal insulin secretion, but deletion of the IA-2 gene in NOD mice did not alter diabetes incidence, suggesting a dispensable role in driving  $\beta$  cell autoimmunity (137). Additionally, NOD mice deficient in GAD65 expression (138, 139) have similar diabetes incidence compared to controls. This particular outcome was surprising, given that GAD65 is associated with early immune responses in the islets, and that GAD65 administration induces Treg and prevents diabetes in NOD mice (140, 141). Studies have indicated that administration of insulin B chains to pre-diabetic NOD mice also efficiently protects against diabetes (129). As a result, clinical trials have investigated the usefulness of insulin-based immunotherapy to suppress  $\beta$  cell autoimmunity in at risk and diabetic patients, with at best modest results (133, 142, 143). To further complicate matters, as of yet unidentified  $\beta$  cell autoantigen are thought to also contribute to pathogenicity (144).

The large number of  $\beta$  cell autoantigens targeted in T1D at late preclinical and clinical stages provide a major challenge in developing immunotherapies to directly tolerize pathogenic effector T cells (Teff) (135, 136). Accordingly, an emphasis has been placed on strategies that efficiently manipulate the Treg pool in a  $\beta$  cell-specific manner (136, 145-147).

### **1.10 The Role of IL-2 in Autoimmunity**

IL-2 is one member of a family of cytokines bound by receptors containing the common  $\gamma$ -chain, which includes IL-4, IL-7, IL-9, IL-15 and IL-21 (148). Like most members of this cytokine family, IL-2 has pleiotropic effects on the immune system. While the majority of studies have investigated the effect of IL-2 on T cells, it is also known to induce signaling in B cells, NK cells and eosinophils, among others (149-151). IL-2 is primarily secreted by activated Teff and is required for the proliferation and survival of conventional T cells (87, 152), influencing for instance the expression of several anti-apoptotic markers, including Bcl-2 (153). Conversely, high levels of IL-2 are known to induce apoptosis in Teff through a process known as activated induced cell death (AICD) (154). Evidence also suggests DCs are a minor source of IL-2 *in vivo* (155). Earlier studies investigating IL-2 showed that IL-2 deficiency surprisingly resulted in autoimmunity (156, 157). Furthermore, in mice lacking expression of either CD25 or CD122, which are components of the IL-2 receptor, autoimmunity was also observed, suggesting a critical role for the IL-2-IL-2R pathway in establishing and/or maintaining peripheral self-



tolerance (158, 159). The emergence of autoimmunity in the absence of IL-2 was later shown to be linked to the lack of FoxP3<sup>+</sup> Treg development and/or maintenance (87, 160, 161). In addition, antibody blockade of IL-2 was also shown to reduce FoxP3<sup>+</sup> Treg numbers, resulting in systemic autoimmunity (162). Moreover, ectopic expression of FoxP3 (163) or adoptive transfer of wild-type FoxP3<sup>+</sup> Treg (160) into CD122-deficient mice restored FoxP3<sup>+</sup> Treg function and immune homeostasis.

As previously stated, FoxP3<sup>+</sup> Treg constitutively express high levels of CD25 (54). CD25 is also up-regulated on recently activated Teff and B cells, but only transiently, making high levels of CD25 expression a reliable marker for Treg identification and/or isolation (87, 149). Additionally, FoxP3<sup>+</sup> Treg suppressor function has been directly linked to an enhanced ability to “soak-up” exogenous IL-2 from Teff cells *in vivo*, due to constitutive CD25 expression (86). After binding to the IL-2R, IL-2 induces the phosphorylation of STAT5 (164), which regulates the expression of various FoxP3<sup>+</sup> Treg related genes, including FoxP3 itself (165, 166). Furthermore, ablation of STAT5 in mice results in a reduced FoxP3<sup>+</sup> Treg pool (166, 167).

Studies in human T1D patients have shown a reduction in FoxP3<sup>+</sup> Treg function and/or number (168, 169). In addition, polymorphisms in IL-2, CD25, and CD122 have been linked to T1D and other autoimmune diseases (170, 171). NOD mice are known to express low levels of IL-2 (153, 172, 173), which is associated with the IL-2 gene containing *idd3* locus (173-176). A direct link between  $\beta$  cell autoimmunity and reduced FoxP3<sup>+</sup> Treg over time has also been shown (177, 178). Interestingly, our laboratory has

shown that replacement of the NOD *idd3* locus with one derived from C57BL/6 mice resulted in a significant reduction in diabetes incidence (65). This protection was mediated by enhanced FoxP3<sup>+</sup> Treg function and will be discussed at length in Chapter 2.

Recombinant (r) IL-2 has been successfully used to treat various human diseases. Separate studies showed that graft-versus-host-disease or Hepatitis C vasculitis were suppressed by low dose rIL-2 therapy, and a subsequent increase FoxP3<sup>+</sup> Treg (179, 180). Of note, the majority of patients in these studies were refractory to other treatment regimes. In NOD mice, treatment with low-dose rIL-2 or with rIL-2-anti-IL-2 antibody complexes increased islet FoxP3<sup>+</sup> Treg survival and frequency in pre-diabetic animals (153). However, increased doses of rIL-2 had significant off-target effects, resulting in systemic activation of NK cells, CD8<sup>+</sup>, and CD4<sup>+</sup> T cells and accelerated onset of overt diabetes. Importantly, these findings underscore how critical the dose of IL-2 is for mediating a protective effect. Diabetes remission was also achieved in recent onset NOD mice treated with low dose rIL-2 alone, or in combinatorial therapy (181, 182). Despite the successes achieved in NOD mice, a recent study in human T1D patients showed that combinatorial treatment with rIL-2 and rapamycin did not significantly alter disease progression or remission (183). Early after treatment, a slight, but transient, increase in FoxP3<sup>+</sup> Treg was seen. Nevertheless, NK cells and eosinophils were increased, and  $\beta$  cell autoimmunity exacerbated. Regardless, IL-2 based therapies for the treatment of T1D are still generally considered to be promising approach to manipulate  $\beta$  cell autoimmunity.

### **1.11 Clinical Scenarios for the Treatment of T1D**

Clinically, there are three main scenarios in which immunotherapy can be applied for the prevention and/or treatment of T1D (8, 184-188). The first, and arguably most important, would be the prevention of T1D onset in at-risk patients. At risk individuals are typically identified by the detection of  $\beta$  cell autoantibodies in the serum, in addition to altered responses upon glucose challenge (189).

Secondly, immunotherapy can be applied to rescue residual  $\beta$  cell mass in recent onset diabetics. At the onset of clinical diabetes there is still a sufficient number of  $\beta$  cells remaining so that hyperglycemia can be reversed providing inflammation is effectively and rapidly suppressed (184). Furthermore, protecting residual  $\beta$  cell mass may provide a “starting pool” for strategies that promote  $\beta$  cell expansion (190). Alternatively, other cells within the pancreas, or tissues such as the liver, can be genetically manipulated to become insulin secreting cells, as well (190, 191). In order to promote  $\beta$  cell differentiation or replication, however, it is essential that  $\beta$  cell autoimmunity is suppressed.

Lastly, immunotherapy can be applied to block autoimmune recognition of islet grafts transplanted into diabetic recipients. As previously stated, the 5 year success rate for achieving insulin independence for recent transplant recipients is low, despite the use of potent immunosuppressive drugs (8). Immunotherapies that selectively suppress allogeneic and autoimmune-mediated destruction of transplanted islets would also avoid the severe complications associated with immunosuppression.

### 1.12 Strategies of Immunotherapy

In order to prevent and suppress  $\beta$  cell autoimmunity, immunotherapies to date have focused on re-establishing the functional balance between pathogenic Teff and Treg. This has been accomplished through several strategies, including manipulation of APC populations, dampening of Teff and/or induction or expansion of various Treg subsets. Clinically, administration of non-mitogenic anti-CD3 antibodies to recent onset diabetic patients has been shown to maintain  $\beta$  cell mass (192-194). The overall effectiveness of this strategy, however, is short lived and systemic depletion of T cells leaves patients susceptible to viral infection (192-194).

Administration of  $\beta$  cell antigens and peptides to selectively manipulate diabetogenic T cell activity has also been investigated. Such an approach has been used to specifically deplete and/or induce anergy in diabetogenic Teff and/or stimulate the differentiation and expansion of Treg (135). By targeting  $\beta$  cell-specific T cells only, the overall effect on the immune system is minimal. Previous studies have shown that antigen-specific immunotherapies are highly effective at preventing diabetes in NOD and transgenic models of T1D (141, 195-197). This protection is mediated by the induction of antigen-specific Treg. Unfortunately, the efficiency of antigen-specific immunotherapy in the clinic has been largely disappointing (142, 143, 198). Lack of clinical success, in part, may be attributed to timing, as the pool of pathogenic CD4<sup>+</sup> and CD8<sup>+</sup> Teff is relatively large and multiple autoantigens are targeted at late preclinical and clinical stages of disease (135, 136). The latter is also problematic in terms of inducing

and/or expanding a Treg pool sufficient to suppress the ongoing  $\beta$  cell autoimmunity. To combat this, the focus of various immunotherapies has been the systemic induction/expansion of Treg, employing for instance, cytokine-based strategies (136, 145-147). Since Treg are able to suppress in a non-antigen specific manner, enhancement in Treg number or function would be expected to be beneficial (136, 145, 146, 187, 199). In T1D mouse models, treatment with rIL-4 and rIL-10 enhances Treg populations, and prevents diabetes (200, 201). As stated previously, low dose systemic administration of rIL-2 to NOD mice results in diabetes protection through selective enhancement of islet FoxP3<sup>+</sup> Treg (153). While effective, cytokine-based therapy typically requires continuous dosing of animals, due to the short *in vivo* half-life of cytokines, and may not prove feasible for long-term treatments. Furthermore, since treatment is systemic the pleiotropic effects of most cytokines are likely to result in unwanted off-target effects.

### **1.13 Gene Delivery via Recombinant Adeno-Associated (rAAV) Vectors**

Adeno-associated virus (AAV) is a member of the Parvoviridae family in the genus Dependovirus (202). Structurally, the viral shell is composed of icosahedral subunits that collectively reach approximately 25 nm in diameter and house a 4.7 kb single stranded DNA genome (203). AAV was originally discovered as a contaminant in adenovirus preparations and requires a helper virus for productive replication and infection (204, 205). Recombinant (r) AAV vectors have a number of properties that are amenable for

gene delivery in the clinic and have been effectively used for the treatment of a broad range of diseases, including macular degeneration, rheumatoid arthritis, hemophilia and Parkinson's disease (206-209). rAAV vectors are capable of infecting both dividing and non-dividing cells (210). In addition, AAV has not been directly associated with any human disease, and typically displays low immunogenicity (202). The safety of rAAV vectors has been further increased by vector modifications that prevent self-replication. In conjunction, AAV typically exist as non-integrating circular monomers or concatemers in the nucleus, with minimal risk of genomic insertion (210).

Several capsid protein serotypes, which display a wide tissue tropism, can be used to package the AAV vectors (211, 212). The use of different serotype capsid proteins, in combination with cell- or tissue-specific promoters, permit targeted *in vivo* transgene expression. Recent advances in our understanding of viral receptors and capsid protein structures have resulted in the development of tissue-specific capsids. This has been accomplished through the utilization of both 1) random peptide libraries to generate capsids for previously resistant tissues (213-215), and 2) pseudotyped capsids developed through the swapping of established amino acids sequences for different serotypes (216-218). As a whole, these characteristics may lead to the development of capsids that promote efficient and cell-specific transduction, a primary target of which could be  $\beta$  cells.

Advances in rAAV vector design has resulted in self-complementary or double-stranded (ds) rAAV vectors. dsAAV vectors exhibit more robust and efficient transgene

expression, thereby reducing the overall dose needed in comparison to single-stranded (ss) rAAV vectors; the latter also minimizing the likelihood of eliciting AAV vector-specific immunity (219-222). Lastly, recent changes in both the manufacturing and purification of rAAV vectors have increased the feasibility for clinical use (223).

#### **1.14 Applications of rAAV Vector-Based Immunotherapy for T1D**

Initial studies utilized rAAV vectors to block  $\beta$  cell autoimmunity via systemic expression of the transgene-encoded protein. Intramuscular (I.M.) injection of rAAV vectors encoding the  $\beta$  cell autoantigen GAD65 (224, 225) or IL-10 (226-228) prevented diabetes in NOD mice at both early and late pre-clinical stages of T1D. Protection was mediated primarily through induction and/or expansion of a protective Treg, in addition to suppression of proinflammatory APC. In addition, I.M. delivery of rAAV vectors encoding human  $\alpha$ 1-anti-trypsin (229), a serine protease inhibitor, and heme-oxygenase-1 (230), a stress-response enzyme, have also been shown to suppress established  $\beta$  cell autoimmunity through suppression of innate effector cells. Of note, transgene expression in treated NOD mice persisted for a number of weeks post-injection and was both dose-dependent and stable. This suggests that immune recognition of transduced cells was minimal and highlights one of the distinct advantages rAAV vectors have over other viral-based gene delivery approaches.

rAAV vectors can be engineered to minimize potential complications associated with long-term systemic expression of immunoregulatory proteins through two

strategies. The first involves the use of an inducible promoter, which allows for controlled “on/off” expression of the transgene. Recent work by our group has shown that a rAAV1 vector expressing IL-2 under control of a tetracycline inducible promoter suppressed  $\beta$  cell autoimmunity in NOD mice at a late pre-clinical stage (231). Long-term protection was induced following only a 3 week period of IL-2 expression, which correlated with an increased frequency of islet FoxP3<sup>+</sup> Treg. Importantly, the level and duration of IL-2 expression had no systemic effects on conventional T cells or innate effectors, such as NK cells. Collectively, these findings show that rAAV vector mediated inducible transgene expression can significantly alter  $\beta$  cell autoimmunity, while having minimal effects on the rest of the immune system.

A second strategy to limit the systemic effects of transgene expression *in vivo* is to engineer rAAV vectors with a tissue specific promoter. For the treatment of T1D,  $\beta$  cell-specific promoters, such as the insulin promoter, have been used (232, 233). In a streptozotocin-induced model of T1D, treatment of BALB/c mice with dsAAV8 expressing glucagon-like peptide-1 under control of the mouse insulin promoter (mIP) reversed diabetes (234). Additionally, a recent study showed that administration of a dsAAV vector expressing IL-4 driven by mIP resulted in a reduced diabetes incidence in young NOD mice (235). This protection was also shown to be at least partially due to increased Treg. In addition to limiting complications associated with systemic expression, increased local levels of the transgene product (i.e. cytokine) would be expected to enhance the potency of the protective effect. Indeed, I.M. injection of



rAAV2 vector expressing IL-4 systemically failed to prevent diabetes in NOD mice, in contrast to IL-4 expression targeted to  $\beta$  cells by dsAAV8 mIP-IL4 (226, 235).

While linking transgene expression specifically to the function of  $\beta$  cells is desirable, the ability of rAAV vectors to specifically transduce these cells is of paramount concern. As such, both serotype and route of administration have proven to be critical for influencing the efficiency of  $\beta$  cell transduction. Direct intrapancreatic injection of rAAV8 expressing EGFP resulted in transduction of both acinar cells and  $\beta$  cells (236). Furthermore, rAAV8 was found to be superior to other serotypes, including rAAV1, rAAV2 and rAAV5, through the same route of administration.

### **1.15 Aims of the Dissertation**

IL-2 directly affects a variety of cell types, including B cells, Teff, and FoxP3<sup>+</sup> Treg. Blocking the IL-2-IL-2R signaling pathway results in depletion of FoxP3<sup>+</sup> Treg and ensuing autoimmunity (87). Furthermore, studies have shown that NOD mice have a defect in systemic IL-2 levels compared to nonautoimmune prone strains that correlates with reduced FoxP3<sup>+</sup>Treg and subsequent development of T1D. Despite this, how pancreatic levels of IL-2 impact  $\beta$  cell autoimmunity is poorly understood. Furthermore, although IL-2 therapy has the potential of being a highly effective strategy to suppress  $\beta$  cell autoimmunity, improved approaches that can be safely applied to the clinic are needed. To investigate the effects of pancreatic IL-2 on  $\beta$  cell autoimmunity, NOD and NOD.*idd3* mice were examined temporally for organ-specific FoxP3<sup>+</sup> Treg frequencies and

function. We hypothesized that elevated levels of IL-2 in the pancreas of NOD.*idd3* mice increases islet-resident FoxP3<sup>+</sup> Treg and therefore prevents the onset of overt diabetes.

Treatment with rIL-2 significantly increased the survival and frequency of systemic FoxP3<sup>+</sup> Treg in both pre-diabetic and recent onset NOD mice (153, 181, 182). Due to effects on non-FoxP3<sup>+</sup> Treg, however, disease was exacerbated in NOD mice receiving a high dose of rIL-2. The effects of islet derived IL-2 in comparison to non-targeted systemic IL-2 has not been investigated. Accordingly, we engineered a rAAV8 vector encoding a mIP-driven IL-2 transgene (AAV8mIP-IL2). Administration of AAV8mIP-IL2 permits a direct assessment of the therapeutic effects of localized versus systemic IL-2 in NOD mice on FoxP3<sup>+</sup> Treg and non-FoxP3<sup>+</sup> Treg, and the progression of  $\beta$  cell autoimmunity. We hypothesized that treatment with AAV8mIP-IL2 results in significant increases in islet resident FoxP3<sup>+</sup> Treg function and fitness, resulting in protection from diabetes, while minimizing potential complications associated with systemic IL-2 delivery.

Efforts are ongoing within the field to test combinatorial immunotherapies, with the aim of establishing synergism between the respective strategies and inducing robust tolerance. Accordingly, we investigated the efficacy of AAV8mIP-IL2 combined with  $\alpha$ CD4/ $\alpha$ CD8 non-depleting monoclonal antibodies to reverse diabetes in NOD mice. We hypothesized that  $\alpha$ CD4 and  $\alpha$ CD8 antibody binding to islet resident CD4<sup>+</sup> and CD8<sup>+</sup> Teff, respectively, enhances the efficacy of AAV8mIP-IL2 to induce remission in recent onset

diabetic NOD mice. In summary, these studies provide insight into the role that IL-2 plays in immunoregulation of  $\beta$  cell autoimmunity, in addition to the potential of “targeted” IL-2 expression for the prevention and treatment of T1D.

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## CHAPTER 2

### REDUCED IL-2 EXPRESSION IN NOD MICE LEADS TO A TEMPORAL INCREASE IN CD62L<sup>LO</sup>FOXP3<sup>+</sup>CD4<sup>+</sup> T CELLS WITH LIMITED SUPPRESSOR ACTIVITY

#### 2.1 Summary

IL-2 plays a critical role in the induction and maintenance of FoxP3-expressing regulatory T cells (FoxP3<sup>+</sup> Treg). Reduced expression of IL-2 is linked to T cell-mediated autoimmune diseases such as type 1 diabetes (T1D), in which an imbalance between FoxP3<sup>+</sup> Treg and pathogenic T effectors exists. We investigated the contribution of IL-2 to dysregulation of FoxP3<sup>+</sup> Treg by comparing wildtype NOD mice with animals congenic for a C57BL/6- derived disease-resistant *I2* allele and in which T cell secretion of IL-2 is increased (NOD.B6Idd3). Although NOD mice exhibited a progressive decline in the frequency of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg due to an increase in CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg, CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg were maintained in the pancreatic lymph nodes and islets of NOD.B6Idd3 mice. Notably, the frequency of proliferating CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg was elevated in the islets of NOD.B6Idd3 versus NOD mice. Increasing levels of IL-2 *in vivo* also resulted in larger numbers of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg in NOD mice. These results demonstrate that IL-2 influences the suppressor activity of the FoxP3<sup>+</sup> Treg pool by

regulating the balance between CD62L<sup>lo</sup> and CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg. In NOD mice, reduced IL-2 expression leads to an increase in nonsuppressive CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg, which in turn correlates with a pool of CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg with limited proliferation.

## 2.2 Introduction

The hallmark of type 1 diabetes (T1D) is the T cell-mediated destruction of the insulin-producing  $\beta$  cells in the pancreatic islets (1-3). Based on studies in humans and the NOD mouse, a spontaneous model of T1D, the breakdown of  $\beta$  cell-specific tolerance is in part due to defective peripheral immunoregulation within the T cell compartment. Conventional T cells in NOD mice for instance, exhibit reduced sensitivity to the suppressive effects of immunoregulatory T cells (Treg) (4, 5). The loss of function and/or frequency of Treg has also been implicated in the differentiation and expansion of pathogenic type 1 effector T cells (Teff) specific for  $\beta$  cells (5-7). Several subsets of Treg with distinct phenotypes and effector functions have been identified (8) including: (i) type 2 Treg which predominantly secrete IL-4, (ii) Th3 cells, which primarily secrete IL-4 and TGF $\beta$  (9), (iii) IL-10 secreting Treg (10), and (iv) natural and adaptive CD4<sup>+</sup>CD25<sup>+</sup> T cells which express the transcription factor Forkhead box P3 (FoxP3-expressing regulatory T cells (FoxP3<sup>+</sup> Treg)) (11).

FoxP3<sup>+</sup> Treg are considered to be the most potent subset of Treg, and are characterized by a suppressor function mediated by cell–cell contact-dependent and -independent mechanisms (12). Humans and mice lacking functional FoxP3 protein develop systemic T cell-mediated autoimmunity (13-15). FoxP3<sup>+</sup> Treg suppress T cells through constitutive expression of CTLA-4 and the glucocorticoid-induced TNF receptor (GITR) which block co-stimulatory signals needed for T cell activation (16). Additionally, FoxP3<sup>+</sup> Treg elicit suppression through a bystander effect via TGF $\beta$  (12, 17), which

modulates the function of APC and inhibits production of IFN $\gamma$  and TNF $\alpha$  by type 1 Teff (18).

The phenotype of FoxP3<sup>+</sup> Treg can be further defined based on CD62L expression. For instance, the *in vitro* and/or *in vivo* suppressor function of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg is superior compared with CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg (7, 19, 20). Furthermore, CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg from the pancreatic lymph nodes (PLN) or spleen of NOD mice exhibit an enhanced capacity to prevent diabetes in an adoptive transfer model compared to CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg (19). Increased levels of TGF $\beta$  expression contribute to the enhanced suppressor function of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg versus CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg (7). CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg are thought to reflect an activated phenotype characterized by increased cycling (21-23). Importantly, our group and others have previously shown that the frequency of suppressive CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg decline with age in NOD female mice which corresponds with the progression of  $\beta$  cell autoimmunity (7, 24). The critical events that induce and maintain the frequency of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg, however, are poorly understood.

Recent studies have demonstrated that IL-2 plays a key role in the maintenance of FoxP3<sup>+</sup> Treg homeostasis (25, 26). Mice lacking or having reduced expression of the *Il2* gene develop severe, systemic autoimmunity due to the reduction of FoxP3<sup>+</sup> Treg (27, 28). Furthermore, Sakaguchi and co-workers showed that diabetes is exacerbated in NOD mice when treated with a neutralizing antibody (Ab) specific for IL-2 at an early age (29). Also, IL-2 in combination with TGF $\beta$  is important for the differentiation of naive CD4<sup>+</sup> T cells into adaptive FoxP3<sup>+</sup> Treg *in vitro* (30, 31).

More than 20 chromosomal loci, termed insulin-dependent diabetes (*Idd*) regions, are associated with T1D susceptibility and resistance (32, 33). While no one gene is sufficient for the development of diabetes, the combined effects of susceptibility genes influence the progression of  $\beta$  cell autoimmunity (32, 33). NOD mice congenic for the *Idd3* locus derived from diabetes resistant mouse strains exhibit a reduced incidence and delayed onset of T1D (34-37). *Idd3* contains genes encoding immunoregulatory molecules including IL-2 and IL-21 (34-37). The NOD *Idd3* locus has been associated with reduced IL-2 expression by T cells and an aberrant FoxP3<sup>+</sup> Treg pool (37, 38). These findings suggest that T1D is influenced by dysregulation of IL-2 expression, which leads to reduced FoxP3<sup>+</sup> Treg frequency and/ or function found in NOD mice. In the current study, NOD mice congenic for a resistant *Idd3* interval derived from C57BL/6 mice (NOD.B6Idd3) were used to further define the role of IL-2 in regulating the peripheral FoxP3<sup>+</sup> Treg pool. We present evidence that reduced IL-2 expression leads to temporal dysregulation of the ratio between suppressor-deficient CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg and suppressor-competent CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg, resulting in a pool of FoxP3<sup>+</sup> Treg insufficient to regulate  $\beta$  cell autoimmunity.

## 2.3 Materials and Methods

### Mice

NOD/LtJ and NOD.CB17-Prkdc<sup>scid</sup>/J (NOD.*scid*) mice were maintained and bred under pathogen-free conditions in an American Association for Laboratory accredited animal facility. NOD.B6c3D mice, provided by Dr. Ed Leiter (The Jackson Laboratory), were established by introgression of an ~17 Mb region of the *Idd3* interval derived from C57BL/6 mice (NOD.B6l<sup>dd3</sup>) for 13 backcross generations. The length of the congenic interval was determined by typing with MIT microsatellite markers and using the MGI posting data from NCBI Build 37 (Table 2.1). Mice were monitored for diabetes by measuring urine glucose levels. All procedures were approved by the University of North Carolina Animal Use and Care Committee.

### T cell isolation and culturing conditions

Single cell suspensions were prepared from the thymus, PLN, and spleen, and filtered with a 70- $\mu$ M strainer (Fisher Scientific). Peripheral blood lymphocytes (PBL) were obtained via submandibular puncture using lancets (Golden Rod) and red blood cells lysed with ACK solution. Islet infiltrating cells were isolated from purified, hand-picked islets. Briefly, pancreases were digested with 2.0 mg/mL collagenase P (Roche) for 20 min at 37°C, and islets purified on a Ficoll (Sigma-Aldrich) gradient. Lymphocytes infiltrating the islets were harvested by dissociating the islets using an enzyme-free cell dissociation solution (Sigma-Aldrich).



Naive CD4<sup>+</sup> T cells were isolated from splenocytes using a bead-based naive CD4 T cell kit (Miltenyi Biotec). Briefly, total lymphocytes were incubated with a biotin-labeled Ab cocktail that selectively enriches for CD4<sup>+</sup> T cells but depletes CD4<sup>+</sup>CD25<sup>+</sup> cells. Enriched CD4<sup>+</sup>CD25<sup>-</sup> T cells were then incubated with CD62L-conjugated microbeads and isolated using a magnetic column.

For general T cell cultures,  $2 \times 10^5$  cells were resuspended in complete RPMI 1640 medium (Gibco) containing 10% heat-inactivated FBS, 100 U/mL penicillin/streptomycin (Gibco), and 50  $\mu$ M 2-ME (Sigma-Aldrich). T cells were stimulated in 96-well plates coated with varying concentrations of purified anti-CD3 Ab (2C11, eBioscience) and soluble, functional-grade anti-CD28 Ab at 2  $\mu$ g/mL (37.51, eBioscience). In some experiments supernatants were collected, diluted 1:3 in 1% BSA in PBS, and IL-2 secretion measured 24 h post stimulation. An anti-IL-2 Ab set (eBioscience) was used at 2  $\mu$ g/mL on a high-binding ELISA plate (Costar).

### **Flow cytometry (FACS)**

Total cells from the respective tissues were stained with a variety of fluorochrome-conjugated monoclonal Ab including: anti-CD3 (2C11), anti-CD4 (L3T4), anti-CD8 (Ly-2), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-CD62L (MEL14), and anti-FoxP3 (FJK.16 kit) (eBioscience). Fc receptors were blocked with a 1/200 dilution of rat Ig prior to staining. Intracellular Ki67 (B56; BD Biosciences) staining was done using cytofix/cytoperm reagents (BD Biosciences) according to the manufacturer's specifications. Data were acquired on a Cyan flow cytometer (DakoCytomation), and analyzed using Summit

software (DakoCytomation). In addition, CD4<sup>+</sup>CD25<sup>+</sup> T cells (CD62L<sup>lo</sup> or CD62L<sup>hi</sup>) were sorted by a MoFlo high-speed sorter (DakoCytomation).

Intracellular cytokine staining was performed on single cell suspensions from pancreatic lymph nodes (PLN) or islet-infiltrating cells as previously described (39). Briefly, lymphocytes were stimulated with 10 ng/mL PMA (Sigma-Aldrich) and 150 ng/mL ionomycin (Sigma-Aldrich) in complete RPMI 1640 medium for 6 h at 37°C; 10 µg/mL of Brefeldin A (Sigma-Aldrich) was added for the final 4 h of incubation. Cells were stained for surface molecules, fixed and permeabilized with cytokfix/cytoperm reagents (BD Biosciences), and stained for intracellular IFNγ (XMG1.2) (eBioscience).

#### ***In vitro* suppression assay**

Different ratios of FACS-sorted CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>LO/Hi</sup> T cells were cultured with cell-trace violet- (Invitrogen) labeled naive 5 x 10<sup>4</sup> CD4<sup>+</sup> T cells, 2 µg/mL anti-CD28, and 2 µg/mL anti-CD3 Ab in 96-well round bottom plates (Costar) for 3 days. Proliferation was assessed in triplicate by FACS analysis as the total percentage of labeled CD4<sup>+</sup>Thy1.2<sup>+</sup> naive cells undergoing at least one round of division.

#### **Cell adoptive transfers**

Diabetogenic NOD splenocytes (2.5 x 10<sup>6</sup>) were suspended in PBS and injected i.p. into 8-wk-old NOD.scid male mice alone or in combination with FACS-sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells (1 x 10<sup>5</sup>) isolated from the PLN of NOD or NOD.B6Idd3 mice. Mice were monitored bi-weekly post transfer for diabetes.

### **Construction of and treatment with AAV encoding NOD IL-2**

Using the forward primer 5'-gaagcttcaggcatgtacagcatgcagctc-3' that includes a HindIII restriction site and the reverse primer 5'-gtcgactagttattgagggtgttgagat-3' that contains an EcoRV restriction site, the *Il2* gene was PCR amplified with PFU Turbo (Promega) from mRNA (Qiagen) of ConA- (Sigma-Aldrich) stimulated NOD lymphocytes. Amplicons were subcloned into the topo-TA vector (Invitrogen) and sequenced. Full-length cDNA encoding *Il2* was subcloned into an AAV-Tet-on vector plasmid (kindly provided by Dr. Sihong Song) using Sall and EcoRV sites. Transgene expression was verified by measuring via ELISA IL-2 secretion by HEK 293 cells transfected with AAV-Tet-on-IL-2 plasmid DNA.

AAV virus production was previously described (40). Briefly, packaged AAV serotype 1 (AAV1) virus was prepared by transfecting 293 cells via calcium phosphate with the adeno helper encoding plasmid (pXX6-80), AAV1 encoding plasmid (pXR-1), and the Tet-on-IL-2 constructs (described above). 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.

NOD female mice were vaccinated with  $5 \times 10^{10}$  viral particles of AAV-Tet-on-IL-2 virus serotype 1 (AAV-Tet-IL-2) in contralateral, hind limb muscles using an insulin syringe. After injection, mice were fed chow containing 200 mg/kg doxycycline (BioServ) for 2 wks.

**Insulitis scoring**

Pancreases were harvested and fixed with formalin for 24 h. Serial sections 90 µm apart were prepared and stained with H&E. More than 100 islets were scored per group.

## 2.4 Results

### 2.4.1 An age-dependent decline in CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg is detected in NOD but not NOD.B6lidd3 mice

Studies have demonstrated that *lidd3* in NOD mice contributes to the progression of  $\beta$  cell autoimmunity by influencing the pool of FoxP3<sup>+</sup> Treg (37, 38). To further study the effect(s) of *lidd3* on FoxP3<sup>+</sup> Treg, NOD.B6lidd3 mice congenic for an ~17Mb interval derived from the C57BL/6 genotype were employed (Table 2.1). This line of NOD.B6lidd3 female mice exhibited a reduced frequency of diabetes and insulinitis relative to NOD female mice (Fig. 2.1), similar to other NOD mouse lines congenic for a resistant *lidd3* locus (37, 38, 41). Consistent with previous findings (38) naive CD4<sup>+</sup> T cells isolated from the spleen of NOD.B6lidd3 mice exhibited increased IL-2 secretion upon *in vitro* stimulation relative to NOD CD4<sup>+</sup> T cells (Fig. 2.2). To determine the influence of *lidd3* on FoxP3<sup>+</sup> Treg, the frequency and number of gated CD4<sup>+</sup>CD3<sup>+</sup> T cells expressing FoxP3 and CD25 (Fig. 2.3A) were assessed in the thymus, spleen, PLN, and islets of age-matched NOD and NOD.B6lidd3 female mice via FACS. No difference in the frequency of FoxP3<sup>+</sup> Treg was detected in the thymus of NOD and NOD.B6lidd3 mice suggesting that thymic development of FoxP3<sup>+</sup> Treg is unaffected by IL-2 expression levels. On the other hand, an increased frequency and number of FoxP3<sup>+</sup> Treg was detected in the PLN and spleen of older NOD.B6lidd3 mice relative to age-matched NOD mice (Fig. 2.3A–C). In addition, the frequency of FoxP3<sup>+</sup> Treg was significantly increased in the islets of 10- and 16-wk-old NOD.B6lidd3 versus NOD female mice (Fig. 2.3B). Notably, however, a greater number of FoxP3<sup>+</sup> Treg were detected in the islets of older NOD mice (Fig. 2.3C)

reflecting increased T cell infiltration of the islets relative to age-matched NOD.B6Idd3 mice. These data demonstrate that the frequency of FoxP3<sup>+</sup> Treg is increased in the PLN and islets of NOD.B6Idd3 mice compared with NOD mice.

We and others have shown that CD62L<sup>hi</sup>- versus CD62L<sup>lo</sup>- expressing FoxP3<sup>+</sup> Treg exhibit increased suppressor activity (7, 19). Accordingly, CD62L<sup>hi</sup>- and CD62L<sup>lo</sup>- expressing FoxP3<sup>+</sup> Treg were examined temporally in age-matched NOD.B6Idd3 and NOD female mice. Interestingly, age-dependent differences in the frequency and number of CD62L<sup>hi</sup>- and CD62L<sup>lo</sup>-expressing FoxP3<sup>+</sup> Treg were detected in the PLN and islets of the respective groups of mice. NOD female mice exhibited a temporal decrease in the frequency of CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg and a concomitant increase in CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg in PLN (Fig. 2.4B). Although the number of CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg progressively increased in the PLN of NOD female mice ( $5.2 \times 10^4$  (4 wk) versus  $9.0 \times 10^4$  (16 wk)), a greater increase in CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg numbers was detected ( $6.3 \times 10^4$  (4 wk) versus  $14.9 \times 10^4$  (16 wk)) (Fig. 2.4C). In the PLN of NOD.B6Idd3 mice, however, the frequency and number of CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg showed no marked change with age, which were increased relative to age matched NOD females (Fig. 2.4B and C).

A similar scenario was observed in the islets of NOD and NOD.B6Idd3 female mice. A temporal increase in the frequency of CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg was detected in the islets of NOD female mice which was due to elevated numbers relative to CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg (Fig. 2.4D and E). Despite a progressive decline, the frequency of CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg in the islets of NOD.B6Idd3 female mice was elevated relative to age-matched NOD female mice (Fig. 2.4D and E). FACS analysis showed similar levels of

CD25, glucocorticoid-induced TNF receptor, and CTLA-4 surface expression between CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg and CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg infiltrating the islets of either NOD or NOD.B6Idd3 mice (data not shown).

Differences in the proliferative status of CD62L<sup>hi</sup>- versus CD62L<sup>lo</sup>-expressing FoxP3<sup>+</sup> Treg could explain the distinct FoxP3<sup>+</sup> Treg profiles seen in the islets of NOD and NOD.B6Idd3 mice. To investigate this possibility, proliferation of CD62L<sup>hi</sup> CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD62L<sup>lo</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells was assessed via Ki67 staining in the islets of 12-wk-old NOD and NOD.B6Idd3 female mice. Regardless of the genotype, the frequency of proliferating CD62L<sup>lo</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells was elevated relative to CD62L<sup>hi</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells (Fig. 2.5B). Importantly, however, the frequency of proliferating CD62L<sup>hi</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells (Fig. 2.5B) and the ratio of Ki67-staining CD62L<sup>hi</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> to CD62L<sup>lo</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells (Fig. 2.5C) were increased in the islets of NOD.B6Idd3 versus NOD female mice. Together, these results indicate that within the pool of FoxP3<sup>+</sup> Treg a significant shift from CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg to CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg occurs in the PLN and islets of NOD but to a lesser extent in NOD.B6Idd3 female mice, which correlates with a decreased proliferative status of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg in NOD and NOD.B6Idd3 mice.

#### **2.4.2 Increased numbers of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg enhance the suppressor activity of Treg from NOD.B6Idd3 versus NOD mice**

Elevated numbers of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg in NOD.B6Idd3 mice would be expected to enhance suppression of pathogenic Teff in the respective tissues. Indeed, at 16 wks of

age the frequency of insulinitis is reduced in 16-wk-old NOD.B6Idd3 versus NOD female mice (Fig. 2.1B). Consistent with the latter, the ratio of CD62L<sup>hi</sup>FoxP3<sup>+</sup>Treg versus IFN $\gamma$  secreting CD4<sup>+</sup> T cells in the islets and PLN was significantly increased in 16-wk-old NOD.B6Idd3 versus NOD female mice (Fig. 2.6A). The overall frequency of proliferating T cells was reduced in the islets of 16-wk-old NOD.B6Idd3 versus NOD female mice (Fig. 2.6B). To directly assess the *in vivo* suppressor activity of NOD and NOD.B6Idd3 FoxP3<sup>+</sup>Treg, co-adoptive transfer experiments were carried out. CD4<sup>+</sup>CD25<sup>+</sup> T cells were prepared from PLN of 16-wk-old NOD.B6Idd3 or NOD female mice, co-injected with splenocytes from diabetic NOD donors into NOD.*scid* mice, and diabetes monitored. Importantly, the frequency of FoxP3-expressing cells in the pool of sorted CD4<sup>+</sup>CD25<sup>+</sup> T cells was similar between NOD and NOD.B6Idd3 donors (72  $\pm$  5% and 75  $\pm$  3%, respectively; average of 3 separate experiments). As expected all NOD.*scid* mice receiving diabetogenic splenocytes alone developed diabetes (Fig. 2.6C). Similarly, the entire group of NOD.*scid* mice injected with a mixture of diabetogenic splenocytes plus NOD CD4<sup>+</sup>CD25<sup>+</sup> T cells developed diabetes albeit with delayed kinetics (Fig. 2.6C). In contrast, NOD.*scid* mice receiving NOD.B6Idd3 CD4<sup>+</sup>CD25<sup>+</sup> T cells plus diabetogenic splenocytes exhibited a significantly delayed onset and reduced frequency of diabetes relative to recipients of the cell mixture containing NOD CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 2.6C). Therefore, CD4<sup>+</sup>CD25<sup>+</sup> T cells from NOD.B6Idd3 mice exhibit an increased suppressor activity compared to NOD CD4<sup>+</sup>CD25<sup>+</sup> T cells.

To determine whether the protection mediated by NOD.B6Idd3 CD4<sup>+</sup>CD25<sup>+</sup> T cells was due to quantitative or qualitative differences within the pool of



CD62L<sup>hi</sup>FoxP3<sup>+</sup>Treg, the suppressor activity of these immunoregulatory effectors was tested *in vitro*. CD62L<sup>lo</sup>- and CD62L<sup>hi</sup>-expressing CD4<sup>+</sup>CD25<sup>+</sup> T cells were FACS sorted from the PLN of 16-wk-old NOD.B6Idd3 and NOD female mice, and then cultured at various ratios with naive CD4<sup>+</sup> T cells from the spleen of NOD mice. As expected, CD62L<sup>lo</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells from either NOD.B6Idd3 or NOD female mice were inefficient at suppressing proliferation of the stimulated CD4<sup>+</sup> T cells (Fig. 2.6D). On the other hand, CD62L<sup>hi</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells effectively suppressed proliferation of the responder CD4<sup>+</sup> T cells. Furthermore, no significant difference in suppressor activity of NOD.B6Idd3 and NOD CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg was detected (Fig. 2.6D). Therefore, the enhanced suppressor activity detected in the PLN of NOD.B6Idd3 mice is due to an increased number of CD62L<sup>hi</sup> FoxP3<sup>+</sup>Treg, consistent with results obtained in the above co-adoptive transfer experiments (Fig. 2.6C).

#### **2.4.3 The frequency of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg is increased *in vivo* in NOD mice treated with IL-2**

Since IL-2 secretion by conventional T cells is limited in NOD mice compared with NOD.B6Idd3 animals (Fig. 2.2) (38), then increasing the level of “endogenous” IL-2 would be expected to enhance the frequency of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg *in vivo*. To test this hypothesis, 10-wk-old NOD female mice were injected intramuscularly with a doxycycline inducible adeno-associated virus (AAV) recombinant encoding IL-2 (AAV-Tet-IL-2). No difference was detected in the frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in AAV-Tet-IL-2 treated but uninduced NOD mice or animals left untreated (Fig. 2.7A and

B). In contrast, NOD mice treated with AAV-Tet-IL-2 and in which IL-2 transgene expression was induced exhibited an increased frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> in all tissues tested (Fig. 2.7A and B), and showed a significant increase in CD62L<sup>hi</sup>-expressing CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in the PLNs (Fig. 2.7C). Furthermore, addition of IL-2 to FACS-sorted CD62L<sup>lo</sup>-expressing CD4<sup>+</sup>CD25<sup>+</sup> T cells upregulated expression of CD62L *in vitro* (Fig. 2.7D). These results indicate that: (i) IL-2 availability *in vivo* regulates the frequency of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg, and (ii) IL-2 can “convert” CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg into CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg *in vitro*.

## 2.5 Discussion

Analyses of NOD mice congenic for protective *Idd3* intervals have shown that aberrant expression of IL-21 and IL-2 influences various aspects of  $\beta$  cell autoimmunity in NOD mice (34-38). Increased expression of IL-21 and IL-21R by T cells is associated with enhanced development of pathogenic Teff in NOD mice through, for instance, disruption of T cell homeostasis (34, 36, 42-44). IL-21 has also been reported to render conventional T cells resistant to the suppressor effects of FoxP3<sup>+</sup> Treg (45, 46). In NOD mice the resistance of conventional T cells to Treg-mediated suppression, however, appears to be independent of *Idd3* (47). On the other hand, decreased transcription of the *Il2* gene in NOD mice has been linked to a reduced frequency of FoxP3<sup>+</sup> Treg in the PLNs, decreased intra-islet survival, a limited suppressor function of FoxP3<sup>+</sup> Treg, in addition to an impaired capacity of FoxP3<sup>+</sup> Treg to expand in the islets (24, 37, 38). Differences in glycosylation of IL-2 between C57BL/6 and NOD mice, however, have no effect on diabetes development (48). The current study provides new insight into how dysregulation of IL-2 adversely influences the pool of FoxP3<sup>+</sup> Treg in NOD mice as T1D progresses. We show that reduced IL-2 expression in NOD mice is associated with a temporal shift favoring CD62L<sup>lo</sup>- versus CD62L<sup>hi</sup>-expressing FoxP3<sup>+</sup> Treg (Fig. 2.4) thereby altering the composition and diminishing the suppressor function of the overall pool of FoxP3<sup>+</sup> Treg (Fig. 2.6).

Previous work by our group (7) and others (38) demonstrated that the progression of  $\beta$  cell autoimmunity correlates with an age-dependent decrease in the frequency of CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg in NOD female mice. The current study shows that this

decrease is due to an inverse relationship between CD62L<sup>hi</sup>- and CD62L<sup>lo</sup>- expressing FoxP3<sup>+</sup> Treg that is dependent on the level of IL-2 expression. A direct role for IL-2 in regulating the balance between CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg and CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg was seen *in vitro* and *in vivo*. Supplementing cultures of sorted CD62L<sup>lo</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells with IL-2, for instance, increased the frequency of CD62L<sup>hi</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 2.7D). In addition, an increase in the frequency of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg was detected in the PLN of NOD mice following a brief induction of AAV encoded IL-2 (Fig. 2.7C). This *in vivo* pulse of ectopic IL-2 also resulted in effective suppression of  $\beta$  cell autoimmunity and prevention of overt diabetes in treated NOD mice (49).

The above results are consistent with IL-2 providing critical signals for the maintenance of the FoxP3<sup>+</sup> Treg compartment in general (24, 25), and specifically CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg. Our findings demonstrate that the temporal shift in the composition of FoxP3<sup>+</sup> Treg in NOD mice correlates with the proliferative status of CD62L<sup>hi</sup>- versus CD62L<sup>lo</sup>- expressing FoxP3<sup>+</sup>Treg. In the islets of NOD mice a greater than two-fold increase in the frequency of proliferating cells is detected in CD62L<sup>lo</sup> (45%)- versus CD62L<sup>hi</sup> (17%)-expressing FoxP3<sup>+</sup> Treg (Fig. 2.5A and B). However, the frequency of proliferating CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg is increased twofold in the islets of NOD.B6Idd3 (33%) versus NOD (17%) mice (Fig. 2.5A and B), resulting in a significantly increased ratio of dividing CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg to CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg in NOD.B6Idd3 islets (Fig. 2.5C). A similar trend was detected in the islets of NOD mice treated with AAV-Tet-IL-2 and fed doxycycline (Fig. 2.S1). Increased proliferation in NOD.B6Idd3 mice would be expected to promote more efficient maintenance of the CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg pool compared with

NOD mice. Although the frequency of proliferating CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg was also increased in the islets of NOD.B6Idd3 (55%) versus NOD (45%) mice, the difference between the two was not as great as that seen between the respective CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg pools (Fig. 2.5A and B). This finding suggests that CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg are more sensitive to changes in the level of IL-2 than CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg. Elevated IL-2 expression by conventional T cells in NOD.B6Idd3 mice may therefore selectively increase proliferation (Fig. 2.5) and survival (24) of suppressor-efficient CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg residing in the islets. IL-2 also has direct effects on CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg. As noted above, IL-2 converts a significant number of sorted CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg into CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg *in vitro* (Fig. 2.7D), possibly reflecting downregulation of the activation status of CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg. Indeed, IL-2 mediates both positive and negative effects on conventional T cells depending on the activational status of the cells (28, 50). Finally, APC may also influence the CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg to CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg ratio *in vivo*. The type and activational status of professional APC can have a marked effect on FoxP3<sup>+</sup> Treg induction/expansion. Groups have shown that macrophages and DCs exhibit an increased tolerogenic capacity in NOD.Idd3 versus NOD mice (51, 52); the mechanistic basis for this enhanced tolerogenic effect, however, has yet to be determined.

Recent studies with NOD.Idd3 congenic lines have shown that NOD-derived FoxP3<sup>+</sup> Treg exhibit an impaired suppressor function (37, 38). Our results demonstrate that the limited suppressor activity reported for NOD FoxP3<sup>+</sup> Treg is due to an increased number and frequency of suppressor-deficient CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg, which “dilute out” the suppressor-competent CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg. The limited suppressor function of

sorted NOD or NOD.B6lidd3 CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg was demonstrated *in vitro* (Fig. 2.6D), consistent with an earlier report (7). These results, however, differ from work published by Szanya *et al.*, that demonstrated that CD62L<sup>hi</sup>CD4<sup>+</sup>CD25<sup>+</sup> and CD62L<sup>lo</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells from the spleen of NOD mice differ in suppressor activity only in *in vivo*, but not *in vitro*, assays (19). The level of anti-CD62L Ab-binding and the gating scheme may account for differences in the frequency of and, in turn the *in vitro* suppressor activity of, the pool of CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg in the respective studies. In addition, Szanya *et al.* examined splenic-derived CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg, whereas in this study CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg were prepared from PLN; “tissue residency” may also influence the suppressor activity of these T cells and contribute to the disparity between the studies. Reduced TGFβ1 (7) expression relative to CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg, however, is consistent with a diminished suppressor activity by CD62L<sup>lo</sup>FoxP3<sup>+</sup> Treg. In contrast to NOD mice, the increased frequency of CD62L<sup>hi</sup>-FoxP3<sup>+</sup> Treg in the PLN and islets of NOD.B6lidd3 mice efficiently blocks β cell autoimmunity (Fig. 2.6B and C). Supporting this model is the marked increase in the ratio of FoxP3<sup>+</sup> Treg to Teff detected in the PLN and islets of NOD.B6lidd3 mice relative to age-matched NOD female mice (Fig. 2.6A). In addition, CD4<sup>+</sup>CD25<sup>+</sup> T cells from the PLN of NOD.B6lidd3 mice proved to be more effective at suppressing the adoptive transfer of diabetes relative to NOD CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 2.6C). One caveat with the latter finding is that, despite similar numbers of activated Teff (e.g. FoxP3<sup>-</sup> CD4<sup>+</sup>CD25<sup>+</sup> T cells) in the transferred NOD and NOD.B6lidd3 CD4<sup>+</sup>CD25<sup>+</sup> T cells, an increased frequency of β cell-specific pathogenic Teff may have limited the efficacy the NOD Treg pool. A previous study, however, showed that proliferation of transferred

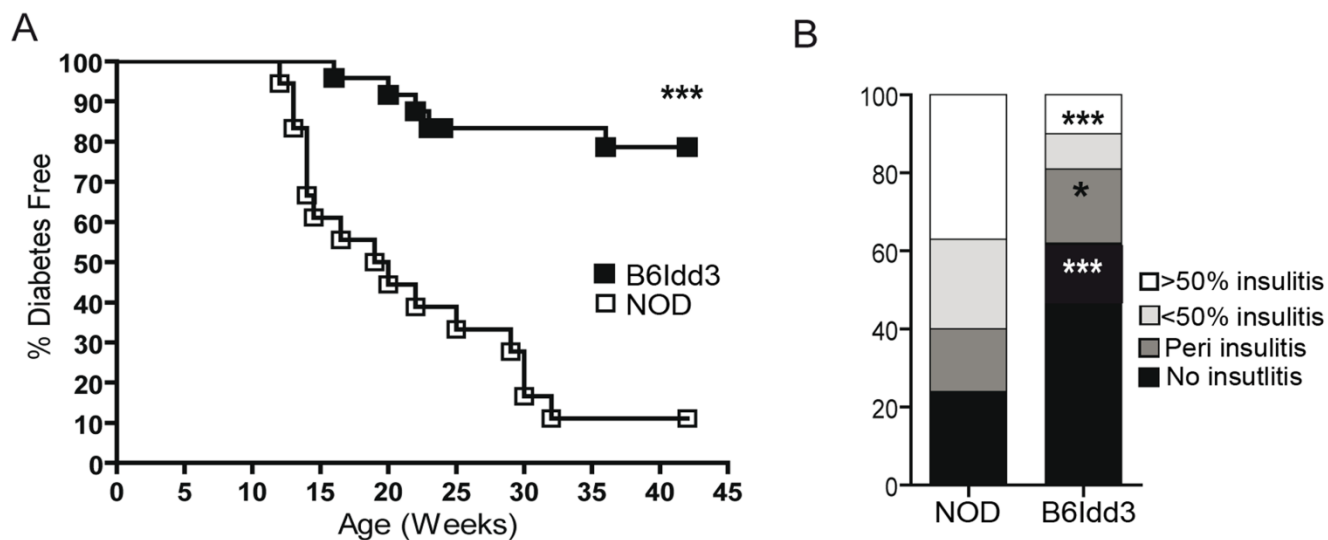
diabetogenic CD4<sup>+</sup> T cells was significantly reduced in the PLN of NOD.B6lidd3 versus NOD recipients (38), which is consistent with NOD.B6lidd3 mice having enhanced suppressor activity. Noteworthy is that no difference was detected in the *in vitro* suppressor activity of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg from NOD and NOD.B6lidd3 mice (Fig. 2.5C); in addition, similar *in vivo* suppressor activity was detected for the respective CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg as determined by co-adoptive transfer experiments (M. C. J. and R. T.; unpublished data). These observations argue that quantitative and not qualitative differences in CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg explain the distinct suppressor activity of the FoxP3<sup>+</sup> Treg pool detected in NOD and NOD.B6lidd3 mice (Fig. 2.6B). It is important to note that the frequency of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg decreased with age in the islets of NOD.B6lidd3 albeit to a lesser extent than seen in NOD islets (Fig. 2.4D). NOD.B6lidd3 mice develop insulinitis and diabetes but at a reduced frequency and a delayed onset compared with NOD mice (Fig. 2.1). Therefore, in addition to IL-2, other factors contribute to the homeostasis and function of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg.

In summary, we demonstrate that reduced IL-2 expression impacts FoxP3<sup>+</sup> Treg in NOD mice by altering the ratio of CD62L<sup>hi</sup> to CD62L<sup>lo</sup> FoxP3<sup>+</sup> Treg and in turn reducing the suppressor activity of the FoxP3<sup>+</sup> Treg compartment. These findings provide further rationale for the development of IL-2- based immunotherapy as a means to manipulate FoxP3<sup>+</sup> Treg for the prevention and suppression of  $\beta$  cell autoimmunity.

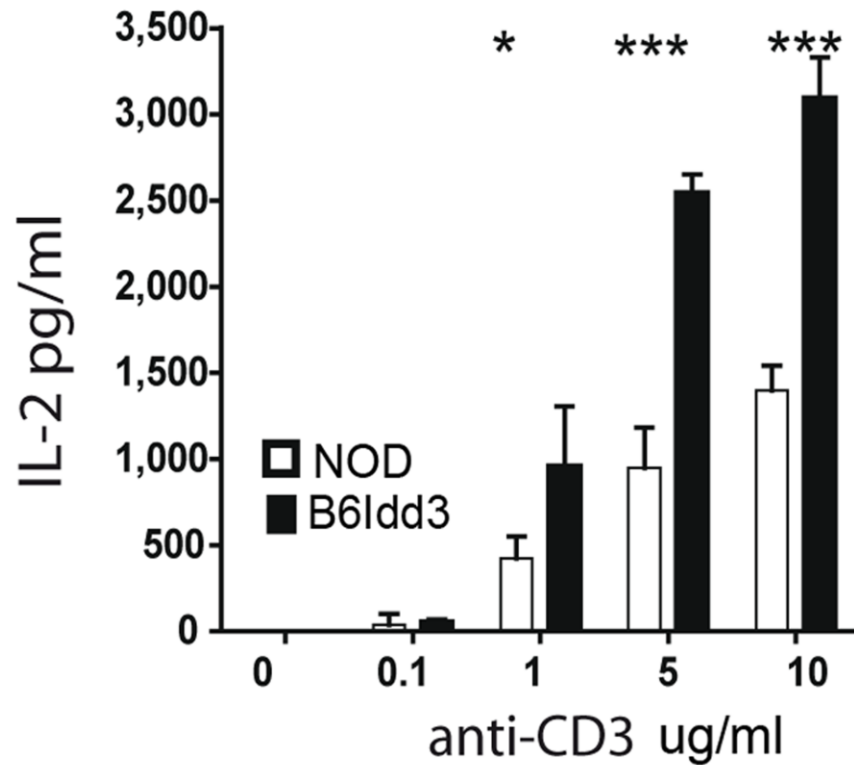
## **2.6 Acknowledgments**

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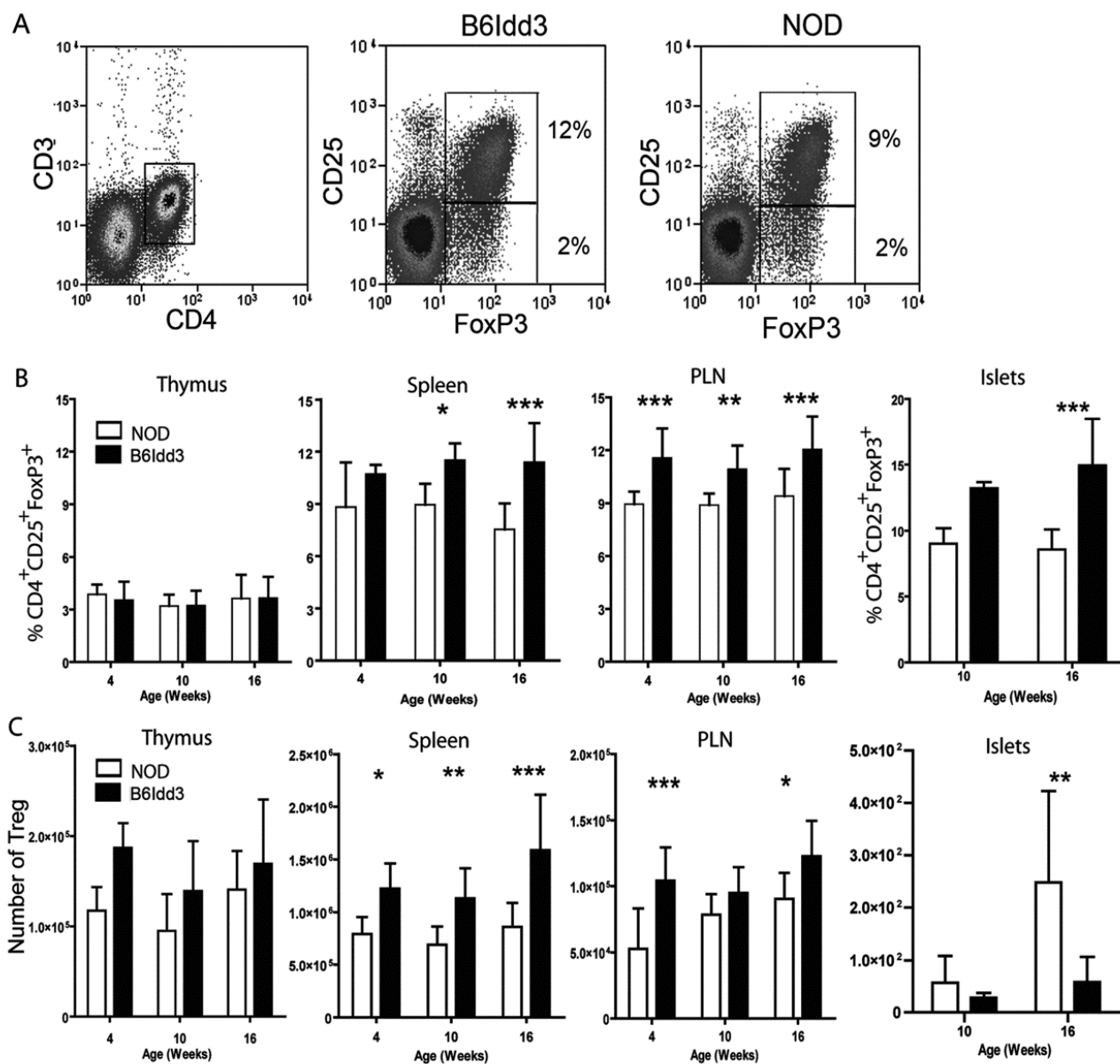




**Figure 2.1 NOD.B6Idd3 female mice exhibit a reduced frequency of diabetes and insulinitis compared to NOD female mice.** (A) Female NOD (open square,  $n = 20$ ) and NOD.B6Idd3 (black square,  $n = 19$ ) were monitored for diabetes incidence. \*\*\* $p < 0.001$  (Kaplan–Meier logrank test). (B) The frequency of insulinitis in the pancreas of 16-wk-old NOD ( $n = 7$ ) and NOD.B6Idd3 ( $n = 6$ ) female mice was determined via H&E staining. \* $p < 0.05$ , \*\*\* $p < 0.001$ ; NOD versus NOD.B6Idd3 for a given type of insulinitis (Chi square).

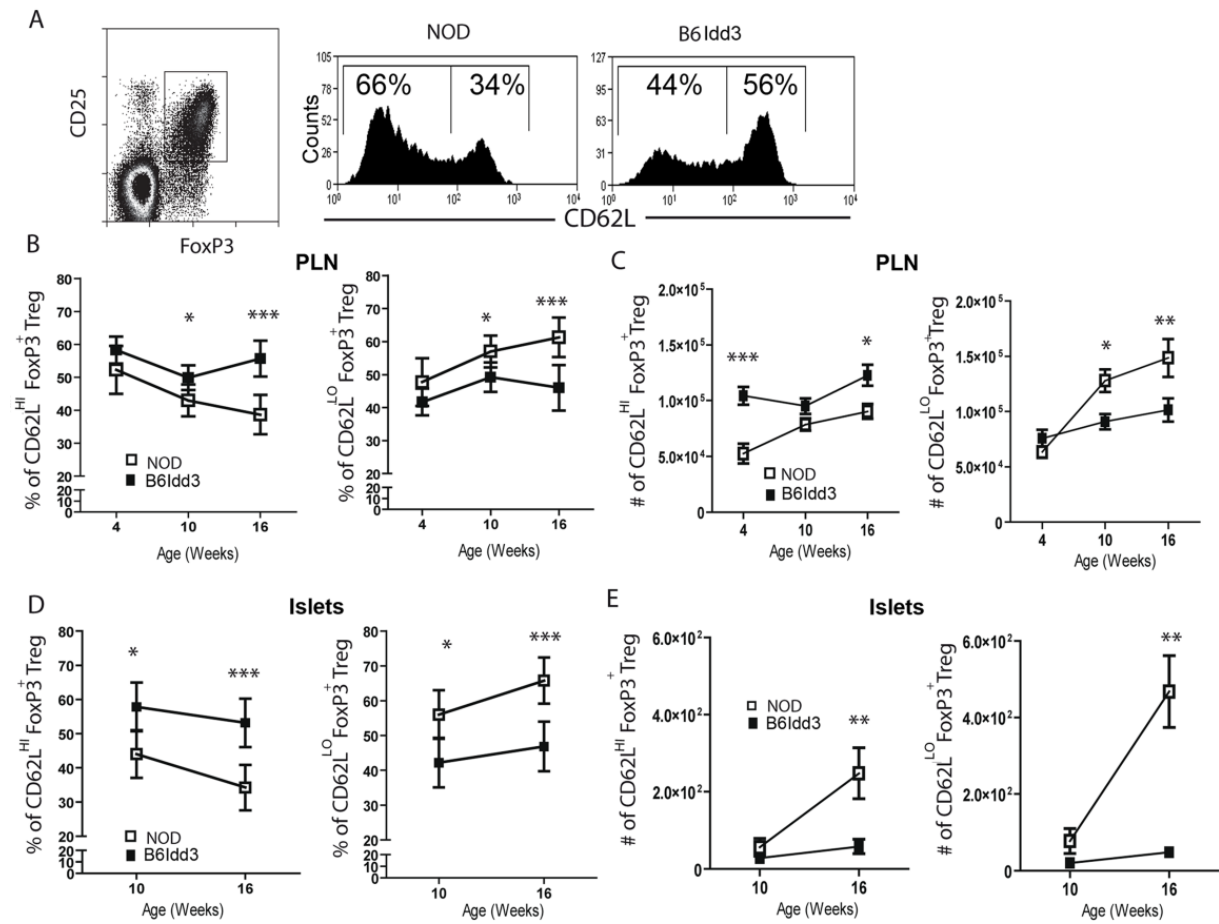


**Figure 2.2 NOD.B6lidd3 versus NOD naïve CD4<sup>+</sup> T cells secrete more IL-2 upon stimulation.** Naïve CD4<sup>+</sup> T cells from the spleen of 4-wk old NOD and NOD.B6lidd3 female mice were stimulated with varying concentrations of plate-bound anti-CD3 Ab and 2 µg/ml anti-CD28, and IL-2 secretion measured 24 h post-stimulation by ELISA using RPMI complete medium. \*, p<0.05; \*\*\*, p<0.001; NOD.B6lidd3 versus NOD cultures for give anti-CD3 Ab concentration (2-way ANOVA, error bars indicate ±SEM).

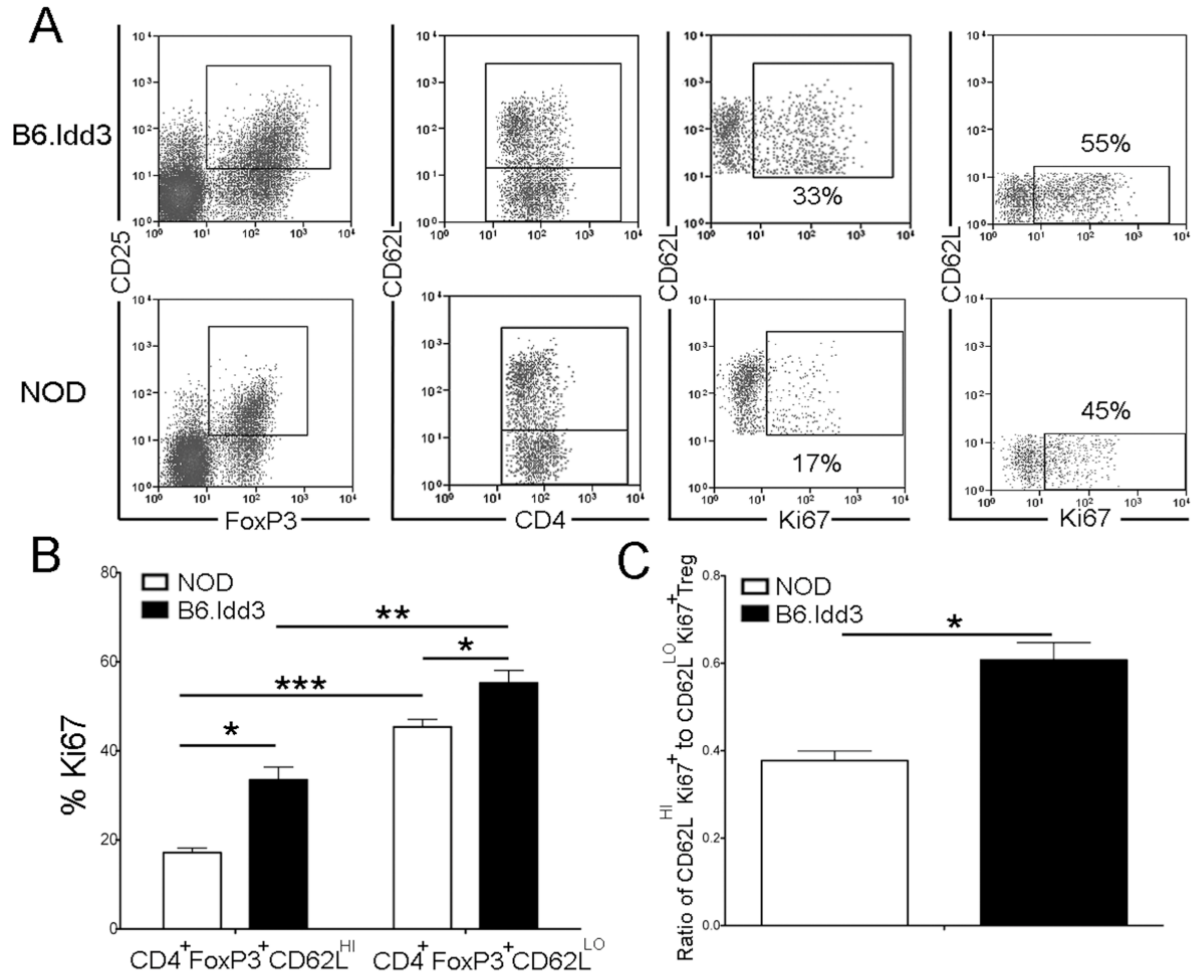


**Figure 2.3 NOD.B6Idd3 mice have an increased frequency of peripheral FoxP3<sup>+</sup>Treg compared to age-matched NOD mice.** (A) Representative FACS plots of NOD and NOD.B6Idd3 PLN at 16 wks of age staining for CD3<sup>+</sup>CD4<sup>+</sup>T cells (left column) used to gate CD25<sup>+</sup>FoxP3<sup>+</sup>T cells (right columns) with the average percentage of the indicated populations shown to the right of each gate. The (B) frequency and (C) absolute number of CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>T cells in the thymus, spleen, PLN, and islets were measured in

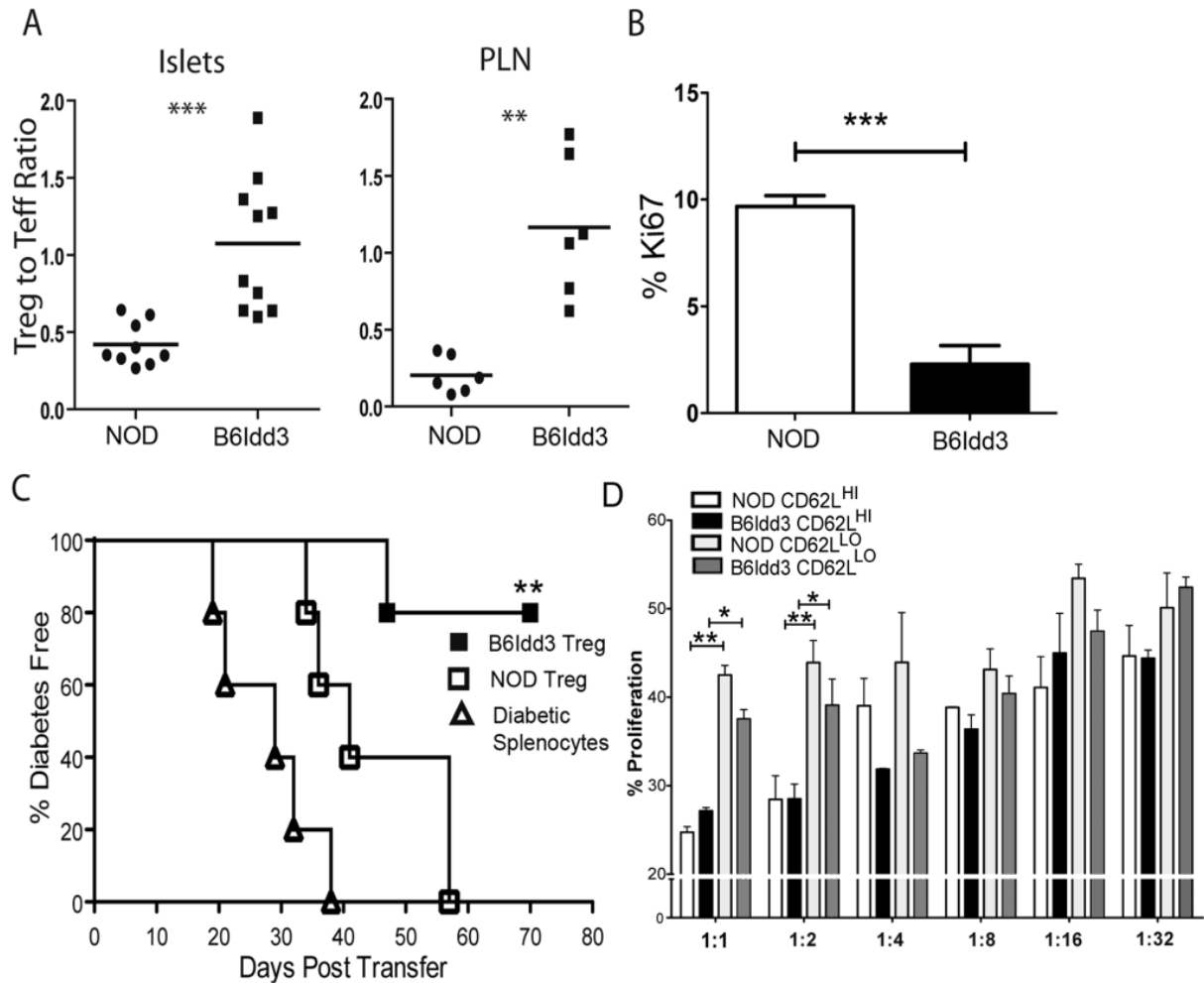
female NOD (n = 8–15/age group) and NOD.B6Idd3 mice (n = 7–16/age group) at 4, 10, and 16 wks of age. The absolute number in each respective tissue was determined by multiplying the total number of counted cells by the percentage of T cells determined by FACS. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; NOD.B6Idd3 versus NOD for a given tissue (2-way ANOVA, data are mean  $\pm$  SEM).



**Figure 2.4 A temporal shift in CD62L<sup>hi</sup>- versus CD62L<sup>lo</sup>-expressing FoxP3<sup>+</sup> Treg is detected in NOD but not NOD.B6lidd3 female mice.** (A) Representative FACS-staining profile of CD62L expression on gated CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells in the PLN of 16-wk-old NOD and NOD.B6lidd3 mice. The (B and D) frequency and (C and E) number of CD62L<sup>hi</sup>- and CD62L<sup>lo</sup>-expressing CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells in the (B and C) PLN and (D and E) islets of 4-, 10-, and/or 16-wk-old NOD (n = 7–10/age group) and NOD.B6lidd3 (n = 8–10/age group) female mice. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; NOD.B6lidd3 versus NOD for a given tissue (2-way ANOVA, data are mean  $\pm$  SEM).



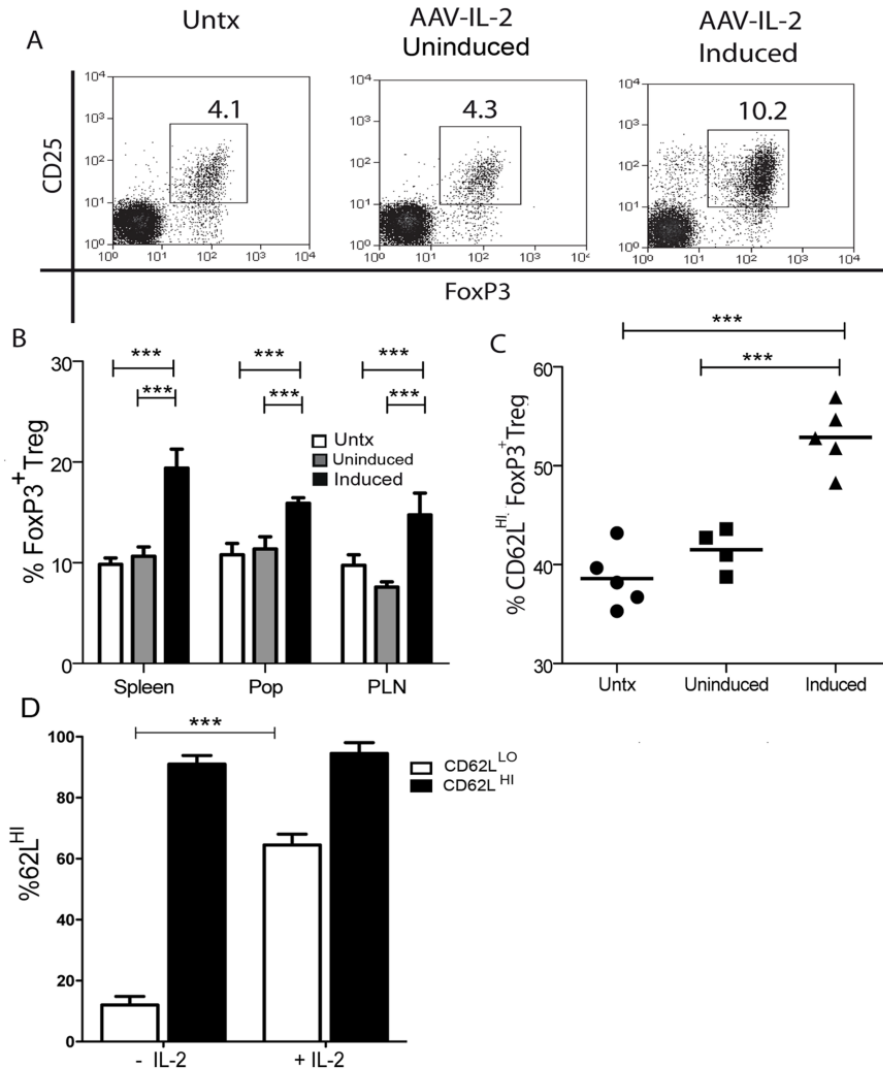
**Figure 2.5 Proliferation of CD62L<sup>hi</sup> FoxP3<sup>+</sup> Treg is increased in the islets of NOD.B6Idd3 versus NOD female mice.** (A) Representative FACS plots for the gating scheme used to assess the frequency of islet infiltrating Ki67-staining CD62L<sup>hi</sup> versus CD62L<sup>lo</sup> in CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells of 12-wk-old NOD and NOD.B6Idd3 female mice. (B) Average percent and (C) the ratio of Ki67-staining CD62L<sup>hi</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD62L<sup>lo</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells from the islets of 12-wk-old NOD (n = 5) and NOD.B6Idd3 (n = 5) female mice. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001 (2-way ANOVA, data are mean + SEM).



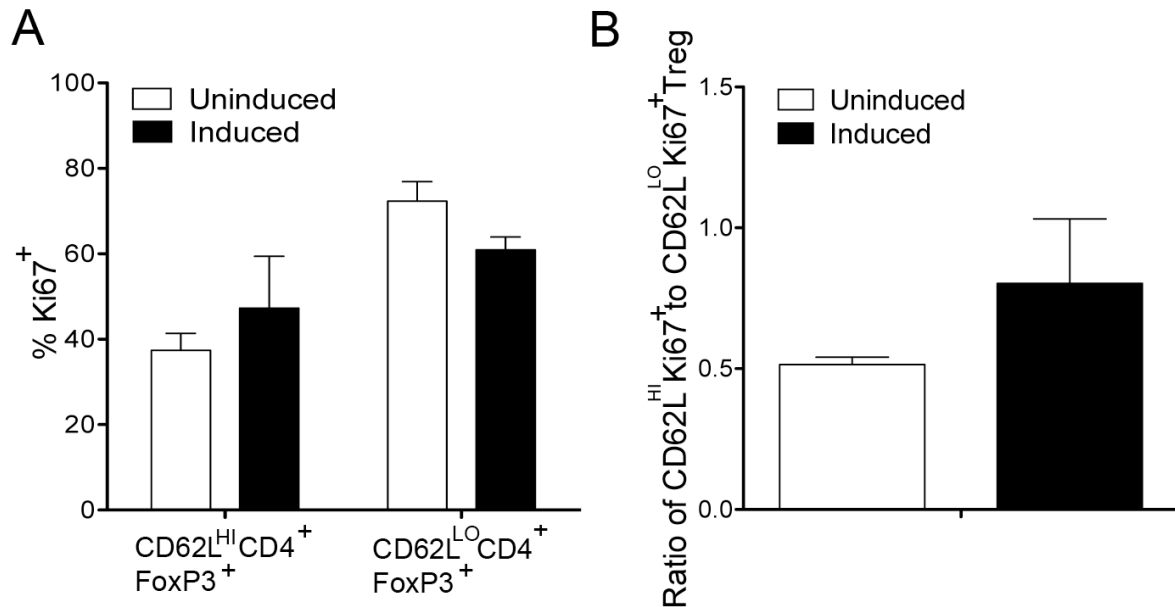
**Figure 2.6 The FoxP3<sup>+</sup> Treg pool in 16-wk-old NOD.B61dd3 versus NOD female mice exhibits increased suppressor activity.** (A) The ratio of CD62L<sup>hi</sup> expressing CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells to CD3<sup>+</sup>CD4<sup>+</sup> T cells expressing intracellular IFN $\gamma$  (e.g. type 1 T effectors (Teff)) was determined in the islets and PLN of 16-wk-old NOD (n = 9 and 6, respectively) and NOD.B61dd3 (n = 10 and 6, respectively) female mice via FACS. Each data point represents an individual mouse, horizontal bar represents the mean. \*\*p<0.01; \*\*\*p<0.001; NOD.B61dd3 versus NOD (Student's t-test). (B) Ki67staining for T cell proliferation of CD3<sup>+</sup> T cells in the islets of 16-wk-old NOD.B61dd3 (n = 6) versus NOD (n = 5) female mice; \*\*\*p<0.001 (Student's t-test). (C) CD4<sup>+</sup>CD25<sup>+</sup> T cells sorted from pooled PLN of 16-wk-old NOD or NOD.B61dd3 female mice were transferred with diabetogenic NOD splenocytes into NOD.scid mice (n = 5 per group) and diabetes monitored; as a control, diabetogenic splenocytes alone were transferred into NOD.scid recipients; \*\*p<0.001, NOD.B61dd3 versus NOD CD4<sup>+</sup>CD25<sup>+</sup> T cells (Kaplan–Meier logrank test). (D) The *in vitro* suppressor activity was compared between CD62L<sup>hi</sup>-

and CD62L<sup>lo</sup>-expressing CD4<sup>+</sup>CD25<sup>+</sup> T cells sorted from the PLN of 16-wk-old NOD and NOD.B6Idd3 female mice. Suppressor activity was determined by measuring via FACS proliferation of cell-trace violet dye-labeled CD4<sup>+</sup>CD25<sup>-</sup> responder T cells stimulated with anti-CD3 plus anti-CD28 Ab, and cultured with varying numbers of CD62L<sup>hi</sup>- or CD62L<sup>lo</sup>-expressing CD4<sup>+</sup>CD25<sup>+</sup> T cells; data are the average percent of proliferation from 2 wells  $\pm$  SEM. \*\*p<0.01; \*p<0.05; CD62L<sup>hi</sup> versus CD62L<sup>lo</sup> in NOD and NOD.B6Idd3 (2-way ANOVA). Data are representatives of three independent experiments.

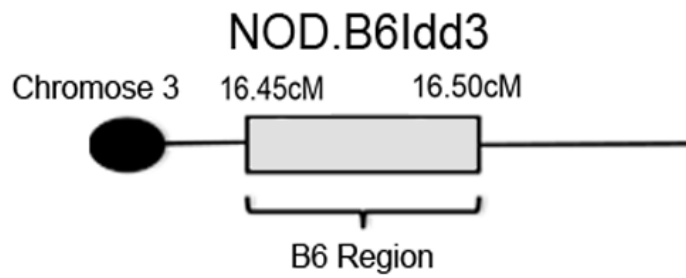




**Figure 2.7 Increased IL-2 induces an elevated frequency of CD62L<sup>hi</sup>FoxP3<sup>+</sup> Treg *in vivo* and *in vitro*.** (A) A representative FACS plot of the frequency of CD3<sup>+</sup>CD4<sup>+</sup> gated T cells expressing CD25 and FoxP3 in PBL prepared from NOD female mice left untreated (Untx; n = 5) or injected at 10 wks of age with AAV-Tet-IL-2 and given doxycycline-containing chow for 2 wks (induced; n = 5) or not (uninduced; n = 4) 3 wks post induction (average percentages per group are inlayed in the dot plots). Similarly the frequency of (B) CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells in the spleen, popliteal lymph nodes (Pop) and PLN, and (C) CD62L<sup>hi</sup>- expressing CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells in the PLN were determined via flow cytometry in the respective groups of NOD mice. (C) Each data point represents an individual mouse, horizontal bars represent the mean. \*\*\*p<0.001 (2-way ANOVA, data are mean +/- SEM). (D) CD62L expression after culturing for 48 h sorted CD62L<sup>hi</sup> or CD62L<sup>lo</sup> CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells in the presence or absence of 20 ng/mL of IL-2. \*\*\*p<0.001, CD62L<sup>lo</sup> (-IL-2) versus CD62L<sup>lo</sup> (+IL-2) (Student's t-test, data are mean ± SEM).



**Figure 2.S1 The frequency of proliferating CD62L<sup>HI</sup>FoxP3<sup>+</sup> Treg is increased in AAV-Tet-IL-2 treated NOD mice fed doxycycline.** (A) The frequency of islet infiltrating Ki67<sup>+</sup> cells in CD62L<sup>HI</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> and CD62L<sup>LO</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> T cells was determined by FACS in NOD female mice treated with AAV-Tet-IL-2 at 10 wks of age and fed doxycycline-containing (Induced; n=4) or normal chow (Uninduced; n=4) for 2 wks, after which islets were harvested. The bars represent the average of individual mice  $\pm$  SEM. (B) The ratio of FoxP3<sup>+</sup>CD62L<sup>HI</sup>Ki67<sup>+</sup> (CD62L<sup>+</sup>Ki67<sup>+</sup> Treg) to FoxP3<sup>+</sup>CD62L<sup>LO</sup>Ki67<sup>+</sup> (CD62L<sup>-</sup>Ki67<sup>+</sup> Treg) for uninduced and induced animals was determined by dividing the percentage of the former population by the later.



		NOD	B6	NOD.B6Idd3
Markers (D3Mit)	Mb		(16.45-16.5cM)	
92	25.95	N	B	N
93	28.8	N	B	N
118	28.8	N	B	N
151	31	N	B	
167	32.05	N	B	B
206	33.05	N	B	B
IL-2 (Nds6)	36.91	N	B	B
IL-21	37.02			
95	46.26	N	B	N
241	66.8	N	B	N
22	70.06	N	B	N
51	77.9	N	B	N

**Table 2.1 NOD.B6Idd3 interval.** The microsatellite markers used to define the interval of the B6.Idd3 region introgressed onto the NOD background.

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## CHAPTER 3

### ADENO-ASSOCIATED VIRUS VECTOR MEDIATED $\beta$ CELL SPECIFIC IL-2 EXPRESSION SUPPRESSES TYPE I DIABETES IN NOD MICE

#### 3.1 Summary

IL-2 is a critical cytokine that regulates T cell survival, activation, and proliferation. Studies utilizing the NOD mouse, a model of human type 1 diabetes (T1D), have shown decreased survival of FoxP3-expressing immunoregulatory T cells (FoxP3<sup>+</sup> Treg) in the pancreas, which has been directly linked to reduced IL-2 secretion. As such, administration of IL-2 offers an attractive approach to manipulate FoxP3<sup>+</sup> Treg for the treatment of T1D and other T cell mediated diseases. The effectiveness of IL-2 therapy, however, is dependent on the dose and route of administration, length of treatment and local cytokine levels that can be achieved. In the current study, we show that treatment with a recombinant adeno-associated virus (rAAV) 8 vector expressing IL-2 driven by the mouse insulin promoter, mIP (AAV8mIP-IL2), effectively suppresses ongoing  $\beta$  cell autoimmunity in NOD mice at a late preclinical stage of T1D.  $\beta$  cell specific expression of IL-2 resulted in increased FoxP3<sup>+</sup> Treg within the pancreatic islets that corresponded with a reduced ratio of pathogenic islet effector T cells to FoxP3<sup>+</sup> Treg. Notably, islet resident FoxP3<sup>+</sup> Treg exhibited enhanced suppressor function, which

correlated with increased expression of both contact-dependent and secreted suppressor molecules. Furthermore, islet FoxP3<sup>+</sup> Treg expressed significantly higher levels of anti-apoptotic markers, resulting in enhanced survival after AAV8mIP-IL2 administration. In contrast, FoxP3<sup>+</sup> Treg in the draining pancreatic lymph node were unaffected indicating that treatment with AAV8mIP-IL2 was islet-specific. These findings demonstrate that AAV8mIP-IL2 administration induces robust protection against T1D due to enhanced survival and function of islet FoxP3<sup>+</sup> Treg.

### 3.2 Introduction

Type I diabetes (T1D) is an autoimmune disease characterized by the T cell-mediated destruction of the insulin secreting  $\beta$  cells located in the pancreatic islets of Langerhans (1-4). The disease is characterized as a chronic inflammatory response within the islets, typically progressing over a number of years in patients until the remaining  $\beta$  cell mass is insufficient to maintain insulin levels needed for blood glucose regulation. Breakdown of self-tolerance towards  $\beta$  cells is mediated by both genetic and environmental factors, leading to dysregulation within the T cell compartment (5-7). Pathogenic  $CD8^+$  and  $CD4^+$  T effectors (Teff) are typically characterized by the secretion of  $IFN\gamma$  and  $TNF\alpha$ , although IL-17 secreting Th17 cells have also been implicated in the disease process (8-12). Skewed differentiation of naïve T cells toward pathogenic Teff correlates with a reduced number and/or function of regulatory  $CD4^+$  T cells (FoxP3<sup>+</sup> Treg), and a limited sensitivity to FoxP3<sup>+</sup> Treg-mediated suppression (13-18). As a result, current immunotherapy approaches for the treatment of T1D and other T cell-mediated autoimmune diseases have focused on the induction and/or expansion of FoxP3<sup>+</sup> Treg to restore self-tolerance.

Interleukin-2 (IL-2) is a cytokine that affects a number of other cell types including NKT cells, B cells and dendritic cells (DCs), and acts in an autocrine fashion to enhance the expansion and survival of Teff (19-22). Notably, IL-2 is also necessary for the *in vivo* maintenance of thymically derived natural FoxP3<sup>+</sup> Treg (23-27), in addition to contributing to the formation of adaptive FoxP3<sup>+</sup> Treg from non-FoxP3 expressing precursors in the periphery (28-30). Unlike conventional T cells, FoxP3<sup>+</sup> Treg are unable

to synthesize IL-2 and are therefore dependent on both Teff and DCs *in vivo* as sources for IL-2 (27, 31). The importance of FoxP3<sup>+</sup> Treg in maintaining self-tolerance is highlighted by studies demonstrating that defects within the FoxP3<sup>+</sup> Treg compartment result in systemic autoimmunity (32, 33). Furthermore, previous reports have shown that non-obese diabetic (NOD) mice, a spontaneous model of human T1D, have deficiencies in the number and/or function of FoxP3<sup>+</sup> Treg, contributing to the progression of  $\beta$  cell autoimmunity (15, 18, 34). Dysregulation of FoxP3<sup>+</sup> Treg in NOD mice is in part attributed to reduced IL-2 secretion by Teff (35-37). NOD.Idd3 congenic mice, in which protective insulin dependent diabetes loci 3 (*Idd3*) encoding the *Il-2* gene from C57BL/6 mice has been introgressed, remain diabetes-free due to increased IL-2 expression and enhanced FoxP3<sup>+</sup> Treg in the islets (37-41). Interestingly, FoxP3<sup>+</sup> Treg isolated from T1D patients exhibit reduced sensitivity to IL-2 (42).

IL-2 has been successfully utilized for the treatment and prevention of T1D. Initial studies have shown that low-dose recombinant (r) IL-2, and administration of IL-2- $\alpha$ -IL-2 complexes promoted islet-resident FoxP3<sup>+</sup> Treg survival, resulting in protection from T1D in NOD mice (36). Furthermore, administration of low dose rIL-2 induced remission in recent onset diabetic NOD mice through FoxP3<sup>+</sup> Treg dependent mechanisms (43, 44). Importantly, high doses of rIL-2 resulted in systemic activation of conventional T cells, Teff, and NKT cells in NOD mice, emphasizing the importance of regulating systemic IL-2 levels (36, 43). Clinically, rIL-2 therapy has been shown to ameliorate both graft-versus-host-disease and Hepatitis C induced vasculitis in patients that were refractory to other treatments (45, 46). In both studies, the beneficial effects

of rIL-2 treatment were attributed to enhanced systemic FoxP3<sup>+</sup> Treg levels. On the other hand, a recent trial administering rIL-2 and rapamycin to recent onset T1D patients showed only a transient increase in FoxP3<sup>+</sup> Treg, concomitant with an increase in activated NKT cells and eosinophils, and accelerated loss of insulin C peptide levels, suggesting exacerbated  $\beta$  cell autoimmunity (47). Together, these studies indicate IL-2 therapy can be effective but improved strategies of IL-2 administration are required to minimize potential severe side effects.

Accordingly, we have assessed the use of an adeno-associated virus (rAAV) vector to selectively target IL-2 transgene expression to  $\beta$  cells *in vivo*. rAAV vectors offer an attractive immunotherapeutic strategy for several reasons. rAAV vectors exhibit limited toxicity and exist as nonintegrating circular monomers or concatemers in the nucleus thereby limiting the risk of genomic insertion (48). The virus is capable of infecting both dividing and non-dividing cells, in addition to offering a wide range of tissue tropism due to the diversity of capsid proteins (49, 50). Additionally, pairing rAAV vectors with tissue specific promoters allows for targeted expression of the desired transgene (51). Coupled with the development of self-complementary or double stranded (ds) AAV vectors, as well as the recent advances in manufacturing and purification, rAAV vectors provide an approach to sustain long-term transgene expression while limiting virus-specific immune responses (52-56). rAAV vectors are being used in several clinical trials to complement various genetic disorders, such as hemophilia B(57), Parkinson's (58), macular degeneration (59) and Rheumatoid arthritis (60). In addition, a tetracycline inducible rAAV vector IL-2 system has been shown to be

efficacious in altering  $\beta$  cell autoimmunity, suggesting the feasibility of such a targeted approach (61).

In the current study, we demonstrate that vaccination with a rAAV8 vector expressing IL-2 driven by the mouse insulin promoter, mIP (AAV8mIP-IL2), selectively enhances the frequency and function of islet FoxP3<sup>+</sup> Treg to effectively suppress  $\beta$  cell autoimmunity long-term.

### 3.3 Materials and Methods

#### Mice

NOD/LtJ, NOD.CB17-Prkdcscid/J (NOD.*scid*), NOD.Cα<sup>-/-</sup>, NOD.BDC (62), NOD.BDC.FoxP3.GFP (63), C57BL/6, and NOD.8.3 (64) mice were bred and maintained under specific pathogen-free conditions in an American Association for Laboratory-accredited animal facility. All procedures were approved by the University of North Carolina Animal Use and Care Committee.

#### rAAV vector engineering, packaging and vaccination

Full length cDNA encoding murine *il2* (of the NOD genotype) and enhanced green fluorescence protein (*EGFP*) were PCR amplified and subsequently subcloned into TOPO2.1 (Invitrogen) via the manufacturer's recommendations. After sequencing, transgenes were excised and ligated into a rAAV vector mIP plasmid. IL-2 transgene expression was confirmed by ELISA, while EGFP transgene was confirmed by fluorescent microscopy, after Fugene 6 (Roche) mediated transfection of NIT-1 cells *in vitro* according to the manufacturer's directions.

AAV production was completed as previously described (65). Briefly, HEK 293T cells were transfected via polyethyleneimine (PEI) with adeno helper encoding plasmid (pXX6-80), AAV8 capsid encoding plasmid and the AAV8mIP-IL2/EGFP plasmids in order to package AAV serotype 8 (AAV8) virus. Nuclear fractions were harvested 72 hours

post-transfection and virus was purified with a Cesium Chloride (RPI) gradient. The virus containing fractions and titers were determined by Southern dot blot.

10-12 week old NOD female mice were vaccinated with  $2.5 \times 10^{10}$  viral particles (VP) of AAV8mIP-IL2 or AAV8mIP-EGFP or  $1 \times 10^{10}$  VP AAV8mIP-IL2 intra-peritoneally (I.P.) using an insulin syringe.

## **ELISA**

To assess *in vivo* systemic IL-2 levels, serum was collected and diluted 1:2 in RPMI 1640 media containing 10% heat-inactivated FBS, 1 mM sodium pyruvate (Gibco), 2 mM Hepes, 100 U/ml penicillin/streptomycin (Gibco), 50  $\mu$ M  $\beta$  mercaptoethanol (Sigma-Aldrich) (RPMI complete medium). IL-2 was also measured in supernatants collected from *in vitro* cultured islets after *in vivo* transduction with AAV8mIP-IL2, AAV8mIP-EGFP or from responder T cells. The  $\alpha$ IL-2 Ab set (JES6-1 and JES6-5; eBioscience) was used at a concentration of 2  $\mu$ g/ml on a high binding ELISA plate (Costar).

Measurements of IFN $\gamma$  in supernatants were conducted using the mouse IFN $\gamma$  ELISA set (BD optEIA) as per the manufacturer's recommendations. Supernatants were diluted 1:2 in RPMI complete medium.

## **Islet isolation**

Pancreas specimens were perfused with 2 mg/ml Collagenase P (Roche) and digested for 30 min at 37°C. Islets were purified via Ficoll gradient, hand-picked and counted. For flow cytometry analysis, lymphocytes were collected from freshly isolated islets after



culturing in RPMI complete medium for a minimum of 4 hours at 37°C. Cell debris were removed with a 70 µM nylon filter and stained.

### **Streptozotocin treatment (STZ)**

NOD mice were treated I.P. with 200 mg/kg of STZ (Sigma) resuspended in fresh sodium citrate buffer (pH = 4.0). 72 hours post-STZ, animals were vaccinated I.P. with  $2.5 \times 10^{10}$  VP AAV8mIP-IL2 or left untreated. Blood glucose levels and serum IL-2 were assessed over 10 days post-rAAV vector vaccination. NOD mice treated with  $2.5 \times 10^{10}$  VP AAV8mIP-IL2 alone served as positive controls.

### **Flow cytometry**

Total cells from the respective tissues were stained with a variety of previously titrated fluorochrome-conjugated monoclonal antibodies specific for: CD3 (145-2C11), CD4 (GK1.5), CD8 (Ly-2), CD25 (PC61.5), CD62L (MEL-14), GITR (DTA-1), ICOS (7E.17G9), CTLA-4 (UC10-4F10-11), HELIOS (22F6) and FoxP3 (FJK-16s). Fc receptors were blocked with rat  $\alpha$ -mouse CD16/32 (2.4G2) (BD Biosciences) prior to staining. Intracellular Ki67 (B56: BD Biosciences) and Bcl-2 (3F11: BD Biosciences) staining was done using the Fix/Perm and Perm/Wash reagents (ebioscience) according to the manufacturers recommendations.

Intracellular cytokine staining was performed on single cell suspensions as previously described (66). Briefly, lymphocytes were stimulated with 500 ng/ml PMA (Sigma) and 1000 ng/ml ionomycin (Sigma) in complete RPMI complete medium for 5

hours at 37°C; 10 µg/ml Brefeldin A (Sigma) was added to the culture for the last 4 hours of incubation. Cells were then stained for surface molecules, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained for intracellular IFN $\gamma$  (XMG1.2) or IL-10 (JES5-16E3).

For phosphotyrlated STAT5 (pSTAT5) staining, organs were harvested and immediately processed in PBS containing 2% paraformaldehyde for fixation. Single cell suspensions were incubated on ice for 20 minutes, followed by centrifugation and re-suspension in ice cold methanol for 30 minutes for permeabilization. Cells were then counted, washed twice in 1% BSA in PBS, and stained with cell surface, intracellular and pSTAT5 (BD Biosciences) antibodies for a minimum of 1 hour on ice.

Data were acquired on a Cyan flow cytometer (DakoCytomation) and analyzed using Summit software (DakoCytomation).

### **Diabetes monitoring and insulinitis scoring**

NOD mice were monitored weekly for diabetes by urine analysis via Keto-Diastix (Bayer). In urine positive animals, blood glucose levels were subsequently checked for confirmation. NOD mice were classified as diabetic after three consecutive blood glucose readings over 250 mg/dL.

For insulinitis scoring, pancreases were harvested and fixed with formalin for 48 hours at room temperature. Serial sections 100 µm apart were prepared and stained with Hematoxylin and Eosin (H and E). A minimum of 100 islets were scored per group.

### ***In vitro* assays**

Spleens were pooled from NOD.FoxP3.BDC.GFP animals treated with  $2.5 \times 10^{10}$  VP AAV8mIP-IL2 or AAV8mIP-EGFP. After culturing, single cell suspensions were filtered and stained with antibodies for CD4 and CD3 respectively in PBS supplemented with 2.5% BSA and 2 mM EDTA. CD4<sup>+</sup>CD3<sup>+</sup>FoxP3.GFP<sup>+</sup> Treg were sorted on a MoFlo sorter (Dako Cytomation).

For T cell responders, spleens from NOD.BDC mice were pooled. Naïve CD4<sup>+</sup>CD62L<sup>+</sup> splenocytes were isolated using the CD4<sup>+</sup>CD62L<sup>+</sup> T cell isolation kit II (Miltenyi Biotec) and subsequently labeled with Cell Trace Violet (Invitrogen) as per the manufacturer's recommendations. Cell purity in naïve populations was >95% by flow cytometry.

NOD.Cα<sup>-/-</sup> mice were utilized as antigen presenting cells (APCs). Briefly, spleens were harvested, processed and re-suspended at a concentration of  $1.0 \times 10^7$  cells/ml in RPMI complete medium. sBDC peptide was added to a final concentration of 100 ng/ml or 1 µg/ml. Cells were then incubated at 37°C for 2 hours, washed twice in RPMI complete medium and re-suspended at  $4 \times 10^6$  cells/ml.

Cells were co-cultured at a starting ratio of 1:1:1 (FoxP3<sup>+</sup> Treg: T cell responders: APCs) in RPMI complete medium.  $2 \times 10^5$  responder cells/well, with FoxP3<sup>+</sup> Treg titrated down to a 1:8 ratio compared to T cell responders. 72 hours later, supernatants were obtained for IL-2 and IFNγ ELISAs as described above. Cell proliferation in the T cell responder population was assessed by total percentage of cells undergoing cell violet dilution using flow cytometry.

### 3.4 Results

#### 3.4.1 Targeting IL-2 expression to $\beta$ cells *in vivo* via AAV8mIP-IL2 vaccination

IL-2 has pleiotropic effects on the immune system and has been utilized to suppress autoimmunity, including T1D (36, 43, 45). Systemic administration of IL-2, however, can result in significant off-target effects (36, 43). In addition, the efficacy of IL-2 in a localized tissue environment versus systemic administration is poorly defined. With this in mind, we assessed the effects of islet specific IL-2 on  $\beta$  cell autoimmunity using the AAV8mIP-IL2 vector.

To initially show that AAV8mIP-IL2 mediated transgene expression was  $\beta$  cell specific, NOD.*scid* mice were treated with  $2.5 \times 10^{10}$  VP AAV8mIP-IL2,  $1 \times 10^{10}$  VP AAV8mIP-IL2, or were left untreated. At 2 weeks post-AAV vector injection, islets were isolated from individual recipients and cultured for 96 hours. Increased IL-2 was detected in the supernatants in an AAV vector dose dependent manner (Fig. 3.1A). Furthermore, no IL-2 was detected in supernatants from single cell suspensions of liver or heart tissue (data not shown), which are known to be transduced *in vivo* by rAAV8 vectors (67, 68).

To assess systemic IL-2 levels after AAV8mIP-IL2 treatment, 10-12 week old NOD mice were vaccinated I.P. with  $2.5 \times 10^{10}$  VP AAV8mIP-IL2,  $1 \times 10^{10}$  VP AAV8mIP-IL2,  $2.5 \times 10^{10}$  VP AAV8mIP-EGFP, or were left untreated. At 10 days post-treatment, IL-2 was detected in the serum of AAV8mIP-IL2 treated NOD mice and maintained over 24 days (Fig. 3.1B), while no IL-2 was detected in the serum of untreated or AAV8mIP-EGFP controls.

To further demonstrate specificity of IL-2 transgene expression, NOD mice were treated with STZ to induce  $\beta$  cell death, and 72 hours later, vaccinated with  $2.5 \times 10^{10}$  VP AAV8mIP-IL2 or were left untreated. As early as 24 hours post-STZ treatment, NOD mice exhibited elevated blood glucose levels (data not shown). Serum IL-2 levels in STZ-treated NOD mice vaccinated with AAV8mIP-IL2 were significantly lower than AAV8mIP-IL2 only treated controls at day 10 and 14 post-streptozotocin treatment (Fig. 3.1C). This data indicates that levels of IL-2 secretion are dependent on the amount of  $\beta$  mass at the time of AAV8mIP-IL2 treatment.

#### **3.4.2 AAV8mIP-IL2 administration increases islet FoxP3<sup>+</sup> Treg frequency and number**

To directly evaluate the effect of increased local IL-2 on islet FoxP3<sup>+</sup> Treg, NOD mice were treated with AAV8mIP-IL2, AAV8mIP-EGFP or left untreated. At 4 weeks post-treatment, the frequency (Fig. 3.2A) and number (Fig. 3.2B) of FoxP3<sup>+</sup> Treg in the draining pancreatic lymph node (PLN) and islets from the respective treatment groups were determined. Both the frequency and number of FoxP3<sup>+</sup> Treg in NOD mice treated with  $2.5 \times 10^{10}$  VP AAV8mIP-IL2 were significantly increased compared to untreated and AAV8mIP-EGFP treated controls. Furthermore, the magnitude of the effect on islet FoxP3<sup>+</sup> Treg in vaccinated NOD mice directly correlated with AAV8mIP-IL2 dose. Notably, no significant effect on the PLN resident FoxP3<sup>+</sup> Treg was detected after treatment (Fig. 3.2A), suggesting that AAV8mIP-IL2 administration preferentially impacts the islets.

In addition to FoxP3<sup>+</sup> Treg, FoxP3<sup>-</sup>CD4<sup>+</sup> T cells were assessed in the PLN and islets of AAV8mIP-IL2 treated NOD mice. There was no marked change in the frequency of

CD25<sup>+</sup>FoxP3<sup>-</sup>CD4<sup>+</sup> T cells in the PLN or islets of AAV8mIP-IL2 treated NOD mice compared to AAV8mIP-EGFP or untreated controls 4 weeks post-treatment (Fig. 3.2C). On the other hand, the frequency of islet-resident CD25<sup>-</sup>FoxP3<sup>-</sup>CD4<sup>+</sup> T cells was significantly reduced in AAV8mIP-IL2 treated NOD mice (Fig. 3.2D). T1D is primarily mediated by  $\beta$  cell specific IFN $\gamma$ -expressing CD4<sup>+</sup> and CD8<sup>+</sup> Teff (8, 10, 11). No effect on the ratio between IFN $\gamma$ <sup>+</sup> Teff and FoxP3<sup>+</sup> Treg was seen in the PLN, regardless of treatment (Fig. 3.2E). In contrast, the ratio of IFN $\gamma$ <sup>+</sup> Teff to FoxP3<sup>+</sup> Treg in the islets was significantly decreased after AAV8mIP-IL2 treatment compared to untreated and AAV8mIP-EGFP controls.

### **3.4.3 IL-2 receptor signaling by islet FoxP3<sup>+</sup> Treg is increased by AAV8mIP-IL2 administration**

IL-2 binding to the IL-2R preferentially induces the phosphorylation of signal transducer and activator of transcription 5 (pSTAT5), leading to downstream signaling events (69). To correlate IL-2 signaling with enhanced FoxP3<sup>+</sup> Treg frequencies, pSTAT5 levels were measured in CD4<sup>+</sup>FoxP3<sup>+/-</sup> populations in the PLN and islets 4 weeks post-AAV vector vaccination. The frequency of pSTAT5<sup>+</sup> islet FoxP3<sup>+</sup> Treg was increased 5- to 10-fold in AAV8mIP-IL2 versus control groups (Fig. 3.3A). A modest 2-fold increase in the frequency of pSTAT5-positive cells was detected in islet CD4<sup>+</sup>FoxP3<sup>-</sup> T cells in NOD mice vaccinated with  $2.5 \times 10^{10}$  VP AAV8mIP-IL2 (Fig. 3.3B). Furthermore, no effect was seen on either FoxP3<sup>+</sup> Treg or CD4<sup>+</sup>FoxP3<sup>-</sup> populations in the PLN (Fig. 3.3A, 3.3B). Therefore,

the increased frequency and number of FoxP3<sup>+</sup> Treg in the islets of AAV8mIP-IL2-treated NOD mice correlates with enhanced levels of pSTAT5.

#### **3.4.4 FoxP3<sup>+</sup> Treg survival and proliferation are increased by AAV8mIP-IL2 vaccination**

IL-2 plays a key role in the maintenance and expansion of natural FoxP3<sup>+</sup> Treg (23-27), as well as in the differentiation of naïve T cells into adaptive FoxP3<sup>+</sup> Treg from naïve precursors (28-30). Therefore, the composition of the expanded islet FoxP3<sup>+</sup> Treg pool after AAV8mIP-IL2 administration was investigated. NOD mice were treated with AAV8mIP-IL2 or AAV8mIP-EGFP, and the frequency of HELIOS expressing cells within FoxP3<sup>+</sup> Treg was assessed at 4 weeks post-treatment. Approximately 80% of FoxP3<sup>+</sup> Treg in the PLN expressed HELIOS, whereas >94% of FoxP3<sup>+</sup> Treg were HELIOS<sup>+</sup> in the islets (Fig. 3.4A). No difference in the frequency of HELIOS expressing FoxP3<sup>+</sup> Treg between AAV8mIP-IL2 and AAV8mIP-EGFP treated recipients was observed (Fig. 3.4A).

The proliferative status of islet and PLN resident FoxP3<sup>+</sup> Treg after AAV8mIP-IL2 administration was analyzed by KI67 staining. As early as 5 days post-treatment, the frequency of KI67<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg was increased in the islets, with a significant increase at day 8 post-treatment (Fig. 4.4B). Interestingly, the frequency of KI67<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg in the islets was reduced to levels below those found in untreated animals by 4 weeks, despite significant increases in islet FoxP3<sup>+</sup> Treg frequency and number (Fig. 3.4B). Comparatively, the frequency of KI67<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg in the PLN remained unchanged over time (Fig. 3.4B). To determine if AAV8mIP-IL2 vaccination increased the survival of islet FoxP3<sup>+</sup> Treg, expression of the anti-apoptotic

protein Bcl-2 was measured. Islet FoxP3<sup>+</sup> Treg expressed significantly higher levels of Bcl-2 at days 5 and 8 post-treatment based on mean fluorescent intensity (MFI) (Fig. 3.4C), which correlated with an increased frequency of Bcl-2 expressing FoxP3<sup>+</sup> Treg (Fig. 3.4D). In contrast, no change in Bcl-2 expression was detected in PLN FoxP3<sup>+</sup> Treg (Fig. 3.4C, 3.4D) or in islet CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> and CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> Teff (Fig. 3.4E), indicating that IL-2 is specifically affecting FoxP3<sup>+</sup> Treg. These results demonstrate that the increased number and frequency of FoxP3<sup>+</sup> Treg after AAV8mIP-IL2 treatment is associated with enhanced survival and proliferation.

#### **3.4.5 T1D is averted at a late pre-clinical stage in NOD mice treated with AAV8mIP-IL2**

Since localized IL-2 expression increased the islet FoxP3<sup>+</sup> Treg pool, the ability of AAV8mIP-IL2 immunotherapy to suppress ongoing  $\beta$  cell autoimmunity and prevent overt diabetes was examined. NOD female mice 10-12 weeks of age, and representing a late preclinical stage of T1D, were treated with AAV8mIP-IL2, AAV8mIP-EGFP or left untreated and monitored for diabetes. Both serum IL-2 (Fig. 3.5C) and the frequency of FoxP3<sup>+</sup> Treg (Fig. 3.5D) in peripheral blood lymphocytes (PBL) were significantly enhanced at 1 week post-treatment with AAV8mIP-IL2 and were maintained in normoglycemic animals until the conclusion of the study. The incidence of diabetes was significantly reduced in NOD female mice treated with  $2.5 \times 10^{10}$  VP AAV8mIP-IL2 (0/9) or  $1 \times 10^{10}$  VP AAV8mIP-IL2 (2/8) compared to untreated controls (18/22) (Fig. 3.5A). NOD mice treated with  $2.5 \times 10^{10}$  VP AAV8mIP-EGFP (10/10) developed diabetes by 20 weeks of age, indicating that diabetes protection was not attributed to a nonspecific



effect of AAV vector transduction of  $\beta$  cells (Fig. 3.5A). Consistent with diabetes incidence, histological analyses of pancreases from 18 week old NOD mice showed a significant decrease in the frequency of insulitis in AAV8mIP-IL2 treated versus age-matched normoglycemic AAV8mIP-EGFP treated controls (Fig. 3.5B).

After 35 weeks, the PLN and islets from NOD mice were harvested and assayed for the frequency of FoxP3<sup>+</sup> Treg and IFN $\gamma$ <sup>+</sup> Teff. AAV8mIP-IL2 vaccinated NOD mice displayed a significantly higher frequency of FoxP3<sup>+</sup> Treg in the islets compared to untreated controls, while no significant difference in the PLN was observed (Fig. 3.5E). Furthermore, the frequency of CD62L<sup>HI</sup> expressing islet FoxP3<sup>+</sup> Treg was increased in AAV8mIP-IL2 treated NOD mice (Fig. 3.5F). Additionally, the ratio between IFN $\gamma$ <sup>+</sup> Teff to FoxP3<sup>+</sup> Treg in the PLN and islets of long-term treated recipients was evaluated. Strikingly, the ratio of IFN $\gamma$ <sup>+</sup> Teff to FoxP3<sup>+</sup> Treg was decreased in the islets of AAV8mIP-IL2 treated NOD mice compared to normoglycemic untreated controls (Fig. 3.5G); The IFN $\gamma$ <sup>+</sup> Teff to FoxP3<sup>+</sup> Treg ratio in the PLN was unaffected by AAV8mIP-IL2 vaccination.

#### **3.4.6 Islet FoxP3<sup>+</sup> Treg are functionally enhanced after AAV8mIP-IL2 treatment**

In addition to quantitative changes, we assessed whether AAV8mIP-IL2 treatment also induced qualitative changes in islet FoxP3<sup>+</sup> Treg. FoxP3<sup>+</sup> Treg are known to inhibit through various contact dependent and independent mechanisms, including the expression of cell surface GITR (70), CTLA-4 (71), and ICOS (72), as well through secretion of IL-10 (73), among others. At both day 5 and 8 post-treatment with AAV8mIP-IL2, the frequency of IL-10<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg was significantly increased in the

islets compared to control NOD mice (Fig. 3.6A), while no marked effect on the corresponding PLN populations was observed.

ICOS expression has been previously reported to correlate with enhanced synthesis of IL-10 in FoxP3<sup>+</sup> Treg (72). Therefore, the frequency of islet resident IL-10<sup>+</sup>FoxP3<sup>+</sup> Treg was examined in relation to ICOS expression. The frequency of ICOS<sup>+</sup>IL-10<sup>+</sup> Treg in the islets was higher than ICOS<sup>-</sup>IL-10<sup>+</sup> populations in all groups analyzed (Fig. 3.6B). Interestingly, after AAV8mIP-IL2 treatment, the frequency of both ICOS<sup>+</sup>IL-10<sup>+</sup> and ICOS<sup>-</sup>IL-10<sup>+</sup> FoxP3<sup>+</sup> Treg was increased at both day 5 and 8, while maintaining a higher overall frequency of ICOS<sup>+</sup>IL-10<sup>+</sup> in the islets. Furthermore a significant increase in the expression of CD25 and a trending increase in GITR and CTLA-4 expression was detected in islet FoxP3<sup>+</sup> Treg of AAV8mIP-IL2 treated NOD mice (Fig. 3.6C-F). Combined these data would strongly indicate that increased IL-2 in the islets mediated by AAV8mIP-IL2 treatment alters expression of FoxP3<sup>+</sup> Treg-associated suppressor molecules.

To determine if the above phenotype changes correlated with enhanced function, the suppressor activity of FoxP3<sup>+</sup> Treg from AAV8mIP-IL2 treated NOD mice was assessed. For this experiment, FoxP3<sup>+</sup> Treg were sorted from the spleens of AAV8mIP-IL2 or AAV8mIP-EGFP vaccinated NOD.FoxP3.BDC.GFP mice 4 weeks post-vaccination. Isolated FoxP3<sup>+</sup> Treg were then co-cultured *in vitro* with labeled naïve BDC2.5 responder T cells and peptide pulsed APCs for 72 hours, starting at a ratio of 1:1:1. FoxP3<sup>+</sup> Treg from AAV8mIP-IL2 donors showed a significantly increased suppressor function compared to FoxP3<sup>+</sup> Treg isolated from AAV8mIP-EGFP donors (Fig. 3.7A). Proliferation of responder T cells was significantly reduced at both peptide

concentrations tested in cultures containing FoxP3<sup>+</sup> Treg from AAV8mIP-IL2 versus AAV8mIP-EGFP at 1 to 1 and 1 to 2 FoxP3<sup>+</sup> Treg to Teff ratios. Furthermore, IFN $\gamma$  (Fig. 3.7B) levels were similarly reduced in these cultures. Therefore, vaccination with AAV8mIP-IL2 induces a significantly more suppressive FoxP3<sup>+</sup> Treg pool that inhibits  $\beta$  cell specific Teff *in vitro*.

### 3.5 Discussion

IL-2 is essential for the induction and maintenance of FoxP3<sup>+</sup> Treg both *in vivo* and *in vitro* (23-25, 27-30). Furthermore, administration of rIL-2 has been effective in preventing ongoing  $\beta$  cell autoimmunity (36). Due to the effects of IL-2 *in vivo*, however, the efficacy of rIL-2 in inducing a protective versus proinflammatory population is highly dependent on the dose, half-life and localization of the treatment *in vivo*. Our results demonstrate that AAV8mIP-IL2 treatment of NOD mice suppresses ongoing  $\beta$  cell autoimmunity and prevents diabetes.

Previous studies have shown that transgene expression driven by an AAV8mIP expressing vector results in reduced diabetes incidence compared to systemic expression of the same transgene by an AAV vector injected intramuscularly (51, 74). The enhanced protection afforded by AAV8mIP driven vectors is due to increased islet localized transgene expression. Importantly, in our studies islet (Fig. 3.1A) IL-2 levels in AAV8mIP-IL2 vaccinated NOD mice were increased in a vector dose-dependent manner. Furthermore, STZ treated NOD mice subsequently vaccinated with AAV8mIP-IL2 (Fig. 3.1C), and failed long term treated recipients (data not shown), exhibited significantly reduced IL-2 levels in the serum compared to normoglycemic counterparts, showing that  $\beta$  cells are both the source of IL-2 transgene expression and survival of  $\beta$  cells is necessary to maintain IL-2 transgene expression after AAV8mIP-IL2 vaccination. Notably, systemic IL-2 levels in our AAV8mIP-IL2 vaccinated NOD mice (Fig. 3.1B) were significantly reduced compared to our previous reports, which utilized a tetracycline-inducible system (61) and in NOD recipients receiving high-dose rIL-2 systemically (36).

As a result, there were no detectable off-target effects on other cell types, including NKT cells or B cells (data not shown). Clinically, the limiting of transgene expression locally is imperative for dampening the autoimmune process, while leaving the rest of the immune system functional. This was highlighted in recent failed clinical trials utilizing rIL2 and rapamycin for the treatment of recent onset human type 1 diabetic patients (47). In addition, transgene expression was both constant and maintained in treated normoglycemic NOD mice, indicating that AAV transduction did not alter survival or function of  $\beta$  cells (Fig. 3.5C). Given that at the time of diagnosis, only 10-20% of  $\beta$  mass is left, the ability to maintain that population functionally is of paramount importance for treatment.

Despite increasing localized islet IL-2 levels, the ability of AAV8mIP-IL2 vaccination to affect islet-resident lymphocyte populations was unknown. It has been shown that FoxP3<sup>+</sup> Treg preferentially respond to IL-2 in the absence of antigen stimulation, whereas naïve CD4<sup>+</sup> T cells and Teff require antigen stimulation in combination with IL-2 (75). Islet resident FoxP3<sup>+</sup> Treg showed enhanced levels of pSTAT5 (Fig. 3.3A), indicative of constitutive IL-2 signaling after AAV8mIP-IL2 treatment. This persistent IL-2R signaling resulted in an increased frequency of thymically derived natural FoxP3<sup>+</sup> Treg in both short- (Fig. 3.2A) and long- (Fig. 3.5E) term treated NOD mice, as determined by maintenance of HELIOS<sup>+</sup>FoxP3<sup>+</sup> Treg frequency after vaccination (Fig. 3.4A). This shows that IL-2 is sufficient to increase the frequency of thymically derived islet FoxP3<sup>+</sup> Treg *in vivo*, while suggesting that ectopic expression of IL-2 alone is unable to induce the formation of adaptive FoxP3<sup>+</sup> Treg. Importantly, this indicates that

FoxP3<sup>+</sup> Treg in NOD mice can be induced to provide sufficient protection and are not permanently defective *per se*.

Mechanistically, the expanded islet FoxP3<sup>+</sup> Treg pool after AAV8mIP-IL2 vaccination was preceded by an increased frequency of KI67<sup>+</sup> cells (Fig. 3.4B), a proliferation marker, between day 5 and 8 post-AAV vaccination. This initial burst in proliferating islet FoxP3<sup>+</sup> Treg was compounded by increased survival of the expanded FoxP3<sup>+</sup> Treg pool, as determined by expression of the anti-apoptotic marker Bcl-2 (Fig. 3.4C, 3.4D) after AAV8mIP-IL2 vaccination. In addition to quantitative changes, AAV8mIP-IL2 vaccination altered the phenotype of islet FoxP3<sup>+</sup> Treg as determined by increased synthesis of IL-10 (Fig. 6A), as well as increased expression of various contact-dependent FoxP3<sup>+</sup> Treg markers, including ICOS (Fig. 6B), CD25 (Fig. 6D), GITR (Fig. 6E) and CTLA-4 (Fig. 6F). Notably, the frequency of CD62L<sup>HI</sup>FoxP3<sup>+</sup> Treg was increased in the islets after AAV8mIP-IL2 vaccination (Fig. 5F), which have previously been shown to be more protective compared to CD62L<sup>LO</sup> counterparts (39, 76-78). The complexity of these changes shows a robust alteration in the islet FoxP3<sup>+</sup> Treg compartment that is suggestive of a functionally enriched FoxP3<sup>+</sup> Treg pool. Along these lines, FoxP3<sup>+</sup> Treg derived from AAV8mIP-IL2 vaccinated NOD mice were better at suppressing the expansion of BDC2.5 specific responders *in vitro* (Fig. 7A), that correlated with reduced IFN $\gamma$  secretion (Fig. 7B). Collectively, this suggests that AAV8mIP-IL2 acts in multiple ways to alter islet FoxP3<sup>+</sup> Treg, specifically by enhancing expansion, survival, and functionality.

In long-term studies, qualitatively and quantitatively enhanced islet FoxP3<sup>+</sup> Treg reduced diabetes incidence in NOD female mice after AAV8mIP-IL2 vaccination (Fig. 5A). This is attributed to both reduced insulitis (Fig. 5B) and an altered Teff to FoxP3<sup>+</sup> Treg ratio in the islets of both long- (Fig. 5G) and short-term (Fig. 2E) AAV8mIP-IL2 vaccinated recipients. Moreover, AAV8mIP-IL2 vaccination of NOD.8.3 mice, a CD8<sup>+</sup> T cell transgenic mouse model of T1D, showed no change in diabetes incidence compared to AAV8mIP-EGFP controls, indicating FoxP3<sup>+</sup> Treg are required to mediated protection (Fig. 3.S1). Collectively, these results are consistent with a model that suggests that once established islet FoxP3<sup>+</sup> Treg can block further islet infiltrates after AAV8mIP-IL2 vaccination.

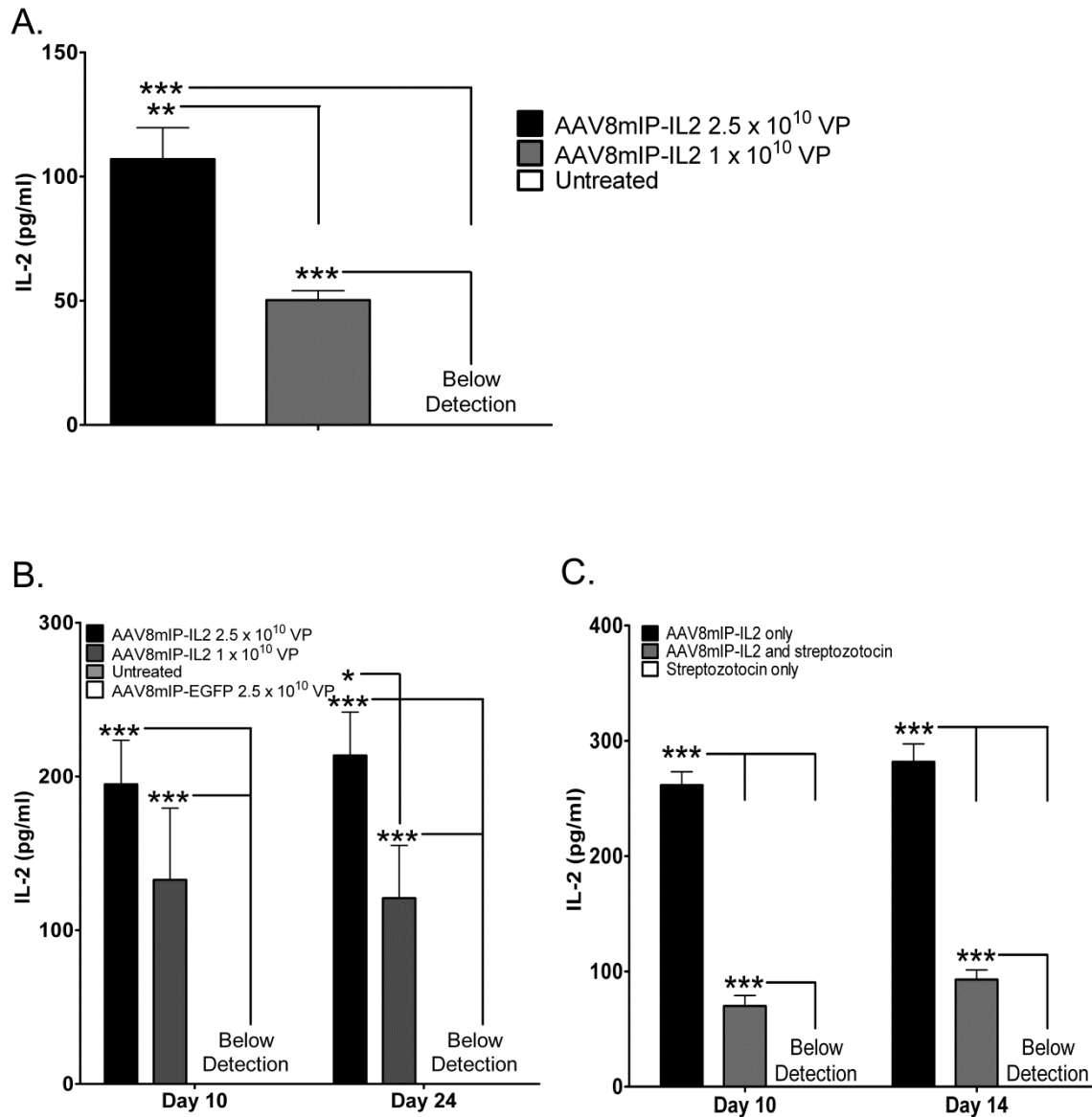
Surprisingly, AAV8mIP-IL2 treatment did not alter FoxP3<sup>+</sup> Treg frequency or function within the PLN or other lymph nodes (Fig. 3.S2). Possibly, AAV8mIP-IL2 transgene expression in the islets may bypass the lymphatics and directly enter the bloodstream. This hypothesis is supported by the significantly increased frequency of FoxP3<sup>+</sup> Treg in PBL (Fig. 3.5C) and detectable serum IL-2 (Fig. 3.5D) levels in long term AAV8mIP-IL2 recipient NOD mice. Additionally, IL-2 was not detected in supernatants of *in vitro* plated PLN cells from AAV8mIP-IL2 recipients (data not shown).

Despite the success of AAV8mIP-IL2 to prevent the onset of T1D in late preclinical NOD female mice, the ability of this treatment to reverse diabetes in recent onset mice is unknown. Presumably, the limited  $\beta$  cell mass existing at the time of clinical onset may be insufficient to drive the necessary transgene expression required

to induce remission, despite a reduction in the autoimmune response. Alternatively, other groups work showing the conversion of different cell types within the pancreas to become insulin secreting cells provides an interesting avenue of investigation (79). Furthermore, utilizing AAV8mIP-IL2 in combinatorial treatment, particularly in the context of non-depleting monoclonal antibody administration or diabetogenic plasmid DNA vaccination, which have both been utilized successfully in prevention and treatment studies, is noteworthy (80-83).

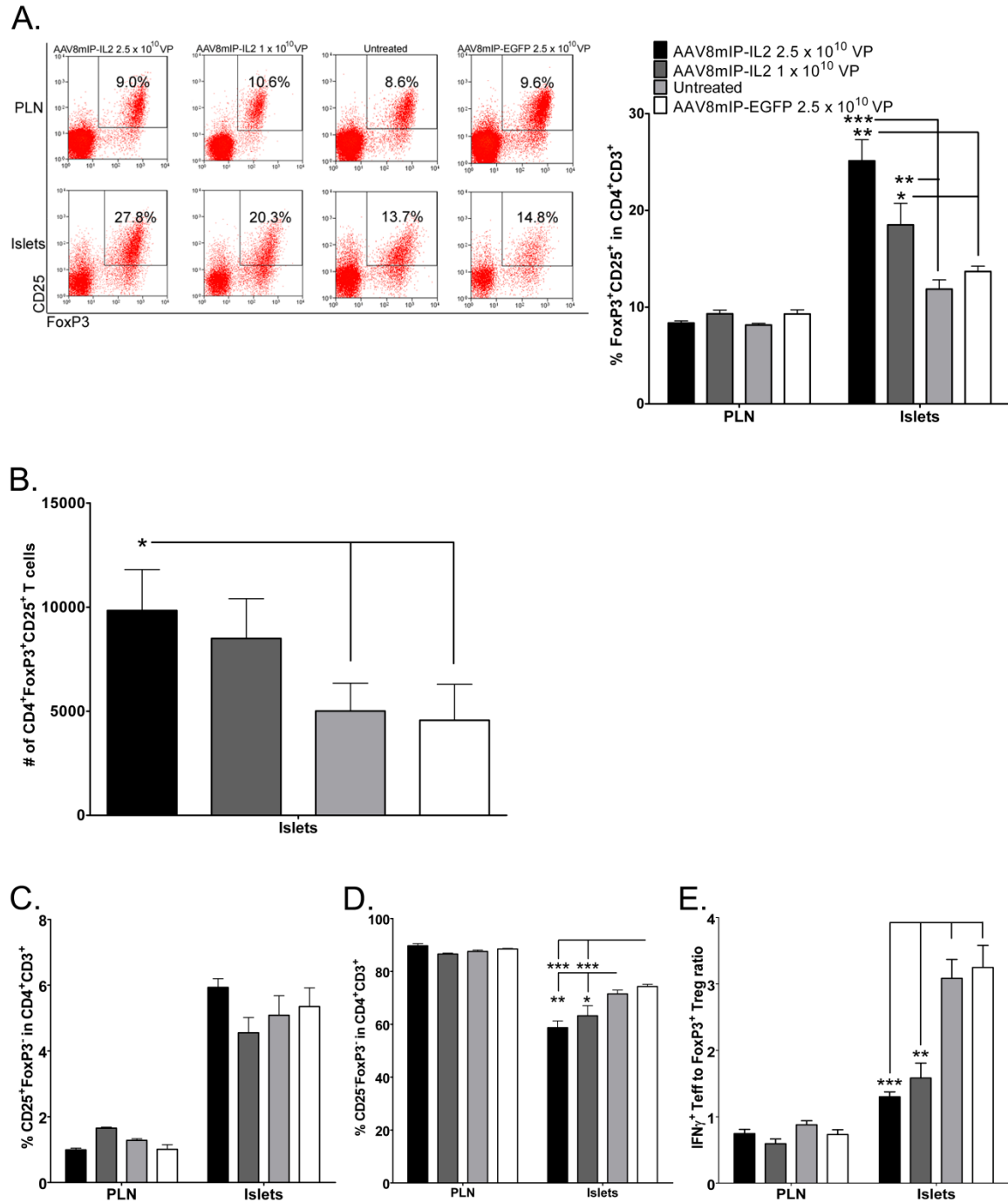
In conclusion, we have demonstrated the AAV8mIP-IL2 treatment is sufficient to prevent ongoing  $\beta$  cell autoimmunity in a fashion that correlates with AAV vector dose. This protection is mediated through effects on the islet FoxP3<sup>+</sup> Treg, resulting in both quantitatively and qualitatively altered islet FoxP3<sup>+</sup> Treg. Furthermore, the effect of AAV8mIP-IL2 was limited strictly to islet FoxP3<sup>+</sup> Treg, significantly reducing the off-target effects. The research presented demonstrates that localized, viral driven transgene expression may prove an effective and safe immunotherapeutic strategy for the treatment of T1D and other T cell mediated autoimmune diseases.





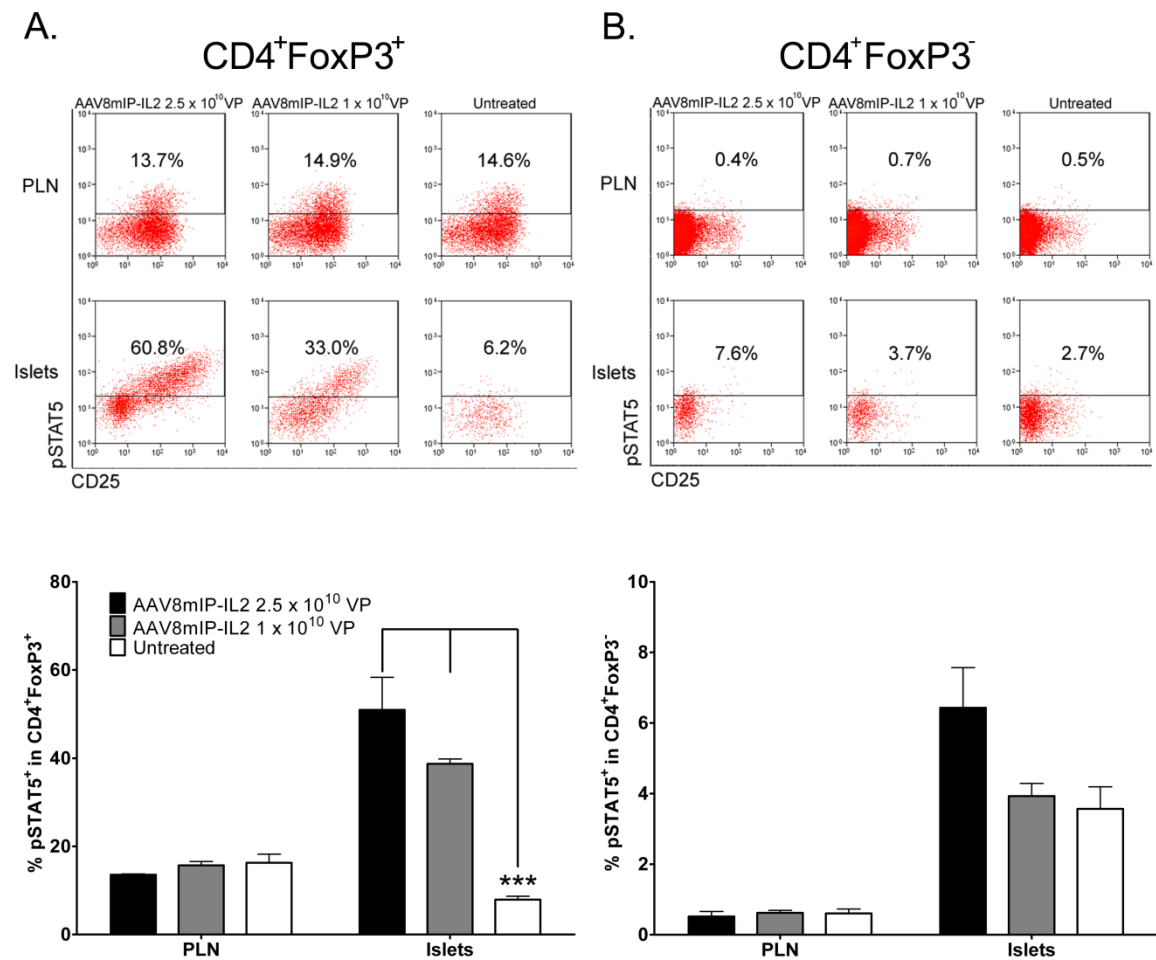
**Figure 3.1  $\beta$  cell specificity of IL-2 transgene expression after AAV8mIP-IL2 vaccination.** (A) IL-2 levels in supernatants from islets isolated from NOD.scid mice vaccinated with AAV8mIP-IL2  $2.5 \times 10^{10}$  VP, AAV8mIP-IL2  $1 \times 10^{10}$  VP, or left untreated and cultured for 96 hours. \*\*\* $p < 0.001$  (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP and AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated), \*\* $p < 0.01$  (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus AAV8mIP-IL2  $1 \times 10^{10}$  VP) (one way ANOVA  $\pm$  SEM). (B) IL-2 levels in serum of 10-12 week old pre-diabetic NOD mice treated with AAV8mIP-IL2  $2.5 \times 10^{10}$  VP, AAV8mIP-IL2  $1 \times 10^{10}$  VP, AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP or left untreated at day 10 and 24 post-treatment. \*\*\* $p < 0.001$  (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP and AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated and AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP), \* $p < 0.05$  (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus AAV8mIP-IL2  $1 \times 10^{10}$  VP at day 24) (two way ANOVA  $\pm$  SEM). (C) Serum IL-2 levels in NOD mice treated with streptozotocin alone, streptozotocin followed by AAV8mIP-IL2  $2.5 \times 10^{10}$  VP 72 hours later, or AAV8mIP-IL2  $2.5 \times 10^{10}$  VP alone at day 10 and 14 post-streptozotocin.

\*\*\*p < 0.001 (AAV8mIP-IL2 only versus AAV8mIP-IL2 and streptozotocin and streptozotocin only and AAV8mIP-IL2 and streptozotocin versus streptozotocin only) (two way ANOVA  $\pm$  SEM).

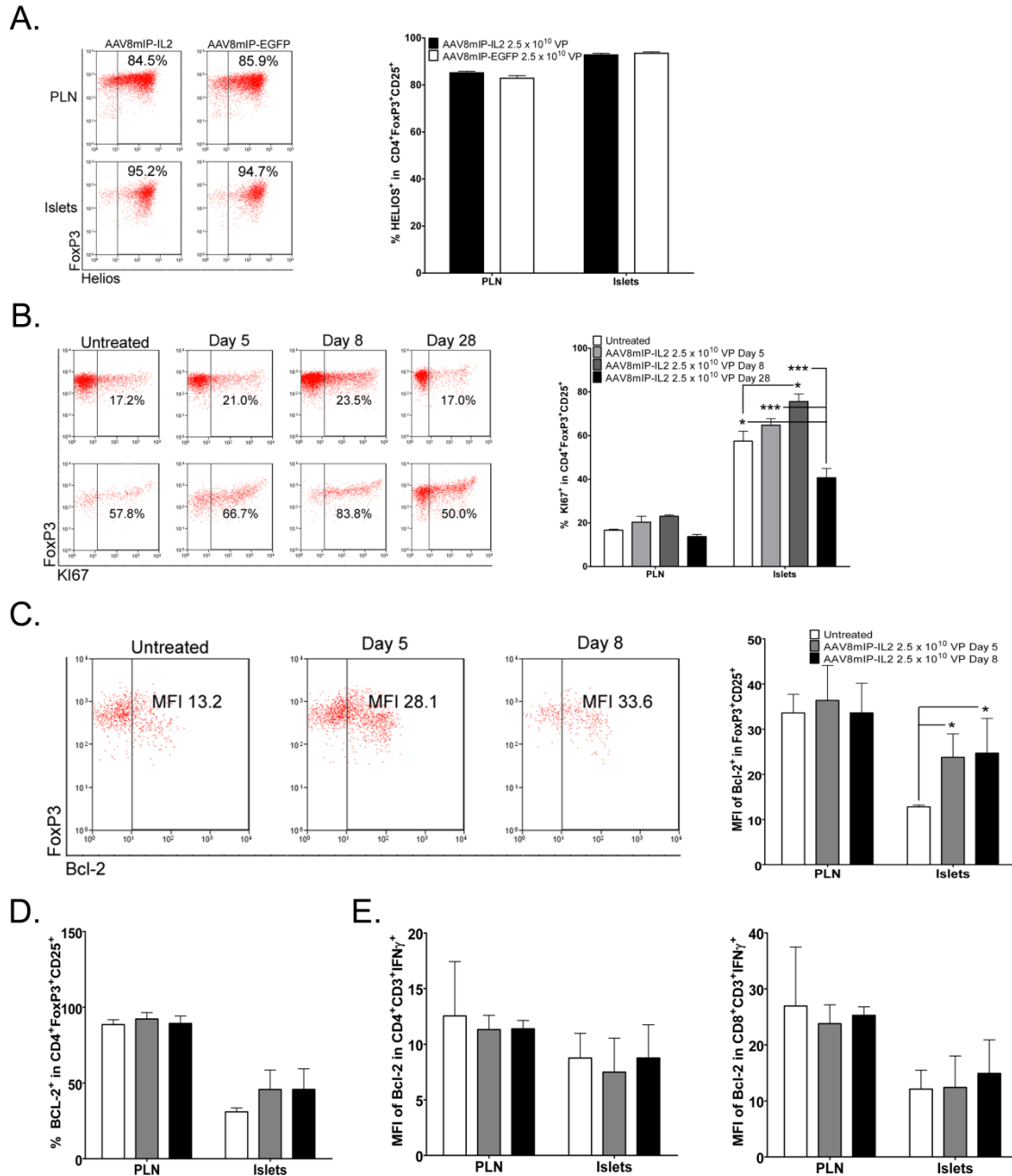


**Figure 3.2 The frequency and number of islet FoxP3<sup>+</sup> Treg are increased after short term AAV8mIP-IL2 treatment.** (A) Representative FACS plots and corresponding graphs for the frequency of CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg in the PLN and islets of groups of 6-10 NOD mice at 4 weeks post-treatment with AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP, AAV8mIP-IL2 1 x 10<sup>10</sup> VP, AAV8mIP-EGFP 2.5 x 10<sup>10</sup> VP, or untreated. \*\*\*p < 0.001 (AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP versus Untreated), \*\*p < 0.01 (AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP versus AAV8mIP-EGFP 2.5 x 10<sup>10</sup> VP and AAV8mIP-IL2 1 x 10<sup>10</sup> VP versus Untreated), \*p < 0.05 (AAV8mIP-IL2 1 x 10<sup>10</sup> VP versus Untreated).

VP versus AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP) (two-way ANOVA  $\pm$  SEM) (B) Number of islet CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg recovered from on average 70-100 islets/mouse. \* $p < 0.05$  (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus untreated and AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP) (one-way ANOVA  $\pm$  SEM). Frequency of (C) CD4<sup>+</sup>FoxP3<sup>-</sup>CD25<sup>+</sup> and (D) CD4<sup>+</sup>FoxP3<sup>-</sup>CD25<sup>-</sup> cells in the same short term treated NOD mice, \*\*\* $p < 0.001$  (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP and AAV8mIP-IL2  $1 \times 10^{10}$  VP versus AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP in islets), \*\* $p < 0.01$  (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus untreated in islets), \* $p < 0.05$  (AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated in islets) (two-way ANOVA  $\pm$  SEM). (E) The ratio of IFN $\gamma$ <sup>+</sup> Teff to FoxP3<sup>+</sup> Treg in the PLN and islets between treatment groups. \*\*\* $p < 0.001$  (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus untreated and AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP in islets), \*\* $p < 0.01$  (AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated and AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP in islets) (two-way ANOVA  $\pm$  SEM).

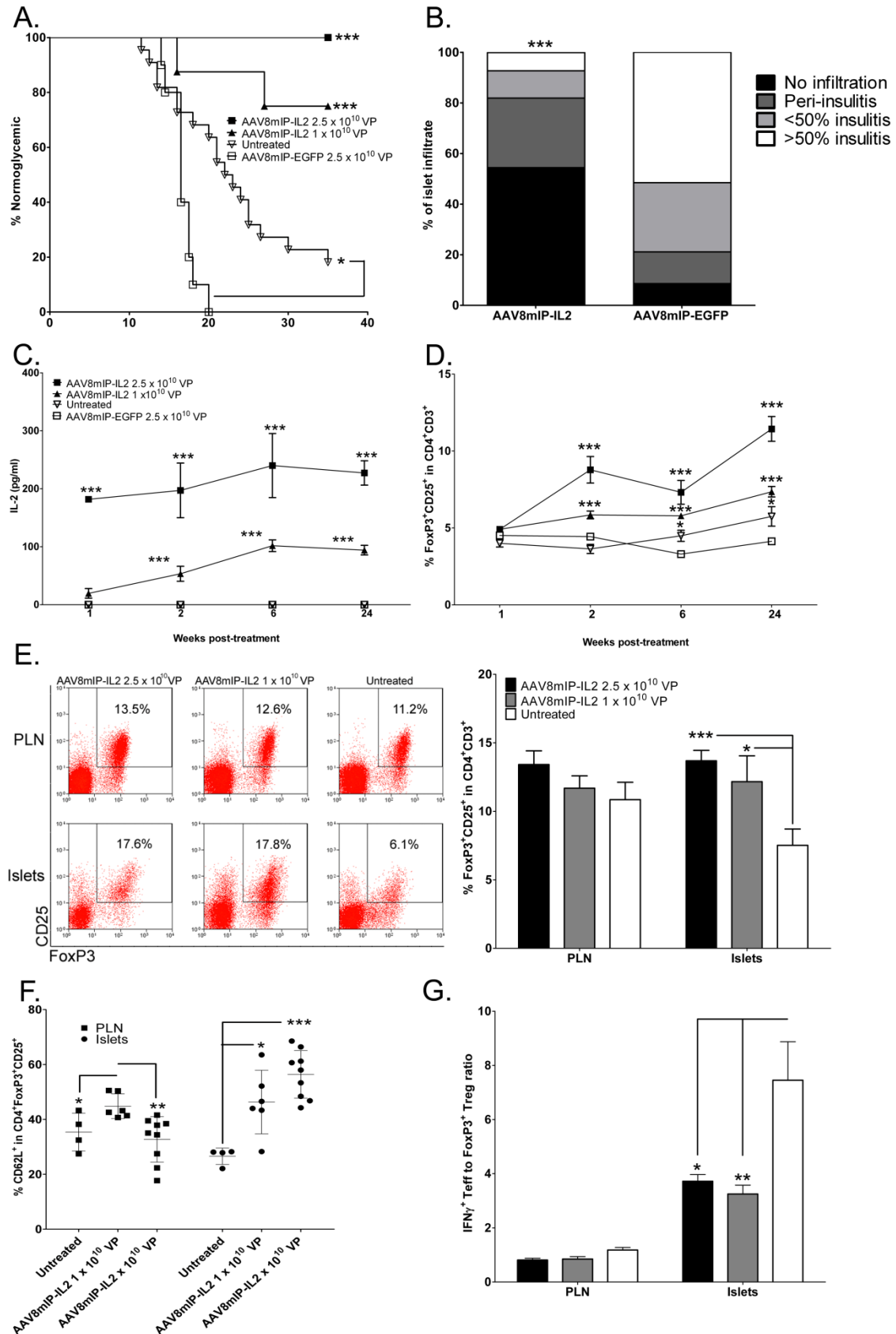


**Figure 3.3 Treatment with AAV8mIP-IL2 increases the frequency of pSTAT5<sup>+</sup> islet FoxP3<sup>+</sup> Treg.** Representative FACS plots of and corresponding graphs for the frequency of pSTAT5<sup>+</sup> cells within the PLN and islet (A) CD4<sup>+</sup>FoxP3<sup>+</sup> or (B) CD4<sup>+</sup>FoxP3<sup>-</sup> T cell in groups of 5 NOD mice vaccinated with AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP, AAV8mIP-IL2 1 x 10<sup>10</sup> VP, or left untreated. Samples were analyzed 4 weeks-post AAV vector administration, \*\*\*p < 0.001 (AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP and AAV8mIP-IL2 1 x 10<sup>10</sup> VP versus untreated in islets) (two-way ANOVA ± SEM).



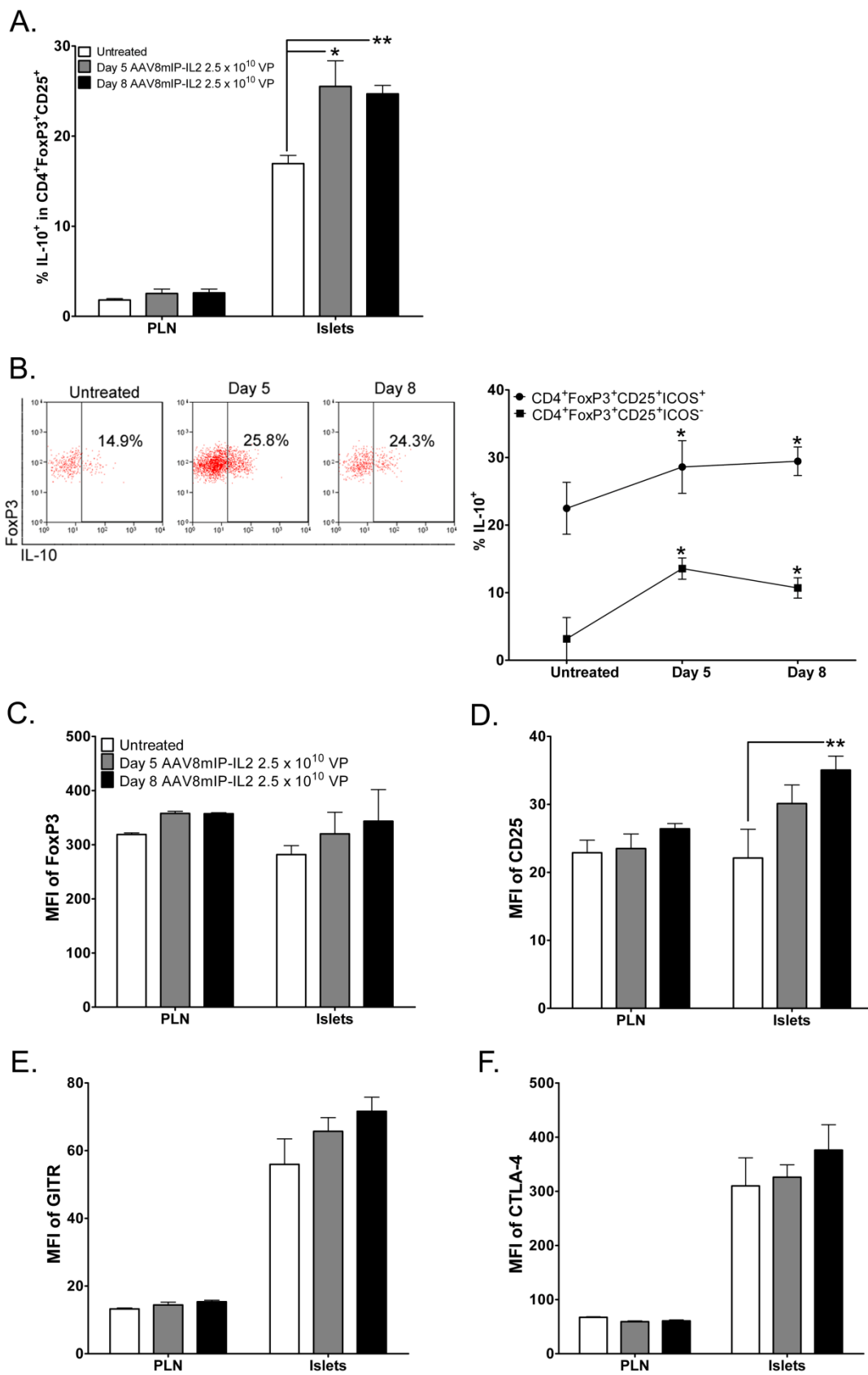
**Figure 3.4 Increased expansion and survival of islet FoxP3<sup>+</sup> Treg after AAV8mIP-IL2 treatment.** Representative FACS plots and corresponding graphs for the frequency of HELIOS<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> (A) or KI67<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> (B) cells in the PLN and islets of groups of 5 NOD mice vaccinated with AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP or AAV8mIP-EGFP 2.5 x 10<sup>10</sup> VP 4 weeks post-treatment. \*\*\*p < 0.001 (AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP at day 5 and day 8 versus AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP at day 28 in islets), \*p < 0.05 (AAV8mIP-IL2 2.5 x

$10^{10}$  VP at day 8 versus untreated and untreated versus AAV8mIP-IL2  $2.5 \times 10^{10}$  VP at day 28 in islets) (two-way ANOVA  $\pm$  SEM). Representative islet FACS plots and graphs for the MFI of Bcl-2 (C) and frequency (D) of Bcl-2<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg in the PLN and islets of untreated NOD mice or at day 5 and 8 post-vaccination with of AAV8mIP-IL2  $2.5 \times 10^{10}$  VP. \* $p < 0.05$  (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP day 5 and 8 versus untreated in islets) (two-way ANOVA  $\pm$  SEM). The MFI of Bcl-2 in CD4<sup>+</sup>CD3<sup>+</sup>IFN $\gamma$ <sup>+</sup> (E) and CD8<sup>+</sup>CD3<sup>+</sup>IFN $\gamma$ <sup>+</sup> (F) was also assessed in the PLN and islets from groups of 4 NOD mice.

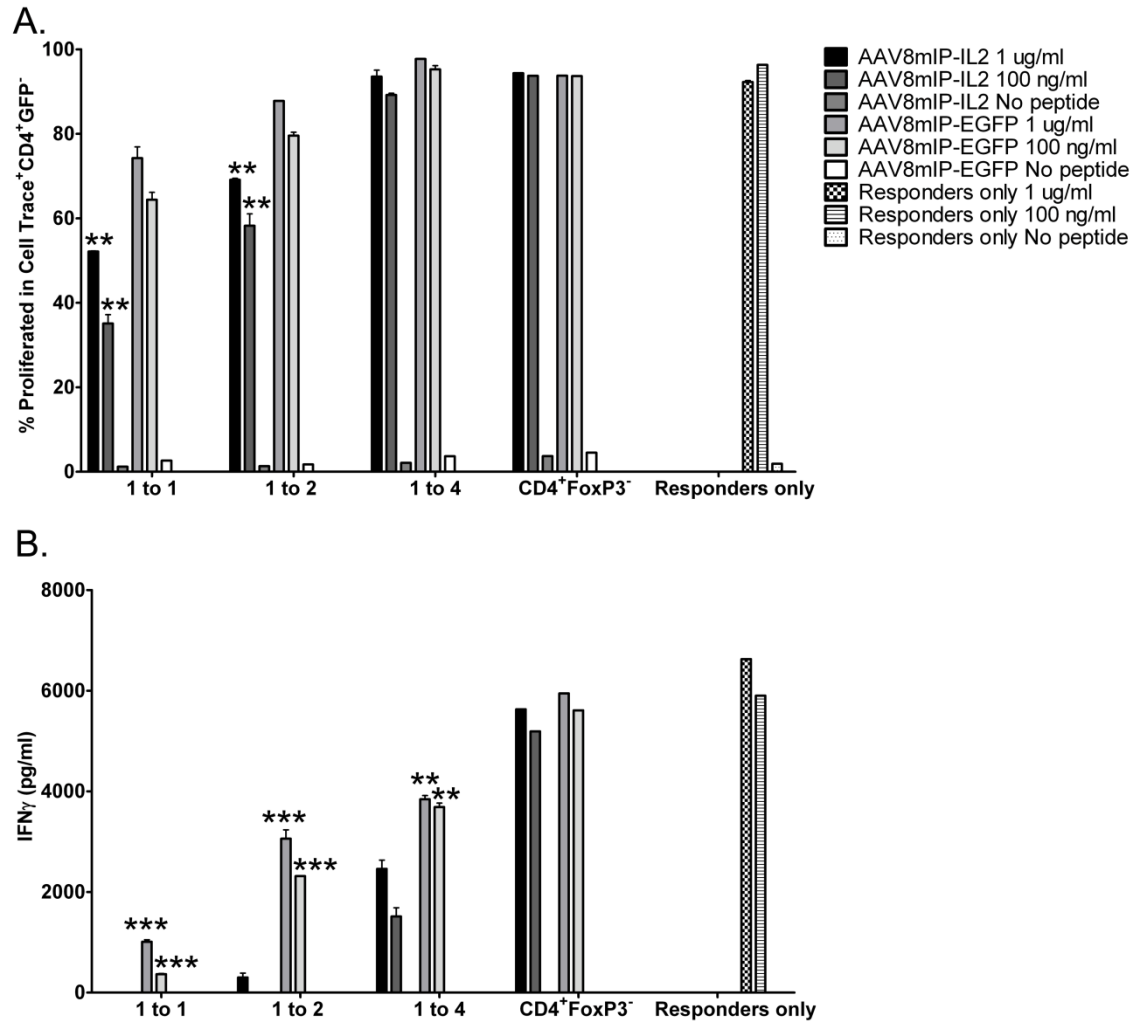




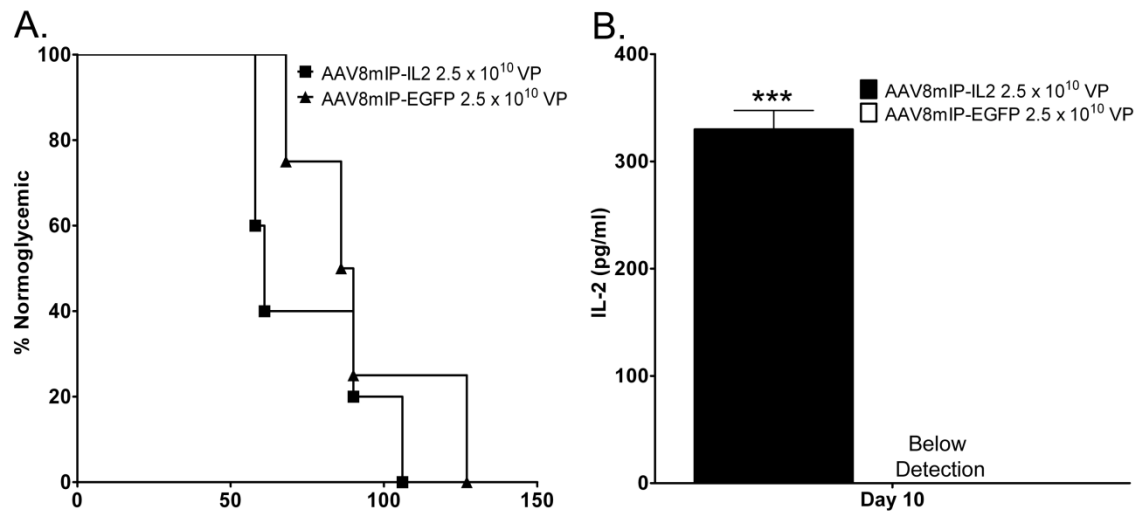
**Figure 3.5 AAV8mIP-IL2 vaccination prevents diabetes in NOD mice at a late pre-clinical stage of T1D by increased islet FoxP3<sup>+</sup> Treg.** (A) 10-12 week NOD female mice were vaccinated with AAV8mIP-IL2  $2.5 \times 10^{10}$  VP (n = 9), AAV8mIP-IL2  $1 \times 10^{10}$  VP (n = 8), AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP (n = 10), or left untreated (n = 22) and then monitored for overt diabetes. \*\*\*p < 0.001 (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP and AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated and AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP), \*p < 0.05 (untreated versus AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP) (Kaplan Meier log rank test). (B) The frequency of insulinitis was determined in groups of 5 pre-diabetic NOD mice treated at 10 weeks with AAV8mIP-IL2  $2.5 \times 10^{10}$  VP or AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP and harvested at 18 weeks. \*\*\* p < 0.001 (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP) (two way ANOVA  $\pm$  SEM) (C) Serum IL-2 levels {\*\*\*p < 0.001 (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP and AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated and AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP) (one-way ANOVA  $\pm$  SEM)}. (D) Frequency of CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg in PBL were assessed from NOD mice treated in (A) up to 35 weeks of age, \*\*\*p < 0.001 (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP and AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated and AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP), \*p < 0.05 (Untreated versus AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP) (one-way ANOVA  $\pm$  SEM), (E) At 35 weeks, representative FACS plots and corresponding graphs are shown for the frequency of CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg in the PLN and islets of NOD mice from (A). \*\*\*p < 0.001 (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus untreated in islets), \*p < 0.05 (AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated in islets) (two-way ANOVA  $\pm$  SEM) (F) CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg were assessed for expression of CD62L. \*\*\*p < 0.001 (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus untreated in islets), \*\*p < 0.01 (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus AAV8mIP-IL2  $1 \times 10^{10}$  VP in PLN), \*p < 0.05 (AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated in islets or AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated in PLN) (two-way ANOVA  $\pm$  SEM). (G) The ratio of IFN $\gamma$ <sup>+</sup> Teff to FoxP3<sup>+</sup> Treg was compared between treatment groups in PLN and islets. \*\*p < 0.01 (AAV8mIP-IL2  $1 \times 10^{10}$  VP versus untreated in islets), \*p < 0.05 (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus untreated in islets) (two-way ANOVA  $\pm$  SEM).



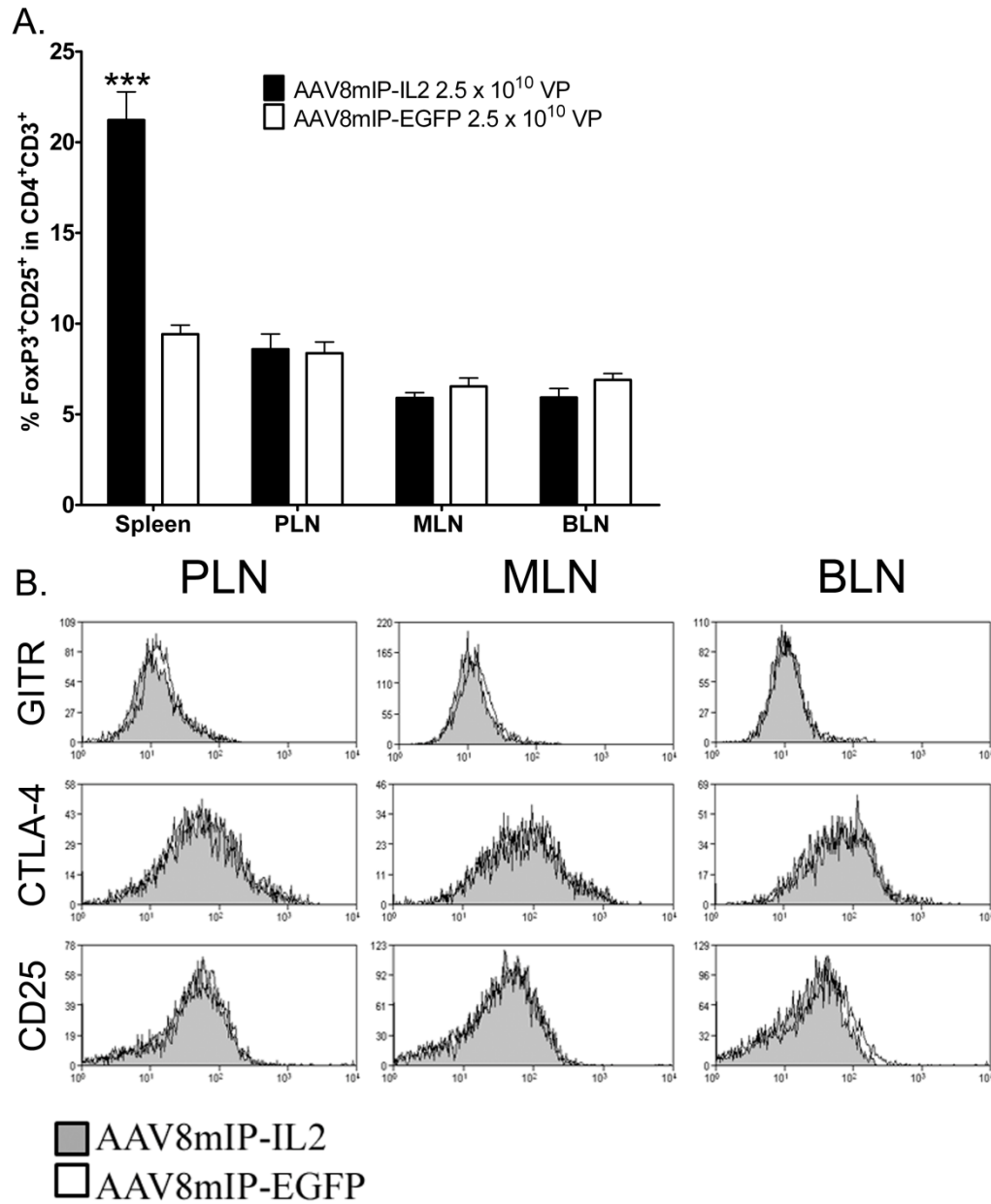
**Figure 3.6 Qualitative changes in islet FoxP3<sup>+</sup> Treg after AAV8mIP-IL2 treatment.** (A) Frequency of IL-10<sup>+</sup> cells within CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> Treg in the islets of NOD mice day 5 and 8 post-treatment with AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP or untreated, \*\*p < 0.01 (Day 8 AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP versus untreated islets), \*p < 0.05 (Day 5 AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP islets versus untreated islets) (two-way ANOVA ± SEM). (B) Representative FACS plots and corresponding graphs for the frequency of IL-10<sup>+</sup>ICOS<sup>+/−</sup> cells within islet FoxP3<sup>+</sup>CD25<sup>+</sup> Treg. \*p < 0.05 (CD4<sup>+</sup>FoxP3<sup>+</sup> CD25<sup>+</sup>ICOS<sup>+</sup> and CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup>ICOS<sup>−</sup> at day 5 and 8 versus untreated) (two-way ANOVA ± SEM). (C-F) MFI of FoxP3, CD25, GITR and intracellular CTLA-4 expression on CD4<sup>+</sup>FoxP3<sup>+</sup> Treg from untreated or NOD mice treated with AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP at day 5 and 8 days post-vaccination. \*\*p < 0.01 (Day 8 AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP versus untreated islets for MFI of CD25) (two-way ANOVA ± SEM).



**Figure 3.7 FoxP3<sup>+</sup> Treg from AAV8mIP-IL2 vaccinated mice have enhanced suppressive capabilities.** (A) CD4<sup>+</sup>CD3<sup>+</sup>GFP<sup>+</sup> splenocytes were isolated from the spleens of AAV8mIP-IL2  $2.5 \times 10^{10}$  VP or AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP treated NOD.FoxP3.BDC.GFP mice and co-cultured at various ratios with cell trace labeled NOD.BDC CD4<sup>+</sup>CD62L<sup>+</sup> cells and peptide pulsed APCs for 72 hours. Conditions were done in triplicate wells. \*\*p < 0.01 (1 to 1 and 1 to 2 AAV8mIP-IL2 1 ug/ml and AAV8mIP-IL2 100 ng/ml versus AAV8mIP-EGFP 1 ug/ml and AAV8mIP-EGFP 100 ng/ml) (two-way ANOVA  $\pm$  SEM). (B) Supernatants were harvested at 72 hours and assayed via ELISA for IFN $\gamma$ . \*\*\*p < 0.001 (1 to 1 and 1 to 2 AAV8mIP-EGFP 1 ug/ml and AAV8mIP-EGFP 100 ng/ml versus AAV8mIP-IL2 1 ug/ml and AAV8mIP-IL2 100 ng/ml), \*\*p < 0.01 (1 to 4 AAV8mIP-EGFP 1 ug/ml and AAV8mIP-EGFP 100 ng/ml versus AAV8mIP-IL2 1 ug/ml and AAV8mIP-IL2 100 ng/ml) (two-way ANOVA  $\pm$  SEM).



**Figure 3.S1 AAV8mIP-IL2 vaccination does not prevent diabetes in CD8<sup>+</sup> T cell transgenic NOD.8.3 female mice.** (A) Cohorts of 5 NOD.8.3 mice were treated with AAV8mIP-IL2  $2.5 \times 10^{10}$  VP or AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP at 8 weeks of age and monitored for the development of overt diabetes. (B) At 10 days post-treatment, serum IL-2 was detected via ELISA. \*\*\*p < 0.001 (AAV8mIP-IL2  $2.5 \times 10^{10}$  VP versus AAV8mIP-EGFP  $2.5 \times 10^{10}$  VP) (Student t test  $\pm$  SEM).



**Figure 3.S2 AAV8mIP-IL2 vaccination has no effect on the frequency or phenotype of FoxP3<sup>+</sup> Treg in the lymphatics.** (A) Frequency of FoxP3<sup>+</sup>CD25<sup>+</sup> Treg in the spleen, PLN, mesenteric lymph node (MLN) and brachial lymph node (BLN) in groups of 5 NOD mice treated with AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP or AAV8mIP-EGFP 2.5 x 10<sup>10</sup> VP 4 weeks earlier. \*\*\*p < 0.001 (AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP versus AAV8mIP-EGFP 2.5 x 10<sup>10</sup> VP in spleen) (two-way ANOVA ± SEM). (B) FoxP3<sup>+</sup>CD25<sup>+</sup> Treg from AAV8mIP-EGFP 2.5 x 10<sup>10</sup> VP (white area) or AAV8mIP-IL2 2.5 x 10<sup>10</sup> VP (gray area) treated NOD mice were compared for the expression of surface GITR, intracellular CTLA-4, and CD25 by MFI.

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## CHAPTER 4

### COMBINATORIAL IMMUNOTHERAPY FOR THE PREVENTION AND TREATMENT OF TYPE I DIABETES

#### 4.1 Summary

Type I diabetes (T1D) is a T cell-mediated autoimmune disease characterized by the destruction of the insulin producing  $\beta$  cells located in the islets of Langerhans. Differentiation and expansion of pathogenic type 1  $CD4^+$  and  $CD8^+$  T effectors (Teff) correlates with an aberrant FoxP3-expressing regulatory T cell (Foxp3<sup>+</sup> Treg) pool in NOD mice and diabetic human patients. Previous work in our laboratory has shown that a short course of non-depleting  $\alpha CD4$  (YTS177) and  $\alpha CD8$  (YTS105) monoclonal antibodies induces rapid remission in recent onset diabetic NOD mice. Induction of diabetes reversal is attributed to T cell purging of the islets, and long-term maintenance of remission is FoxP3<sup>+</sup> Treg-dependent. We have also found that targeting IL-2 expression to the islets via administration of an adeno-associated virus (AAV) vector encoding IL-2 (AAV8mIP-IL2) prevents diabetes in NOD mice at a late preclinical stage of T1D. In this study we assessed whether the efficacy of YTS177 and YTS105 treatment to induce and maintain remission in recent onset diabetic NOD mice under suboptimal conditions is enhanced by AAV8mIP-IL2 vaccination. Notably, the frequency and duration of

remission established by YTS antibody administration was significantly increased by co-treatment with AAV8mIP-IL2. Furthermore, YTS antibody binding had no effect on IL-2 receptor signaling *in vitro* or *in vivo*. Collectively, these results indicate that AAV8mIP-IL2 vaccination can be used in a combinatorial approach to enhance the efficacy of YTS antibody treatment, and possibly other strategies of immunotherapy.



## 4.2 Introduction

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease characterized by the progressive infiltration and destruction of  $\beta$  cells (1-4). Studies in T1D patients and the NOD mouse have demonstrated that pathogenic  $\beta$  cell-specific CD4<sup>+</sup> and CD8<sup>+</sup> effector T cells (Teff) typically exhibit a type 1 phenotype characterized by the secretion of IFN $\gamma$  and TNF $\alpha$ , although Th17 cells may also contribute to the diabetogenic response (5-9). Preferential expansion and differentiation of pathogenic  $\beta$  cell-specific Teff is in part attributed to dysregulation within the immunoregulatory Foxp3-expressing CD4<sup>+</sup> T cell (Foxp3<sup>+</sup>Treg) pool (10-12). Accordingly, immunotherapies are being developed to reestablish the functional balance between pathogenic Teff and Foxp3<sup>+</sup>Treg in order to suppress ongoing  $\beta$  cell autoimmunity (13-20). To date, however, various immunotherapies tested in the clinic have at best exhibited modest efficacy over relatively short periods of time (21-25). Currently, there is a growing consensus within the field that combinatorial immunotherapies may prove to be more effective at suppressing  $\beta$  cell autoimmunity in an additive or synergistic manner.

Our group has been studying 2 distinct approaches of immunotherapy. The first entails the use of adeno-associated virus (AAV) vectors to target cytokine transgene expression to  $\beta$  cells using the mouse insulin promoter (mIP). Treatment of NOD mice at a late preclinical stage of T1D with an AAV8 vector encoding IL-2 (AAV8mIP-IL2) suppressed ongoing  $\beta$  cell autoimmunity and prevented the onset of diabetes (Chapter 3). IL-2 plays a critical role in the induction and maintenance of both natural (26-29) and adaptive FoxP3<sup>+</sup> Treg (30-32), and NOD mice are known to have a defect in IL-2

secretion, which correlates with a reduced FoxP3<sup>+</sup> Treg pool (33-35). Indeed, we found that protection induced by AAV8mIP-IL2 vaccination correlated with significant increases in the frequency and functionality of islet resident FoxP3<sup>+</sup> Treg. The ability of AAV8mIP-IL2 to induce remission in recent onset diabetic NOD mice, however, is unknown. In addition, we have recently demonstrated that a short course of the nondepleting  $\alpha$ CD4 and  $\alpha$ CD8 monoclonal antibodies, YTS177 and YTS105, respectively, efficiently induces remission in recent onset diabetic NOD mice (36). The protective effects of YTS antibody-mediated remission were characterized by the rapid purging of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from the islets and draining pancreatic lymph nodes (PLN). Maintenance of YTS antibody-induced remission was also found to be FoxP3<sup>+</sup> Treg dependent.

In the current study we investigated whether a combinatorial approach consisting of AAV8mIP-IL2 and suboptimal YTS antibody treatment efficiently induced and maintained remission in recent onset NOD mice. Demonstration of enhanced efficacy with the combinatorial treatment may permit administration of lowered doses of the YTS antibodies and AAV8mIP-IL2 vector, thereby limiting off-target effects and unwanted complications while maintaining long-term remission.

### **4.3 Materials and Methods**

#### **Mice**

NOD/LtJ were bred and maintained under specific pathogen-free conditions in an American Association for Laboratory-accredited animal facility. All procedures were approved by the University of North Carolina Animal Use and Care Committee. Mice were monitored weekly for diabetes by urine analysis via Keto-Diastix (Bayer). In urine positive NOD mice, blood glucose levels were subsequently checked for confirmation. Mice were classified as diabetic after 3 consecutive blood glucose readings over 250 mg/dL.

#### **Nondepleting monoclonal antibody production**

YTS105 and YTS177 hybridoma lines were cultured in Cell Line 1000 hybridoma flasks (Wilson Wolf) using BD animal component-free hybridoma medium (BD Falcon). After culturing for 15 days, antibody was precipitated from supernatants with 45% saturated ammonium sulfate, desalted with PBS, sterilized by 0.22  $\mu$ m filtration, and quantified via ELISA. Purity was then assessed by denaturing SDS-PAGE.

#### **T cell isolation and *in vitro* stimulation**

Total CD4<sup>+</sup> or naïve CD4<sup>+</sup>CD62L<sup>+</sup> fractions were isolated from NOD mice using the CD4<sup>+</sup> T cell isolation kit or CD4<sup>+</sup>CD62L<sup>+</sup> T cell isolation kit II (Miltenyi Biotec), with cell purity between 90-95% as confirmed by flow cytometry. For phosphorylated STAT5 (pSTAT5)

analysis, bulk CD4<sup>+</sup> T cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 1 mM sodium pyruvate (Gibco), 2 mM Hepes, 100 U/ml penicillin/streptomycin (Gibco), 50  $\mu$ M  $\beta$  mercaptoethanol (Sigma-Aldrich) (RPMI complete medium) supplemented with 2000 or 200 pg/ml recombinant (r) IL-2 (Peprotech) in the presence or absence of 1  $\mu$ g/ml YTS177 monoclonal antibody for the time points indicated. For *in vitro* FoxP3<sup>+</sup> Treg induction assays, naïve CD4<sup>+</sup>CD62L<sup>+</sup> fractions were cultured for 72 hours in RPMI complete medium supplemented with 2  $\mu$ g/ml functional grade  $\alpha$ CD3 (145-2C11;eBioscience) and  $\alpha$ CD28 (37.51;eBioscience), TGF $\beta$ 1 (Peprotech), rIL-2, and/or 0.5-5.0  $\mu$ g/ml YTS177 antibody. Cells were then spun down, harvested and prepared for flow cytometry.

### **Flow Cytometry (FACS)**

T cells were stained with previously titrated antibodies specific for: CD3 (145-2C11), CD4 (GK1.5), CD8 (Ly-2), CD25 (PC61.5), and FoxP3 (FJK-16s). Fc receptors were blocked with rat  $\alpha$ -mouse CD16/32 (2.4G2) (BD Biosciences) prior to staining. Intracellular cytokine staining was performed on single cell suspensions as previously described (37). Briefly, lymphocytes were stimulated with 500 ng/ml PMA (Sigma) and 1000 ng/ml ionomycin (Sigma) in complete RPMI complete medium for 5 hours at 37°C; 10  $\mu$ g/ml Brefeldin A (Sigma) was added to the culture for the last 4 hours of incubation. Cells were then stained for surface molecules, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained for intracellular IFN $\gamma$  (XMG1.2)

For pSTAT5 staining, organs were harvested and immediately processed in PBS containing 2% paraformaldehyde for fixation. Single cell suspensions were incubated on ice for 20 minutes, followed by centrifugation and re-suspension in ice-cold methanol for 30 minutes for permeabilization. Cells were then counted, washed twice in 1% BSA in PBS, and stained with cell surface, intracellular and pSTAT5 (BD Biosciences) antibodies for a minimum of 1 hour on ice. Data were acquired on a Cyan flow cytometer (DakoCytomation) and analyzed using Summit software (DakoCytomation).

#### **rAAV vector engineering and packaging**

Full-length cDNA encoding murine *IL2* (of the NOD genotype) was PCR amplified and subcloned into TOPO2.1 (Invitrogen) via the manufacturer's recommendations. After sequencing, transgenes were excised and ligated into the rAAV vector mIP plasmid.

rAAV vector production was completed as previously described (38). Briefly, HEK 293T cells were transfected via polyethyleneimine (PEI) with the adeno helper encoding plasmid (pXX6-80), an AAV8 capsid encoding plasmid, and the double stranded mIP IL-2 plasmid in order to package AAV serotype 8 (AAV8) vector (AAV8mIP-IL2). Nuclear fractions were harvested 72 hours post-transfection and vector purified with a Cesium Chloride (RPI) gradient. AAV vector-containing fractions and titers were determined by Southern dot blot.

## ELISA

Serum was collected and diluted 1:2 in RPMI complete medium to assess systemic transgene levels. The  $\alpha$ IL-2 Ab set (JES6-1 and JES6-5; eBioscience) was used at a concentration of 2  $\mu$ g/ml on a high binding ELISA plate (Costar).

## ***In vivo* monoclonal antibody combinatorial treatment regime**

For *in vivo* pSTAT5 analysis, 10-12 week old pre-diabetic NOD mice were vaccinated on day 0 and day 2 with 600  $\mu$ g of YTS177 and YTS105 I.P. or left untreated. NOD mice were injected with  $2.5 \times 10^{10}$  VP I.P. AAV8mIP-IL2 on day 2 either alone or in combination with antibody administration.

In remission studies, recent onset diabetic NOD mice were treated with 600  $\mu$ g of YTS177 and YTS105 or 1000  $\mu$ g YTS177-only on day 0 and 2, and/or  $2.5 \times 10^{10}$  VP of AAV8mIP-IL2 on day 2. Blood glucose was then monitored twice weekly.

## 4.4 Results

### 4.4.1 Addition of YTS177 *in vitro* does not inhibit the effect of exogenous IL-2 on

#### FoxP3<sup>+</sup> Treg

IL-2 binding to the IL-2R induces pSTAT5, which is critical for the expression of various Foxp3<sup>+</sup> Treg associated genes, including FoxP3 (39). Therefore, to determine if the addition of YTS177 *in vitro* altered the IL-2 mediated pSTAT5 induction in FoxP3<sup>+</sup> Treg, CD4<sup>+</sup> T cells were magnetically isolated from the spleens of NOD mice and stimulated with varying concentrations of rIL-2 for up to 1 hour. Treatment with rIL-2-alone resulted in a marked increase in the frequency of pSTAT5<sup>+</sup> FoxP3<sup>+</sup> Treg in a time and dose dependent manner (Fig 4.1A). Interestingly, the addition of YTS177 prior to rIL-2 stimulation did not affect the frequency of pSTAT5<sup>+</sup> FoxP3<sup>+</sup> Treg at any time point analyzed (Fig 4.1A), despite antibody coating of all responding cells (data not shown). Additionally, although the magnitude of the response was lower, a similar trend in frequency was seen within the FoxP3<sup>-</sup>CD4<sup>+</sup> responding pool (Fig. 4.1B). Collectively, these data indicate that YTS177 binding *in vitro* does not affect IL-2-mediated pSTAT5 induction in FoxP3<sup>+</sup> Treg or FoxP3<sup>-</sup>CD4<sup>+</sup> T cells.

Differentiation of adaptive Treg from naïve precursors *in vitro* requires both IL-2 and TGFβ (31). To determine if the presence of YTS177 altered adaptive Treg differentiation *in vitro*, naïve CD4<sup>+</sup>CD62L<sup>+</sup> T cells were isolated from the spleens of NOD mice and cultured with TGFβ alone or both TGFβ and rIL-2. When both cytokines were added, the frequency of FoxP3<sup>+</sup> Treg was similar regardless of the presence or absence of YTS177 (Fig. 4.1C). Surprisingly, the frequency of TGFβ-alone induced FoxP3<sup>+</sup> Treg was

significantly decreased by the addition of YTS177 in a concentration dependent manner (Fig 4.1C). This would suggest that YTS177 inhibits adaptive FoxP3<sup>+</sup> Treg formation in the absence of exogenous IL-2 *in vitro*, but does not block the ability of naïve precursors to differentiate into adaptive FoxP3<sup>+</sup> Treg under the appropriate culture conditions.

#### **4.4.2 AAV8mIP-IL2 co-administration enhances Foxp3<sup>+</sup>Treg expansion by YTS105 and YTS177 treatment**

To complement the above *in vitro* findings, prediabetic NOD mice were treated with combinations of AAV8mIP-IL2, YTS177 and/or YTS105, or left untreated. Serum IL-2 levels in AAV8mIP-IL2-only and AAV8mIP-IL2 plus YTS antibody treated NOD mice were similar, and as expected significantly higher than animals receiving YTS177 and YTS105-only (Fig. 4.2A). The frequency of FoxP3<sup>+</sup> Treg in the spleen, PLN, and islets was significantly higher in AAV8mIP-IL2-only treated controls compared to other treatment groups (Fig. 4.2B). Comparatively, FoxP3<sup>+</sup> Treg frequency in YTS177 and YTS105-only treated mice was significantly reduced compared to untreated controls, consistent with our previous findings (36). Of note, combination treated mice exhibited a significantly increased FoxP3<sup>+</sup> Treg frequency compared to both untreated and YTS177 and YTS105-only controls, albeit to a lesser level in comparison to AAV8mIP-IL2 only treated mice (Fig. 4.2B). This suggests that AAV8mIP-IL2 treatment minimizes the decrease in FoxP3<sup>+</sup> Treg typically induced by YTS177 and YTS105.



In addition, FoxP3<sup>+</sup> Treg and FoxP3<sup>-</sup>CD25<sup>-</sup>CD4<sup>+</sup> T cells were analyzed for the induction of pSTAT5. Similar to *in vitro* studies, there was a significant increase in the frequency pSTAT5<sup>+</sup>FoxP3<sup>+</sup> Treg in the spleens and islets of both AAV8mIP-IL2 and combination treated mice compared to YTS177 and YTS105-only treated and untreated groups (Fig. 4.2C). Furthermore, the magnitude of induction was similar in both the AAV8mIP-IL2 and combination treated groups (Fig. 4.2C). Interestingly, a significant decrease in the frequency of splenic pSTAT5<sup>+</sup>FoxP3<sup>+</sup> Treg was also seen in the YTS177 and YTS105 group compared to untreated controls, a trend which was also observed in the islets (Fig. 4.2C). This indicates that YTS177 binding *in vivo* alters pSTAT5 induction within the FoxP3<sup>+</sup> Treg compartment in the absence of ectopic IL-2. In contrast, the frequency of pSTAT5<sup>+</sup>FoxP3<sup>-</sup>CD25<sup>-</sup>CD4<sup>+</sup> T cells within the spleen, PLN and islets did not differ significantly among any group analyzed (Fig. 4.2D).

#### **4.4.3 AAV8mIP-IL2 treatment augments the protective effect of YTS177 and YTS105 under suboptimal conditions in recent onset diabetic NOD mice**

Previous work by our group has shown that co-administration of YTS177 and YTS105 induces rapid and long-term remission in recent onset diabetic NOD mice (36).

Additionally, AAV8mIP-IL2 administration to pre-diabetic NOD mice suppresses  $\beta$  cell autoimmunity and establishes long-term protection from T1D. Therefore, the ability of AAV8mIP-IL2 to induce remission, either alone or in combination with YTS177 and/or YTS105 was investigated. In a small cohort of recent onset diabetic NOD mice,

treatment with AAV8mIP-IL2 alone failed to reverse diabetes (Fig. 4.3A). Additionally, the relative level of transgene expression was lower than that typically seen in prediabetic NOD mice, and waned within 2 weeks post-treatment in the majority of vaccinated diabetic NOD mice (data not shown). Treatment with 1000  $\mu$ g of YTS177 in combination with AAV8mIP-IL2 also proved to be insufficient for inducing remission (Fig. 4.3B). In a cohort of recent onset NOD mice treated with a suboptimal dose (e.g. 600  $\mu$ g) of YTS177 and YTS105 alone, diabetes was reversed in 4/6 NOD mice but remission was maintained beyond 60 days in only 1/4 animals with a median of 44 days post-treatment (Fig. 4.3C) (A.M. and R.T., unpublished results). In contrast, treatment with the combination of AAV8mIP-IL2 and of YTS177 and YTS105 elicited long-term remission in all treated NOD mice (Fig. 4.3D). Notably, serum levels of IL-2 in all combinatorial treated long-term remission NOD mice were stable over the course of investigation (Fig. 4.4A).

To determine the mechanism of protection in long-term remission NOD mice receiving the combinatorial therapy, the frequency of tissue-specific FoxP3<sup>+</sup> Treg and IFN $\gamma$ <sup>+</sup> Teff was determined by flow cytometry. There was a significant increase in the frequency of FoxP3<sup>+</sup> Treg in the spleen of long-term remission NOD mice compared to normoglycemic, untreated controls (Fig. 4.4B). Unexpectedly, no difference in the frequency of FoxP3<sup>+</sup> Treg was seen in either the PLN or islets. Additionally, the IFN $\gamma$ <sup>+</sup>CD4<sup>+</sup> and IFN $\gamma$ <sup>+</sup>CD8<sup>+</sup> Teff populations were also compared. In both the spleen and PLN, there was no effect on the frequency of either IFN $\gamma$ <sup>+</sup>CD4<sup>+</sup> or IFN $\gamma$ <sup>+</sup>CD8<sup>+</sup> Teff populations between groups. Of interest, there was a significant decrease in the

frequency of islet resident IFN $\gamma$ <sup>+</sup>CD4<sup>+</sup> Teff in long-term remission NOD mice, suggesting that the induction of long-term remission through combinatorial treatment may be maintained through preferential effects on islet resident IFN $\gamma$ <sup>+</sup>CD4<sup>+</sup> Teff.

## 4.5 Discussion

We show that AAV8mIP-IL2 vaccination enhances the efficacy of suboptimal YTS177 and YTS105 treatment in recent onset diabetic NOD mice. Although only a small cohort of NOD mice was tested, remission was induced in all recipients for >100 days (Fig. 4.3D). Notably, levels of serum IL-2 were stable over time (Fig. 4.4A), indicating that  $\beta$  cell mass was maintained after treatment with the combinatorial therapy.

Somewhat surprisingly, AAV8mIP-IL2 treatment alone or with YTS177-only failed to induce remission in recent onset diabetic NOD mice (Fig. 4.3A, B). Although IL-2 was detected after AAV8mIP-IL2 administration, systemic levels were notably lower than seen in prediabetic NOD mice treated with a similar vector dose (Data not shown). In addition, detectable serum IL-2 levels quickly decreased in the majority of treated recipients, suggesting continued destruction of residual  $\beta$  cell mass. Furthermore, treatment with suboptimal doses of YTS177 and YTS105 alone was sufficient to induce remission in recipient NOD mice, but the median induction time was only 44 days (Fig. 4.3C) (A.M. and R.T., unpublished results). At clinical diagnosis, 80-90% of  $\beta$  cells have already been destroyed (40), and the surviving functional population may prove insufficient to synthesize the amount of IL-2 required to induce a protective islet resident FoxP3<sup>+</sup> Treg pool.

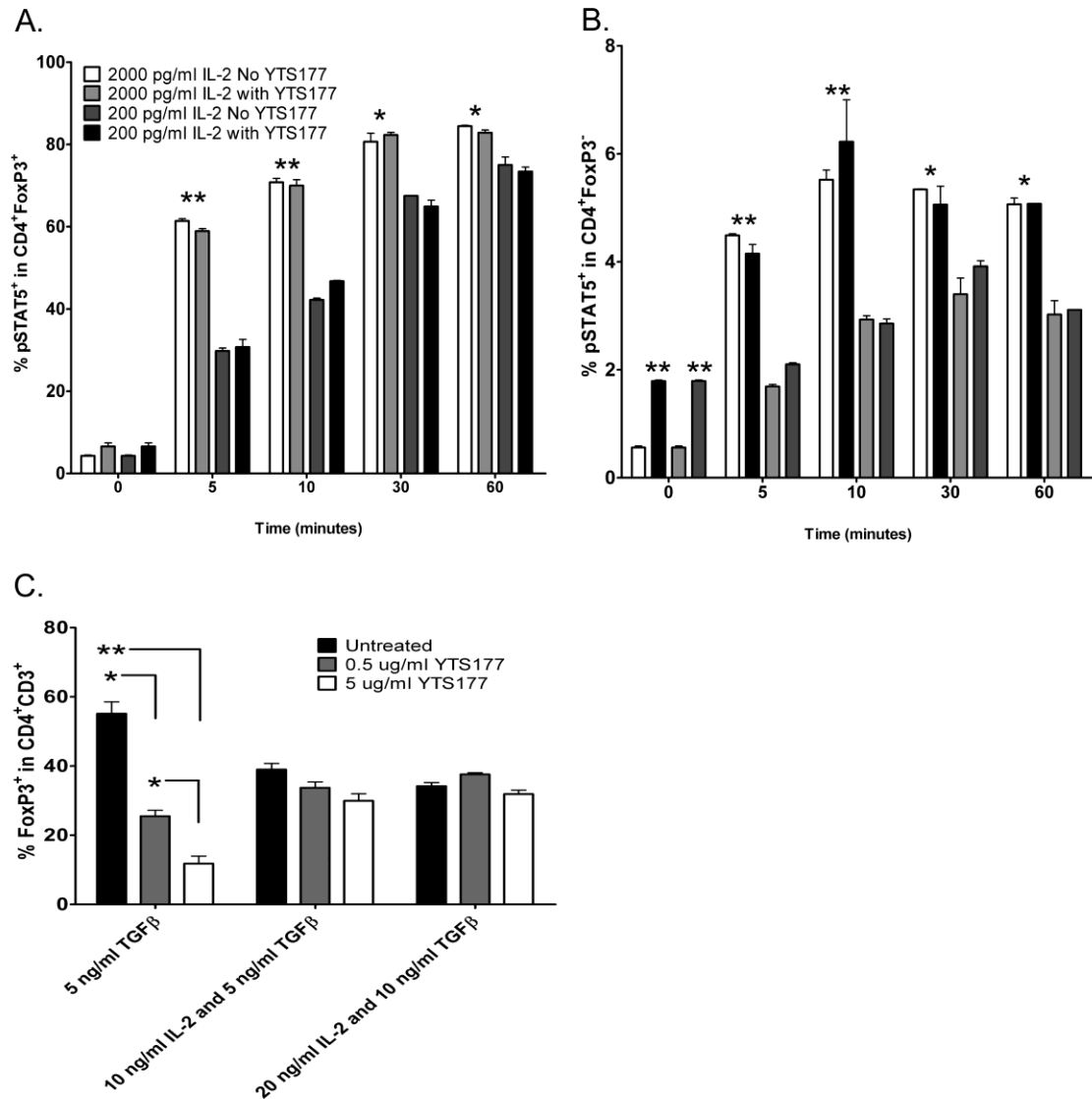
Previous work by our group has shown that a short course of YTS177 and YTS105 was sufficient to induce remission in >80% of recent onset diabetic NOD mice (36). Notably, remission induction was associated with purging of islet-resident CD4<sup>+</sup> and

CD8<sup>+</sup> T cells, including FoxP3<sup>+</sup> Treg (36). In prediabetic NOD mice treated with YTS177, YTS105, and AAV8mIP-IL2, the frequency of FoxP3<sup>+</sup> Treg in various organs, including the islets, were maintained at a significantly higher level compared to both untreated and YTS177 and YTS105-only recipients (Fig. 4.2B). Since FoxP3<sup>+</sup> Treg are thought to be required for the maintenance of remission after YTS administration, the increased islet FoxP3<sup>+</sup> Treg pool likely enhances the efficacy of YTS177 and YTS105 administered at a suboptimal dose. Functionally, it is important to note that induction of pSTAT5, which functions downstream of the IL-2R, was not affected *in vitro* (Fig. 4.1A) or *in vivo* (Fig. 4.2C) by YTS177 and YTS105 binding. Since pSTAT5 regulates the expression of various FoxP3<sup>+</sup> Treg genes, including *FoxP3*, maintaining the function of this pathway is important for FoxP3<sup>+</sup> Treg function. Of interest, the presence of YTS177 *in vitro* did significantly decrease the induction of adaptive FoxP3<sup>+</sup> Treg from naïve precursors, but only in the absence of exogenous IL-2 (Fig. 4.1C). Mechanistically, this is likely due to down-regulated T cell activation caused by YTS177 binding, thereby decreasing the available pool of autocrine/paracrine IL-2. In fact, decreased IL-2 was also seen in the pancreases of recent onset diabetic NOD mice treated with YTS177 and YTS105 (36). Since IL-2 is required for adaptive FoxP3<sup>+</sup> Treg induction, the decreased frequency of FoxP3<sup>+</sup> Treg in that assay was not surprising.

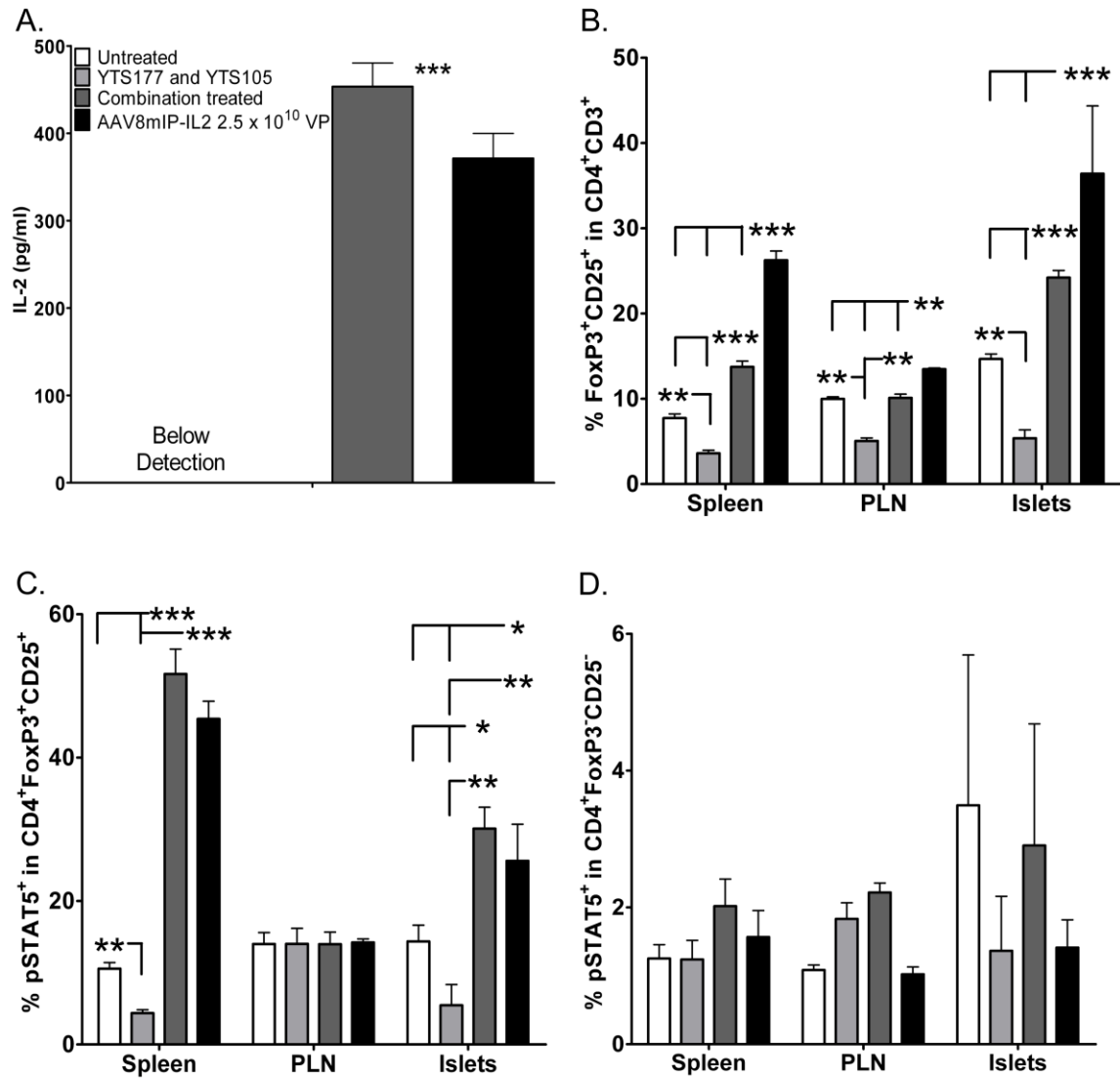
The frequency of FoxP3<sup>+</sup> Treg in long term remission NOD mice was not significantly altered, except in the spleen (Fig. 4.4B). Given the robust effects seen in the islets of prediabetic NOD mice treated with AAV8mIP-IL2, this result was unanticipated. Rather, there was a significant decrease in islet resident CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> Teff (Fig. 4.4C), which

mirrors the effects seen in the PLN of long-term remission NOD mice treated with YTS177 and YTS105-alone (36). Perhaps, the initial purging of FoxP3<sup>+</sup> Treg from the islets after YTS177 and YTS105 treatment is sufficient to off-set the expansion of islet resident FoxP3<sup>+</sup> Treg after vaccination with AAV8mIP-IL2, resulting in the maintenance of FoxP3<sup>+</sup> Treg at a level that is equivalent to that of normoglycemic NOD mice. Since T cells are thought to egress from sites such as the PLN and islets after YTS treatment, this may explain why a significant increase in FoxP3<sup>+</sup> Treg was only seen in the spleen after combinatorial therapy. Regardless, the interplay between these treatments, while effective, still merits further investigation.

In summary, these results show that vaccination with AAV8mIP-IL2 enhances suboptimal doses of YTS antibody therapy to induce long term remission in recent onset NOD mice (Fig. 4.5). Importantly, our findings suggest that manipulating the islet pool of FoxP3<sup>+</sup> Treg via AAV8mIP-IL2 vaccination may prove to be an effective strategy to enhance the efficacy of other antibody- and/or antigen-specific based immunotherapies.



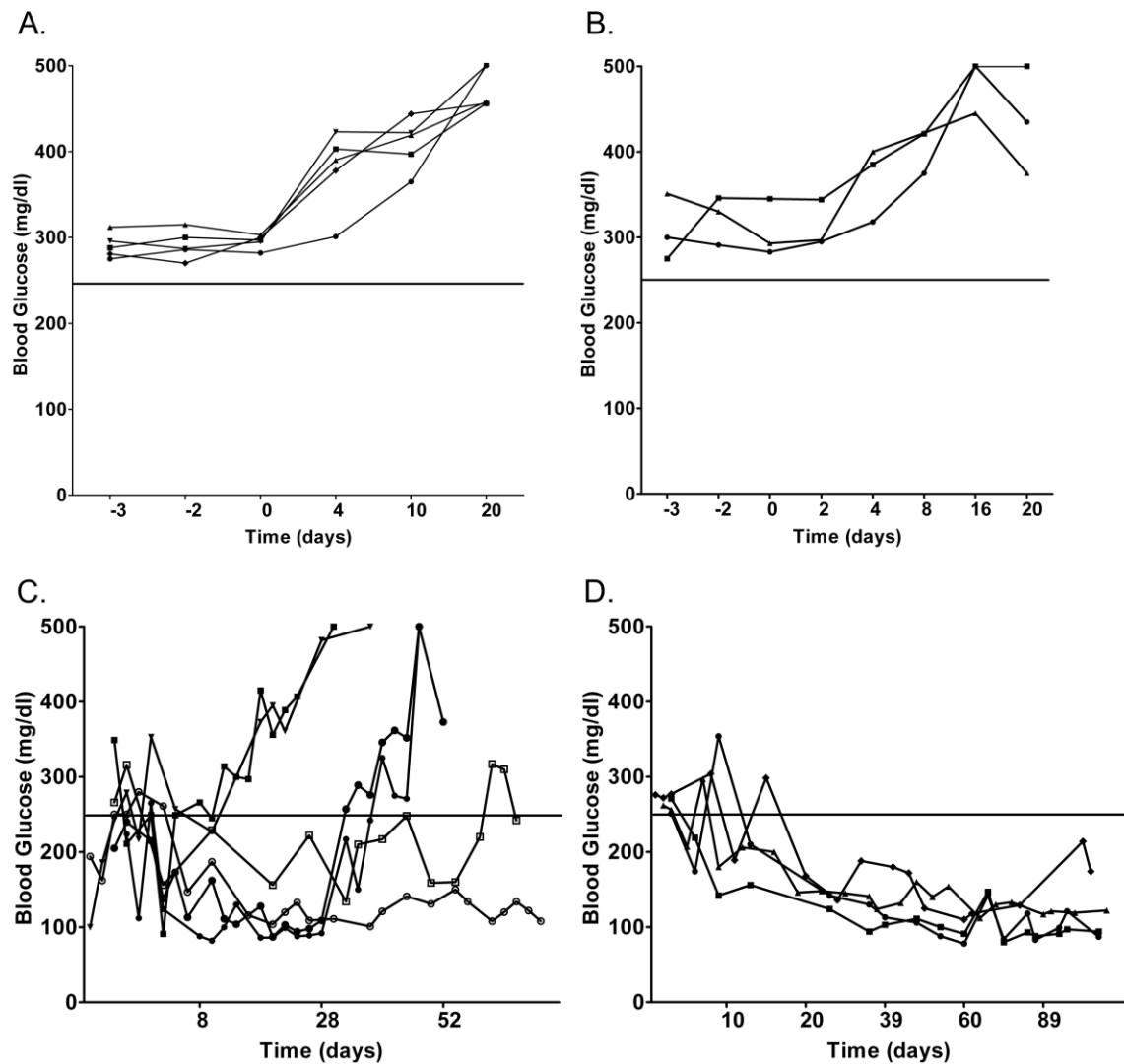
**Figure 4.1 The effect of exogenous IL-2 is not altered by the presence of YTS177 *in vitro*.** (A) The frequency of pSTAT5<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> cells after addition of exogenous IL-2 to isolated CD4<sup>+</sup> NOD splenocytes *in vitro*. All conditions were assayed in triplicate. \*p<0.05; \*\*p<0.01; 2000 pg/ml No YTS177 and with YTS177 versus 200 pg/ml No YTS177 and with YTS177 (2-way ANOVA, data are mean ± SEM). (B) Comparative pSTAT5<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>-</sup> populations in the same wells. \*p<0.05; \*\*p<0.01; 2000 pg/ml and 200 pg/ml with YTS177 versus No YTS177 at time 0, 2000 pg/ml No YTS177 and with YTS177 versus 200 pg/ml No YTS177 and with YTS177 at 5, 10, 30 and 60 minutes (2-way ANOVA, data are mean ± SEM). (C) *In vitro* adaptive FoxP3<sup>+</sup> Treg induction assay using isolated CD4<sup>+</sup>CD62L<sup>+</sup> naïve NOD splenocytes cultured for 72 hours. \*p<0.05; \*\*p<0.01; Untreated versus 0.5 µg/ml YTS177 and 5 µg/ml YTS177 and 0.5 µg/ml YTS177 versus 5 µg/ml YTS177 (2-way ANOVA, data are mean ± SEM).



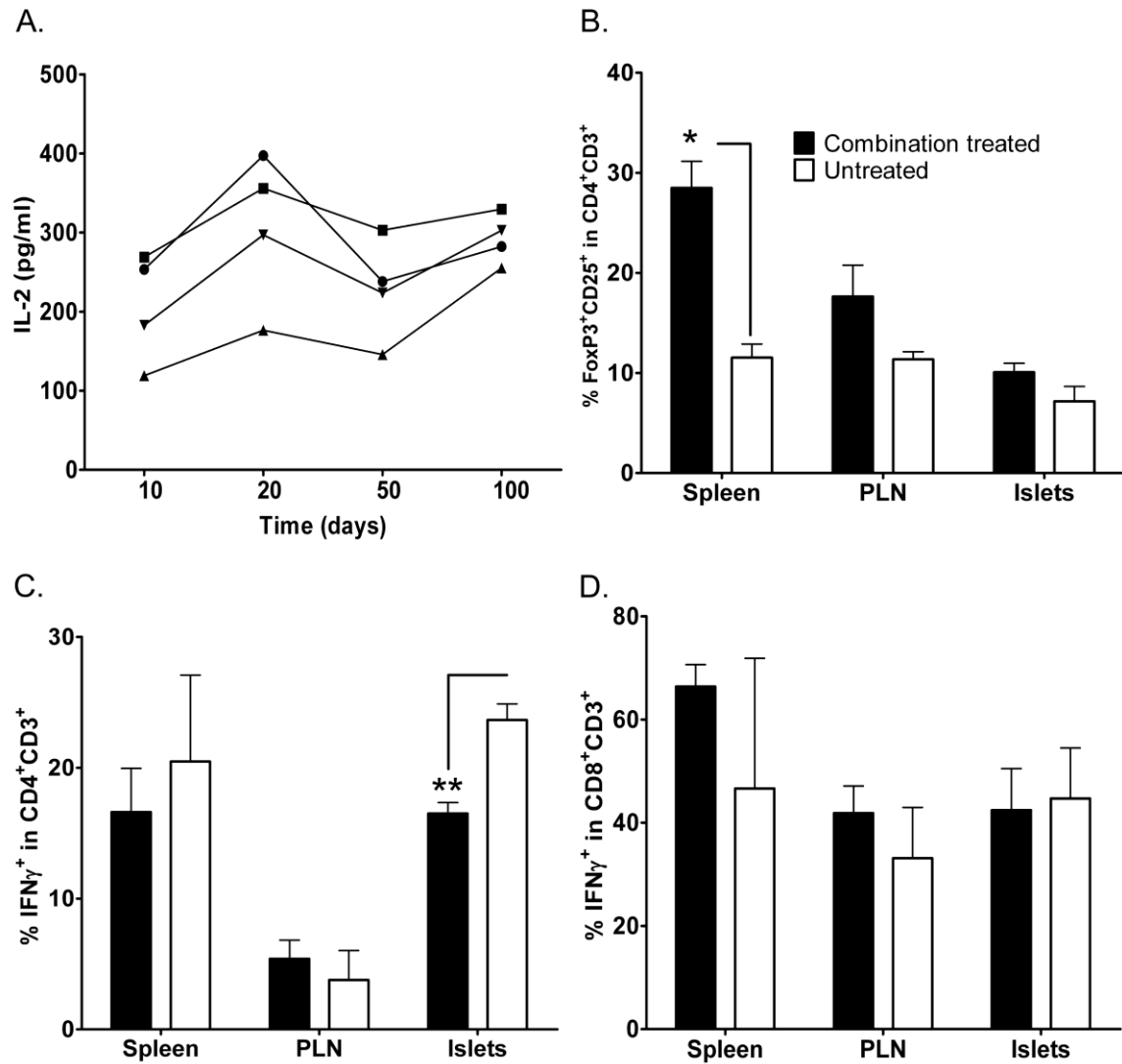
**Figure 4.2 Significantly increased FoxP3<sup>+</sup> Treg frequency and pSTAT5 signaling in combination treated normoglycemic recipients.** (A) Serum IL-2 levels at day 7 post-AAV8mIP-IL2 treatment measured via ELISA in mice treated with YTS177 and YTS105 alone, AAV8mIP-IL2 alone, combination treated or left untreated (n = 4; all panels). \*\*\*p<0.001; AAV8mIP-IL2 and combination treated versus YTS177 and YTS105 and untreated. (1-way ANOVA, data are mean  $\pm$  SEM). (B) 2 weeks post-treatment, frequency of FoxP3<sup>+</sup>CD25<sup>+</sup> Treg was determined by flow cytometry in the spleen, PLN and islets of mice from different treatment groups. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; MIP IL-2 versus combination, YTS177 and YTS105 and untreated, combination treated versus YTS177 and YTS105 and untreated, and untreated versus YTS177 and YTS105 treated (2-way ANOVA, data are mean  $\pm$  SEM). In addition, the frequency of pSTAT5<sup>+</sup> cells was measured in the CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> (C) and CD4<sup>+</sup>FoxP3<sup>-</sup>CD25<sup>-</sup> (D) populations within the same organs. \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; Spleen and islet resident



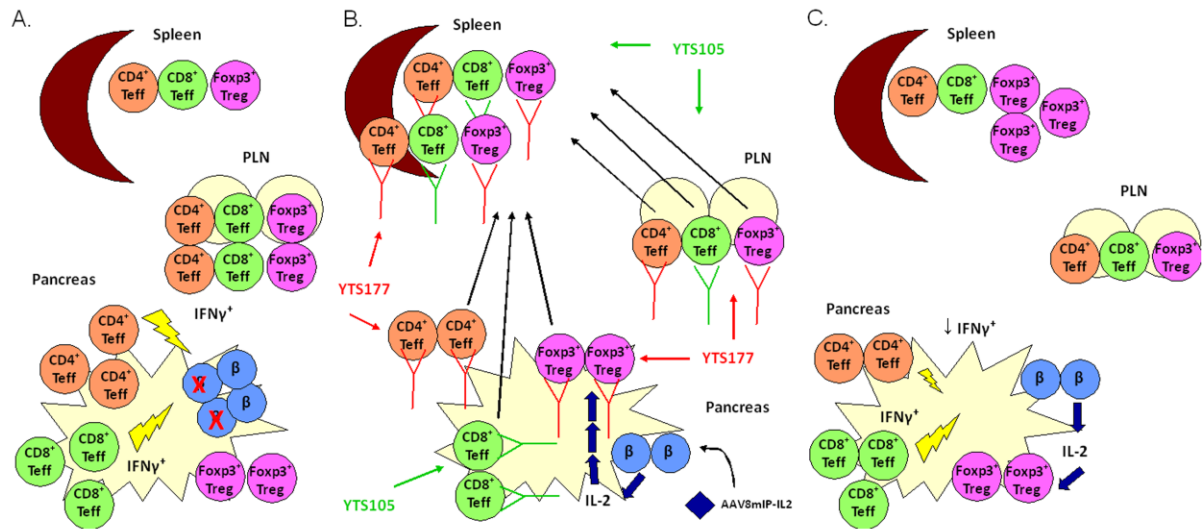
CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> cells in AAV8mIP-IL2 and combination treated mice versus YTS177 and YTS105 and untreated and CD4<sup>+</sup>FoxP3<sup>+</sup>CD25<sup>+</sup> cells in untreated versus YTS177 and YTS105 controls (2-way ANOVA, data are mean  $\pm$  SEM).



**Figure 4.3 AAV8mIP-IL2, YTS177 and YTS105 short course combination treatment induces long term remission in recent onset diabetic NOD mice.** Recent onset diabetic NOD mice were treated with AAV8mIP-IL2 alone (A) (n = 6), AAV8mIP-IL2 and 1000  $\mu$ g YTS177 (2x) (B) (n = 3), 600  $\mu$ g YTS105 (2X) and YTS177 (2X) (C) (n = 6), or AAV8mIP-IL2, 600  $\mu$ g YTS105 (2X) and YTS177 (2X) (D) (n = 4) and were monitored for remission by analysis of blood glucose levels >100 days.



**Figure 4.4 Long term remission NOD mice have altered FoxP3<sup>+</sup>Treg and CD4<sup>+</sup>IFNγ<sup>+</sup> Teff frequencies.** (A) Temporal measurement of serum IL-2 in long term remission mice via ELISA. (B) At >100 days post-remission, the frequencies of FoxP3<sup>+</sup>CD25<sup>+</sup> Treg (B), CD4<sup>+</sup>IFNγ<sup>+</sup> Teff (C) and CD8<sup>+</sup>IFNγ<sup>+</sup> Teff (D) were compared between long term remission mice and controls (n = 4; all groups). \*p<0.05; \*\*p<0.01; Splenic FoxP3<sup>+</sup>CD25<sup>+</sup> Treg in combination treated versus untreated mice and islet resident CD4<sup>+</sup>IFNγ<sup>+</sup> Teff in combination treated versus untreated mice (2-way ANOVA, data are mean ± SEM).



**Figure 4.5 A model of AAV8mIP-IL2 and YTS induced remission in recent onset diabetic NOD mice.** (A) Prior to treatment, destruction of  $\beta$  cells is primarily mediated by a pool of activated  $\beta$  cell specific  $CD4^+$  and  $CD8^+$  Teff resulting in diabetes onset. The existing islet resident  $FoxP3^+$  Treg are both quantitatively and qualitatively insufficient to mediate protection. (B) Co-administration of sub-optimal levels of YTS105 ( $\alpha CD8$ ) and YTS177 ( $\alpha CD4$ ) non-depleting monoclonal antibodies results in purging of  $CD8^+$  and  $CD4^+$  T cells, including  $FoxP3^+$  Treg, from both the islets and PLN into the spleen. At the same time, localized IL-2 expression from AAV8mIP-IL2 transduced  $\beta$  cells results in the expansion of the remaining islet resident  $FoxP3^+$  Treg, maintaining  $FoxP3^+$  Treg frequencies to levels similar to those found in normoglycemic untreated NOD mice. Collectively, YTS177, YTS105 and AAV8mIP-IL2 work synergistically to maintain the existing  $\beta$  cell mass resulting in remission induction. (C) After YTS177 and YTS105 clearance, long-term remission is preserved due to a reduction in total islet  $CD4^+$  and  $CD8^+$  Teff frequencies, as well as the maintenance of islet  $FoxP3^+$  Treg by consistent IL-2 transgene expression from AAV8mIP-IL2 transduced  $\beta$  cells. Additionally, splenic  $FoxP3^+$  Treg frequencies are significantly enhanced due to the collective effects of systemic IL-2 exposure and Teff purging induced by YTS177 and YTS105 administration.

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## CHAPTER 5

### FUTURE PERSPECTIVES

#### 5.1 Increased efficacy of AAV8mIP-IL2 immunotherapy for the treatment of T1D

rAAV vectors expressing a variety of transgenes have been used successfully in the treatment of a multitude of diseases, including T1D (1-5). In this study, we have shown that administration of a rAAV vector expressing IL-2 under control of a  $\beta$  cell specific promoter, mIP (AAV8mIP-IL2), was able to prevent diabetes in NOD mice (Chapter 3). This protection was mediated by specific effects on islet resident FoxP3<sup>+</sup>CD25<sup>+</sup> Treg. Furthermore, in remission studies, AAV8mIP-IL2 vaccination was also shown to work synergistically with the non-depleting  $\alpha$ CD4 and  $\alpha$ CD8 monoclonal antibodies, YTS177 and YTS105.

Mechanistically, the beneficial effects of AAV8mIP-IL2 on long term YTS treated NOD mice do not appear to be limited strictly to effects on FoxP3<sup>+</sup> Treg. However, in prediabetic NOD mice treated with both AAV8mIP-IL2 and the YTS antibodies short term, the frequency of FoxP3<sup>+</sup> Treg in the islets was significantly enhanced compared to YTS-only recipients. This would suggest that AAV8mIP-IL2 can specifically counteract the loss of FoxP3<sup>+</sup> Treg from organs of interest, notably the islets, after YTS treatment. By maintaining a larger frequency of FoxP3<sup>+</sup> Treg in the islets, the beneficial effects of YTS

mediated immunotherapy would be enhanced. Given that systemic IL-2 levels were stably maintained in long term remission animals suggests that any effects on islet FoxP3<sup>+</sup> Treg would be maintained throughout the course of remission. Therefore, since investigations concerning islet FoxP3<sup>+</sup> Treg in long term YTS-only treated animals is lacking, comparisons between combinatorial AAV8mIP-IL2 and YTS and YTS-only long term treated remission NOD mice merit consideration.

The dosing of IL-2 *in vivo* was shown to be intrinsic to therapeutic function in both NOD mice and diabetic human patients. Investigations utilizing both prediabetic and recent onset diabetic NOD mice have shown that low dose rIL-2 therapy acts specifically on FoxP3<sup>+</sup> Treg to induce protection and/or remission (6-8). Importantly, high dose administration resulted in significant off target effects, thereby eliminating any potential benefits of treatment (8). Similarly, a study utilizing rIL-2 combinatorial therapy in recent onset human patients showed expansion of both NK cells and eosinophils, thereby reducing its clinical efficacy, despite modest increases in systemic FoxP3<sup>+</sup> Treg (9). This would suggest that dose is of particular importance for translational approaches.

Although long term systemic transgene levels were low and stable throughout the lifespan of AAV8mIP-IL2 treated NOD mice, constant and increased IL-2 levels *could* potentially have long term adverse effects on the immune system. We have previously shown that a rAAV vector tetracycline inducible (TET-ON) system expressing IL-2 also successfully prevented diabetes in NOD mice, despite a short 3 week induction phase

(2). Both the  $\beta$  cell specific (mIP) and inducible (Tet-ON) rAAV vectors offer distinct advantages over the other. As such, the use of a dual promoter rAAV vector that is both  $\beta$  cell-specific and inducible would be attractive. Development of this vector would allow for localized, short term bursts of transgene to the islets, while significantly curtailing both the magnitude and length of systemic exposure. We recently packaged a rAAV8 vector expressing IL-2 under the control of both the TET-ON promoter and mIP (dual promoter IL-2), which was shown to secrete IL-2 transgene *in vitro* and increase the frequency of pancreas FoxP3<sup>+</sup> Treg *in vivo* (M.C.J. and R.T., unpublished results). Many questions concerning vector dosage and route of administration, systemic versus localized expression of transgene, and the overall effect on diabetes incidence need to be investigated. However, the availability of an immunotherapy that is both targeted and controllable provides an interesting platform for future immunotherapy approaches.

Vaccination with pDNA expressing  $\beta$  cell autoantigens results in the rapid expansion of  $\beta$  cell specific FoxP3<sup>+</sup> Treg (10-12). Increases in antigen-specific FoxP3<sup>+</sup> Treg can prevent diabetes in young NOD mice, but efficacy wanes upon treatment of older animals. Along these lines, we have shown that combinatorial therapy with AAV8mIP-IL2 and pDNA encoding the  $\beta$  cell autoantigen glutamic acid decarboxylase 65 (GAD65) increased the frequency of GAD65 p217-specific FoxP3<sup>+</sup> Treg in the islets of treated NOD mice (data not shown). The ability of this combinatorial approach to prevent the onset of T1D, particularly in older NOD mice, however, was not investigated. Given the success of AAV8mIP-IL2 therapy alone in preventing diabetes in NOD mice treated with a “high”

dose of vector, it would be of particular interest to investigate the efficacy of this strategy with suboptimal levels of AAV8mIP-IL2.

The advent of our dual promoter system raises a novel avenue for the investigation of combinatorial approaches to treat T1D. Multiple  $\beta$  cell autoantigens have been identified as playing a role in the progression of T1D, many of which have been investigated in the context of pDNA vaccination (13-16). By staggering treatment of NOD mice with pDNA encoding different  $\beta$  cell autoantigens, and subsequently coupling the induction of our dual promoter IL-2 system with administration of those different pDNA, the expansion of various antigen-specific FoxP3<sup>+</sup> Treg populations can occur. Potentially, this would establish a substantial pool of protective,  $\beta$  cell specific FoxP3<sup>+</sup> Treg specifically in the islets, that in turn would be expected to overcome some of the limitations associated with pDNA vaccination.

## **5.2 Alternative transgene expression for rAAV vector based immunotherapy**

Potential treatments for T1D involve the dampening of the autoreactive Teff population and/or expansion of the FoxP3<sup>+</sup> Treg pool to re-establish self tolerance. IL-2, which preferentially accomplishes the latter goal, is one of many molecules that has shown efficacy for the treatment of T1D (2, 7, 17, 18). While each molecule offers unique pros and cons in relation to others, a “magic bullet” has yet to be discovered. Therefore, the exploration for alternative approaches is continually warranted.

Interleukin-35 (IL-35) is a recently identified cytokine composed of Epstein-Barr-virus-induced gene 3 and the interleukin-12  $\alpha$  chain that is secreted by FoxP3<sup>+</sup> Treg, but not CD4<sup>+</sup> Teff (19, 20). The secretion of IL-35 from FoxP3<sup>+</sup> Treg is shown to inhibit Teff cell proliferation *in vitro* (19, 21). Furthermore, IL-35, in combination with IL-10, is capable of inducing an “iT<sub>R</sub>35” population, which is characterized by a lack of FoxP3 expression, but constitutive expression of IL-35 (22). This iT<sub>R</sub>35 cell type is thought to contribute to immune regulation and has been shown to be a stable lineage both *in vitro* and *in vivo* (22). IL-35 is beneficial for the treatment of various autoimmune diseases, including IBD and arthritis (19, 23). In addition, a recent study that linked ecotopic expression of IL-35 to  $\beta$  cells through coupling to the rat insulin promoter II showed substantial long term protection from T1D in NOD mice (24). This protection was defined by reduced Teff infiltration and proliferation into the islets, particularly by autoreactive IGRP-specific CD8<sup>+</sup> T cells. Interestingly, however, proliferation of FoxP3<sup>+</sup> Treg was also reduced in the islets of IL-35 expressing animals, as determined by BrdU incorporation (24).

An AAV8mIP vector encoding IL-35 (AAV8mIP-IL35) would offer an alternative approach to IL-2 mediated treatment, since IL-35 preferentially dampens the Teff population, while IL-2 promotes FoxP3<sup>+</sup> Treg. Therefore, the efficacy of AAV8mIP-IL35 treatment in NOD mice for the prevention of T1D could address the feasibility of directly altering islet Teff. In addition, coupling AAV8mIP-IL35 vaccination, with either AAV8mIP-IL2 or AAV8mIP-IL10 treatment may also produce novel results by directly influencing both sides of the autoreactive immune response. For IL-2, the potential dampening of

the islet FoxP3<sup>+</sup> Treg pool by IL-35 could be offset by localized IL-2 expression.

Alternatively, co-administration of IL-10 is particularly noteworthy given that IL-35 and IL-10 collectively induce iT<sub>R</sub>35 (22). Importantly, the ability of different rAAV vectors to transduce and efficiently secrete different transgenes, especially within the restricted  $\beta$  cell mass found in recent onset diabetics, would have to be investigated prior.

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