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CHARACTERIZATION AND IMMUNOMODULATION OF REGULATORY T CELLS IN TYPE 1 DIABETES

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology

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ABSTRACT

Kevin Scott Goudy: Characterization and Immunomodulation of Regulatory T cells in Type 1 Diabetes

"Under the direction of Drs. Roland, Tisch, Stephen H. Clarke, Richard J. Samulski, Jeffrey A. Frelinger, and Zhi Liu."

Type 1 diabetes mellitus (T1D) is a chronic autoimmune disorder characterized by the complete destruction of the insulin-producing pancreatic β cells. The result of β cell loss leads to life-long insulin injections in order to maintain blood glucose levels, and is associated with both micro-vascular and macro-vascular diseases. Disease onset is related to a genetic predisposition, environmental factors, and a cell-mediated immunophenotype. Previous studies using the non-obese diabetic mouse (NOD) suggest that T1D can be prevented by the administration of immunoregulatory proteins that induce/expand both antigen specific and antigen non-specific regulatory cells. The aims of the studies described within are to; i.) test the hypothesis that antigen-specific therapies can prevent diabetes, and ii) to understand the cause(s) of immunoregulatory T cell deficiency in diabetes development.

Results from our first study demonstrate that gene therapy with pDNA encoding the β cell protein gluatamic acid decarboxyasle 65 (GAD65) via gene gun significantly prevents diabetes onset in the NOD mouse. Diabetes protection was attributed to the induction of interleukin-4 secreting immunoregulatory T cells. We also discovered that delivery of the same construct via intra-muscularly injection exacerbated diabetes by the

preferentially induction of pathogenic type 1 T effector cells. Therefore, our findings show that the route of delivery of pDNA encoding autoantigens is integral in shaping the type of effector cell response.

Our second study illustrates that T1D can be regulated by the differential expression of the *il2* gene located in the *Idd3* locus of the NOD mouse genome. We found that reduced IL-2 production by NOD $CD4^+$ T cells resulted in ~two-fold less induction of FoxP3-expressing regulatory T cells that are critical for regulating autoimmunity. Furthermore, we described a new role for IL-21 whereby IL-21 negatively regulates IL-2 production by $CD4^+$ T cells, thus, perpetuating the inhibition of regulatory T cells. Most importantly, we successfully devised an IL-2 therapy regimen for NOD mice that overcomes the regulatory T cell deficiency which could lead to a new therapeutic approach to prevent and/or treat T1D in humans.

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Ag antigen

APC antigen presenting cell

CCR chemokine receptor

CDR3 complementarity determining region 3

ConA concavilin A

CTE cortical thymic epithelial cell

CTL cytotoxic T lymphocyte

CTLA-4 cytotoxic T lymphocyte antigen

DC dendritic cell

DN double-negative

DP double-positive

ELISA enzyme-linked immunosorbent assay

ELISPOT enzyme-linked immuospot asay

FACS fluorescent activated cell sorting

FITC fluorescein isothiocyanate

FoxP3 Forkhead box P3

GAD65 glutamic acid decarboxylase 65

GITR glucocorticoid-induced tumor necrosis factor receptor family-related gene

HA hemagglutinin

HEL hen egg lysozyme

HLA human leukocyte antigen

HRP horseradish peroxidase

IA-2 insulinoma-associated protein 2

Idd insulin-dependent genes

IDDM Insulin dependent diabetes mellitus

IFN interferon

Ig immunoglobulin

IGRP islet-specific glucose-6-phosphatase catalytic subunit–related protein

IL interleukin

InsB insulin B

i.d. intradermal

i.m. intramuscular

i.v. intravenous

MAb monoclonal antibody

MHC major histocompatibility complex

MΦ macrophage

NK natural killer

NOD non-obese diabetic

Ova ovalbumin

PBL peripheral blood lymphocyte

PE phyco-erythrin

PerCP peridinin-chlorophyll protein

PLN pancreatic lymph node

RIP rat insulin promoter

RT-PCR reverse transcription-polymerase chain reaction

SAV streptavidin

mBDC mimetic BDC

scid severe-combined immunodeficient

SP single-positive

T1D type 1 diabetes

TCR t cell receptor

TGF transforming growth factor

Tg transgenic

Th1 type 1 T helper

Th2 type 2 T helper

TNF tumor necrosis factor

Treg regulatory T cell

α alpha

β beta

γ gamma

CHAPTER 1

INTRODUCTION

1.1 Diabetes Classification And Etiology

Diabetes mellitus is a multifactorial set of metabolic diseases caused by insufficient insulin production or insulin resistance (www.diabetes.org). Insulin is a heterodimeric protein produced exclusively by the β cells found in the islets of Langerhans in the pancreas (1). The primary function of insulin is to regulate glucose transport and as a consequence, glucose metabolism within myocytes and adipocytes (2). Thus, any condition hindering insulin production or retarding insulin action interferes with glucose homeostasis.

Type 1 diabetes mellitus (T1D), formerly referred to as juvenile diabetes and insulin-dependent diabetes, is a disease characterized by the autoimmune-mediated destruction of the insulin-producing pancreatic β cells leading to insulin deficiency (www.cdc.gov). Type 2 diabetes mellitus (T2D), formerly known as adult onset diabetes and non-insulin dependent diabetes, results from insulin resistance or insulin secretory defects (www.cdc.gov). Type 3 diabetes mellitus results from factors not involved in T1D or T2D, while Type 4 diabetes mellitus refers to gestational diabetes (www.diabetes.org).

As noted above, T1D is marked by the autoimmune-mediated destruction of the insulin-producing β cells (3, 4). Clinical manifestations of T1D develop as a result of chronic complications related to a hyperglycemic state. Hyperglycemia develops when greater that 80 percent of the insulin-producing β cells have been destroyed (5). The early stages of β cell autoimmunity are characterized by the development of

autoantibodies against islet antigens, autoreactive T cells, and an islet cell infiltrate referred to as insulitis. The development of β cell autoimmunity is influenced by both genetic and environmental factors (6, 7).

Those afflicted with T1D typically suffer from a number of complications associated with inappropriate glucose metabolism, in addition to having a 30% percent reduction in life expectancy (www.massgeneral.org/diabetes,org). Currently, there is no cure for T1D. Pancreatic and islet transplantation is one approach that has been used to "cure" T1D. However, long-term success of these procedures has been limited at best due to eventual graft rejection and/or failure. Currently, the four major obstacles to successful pancreas and islet transplantation are ongoing β cell autoimmunity, allogenic recognition of the graft, complications associated with immunosuppressive drugs, and organ availability. For instance, established autoimmunity in diabetic patients if not suppressed will result in destruction of newly transplanted β cells (8). Furthermore, recipients of allogenic transplants receive potent immunosuppressive drugs, which upon long-term use lead to severe side effects. Current drug therapies for transplant recipients include the use of the immunosuppressive drugs FK506 and/or Cyclosporin A that also exhibit β cell and kidney cytotoxicity (9). Finally, the availability of organs is limited as the number of donors is few, estimated at 1,200 pancreata in the US per year (www.jdrf.org). Further restricting the availability of the organs is the requirement to genetically "match" the graft with the recipient. Despite some promising clinical results, pancreatic and islet transplantation is still too premature to be applied large scale for the

treatment of diabetic individuals. Thus, in the meantime, Type 1 diabetics remain dependent on the use of exogenous insulin therapy.

1.2 The NOD Mouse

The nonobese diabetic (NOD) mouse, a spontaneous model of human T1D has been instrumental for the study of T1D (10). Eighty and ten percent of female and male NOD mice, respectively, develop symptoms that parallel human T1D (11). At three to five weeks of age, insulitis is first detected in NOD mice (11). Insulitis progresses and by 12-16 weeks of age the majority of β cells are destroyed and hyperglycemic blood levels are established (12). Similar to human T1D, the islet infiltrate is heterogeneous consisting of T cells, macrophages, dendritic cells (DC), and B lymphocytes (13). Additionally, NOD mice develop autoantibodies specific for various β cell antigens. Strikingly, several cell specificities targeted by T cells and autoantibodies are shared between NOD mice and diabetic individuals.

1.3 Genetic Factors of Type 1 Diabetes

Since the sequencing and subsequent discovery that the human genome encodes over 30,000 genes, it is clear that finding a singular genetic basis for a disease is a highly challenging endeavor. It is especially complicated for T1D since disease susceptibility is polygenic and also influenced by ill-defined environmental factors. For example, monozygotic twin studies have shown on average 33 percent concordance among diabetic twins (14). The genetic locus with the strongest association with T1D susceptibility and resistance in humans is the human leukocyte antigen region (HLA) (15).

HLA molecules are surface glycoproteins that present antigens to the immune system (16). HLA class I molecules (A, B or C) bind and present peptides derived from antigens to CD8+ T cells (17). The HLA class II locus encodes three subclasses known as HLA-DR, DQ and DP, which bind and present peptides that are recognized by $CD4^+$ T cells (16). Among the HLA class II molecules, it has been found that individuals who have certain HLA-DR or –DQ alleles have an increased risk of developing T1D (15, 18). For instance, ninety-five percent of individuals with T1D have HLA –DR3 or DR4 or both alleles (19).

The NOD mouse has a similar strong association with Major Histocompatibility Complex (MHC) locus and T1D susceptibility and resistance (20). IAg7, the MHC class II allele expressed by NOD mice plays a key role in the initiation and progression of T1D (21). The association of IA^{g7} with T1D is believed to be due to the molecule's unique structure. Its direct role in T1D has been confirmed by introduction of transgenes encoding various IA alleles that prevent or reduce the frequency of diabetes in transgenic NOD mice (22, 23). Furthermore, substitution of the histidine and/or serine residues at □6 and 57 on the β chain with a proline and aspartic acid, respectively, significantly reduces the frequency of insulitis and prevents diabetes in the corresponding lines of transgenic NOD mice (22, 24). Consequently, it has been proposed that the amino acid residues at β56 and β57 influence the peptide binding properties of IA^{g7} (25, 26). Studies have suggested that IA^{g7} binds to peptides weakly, and/or the surface half-life of IA^{g7}-peptide complexes are relatively short-lived (25, 27). Either of these properties may skew the T cell repertoire towards β cell-specific reactivity.

In addition to the HLA and MHC loci several other genetic loci have been identified (Table 1.1 (for review see (7)). These insulin dependent diabetes (IDDM/Idd) loci largely encode gene products that influence the development and/or function of immune effector cells. It is believed that these Idd loci primarily function as "modifiers" of the dominant effects associated with the HLA and MHC loci (18).

Table 1.1. Identified *idd* Loci in NOD Mice

idd1	17	MHC I &II, TNFα/β, Tap1&2
idd2	9	Insulin
idd3	3	IL-2. IL-21, HIPK1, PTPN8
idd4	11	FADD
idd5.1	1	Caspase8, CD28, CTLA4
idd5.2	1	CD152, ICOS
idd6	6	? (localized toD18s87)
idd7	7	GALNT3
idd8	14	? (localized toD6s264)
idd9	4	Jak1, Lck, TnfR2
idd10	3	? (localized toD10s193)
idd11	4	? (localized toD14s67)
idd12	14	? (localized toD6s86)
idd13	2	B_2m
idd14	13	?
idd15	5	?
idd16	17	?
idd17	3	?
idd18	3	?

1.4 *Idd3* and the *il2* gene

Among the more than 20 IDDM/idd HLA- and MHC-independent loci, *IDDM3/idd3* has one of the stronger associations with T1D susceptibility in humans and NOD mice (28). For the purpose of the studies discussed throughout this dissertation, I will focus on the murine *Idd3* locus located on 5' proximal end of chromosome 3. The Idd3 locus is of keen interest due to studies demonstrating an association with the development of β cell-specific effector T cells (29, 30). NOD mice congenic for an Idd3 interval derived from a diabetes resistant strain of mice exhibit a markedly reduced incidence and delayed onset of T1D (31, 32). Mapping of idd3 demonstrated that the interval spans 780 kilobases

(kb) and contains genes that encode a variety of immunoregulatory molecules: IL-2, IL-21, homeodomain interacting protein kinase 1 (HIPK1), and protein tyrosine phosphotase 8 (PTPN8) (33). IL-2 has been the focus of most studies attempting to define the candidate gene(s) in Idd3 (34-36). Podolin et al. first proposed that the function of IL-2 was altered in NOD mice compared to disease resistant strains such C57BL/6 (B6) mice due to differences in glycosylation (33). However comparative analyses showed no differences between NOD versus B6 IL-2 in stimulating T cell proliferation in vitro (33). Nevertheless, it is possible that heavily glycosylated NOD IL-2 is less functional in a "natural" environment due to stereo-hindrance (37, 38). Furthermore, binding of IL-2 to the trimeric IL-2 receptor (IL-2R; α receptor (CD25), β receptor (CD122), γ receptor (CD132)) may be attenuated by the additional N-glycosylated moieties. Studies measuring the binding affinity of NOD versus B6 IL-2 to the trimeric IL-2R would aid in ruling out this possibility (37, 38).

A number of single nucleotide polymorphisms (SNPs) have been identified within and flanking the *il2* gene (39). SNPs have been widely used to identify polymorphisms associated with various disease states, and can affect gene expression and/or protein function. Notably a SNP found -1010 base pairs (bp) upstream of NOD *il2* within a conical binding

Table 1.2. Mutated AP-1 Binding Domain ¯1010 bp Upstream of IL-2 Promoter

	Sequence
Canonical AP-1 Binding motif:	5'- tgagt**C**a -3'
NOD AP-1 Binding site:	5'- tgagt**T**a -3'

motif for the AP-1 transcriptional factor has significant effects on *il2* gene expression (See Table 1.2) (40). This point mutation reduces AP-1 binding and results in a two-fold reduction in NOD *il2* gene expression. This finding is consistent with observations made by Yamanouchi et al. demonstrating that an approximate two-fold reduction in IL-2 expression in NOD mice relative to various NOD.idd3 congenic lines correlates with increased diabetes incidence (33, 41).

The genetic evidence that *idd3* and specifically IL-2 is involved in the development of T1D in both humans and NOD mice raises questions regarding the impact IL-2 expression has on T1D susceptibility. IL-2 is known to have broad effects on T cell biology. The activation of T cells via the T cell receptor (TCR) and costimulatory molecules results in the production of IL-2 and up regulation of IL-2R. The autocrine/paracrine effects of IL-2 on T cells results in cell survival, clonal expansion and the differentiation into effector cells (42). In addition, IL-2 binding of the IL-2R initiates a Stat5-dependent signaling cascade that induces CD25 expression that leads to a positive feed back loop (43). IL-2 also alters the immune response by increasing the susceptibility of activated T cells to apoptosis following strong TCR signaling. The latter, referred to as activation induced cell death (AICD) is in part characterized by increased expression by T cells of death-mediating molecules in the Fas and tumor necrosis factor pathways (44). IL-2 also plays a role in the maintenance and function of memory $CD8^+$ T cells (45).

In the context of autoimmunity, IL-2 serves a key function in establishing and maintaining self-tolerance (For review see (46)). IL-2 knockout mice develop autoimmunity including but not limited to T1D (47, 48), while mice deficient in CD25 have enlarged lymphoid organs in which T and B cells undergo expansion due to defects in AICD (47, 49). Furthermore, anti-CD25 blocking antibodies exacerbate autoimmune disease by preventing IL-2-mediated signaling events in T cells (32). Finally, as noted above elevated IL-2 expression in NOD.idd3 congenic mice reduces the frequency of T1D (32, 50, 51). The mechanism by which IL-2 controls autoimmunity is believed to include the induction and maintenance of CD4+ T regulatory cells, which will be discussed in depth in a later section.

1.5 *il21* Another Candidate Gene In Idd3

Despite some groups believing that il2 is the lone gene accounting for the association of *Idd3* with T1D, the *Idd3* locus also encodes another gene candidate, namely *il21* (31, 52). IL-21 is a member of the common gamma chain signaling family (IL-2Rγ) of cytokines that include IL-2, IL-4, IL-7, IL-9 and IL-15. The IL-21 receptor (IL-21Rα) and IL-2Rγ are expressed on immune cells such as T, B, NK and DC, and have roles in activation, proliferation and survival (53). IL-21 signaling results in activation of the Jak-Stat pathway whereby Stat3 is phosphorylated which in turn promotes up regulation of IL-21Rα expression. The downstream effects of IL-21 signaling have been controversial. IL-21 was once thought to induce the differentiation of CD4+ T cells into T helper 17 (Th17) cells (54). More recently, however, stronger evidence indicates that IL-21 induces differentiation of T follicular helper (Tfh) cells characterized by the production

of IL-21 (55, 56). Tfh cells are recognized for a capacity to control the formation of germinal centers and enhance B cell antibody production (57).

IL-21 may affect T1D in multiple ways. IL-21 is similar to IL-2 in that both are abundantly produced by activated $CD4^+$ T cells (41). Since IL-21 is a key growth factor driving $CD8^+$ effector T cell proliferation, this may preferentially expand autoreactive T cells in a lymphopenic environment; a condition attributed to NOD mice by some (31, 58-60). Additionally, a recent study suggests IL-21 may also have a direct effect on islet generation. Spolski et al. developed a NOD.IL-21Rα-/- knockout mouse in which progression of insulitis was reduced, diabetes prevented, and expression of the regenerating gene (Reg) in islets increased (61). This study described a scenario whereby IL-21 signaling in islets suppresses the Reg gene that is required for islet generation. Finally, IL-21 has known suppressive effects on the generation and function of regulatory T cells that are important in controlling self-tolerance (62).

1.6 Thymic Selection and Self-Tolerance in the T cell Compartment

Autoimmunity occurs when the immune system fails to discriminate between self- and foreign-antigens. T cell self-tolerance is established in the thymus where $CD4^+$CD8+ double positive (DP) thymocytes go through an educational process known as positive and negative selection. During positive selection, DP thymocytes bearing a newly rearranged TCR encounter antigen in the context of MHC molecules on the surface of cortical thymic epithelial cells. This interaction delivers a “positive” signal promoting survival of DP thymocytes and in turn ensures that the expressed TCR is restricted to

self-MHC. DP thymocytes which fail to receive a positive signal undergo apoptosis (63). Positively-selected DP thymocytes then traffick to the cortico-medullary junction and medulla to undergo negative selection (64). Normally, autoreactive DP thymocytes are clonally deleted during negative selection or rendered non-reactive upon subsequent self-antigen encounter (65). Deletion of autoreactive DP thymocytes is determined by the affinity and avidity of the TCR for peptide/MHC complexes on the surface of medullary thymic epithelial cells and/or thymic DC. DP thymocytes expressing TCR with relatively high affinity for peptide/MHC complexes are deleted. Negative selection of autoreactive DP thymocytes is further regulated by the transcription factor AIRE which controls the expression of self-antigens by medullary thymic epithelial cells (66, 67). In the case of T1D, studies in NOD mice suggest that the development of pathogenic β cell-specific T cells is due to inefficient negative thymic selection which has been at least partially attributed to the peptide binding properties of IA^{g7} (10, 68, 69). Less is known about the negative selection process in the human development of T1D.

1.7 β Cell Autoantigens

Islets contain glucagon-producing α cells, somatostatin-producing δ cells, pancreatic poly-peptide producing (PP) cells in addition to insulin-producing β cells. However, only β cell antigens are targeted in the disease process reflected by the development of β cell-specific autoantibodies and T cell reactivity.

Since T cell pathogenesis is critical in T1D, many studies have aimed to identify the important β cell antigens responsible for driving the autoreactive T cell response in

NOD mice and diabetic patients (46, 70). Only a select few β cell autoantigens are targeted early in the disease process (71). However, the diabetogenic T cell response is amplified as inter and intra-epitope spreading occurs. For example, during the initial stages of insulitis in NOD mice CD4+ T cell reactivity specific for insulin B chain and glutamic acid decarboxylase 65 (GAD65) (72) are detected, whereas islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) and insulin B chain appear to be key early targets of $CD8^+$ T cells (73, 74). T cells with other specificities such as IA-2, heat shock protein 60 (HSP60) and dystrophia myotonica kinase are then recruited over time (73, 74).

Insulin is an essential autoantigen in the development of T1D. Insulin is expressed exclusively by β cells in response to increased glucose. It is secreted in a zymogenic form as preproinsulin which undergoes two cleavage modifications yielding active insulin and the metabolite C-peptide. Mice express two isoforms of insulin, insulin 1 and 2 while humans only express one form. Thymic expression of proinsulin 2 is believed to be crucial for inducing self-tolerance to insulin. Nakayama and colleagues demonstrated that insulitis and diabetes are prevented in NOD mice expressing a transgene encoding proinsulin 2 in which the IA^{g7} and $H2K^d$ restricted epitopes had been mutated (78). A similar finding was made by Jaeckel and colleagues using a transgenic model system expressing insulin and other autoantigens in the thymus of NOD mice (79, 80). Tolerance to insulin led to reduced diabetes incidence whereas tolerance to other autoantigens had no effect on disease development.

GAD is another β cell autoantigen thought to have a key albeit undefined role in T1D. GAD is an enzyme with two isoforms GAD65 and GAD67, that catalyzes the synthesis of the neurotransmitter β amino butyric acid (GABA) in the central nervous system (81, 82). While the role of GAD in catalyzing the synthesis of GABA in the brain is well documented, its function in the pancreas is unclear. Of the two isoforms, it is GAD65 that is implicated in the pathogenesis of T1D. In NOD mice, the earliest detectable response to islet extracts coincides with detection of GAD65-specific T cell reactivity. Furthermore, immunization with plasmid DNA (pDNA) encoding GAD65 exacerbates the onset of diabetes in NOD mice, further suggesting a pathogenic role in T1D (85).

1.8 Development of Autoantibodies in T1D

Autoantibodies appear to have no pathological role in β cell destruction. However, detection of β cell-specific autoantibodies has been extensively used to monitor and predict the development of overt diabetes in at risk or prediabetic individuals (86). For instance, greater than 90 percent of at risk individuals presenting with autoantibodies specific for GAD65, insulin and/or islet protein tyrosine phosphatase (IA-2) develop overt diabetes (87). Furthermore, studies have shown that the probability of developing diabetes is also directly proportional to the titer of a given autoantibody. Individuals presenting with islet cell cytoplasmic antigen autoantibodies above 20 JDF units have a 35 percent chance of developing overt diabetes within five years, while those with less than 20 JDF units have only a 5 percent chance (86).

1.9 The Role of T cells in the Pathogenesis of T1D

T cells are the primary mediators of β cell destruction while B cells, macrophages and DC act as APC to drive the immune response in the islets (88-92). A direct role for T cells in β cell destruction was initially shown by depleting T cells via administration of a depleting anti-CD3 antibody, and preventing diabetes in NOD mice (93-96). Furthermore, NOD mice deficient of T cells develop neither insulitis nor diabetes, and diabetes can be adoptively transferred in immunodeficient NOD.scid mice by T cells from diabetic NOD donors. A number of transgenic mouse lines have been established expressing TCR specific for β cell autoantigens that further substantiate the critical role for T cells in the initiation and progression of β cell autoimmunity (97-100)

The relative contribution $CD4^+$ and $CD8^+$ T cells in the diabetogenic response continue to be a controversial topic. A number of studies suggest that $CD4^+$ T cells are necessary for the early and late stages of β cell autoimmunity. Several groups have shown that activated $CD4^+$ T cells alone can transfer diabetes to irradiated NOD or NOD.scid recipients (100). Additionally, depletion of $CD4^+$ T cells in NOD mice via anti-CD4 antibody treatment at early and late preclinical stages of T1D prevents diabetes. Prevention of diabetes, however, is only detected when young but not older NOD mice (>5 weeks of age) are treated with anti-CD8 antibody (12). Together, these findings suggest that $CD8^+$ T cells play an important role in the initiation of □ cell autoimmunity.

The autoimmune process of T1D is mediated and regulated by distinct subsets of $CD4^+$ T cells that are characterized by the types of cytokines secreted. For example, type 1 $CD4^+$ effector T cells are characterized by secretion of interferon gamma (IFNγ) and

tumor necrosis factor-α (TNFα), and are the primary mediators of β cell autoimmunity. In general, type 1 T effectors are associated with cell-mediated immunity that supports macrophage activation, delayed type hypersensitivity responses, and immunoglobulin isotype switching to IgG2a (101, 102). In NOD mice, intra-pancreatic levels of mRNA and the frequency of IFNγ and TNFα secreting T cells are elevated in diabetic animals (3, 103-107). T cells isolated from the blood of diabetic patients typically secrete IFN□ when stimulated with β cell-derived peptides (108).

1.10 The Role of Immunoregulatory T cells in Self-Tolerance and T1D

Self-tolerance within the T cell compartment is maintained by the frequency and function of immunoregulatory T cells. As alluded to above, T1D in humans and NOD mice is attributed to a functional imbalance between β cell-specific $CD4^+$ type 1 and immunoregulatory effectors (106, 109). The first immunoregulatory subset of CD4+ T cells to be defined was the Th2 or type 2 effector (110, 111). Type 2 effector T cells typically mediate humoral immunity, are characterized by the dominate production of IL-4, and to a lesser extent IL-5, IL-10, and IL-13 (112, 113). IFNγ and IL-4 have reciprocal down-regulatory effects on the differentiation of naïve T cells into type 1 or type 2 effectors. For example, IFNγ aids in the differentiation of type 1 T effectors, and blocks type 2 T cell development (114). Conversely, IL-4 promotes and inhibits the differentiation of type 2 and type 1 T cells, respectively (111).

The functional balance between β cell specific type 1 and type 2 effector T cells does not fully explain the immune process involved in T1D. Studies have shown that

T1D requires a prolonged immune reaction against β cells, and possible constant activation of cytotoxic cells in order to result in sufficient β cell death causing T1D. This hypothesis is supported by the observation that progression to T1D can take months, years, or even decades until clinical manifestations occur. Areas of great interest that may help explain the long pre-diabetes stage is the role T regulatory cells play in regulating autoreactive cells. Several subsets of immunoregulatory T cells with distinct phenotypes and mechanisms of action have been identified for their contribution to the prevention of diabetes (115). These subsets include: i) Th3 cells, which primarily secrete IL-4 and transforming growth factor-β (TGFβ) and are induced via mechanisms of oral tolerance (116), ii) T regulatory type 1 cell (Tr1), which secrete high levels of IL-10 (117), and iii) "natural" and "adaptive" CD4+CD25+ regulatory cells which are defined by the expression of the transcription factor Forkhead box P3 ($FoxP3^+$Treg) and exhibit suppressor function mediated by cell-cell contact and secretion of TGFβ and IL-10 (118). More recently, $CD8^+$ T cells exhibiting immunoregulatory function have also been identified (119, 120). For the purpose of this thesis and in view of their potent immunoregulatory function, the following discussion will focus primarily on Tr1 cells and $FoxP3^+$Treg. Discussion of other subsets of immunoregulatory T cells can be obtained in the following reviews (121, 122).

Similar to type 1 and type 2 effector cells, Tr1 cells differentiate from naïve CD4+ T precursors in the presence of the cytokine IL-10. Tr1 cells are characterized by the secretion of high levels of IL-10, low amounts of IL-5, IFNγ, and no IL-2 and IL-4 production (117)(123). Not surprisingly, surprisingly the immunoregulatory function of

Tr1 cells is mediated by secretion of high levels of IL-10 which has potent suppressive effects on both APC and T cells (117). IL-10 blocks the effector function of APC by inhibiting up regulation of costimulatory molecules and pro-inflammatory cytokine secretion, and directly inhibits IL-2 and TNFαproduction by $CD4^+$ T cells (124). Numerous studies have demonstrated that Tr1 cells prevent the development of type 1-mediated autoimmuity and inflammatory bowel diseases (125-127).

$FoxP3^+$Treg are thought to play a major role in regulating the progression of β cell autoimmunity. FoxP3 is a transcription factor that controls the expression of genes encoding key suppressor molecules known to inhibit T effector cell activation and differentiation. FoxP3 controls the expression of CTLA-4 and glucocorticoid-induced TNF receptor (GITR) which are known to control activation of APC through cell-cell contact, and the expression of TGF-β1 and possibly IL-10 which affect both T cells and APC (128).

FoxP3 expression is induced in the thymus and periphery to generate "natural" or "adaptive" $FoxP3^+$Treg, respectively. "Natural" $FoxP3^+$Treg are established in the thymus upon recognition of self-antigen. Even though "natural" $FoxP3^+$Treg arise in the thymus, their phenotype and function is similar to the peripheral-derived "adaptive" $FoxP3^+$Treg. The frequency of both "natural" and "adaptive" $FoxP3^+$Treg is critical for regulation of self tolerance. Notably, the frequency and function of "natural" $FoxP3^+$Treg progressively decline with age in female but not male NOD mice (129-131). In diabetic subjects, $FoxP3^+$Treg exhibit a reduced suppressor activity in vitro (132). Furthermore,

the induction of "adaptive" FoxP3^{+}Treg suppresses ongoing β cell autoimmunity in NOD mice (133-135).

Defects in FoxP3^{+}Treg maintenance, expansion, induction and function have all been cited as potential causes for aberrant FoxP3^{+}Treg immunoregulation in type 1 diabetics and NOD mice. Deficiencies in key immunoregulatory molecules necessary for maintaining proper development and function of FoxP3+Treg can be partly attributed to the genetics of T1D. As mentioned earlier, IL-2 is a critical molecule for the development and maintenance of FoxP3^{+}Treg. Altered expression and/or function of IL-2 would be expected to impact FoxP3^{+}Treg (45, 46). Additionally, the suppressive molecule CTLA-4 found on the surface of FoxP3^{+}Treg also map to the diabetes locus Idd5 and has been shown to be down-regulated in NOD mice (136).

1.11 Induction of Immunoregulation to Reestablish Self-Tolerance

The majority of immunotherapies being tested for the prevention and/or treatment of T1D have largely focused on reestablishing self-tolerance within the T cell compartment. Typical approaches of immunotherapy have included the induction of β cell-specific immunoregulatory T cells, blocking T cell co-stimulation, and depletion of T cells (For review see (137)). In general, two approaches have been used to target T cells for the purpose of immunotherapy: antigen-independent versus antigen-dependent therapies. Antigen-independent immunotherapy has the advantage of tolerizing large numbers of T cells. The latter is a key issue at late stages of the diabetogenic response when the frequency of pathogenic effector T cells is high. However, antigen-independent

immunotherapy fails to discriminate between T cells specific for self versus foreign antigens, and in turn may affect the normal function of the immune system. On the other hand, antigen-dependent immunotherapy provides a strategy to selectively target autoreactive T cells. However, the efficacy of antigen-dependent immunotherapy typically wanes at later stages of disease progression. Recently, "combined" immunotherapies are being considered and tested to exploit the respective strengths of antigen-dependent and –independent approaches

1.12.1 Antigen-Independent Immunotherapy

A variety of antigen-independent approaches have been investigated attempting to modulate the differentiation and/or function of pathogenic β cell-specific T cells. One of the more common approaches has been continuous administration of anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and TGF-β or the use of blocking antibodies to proinflammatory cytokines to prevent diabetes in young NOD mice (138-141). While many of these studies have shown promise, the feasibility and efficacy of cytokine immunotherapy is significantly diminished when considering the costs and practicality of such a therapy. Accordingly, gene therapy has been used to express various cytokines either systemically or in a tissue-specific manner to effectively modulate autoreactive T cell responses in NOD mice (142, 143).

Cell-based immunotherapies are a relatively novel approach being tested to treat T1D. This strategy involves in vitro expansion and adoptive transfer of a given type of regulatory cell. For example, exploiting the tolerogenic properties of immature DC or DC

rendered immature by suppressing the transcription factor NF-κB has shown to induce Tr1 and FoxP3+Treg capable of preventing diabetes in NOD mice (144-147). The use of stem cells is another approach currently being tested in clinical trials for immunological disorders, such as graft-versus-host disease, that in turn could be applied for the treatment of T1D. Mesenchymal stem cells for example, exhibit potent immunomodulatory properties that include suppression of T cell proliferation and induction and/or expansion of Treg (for review see (148)).

T cell-depleting antibodies specific for CD4, CD8, and CD25 have been used in NOD mice and transgenic mouse models of T1D to effectively prevent or induce remission of diabetes (12, 149-153). The most promising antigen-independent immunotherapy to date is the administration of anti-CD3 antibody. Anti-CD3 antibody has been used to treat new onset diabetes in NOD mice, and has exhibited some therapeutic efficacy in recent diabetic patients. For example, long-term remission is induced in the majority of diabetic NOD mice treated with a nonmitogenic anti-CD3 antibody. The anti-CD3 antibody transiently depletes T cells, in addition to inducing Treg that mediate suppression in a TGF-β-dependent manner. Interestingly, these Treg appear to be inducing adaptive FoxP3^{+}Treg from naïve CD4+ T cells not due to the expansion of existing "natural" Treg. Promising results have been obtained in recent clinical studies assessing the safety and efficacy of anti-CD3 antibody treatment in recent onset diabetic individuals. Herold et al. using a short course of anti-CD3 antibody injections within the first 6 weeks of diagnosis showed continued β cell function in the majority of patients up to but not beyond an 18 month period. The potential side effects

from such therapy are obvious as any extended period of immunosuppression opens the possibility of opportunistic infection. Despite potential pitfalls, the ability of anti-CD3 antibody to reestablish protection by clonally deleting β cell-specific, pathogenic T effector cells and inducing FoxP3^{+}Treg makes this therapy the strongest candidate to treat diabetes from the antigen-independent therapeutic approaches.

1.12.2 Antigen-Dependent Immunotherapy

The use of self-antigen as a therapy can affect autoreactive T cells in two ways. First, T cells may undergo clonal anergy and/or deletion upon administration of high doses of soluble self-antigen (154, 155). However, inducing anergy/deletion in a limited set of clonotypes is only marginally effective when multiple autoantigens are targeted, as seen in the late stages of T1D (156, 157). Secondly, self-antigen vaccination can induce/expand immunoregulatory T cells. This result is attractive as once established, immunoregulatory T cells can traffick to the site of inflammation and suppress the differentiation and/or activity of pathogenic T effectors in an antigen-independent manner via cytokine secretion. For example, administration of intact GAD65 or a pool of GAD65-specific peptides (e.g. p217-236, p290-309) induces GAD65-specific immunoregulatory CD4+ T cells in 12 week-old NOD female mice (158, 159). Importantly, the extracellular milieu established by GAD65-specific immunoregulatory T cells also promotes the development of additional immunoregulatory T cells with distinct β cell-specificities to amplify the protective effect (90, 160, 161).

Several factors must be considered when devising an immunotherapy to effectively induce β cell-specific immunoregulatory T cells. The first is the identity of the βcell antigen used for vaccination. At late stages of disease progression when the frequency of pathogenic T cells is high, a sufficient number of immunoregulatory T cells is required to reestablish a functional balance between T cells. Notably, the number of immunoregulatory T cells that can be induced is in part determined by the size of the pool of naïve T cell precursors for a given antigen. Thus, it is important to identify a β cell antigen for which a relatively high frequency of naïve T precursors exists. Studies in the NOD mouse have highlighted this issue. For example, diabetes is readily prevented when young NOD mice are vaccinated with several β cell autoantigens such as insulin, GAD65, and HSP60 (90, 160, 162-164). However, in older NOD mice in which β cell autoimmunity is well established, only administration of intact GAD65 or its derived peptides has consistently induced a sufficient frequency of immunoregulatory T cells to prevent diabetes (90, 158, 159, 165). A possible explanation as to why GAD65 treatment works in older mice may partly be explained by a relatively high frequency of GAD65-specific naïve T cell precursors (137).

The second critical issue is to establish conditions of autoantigen administration that preferentially induce immunoregulatory versus pathogenic effector T cells. Here the major concern is that β cell autoimmunity is exacerbated following antigen vaccination. Adjuvants such as complete Freund's adjuvant or alum have been employed to induce antigen-specific type 2 and Tr1-like cells at a high frequency (166, 167). Similarly, the co-administration of antigen and anti-inflammatory cytokines such as IL-4 and/or IL-10

provides the means to preferentially induce specific subsets of immunoregulatory T cells (85, 168, 169). Tolergenic DC can also be utilized as an adjuvant. Treatment of murine or human immature DC with 1α,25-dihydroxyvitamin D3 and/or dexamethasone blocks secretion of proinflammatory cytokines and promotes IL-10 secretion upon DC activation. Accordingly, adoptive transfer of tolergenic DC pulsed with β cell-derived peptide may prove to be an effective approach to elicit peptide-specific Tr1-like cells and/or Treg.

The route of administration is also a key factor in determining the nature of the immune response induced by β cell antigen vaccination. For instance, targeting mucosal tissues by intranasal or oral administration of insulin and insulin peptides results in preferential induction of type 2 effectors or FoxP3^{+}Treg reactivity and diabetes prevention in NOD mice (170, 171). Furthermore, ingestion of transgenic plants expressing autoantigens such as GAD67 has also been effective at preventing diabetes in NOD mice (172, 173). However, oral administration of insulin to prediabetic individuals in the Diabetes Prevention Trial-1 (DPT-1) showed no significant effect on the development of diabetes or reduction in β cell autoimmunity (174, 175). Although it is unclear why the treatment failed to prevent diabetes, administration of insufficient doses of insulin is thought to be a likely factor (137).

The final parameter of antigen-specific immunotherapy that needs to be considered is the type of immunoregulatory T cells elicited by a given strategy. Induction of different types of immunoregulatory T cells, which exhibit distinct modes of action would be expected to enhance overall immunotherapeutic efficacy. For instance,

vaccination with pDNA encoding GAD65, IL-4 and IL-10 induces protection for syngeneic islet grafts in diabetic NOD recipients (176). Long-term islet graft protection is due to increased numbers of GAD65-specific type 2 effector cells, Tr1 cells and FoxP3^{+}Treg. In contrast, islet grafts are readily destroyed in recipients treated with pDNA encoding GAD65 and IL-4 in which only GAD65-specific type 2 effector cells are established.

1.13 Aims of the Dissertation

The objective of the first project is to establish a clinically amenable method of preventing T1D. The goal here is to determine whether gene-gun delivery enhances the immunotherapeutic efficacy pDNA vaccines. The aim of the second project is to elucidate the roles of IL-2 and IL-21 in the development of β cell autoimmunity in NOD mice. In this way a better understanding of the key events driving the diabetogenic response can be obtained.

1.16 REFERENCES

1. Banting, F.G. 1937. Early Work on Insulin. Science 85:594-596.

2. Bell, P.M., R.G. Firth, and R.A. Rizza. 1986. Assessment of insulin action in insulin-dependent diabetes mellitus using [6(14)C]glucose, [3(3)H]glucose, and [2(3)H]glucose. Differences in the apparent pattern of insulin resistance depending on the isotope used. J Clin Invest 78:1479-1486.

3. Liblau, R.S., S.M. Singer, and H.O. McDevitt. 1995. Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today 16:34-38.

4. Tisch, R., and H. McDevitt. 1996. Insulin-dependent diabetes mellitus. Cell 85:291-297.

5. Foulis, A.K., C.N. Liddle, M.A. Farquharson, J.A. Richmond, and R.S. Weir. 1986. The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. Diabetologia 29:267-274.

6. Akerblom, H.K., O. Vaarala, H. Hyoty, J. Ilonen, and M. Knip. 2002. Environmental factors in the etiology of type 1 diabetes. Am J Med Genet 115:18-29.

7. Maier, L.M., and L.S. Wicker. 2005. Genetic susceptibility to type 1 diabetes. Curr Opin Immunol 17:601-608.

8. Stegall, M.D., K.J. Lafferty, I. Kam, and R.G. Gill. 1996. Evidence of recurrent autoimmunity in human allogeneic islet transplantation. Transplantation 61:1272-1274.

9. Bach, J.F. 1993. Immunosuppressive therapy of autoimmune diseases. Immunol Today 14:322-326.

10. Anderson, M.S., and J.A. Bluestone. 2005. The NOD mouse: a model of immune dysregulation. Annu Rev Immunol 23:447-485.

11. Leiter, E.H. 2001. The NOD mouse: a model for insulin-dependent diabetes mellitus. Curr Protoc Immunol Chapter 15:Unit 15 19.

12. Wang, B., A. Gonzalez, C. Benoist, and D. Mathis. 1996. The role of CD8+ T cells in the initiation of insulin-dependent diabetes mellitus. Eur J Immunol 26:1762-1769.

13. Irving-Rodgers, H.F., A.F. Ziolkowski, C.R. Parish, Y. Sado, Y. Ninomiya, C.J. Simeonovic, and R.J. Rodgers. 2008. Molecular composition of the peri-islet basement membrane in NOD mice: a barrier against destructive insulitis. Diabetologia 51:1680-1688.

14. Barnett, A.H., C. Eff, R.D. Leslie, and D.A. Pyke. 1981. Diabetes in identical twins. A study of 200 pairs. Diabetologia 20:87-93.

15. Undlien, D.E., B.A. Lie, and E. Thorsby. 2001. HLA complex genes in type 1 diabetes and other autoimmune diseases. Which genes are involved? Trends Genet 17:93-100.

16. Appel, H., L. Gauthier, J. Pyrdol, and K.W. Wucherpfennig. 2000. Kinetics of T-cell receptor binding by bivalent HLA-DR.Peptide complexes that activate antigen-specific human T-cells. J Biol Chem 275:312-321.

17. Jarchum, I., J.C. Baker, T. Yamada, T. Takaki, M.P. Marron, D.V. Serreze, and T.P. DiLorenzo. 2007. In vivo cytotoxicity of insulin-specific CD8+ T-cells in HLA-A*0201 transgenic NOD mice. Diabetes 56:2551-2560.

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

19. Bowman, M.A., O.G. Simell, A.B. Peck, J. Cornelius, R. Luchetta, Z. Look, N.K. Maclaren, and M.A. Atkinson. 1996. Pharmacokinetics of aminoguanidine administration and effects on the diabetes frequency in nonobese diabetic mice. J Pharmacol Exp Ther 279:790-794.

20. Lyons, P.A., and L.S. Wicker. 1999. Localising quantitative trait loci in the NOD mouse model of type 1 diabetes. Curr Dir Autoimmun 1:208-225.

21. Hattori, M., J.B. Buse, R.A. Jackson, L. Glimcher, M.E. Dorf, M. Minami, S. Makino, K. Moriwaki, H. Kuzuya, H. Imura, and et al. 1986. The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. Science 231:733-735.

22. Lund, T., L. O'Reilly, P. Hutchings, O. Kanagawa, E. Simpson, R. Gravely, P. Chandler, J. Dyson, J.K. Picard, A. Edwards, and et al. 1990. Prevention of insulin-dependent diabetes mellitus in non-obese diabetic mice by transgenes encoding modified I-A beta-chain or normal I-E alpha-chain. Nature 345:727-729.

23. Nishimoto, H., H. Kikutani, K. Yamamura, and T. Kishimoto. 1987. Prevention of autoimmune insulitis by expression of I-E molecules in NOD mice. Nature 328:432-434.

24. Quartey-Papafio, R., T. Lund, P. Chandler, J. Picard, P. Ozegbe, S. Day, P.R. Hutchings, L. O'Reilly, D. Kioussis, E. Simpson, and et al. 1995. Aspartate at position 57 of nonobese diabetic I-Ag7 beta-chain diminishes the spontaneous incidence of insulin-dependent diabetes mellitus. J Immunol 154:5567-5575.

25. Carrasco-Marin, E., J. Shimizu, O. Kanagawa, and E.R. Unanue. 1996. The class II MHC I-Ag7 molecules from non-obese diabetic mice are poor peptide binders. J Immunol 156:450-458.

26. Latek, R.R., A. Suri, S.J. Petzold, C.A. Nelson, O. Kanagawa, E.R. Unanue, and D.H. Fremont. 2000. Structural basis of peptide binding and presentation by the type I diabetes-associated MHC class II molecule of NOD mice. Immunity 12:699-710.

27. James, E.A., and W.W. Kwok. 2008. Low-affinity major histocompatibility complex-binding peptides in type 1 diabetes. Diabetes 57:1788-1789.

28. Lord, C.J., S.K. Bohlander, E.A. Hopes, C.T. Montague, N.J. Hill, J.B. Prins, R.J. Renjilian, L.B. Peterson, L.S. Wicker, J.A. Todd, and et al. 1995. Mapping the diabetes polygene Idd3 on mouse chromosome 3 by use of novel congenic strains. Mamm Genome 6:563-570.

29. Rainbow, D.B., L. Esposito, S.K. Howlett, K.M. Hunter, J.A. Todd, L.B. Peterson, and L.S. Wicker. 2008. Commonality in the genetic control of Type 1 diabetes in humans and NOD mice: variants of genes in the IL-2 pathway are associated with autoimmune diabetes in both species. Biochem Soc Trans 36:312-315.

30. Dendrou, C.A., and L.S. Wicker. 2008. The IL-2/CD25 Pathway Determines Susceptibility to T1D in Humans and NOD Mice. J Clin Immunol.

31. King, C., A. Ilic, K. Koelsch, and N. Sarvetnick. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 117:265-277.

32. Encinas, J.A., L.S. Wicker, L.B. Peterson, A. Mukasa, C. Teuscher, R. Sobel, H.L. Weiner, C.E. Seidman, J.G. Seidman, and V.K. Kuchroo. 1999. QTL influencing autoimmune diabetes and encephalomyelitis map to a 0.15-cM region containing Il2. Nat Genet 21:158-160.

33. Podolin, P.L., M.B. Wilusz, R.M. Cubbon, U. Pajvani, C.J. Lord, J.A. Todd, L.B. Peterson, L.S. Wicker, and P.A. Lyons. 2000. Differential glycosylation of interleukin 2, the molecular basis for the NOD Idd3 type 1 diabetes gene? Cytokine 12:477-482.

34. Denny, P., C.J. Lord, N.J. Hill, J.V. Goy, E.R. Levy, P.L. Podolin, L.B. Peterson, L.S. Wicker, J.A. Todd, and P.A. Lyons. 1997. Mapping of the IDDM locus Idd3 to a 0.35-cM interval containing the interleukin-2 gene. Diabetes 46:695-700.

35. Lord, C.J., S. Howlett, P.A. Lyons, L.B. Peterson, L.S. Wicker, and J.A. Todd. 2001. The murine type 1 diabetes loci, Idd1, Idd3, Idd5, Idd9, and Idd17/10/18, do not control thymic CD4-CD8-/TCRalphabeta+ deficiency in the nonobese diabetic mouse. Mamm Genome 12:175-176.

36. Ikegami, H., T. Fujisawa, S. Makino, and T. Ogihara. 2002. Genetic dissection of type 1 diabetes susceptibility gene, Idd3, by ancestral haplotype congenic mapping. Ann N Y Acad Sci 958:325-328.

37. Stauber, D.J., E.W. Debler, P.A. Horton, K.A. Smith, and I.A. Wilson. 2006. Crystal structure of the IL-2 signaling complex: paradigm for a heterotrimeric cytokine receptor. Proc Natl Acad Sci U S A 103:2788-2793.

38. Wang, X., M. Rickert, and K.C. Garcia. 2005. Structure of the quaternary complex of interleukin-2 with its alpha, beta, and gammac receptors. Science 310:1159-1163.

39. Lyons, P.A., N. Armitage, F. Argentina, P. Denny, N.J. Hill, C.J. Lord, M.B. Wilusz, L.B. Peterson, L.S. Wicker, and J.A. Todd. 2000. Congenic mapping of the type 1 diabetes locus, Idd3, to a 780-kb region of mouse chromosome 3: identification of a candidate segment of ancestral DNA by haplotype mapping. Genome Res 10:446-453.

40. del Rio, R., R. Noubade, M. Subramanian, N. Saligrama, S. Diehl, M. Rincon, and C. Teuscher. 2008. SNPs upstream of the minimal promoter control IL-2 expression and are candidates for the autoimmune disease-susceptibility locus Aod2/Idd3/Eae3. Genes Immun 9:115-121.

41. Yamanouchi, J., D. Rainbow, P. Serra, S. Howlett, K. Hunter, V.E. Garner, A. Gonzalez-Munoz, J. Clark, R. Veijola, R. Cubbon, S.L. Chen, R. Rosa, A.M. Cumiskey, D.V. Serreze, S. Gregory, J. Rogers, P.A. Lyons, B. Healy, L.J. Smink, J.A. Todd, L.B. Peterson, L.S. Wicker, and P. Santamaria. 2007. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. Nat Genet 39:329-337.

42. Malek, T.R. 2008. The biology of interleukin-2. Annu Rev Immunol 26:453-479.

43. Malek, T.R., A. Yu, P. Scibelli, M.G. Lichtenheld, and E.K. Codias. 2001. Broad programming by IL-2 receptor signaling for extended growth to multiple cytokines and functional maturation of antigen-activated T cells. J Immunol 166:1675-1683.

44. Lenardo, M.J. 1996. Fas and the art of lymphocyte maintenance. J Exp Med 183:721-724.

45. Schoenberger, S.P., and E.M. Janssen. 2006. IL-2 gets with the program. Nat Immunol 7:798-800.

46. Schimpl, A., I. Berberich, B. Kneitz, S. Kramer, B. Santner-Nanan, S. Wagner, M. Wolf, and T. Hunig. 2002. IL-2 and autoimmune disease. Cytokine Growth Factor Rev 13:369-378.

47. Schorle, H., T. Holtschke, T. Hunig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. Nature 352:621-624.

48. Sadlack, B., H. Merz, H. Schorle, A. Schimpl, A.C. Feller, and I. Horak. 1993. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell 75:253-261.

49. Waithman, J., T. Gebhardt, G.M. Davey, W.R. Heath, and F.R. Carbone. 2008. Cutting edge: Enhanced IL-2 signaling can convert self-specific T cell response from tolerance to autoimmunity. J Immunol 180:5789-5793.

50. Teuscher, C., B.B. Wardell, J.K. Lunceford, S.D. Michael, and K.S. Tung. 1996. Aod2, the locus controlling development of atrophy in neonatal thymectomy-induced autoimmune ovarian dysgenesis, co-localizes with Il2, Fgfb, and Idd3. J Exp Med 183:631-637.

51. Boulard, O., G. Fluteau, L. Eloy, D. Damotte, P. Bedossa, and H.J. Garchon. 2002. Genetic analysis of autoimmune sialadenitis in nonobese diabetic mice: a major susceptibility region on chromosome 1. J Immunol 168:4192-4201.

52. van Heel, D.A., L. Franke, K.A. Hunt, R. Gwilliam, A. Zhernakova, M. Inouye, M.C. Wapenaar, M.C. Barnardo, G. Bethel, G.K. Holmes, C. Feighery, D. Jewell, D. Kelleher, P. Kumar, S. Travis, J.R. Walters, D.S. Sanders, P. Howdle, J. Swift, R.J. Playford, W.M. McLaren, M.L. Mearin, C.J. Mulder, R. McManus, R. McGinnis, L.R. Cardon, P. Deloukas, and C. Wijmenga. 2007. A genome-wide association study for celiac disease identifies risk variants in the region harboring IL2 and IL21. Nat Genet 39:827-829.

53. Leonard, W.J., and R. Spolski. 2005. Interleukin-21: a modulator of lymphoid proliferation, apoptosis and differentiation. Nat Rev Immunol 5:688-698.

54. Korn, T., E. Bettelli, W. Gao, A. Awasthi, A. Jager, T.B. Strom, M. Oukka, and V.K. Kuchroo. 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature 448:484-487.

55. Sonderegger, I., J. Kisielow, R. Meier, C. King, and M. Kopf. 2008. IL-21 and IL-21R are not required for development of Th17 cells and autoimmunity in vivo. Eur J Immunol 38:1833-1838.

56. King, C. 2008. T helper cell differentiation: IL-21 and T helper cell differentiation: Jack of all trades? Immunol Cell Biol 86:554-556.

57. Suto, A., D. Kashiwakuma, S. Kagami, K. Hirose, N. Watanabe, K. Yokote, Y. Saito, T. Nakayama, M.J. Grusby, I. Iwamoto, and H. Nakajima. 2008. Development and characterization of IL-21-producing CD4+ T cells. J Exp Med 205:1369-1379.

58. Allard, E.L., M.P. Hardy, J. Leignadier, M. Marquis, J. Rooney, D. Lehoux, and N. Labrecque. 2007. Overexpression of IL-21 promotes massive CD8+ memory T cell accumulation. Eur J Immunol 37:3069-3077.

59. Casey, K.A., and M.F. Mescher. 2007. IL-21 promotes differentiation of naive CD8 T cells to a unique effector phenotype. J Immunol 178:7640-7648.

60. Zeng, R., R. Spolski, S.E. Finkelstein, S. Oh, P.E. Kovanen, C.S. Hinrichs, C.A. Pise-Masison, M.F. Radonovich, J.N. Brady, N.P. Restifo, J.A. Berzofsky, and W.J. Leonard. 2005. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. J Exp Med 201:139-148.

61. Spolski, R., M. Kashyap, C. Robinson, Z. Yu, and W.J. Leonard. 2008. IL-21 signaling is critical for the development of type I diabetes in the NOD mouse. Proc Natl Acad Sci U S A 105:14028-14033.

62. Li, Y., and C. Yee. 2008. IL-21 mediated Foxp3 suppression leads to enhanced generation of antigen-specific CD8+ cytotoxic T lymphocytes. Blood 111:229-235.

63. Nossal, G.J. 1994. Negative selection of lymphocytes. Cell 76:229-239.

64. Page, D.M. 1999. Cutting edge: thymic selection and autoreactivity are regulated by multiple coreceptors involved in T cell activation. J Immunol 163:3577-3581.

65. Anderson, G., K.M. Partington, and E.J. Jenkinson. 1998. Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. J Immunol 161:6599-6603.

66. Kyewski, B., J. Derbinski, J. Gotter, and L. Klein. 2002. Promiscuous gene expression and central T-cell tolerance: more than meets the eye. Trends Immunol 23:364-371.

67. Anderson, M.S., E.S. Venanzi, Z. Chen, S.P. Berzins, C. Benoist, and D. Mathis. 2005. The cellular mechanism of Aire control of T cell tolerance. Immunity 23:227-239.

68. Thomas-Vaslin, V., D. Damotte, M. Coltey, N.M. Le Douarin, A. Coutinho, and J. Salaun. 1997. Abnormal T cell selection on nod thymic epithelium is sufficient to induce autoimmune manifestations in C57BL/6 athymic nude mice. Proc Natl Acad Sci U S A 94:4598-4603.

69. Kishimoto, H., and J. Sprent. 2001. A defect in central tolerance in NOD mice. Nat Immunol 2:1025-1031.

70. Harfouch-Hammoud, E., T. Walk, H. Otto, G. Jung, J.F. Bach, P.M. van Endert, and S. Caillat-Zucman. 1999. Identification of peptides from autoantigens GAD65 and

IA-2 that bind to HLA class II molecules predisposing to or protecting from type 1 diabetes. Diabetes 48:1937-1947.

71. You, S., and L. Chatenoud. 2006. Proinsulin: a unique autoantigen triggering autoimmune diabetes. J Clin Invest 116:3108-3110.

72. Pedersen, A.E., M. Gad, M.R. Walter, and M.H. Claesson. 2004. Induction of regulatory dendritic cells by dexamethasone and 1alpha,25-Dihydroxyvitamin D(3). Immunol Lett 91:63-69.

73. French, M.B., J. Allison, D.S. Cram, H.E. Thomas, M. Dempsey-Collier, A. Silva, H.M. Georgiou, T.W. Kay, L.C. Harrison, and A.M. Lew. 1997. Transgenic expression of mouse proinsulin II prevents diabetes in nonobese diabetic mice. Diabetes 46:34-39.

74. Eisenbarth, G.S., H. Moriyama, D.T. Robles, E. Liu, L. Yu, S. Babu, M.J. Redondo, P. Gottlieb, D. Wegmann, and M. Rewers. 2002. Insulin autoimmunity: prediction/precipitation/prevention type 1A diabetes. Autoimmun Rev 1:139-145.

75. Wong, F.S., J. Karttunen, C. Dumont, L. Wen, I. Visintin, I.M. Pilip, N. Shastri, E.G. Pamer, and C.A. Janeway, Jr. 1999. Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library. Nat Med 5:1026-1031.

76. Kent, S.C., Y. Chen, L. Bregoli, S.M. Clemmings, N.S. Kenyon, C. Ricordi, B.J. Hering, and D.A. Hafler. 2005. Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope. Nature 435:224-228.

77. Krishnamurthy, B., N.L. Dudek, M.D. McKenzie, A.W. Purcell, A.G. Brooks, S. Gellert, P.G. Colman, L.C. Harrison, A.M. Lew, H.E. Thomas, and T.W. Kay. 2006. Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP. J Clin Invest 116:3258-3265.

78. Nakayama, M., N. Abiru, H. Moriyama, N. Babaya, E. Liu, D. Miao, L. Yu, D.R. Wegmann, J.C. Hutton, J.F. Elliott, and G.S. Eisenbarth. 2005. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 435:220-223.

79. Jaeckel, E., L. Klein, N. Martin-Orozco, and H. von Boehmer. 2003. Normal incidence of diabetes in NOD mice tolerant to glutamic acid decarboxylase. J Exp Med 197:1635-1644.

80. Jaeckel, E., M.A. Lipes, and H. von Boehmer. 2004. Recessive tolerance to preproinsulin 2 reduces but does not abolish type 1 diabetes. Nat Immunol 5:1028-1035.

81. Martin, D.L., and K. Rimvall. 1993. Regulation of gamma-aminobutyric acid synthesis in the brain. J Neurochem 60:395-407.

82. Erlander, M.G., N.J. Tillakaratne, S. Feldblum, N. Patel, and A.J. Tobin. 1991. Two genes encode distinct glutamate decarboxylases. Neuron 7:91-100.

83. Kim, J., W. Richter, H.J. Aanstoot, Y. Shi, Q. Fu, R. Rajotte, G. Warnock, and S. Baekkeskov. 1993. Differential expression of GAD65 and GAD67 in human, rat, and mouse pancreatic islets. Diabetes 42:1799-1808.

84. Faulkner-Jones, B.E., D.S. Cram, J. Kun, and L.C. Harrison. 1993. Localization and quantitation of expression of two glutamate decarboxylase genes in pancreatic beta-cells and other peripheral tissues of mouse and rat. Endocrinology 133:2962-2972.

85. Tisch, R., B. Wang, D.J. Weaver, B. Liu, T. Bui, J. Arthos, and D.V. Serreze. 2001. Antigen-specific mediated suppression of beta cell autoimmunity by plasmid DNA vaccination. J Immunol 166:2122-2132.

86. Barker, J.M., K.J. Barriga, L. Yu, D. Miao, H.A. Erlich, J.M. Norris, G.S. Eisenbarth, and M. Rewers. 2004. Prediction of autoantibody positivity and progression to type 1 diabetes: Diabetes Autoimmunity Study in the Young (DAISY). J Clin Endocrinol Metab 89:3896-3902.

87. Tuomilehto, J., P. Zimmet, I.R. Mackay, P. Koskela, G. Vidgren, L. Toivanen, E. Tuomilehto-Wolf, K. Kohtamaki, J. Stengard, and M.J. Rowley. 1994. Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease. Lancet 343:1383-1385.

88. Wicker, L.S., B.J. Miller, and Y. Mullen. 1986. Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice. Diabetes 35:855-860.

89. Bach, J.F. 1994. Insulin-dependent diabetes mellitus as an autoimmune disease. Endocr Rev 15:516-542.

90. Kaufman, D.L., M. Clare-Salzler, J. Tian, T. Forsthuber, G.S. Ting, P. Robinson, M.A. Atkinson, E.E. Sercarz, A.J. Tobin, and P.V. Lehmann. 1993. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature 366:69-72.

91. Baekkeskov, S., H.J. Aanstoot, S. Christgau, A. Reetz, M. Solimena, M. Cascalho, F. Folli, H. Richter-Olesen, and P. De Camilli. 1990. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347:151-156.

92. Baekkeskov, S., J.H. Nielsen, B. Marner, T. Bilde, J. Ludvigsson, and A. Lernmark. 1982. Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins. Nature 298:167-169.

93. Hayward, A.R., and M. Shreiber. 1989. Neonatal injection of CD3 antibody into nonobese diabetic mice reduces the incidence of insulitis and diabetes. J Immunol 143:1555-1559.

94. Chatenoud, L., E. Thervet, J. Primo, and J.F. Bach. 1994. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci U S A 91:123-127.

95. Chatenoud, L., E. Thervet, J. Primo, and J.F. Bach. 1992. [Remission of established disease in diabetic NOD mice induced by anti-CD3 monoclonal antibody]. C R Acad Sci III 315:225-228.

96. Hayward, A.R., and M. Shriber. 1992. Reduced incidence of insulitis in NOD mice following anti-CD3 injection: requirement for neonatal injection. J Autoimmun 5:59-67.

97. Pankewycz, O., T.B. Strom, and V.E. Rubin-Kelley. 1991. Islet-infiltrating T cell clones from non-obese diabetic mice that promote or prevent accelerated onset diabetes. Eur J Immunol 21:873-879.

98. Ohashi, P.S., S. Oehen, K. Buerki, H. Pircher, C.T. Ohashi, B. Odermatt, B. Malissen, R.M. Zinkernagel, and H. Hengartner. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell 65:305-317.

99. Oldstone, M.B., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell 65:319-331.

100. Katz, J.D., B. Wang, K. Haskins, C. Benoist, and D. Mathis. 1993. Following a diabetogenic T cell from genesis through pathogenesis. Cell 74:1089-1100.

101. Mayer, K.D., K. Mohrs, S.R. Crowe, L.L. Johnson, P. Rhyne, D.L. Woodland, and M. Mohrs. 2005. The functional heterogeneity of type 1 effector T cells in response to infection is related to the potential for IFN-gamma production. J Immunol 174:7732-7739.

102. Erb, P., M. Troxler, M. Fluri, D. Grogg, and S.S. Alkan. 1991. Functional heterogeneity of CD4-positive T-cell subsets: the correlation between effector functions and lymphokine secretion is limited. Cell Immunol 135:232-244.

103. Rabinovitch, A. 1994. Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation? Diabetes 43:613-621.

104. Kroemer, G., F. Hirsch, A. Gonzalez-Garcia, and C. Martinez. 1996. Differential involvement of Th1 and Th2 cytokines in autoimmune diseases. Autoimmunity 24:25-33.

105. Healey, D., P. Ozegbe, S. Arden, P. Chandler, J. Hutton, and A. Cooke. 1995. In vivo activity and in vitro specificity of CD4+ Th1 and Th2 cells derived from the spleens of diabetic NOD mice. J Clin Invest 95:2979-2985.

106. Katz, J.D., C. Benoist, and D. Mathis. 1995. T helper cell subsets in insulin-dependent diabetes. Science 268:1185-1188.

107. Overbergh, L., D. Valckx, M. Waer, and C. Mathieu. 1999. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. Cytokine 11:305-312.

108. Wilson, S.B., S.C. Kent, K.T. Patton, T. Orban, R.A. Jackson, M. Exley, S. Porcelli, D.A. Schatz, M.A. Atkinson, S.P. Balk, J.L. Strominger, and D.A. Hafler. 1998. Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. Nature 391:177-181.

109. Haskins, K., and D. Wegmann. 1996. Diabetogenic T-cell clones. Diabetes 45:1299-1305.

110. Coffman, R.L., and S.L. Reiner. 1999. Instruction, selection, or tampering with the odds? Science 284:1283, 1285.

111. Abbas, A.K., K.M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. Nature 383:787-793.

112. Stevens, T.L., A. Bossie, V.M. Sanders, R. Fernandez-Botran, R.L. Coffman, T.R. Mosmann, and E.S. Vitetta. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature 334:255-258.

113. Kopf, M., G. Le Gros, M. Bachmann, M.C. Lamers, H. Bluethmann, and G. Kohler. 1993. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature 362:245-248.

114. Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136:2348-2357.

115. Ramsdell, F. 2003. Foxp3 and natural regulatory T cells: key to a cell lineage? Immunity 19:165-168.

116. Chen, Y., V.K. Kuchroo, J. Inobe, D.A. Hafler, and H.L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. Science 265:1237-1240.

117. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J.E. de Vries, and M.G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 389:737-742.

118. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057-1061.

119. Chang, C.C., R. Ciubotariu, J.S. Manavalan, J. Yuan, A.I. Colovai, F. Piazza, S. Lederman, M. Colonna, R. Cortesini, R. Dalla-Favera, and N. Suciu-Foca. 2002. Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. Nat Immunol 3:237-243.

120. Hu, D., K. Ikizawa, L. Lu, M.E. Sanchirico, M.L. Shinohara, and H. Cantor. 2004. Analysis of regulatory CD8 T cells in Qa-1-deficient mice. Nat Immunol 5:516-523.

121. Weaver, C.T., L.E. Harrington, P.R. Mangan, M. Gavrieli, and K.M. Murphy. 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity 24:677-688.

122. Bluestone, J.A., and H. Boehmer. 2006. Regulatory T cells. Semin Immunol 18:77.

123. Bacchetta, R., C. Sartirana, M.K. Levings, C. Bordignon, S. Narula, and M.G. Roncarolo. 2002. Growth and expansion of human T regulatory type 1 cells are independent from TCR activation but require exogenous cytokines. Eur J Immunol 32:2237-2245.

124. Conti, P., D. Kempuraj, S. Frydas, K. Kandere, W. Boucher, R. Letourneau, B. Madhappan, K. Sagimoto, S. Christodoulou, and T.C. Theoharides. 2003. IL-10 subfamily members: IL-19, IL-20, IL-22, IL-24 and IL-26. Immunol Lett 88:171-174.

125. O'Garra, A., P.L. Vieira, P. Vieira, and A.E. Goldfeld. 2004. IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage. J Clin Invest 114:1372-1378.

126. Roncarolo, M.G., R. Bacchetta, C. Bordignon, S. Narula, and M.K. Levings. 2001. Type 1 T regulatory cells. Immunol Rev 182:68-79.

127. Goudy, K., S. Song, C. Wasserfall, Y.C. Zhang, M. Kapturczak, A. Muir, M. Powers, M. Scott-Jorgensen, M. Campbell-Thompson, J.M. Crawford, T.M. Ellis, T.R. Flotte, and M.A. Atkinson. 2001. Adeno-associated virus vector-mediated IL-10 gene delivery prevents type 1 diabetes in NOD mice. Proc Natl Acad Sci U S A 98:13913-13918.

128. Hill, J.A., M. Feuerer, K. Tash, S. Haxhinasto, J. Perez, R. Melamed, D. Mathis, and C. Benoist. 2007. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. Immunity 27:786-800.

129. Green, E.A., Y. Choi, and R.A. Flavell. 2002. Pancreatic lymph node-derived CD4(+)CD25(+) Treg cells: highly potent regulators of diabetes that require TRANCE-RANK signals. Immunity 16:183-191.

130. Pop, S.M., C.P. Wong, D.A. Culton, S.H. Clarke, and R. Tisch. 2005. Single cell analysis shows decreasing FoxP3 and TGFbeta1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes. J Exp Med 201:1333-1346.

131. Brusko, T.M., C.H. Wasserfall, M.J. Clare-Salzler, D.A. Schatz, and M.A. Atkinson. 2005. Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes. Diabetes 54:1407-1414.

132. Gregori, S., N. Giarratana, S. Smiroldo, and L. Adorini. 2003. Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development. J Immunol 171:4040-4047.

133. You, S., B. Leforban, C. Garcia, J.F. Bach, J.A. Bluestone, and L. Chatenoud. 2007. Adaptive TGF-beta-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment. Proc Natl Acad Sci U S A 104:6335-6340.

134. Bluestone, J.A., and A.K. Abbas. 2003. Natural versus adaptive regulatory T cells. Nat Rev Immunol 3:253-257.

135. Cassis, L., S. Aiello, and M. Noris. 2005. Natural versus adaptive regulatory T cells. Contrib Nephrol 146:121-131.

136. Eggena, M.P., L.S. Walker, V. Nagabhushanam, L. Barron, A. Chodos, and A.K. Abbas. 2004. Cooperative roles of CTLA-4 and regulatory T cells in tolerance to an islet cell antigen. J Exp Med 199:1725-1730.

137. Shoda, L.K., D.L. Young, S. Ramanujan, C.C. Whiting, M.A. Atkinson, J.A. Bluestone, G.S. Eisenbarth, D. Mathis, A.A. Rossini, S.E. Campbell, R. Kahn, and H.T. Kreuwel. 2005. A comprehensive review of interventions in the NOD mouse and implications for translation. Immunity 23:115-126.

138. Cameron, M.J., G.A. Arreaza, P. Zucker, S.W. Chensue, R.M. Strieter, S. Chakrabarti, and T.L. Delovitch. 1997. IL-4 prevents insulitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function. J Immunol 159:4686-4692.

139. Nitta, Y., F. Tashiro, M. Tokui, A. Shimada, I. Takei, K. Tabayashi, and J. Miyazaki. 1998. Systemic delivery of interleukin 10 by intramuscular injection of expression plasmid DNA prevents autoimmune diabetes in nonobese diabetic mice. Hum Gene Ther 9:1701-1707.

140. Piccirillo, C.A., Y. Chang, and G.J. Prud'homme. 1998. TGF-beta1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice. J Immunol 161:3950-3956.

141. Zaccone, P., J. Phillips, I. Conget, R. Gomis, K. Haskins, A. Minty, K. Bendtzen, A. Cooke, and F. Nicoletti. 1999. Interleukin-13 prevents autoimmune diabetes in NOD mice. Diabetes 48:1522-1528.

142. Goudy, K.S., B.R. Burkhardt, C. Wasserfall, S. Song, M.L. Campbell-Thompson, T. Brusko, M.A. Powers, M.J. Clare-Salzler, E.S. Sobel, T.M. Ellis, T.R. Flotte, and M.A. Atkinson. 2003. Systemic overexpression of IL-10 induces CD4+CD25+ cell populations in vivo and ameliorates type 1 diabetes in nonobese diabetic mice in a dose-dependent fashion. J Immunol 171:2270-2278.

143. Song, S., K. Goudy, M. Campbell-Thompson, C. Wasserfall, M. Scott-Jorgensen, J. Wang, Q. Tang, J.M. Crawford, T.M. Ellis, M.A. Atkinson, and T.R. Flotte. 2004. Recombinant adeno-associated virus-mediated alpha-1 antitrypsin gene therapy prevents type I diabetes in NOD mice. Gene Ther 11:181-186.

144. Dhodapkar, M.V., R.M. Steinman, J. Krasovsky, C. Munz, and N. Bhardwaj. 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. J Exp Med 193:233-238.

145. Kared, H., A. Masson, H. Adle-Biassette, J.F. Bach, L. Chatenoud, and F. Zavala. 2005. Treatment with granulocyte colony-stimulating factor prevents diabetes in NOD mice by recruiting plasmacytoid dendritic cells and functional CD4(+)CD25(+) regulatory T-cells. Diabetes 54:78-84.

146. Kared, H., B. Leforban, R. Montandon, A. Renand, E. Layseca Espinosa, L. Chatenoud, Y. Rosenstein, E. Schneider, M. Dy, and F. Zavala. 2008. Role of GM-CSF in tolerance induction by mobilized hematopoietic progenitors. Blood 112:2575-2578.

147. Lee, J.I., and G.J. Burckart. 1998. Nuclear factor kappa B: important transcription factor and therapeutic target. J Clin Pharmacol 38:981-993.

148. Abdi, R., P. Fiorina, C.N. Adra, M. Atkinson, and M.H. Sayegh. 2008. Immunomodulation by mesenchymal stem cells: a potential therapeutic strategy for type 1 diabetes. Diabetes 57:1759-1767.

149. Wang, Y., L. Hao, R.G. Gill, and K.J. Lafferty. 1987. Autoimmune diabetes in NOD mouse is L3T4 T-lymphocyte dependent. Diabetes 36:535-538.

150. Shizuru, J.A., C. Taylor-Edwards, B.A. Banks, A.K. Gregory, and C.G. Fathman. 1988. Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T-helper lymphocytes. Science 240:659-662.

151. Kuttler, B., K. Rosing, M. Lehmann, J. Brock, and H.J. Hahn. 1999. Prevention of autoimmune but not allogeneic destruction of grafted islets by different therapeutic strategies. J Mol Med 77:226-229.

152. Balasa, B., T. Krahl, G. Patstone, J. Lee, R. Tisch, H.O. McDevitt, and N. Sarvetnick. 1997. CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice. J Immunol 159:4620-4627.

153. Lenschow, D.J., S.C. Ho, H. Sattar, L. Rhee, G. Gray, N. Nabavi, K.C. Herold, and J.A. Bluestone. 1995. Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. J Exp Med 181:1145-1155.

154. Liblau, R.S., C.I. Pearson, K. Shokat, R. Tisch, X.D. Yang, and H.O. McDevitt. 1994. High-dose soluble antigen: peripheral T-cell proliferation or apoptosis. Immunol Rev 142:193-208.

155. Liblau, R.S., R. Tisch, K. Shokat, X. Yang, N. Dumont, C.C. Goodnow, and H.O. McDevitt. 1996. Intravenous injection of soluble antigen induces thymic and peripheral T-cells apoptosis. Proc Natl Acad Sci U S A 93:3031-3036.

156. Tian, J., S. Gregori, L. Adorini, and D.L. Kaufman. 2001. The frequency of high avidity T cells determines the hierarchy of determinant spreading. J Immunol 166:7144-7150.

157. Sohnlein, P., M. Muller, K. Syren, U. Hartmann, B.O. Bohm, H.M. Meinck, M. Knip, H.K. Akerblom, and W. Richter. 2000. Epitope spreading and a varying but not disease-specific GAD65 antibody response in Type I diabetes. The Childhood Diabetes in Finland Study Group. Diabetologia 43:210-217.

158. Tisch, R., R.S. Liblau, X.D. Yang, P. Liblau, and H.O. McDevitt. 1998. Induction of GAD65-specific regulatory T-cells inhibits ongoing autoimmune diabetes in nonobese diabetic mice. Diabetes 47:894-899.

159. Tisch, R., B. Wang, and D.V. Serreze. 1999. Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent. J Immunol 163:1178-1187.

160. Tisch, R., X.D. Yang, S.M. Singer, R.S. Liblau, L. Fugger, and H.O. McDevitt. 1993. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature 366:72-75.

161. Elliott, J.F., H.Y. Qin, S. Bhatti, D.K. Smith, R.K. Singh, T. Dillon, J. Lauzon, and B. Singh. 1994. Immunization with the larger isoform of mouse glutamic acid

decarboxylase (GAD67) prevents autoimmune diabetes in NOD mice. Diabetes 43:1494-1499.

162. Daniel, D., and D.R. Wegmann. 1996. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23). Proc Natl Acad Sci U S A 93:956-960.

163. Elias, D., A. Meilin, V. Ablamunits, O.S. Birk, P. Carmi, S. Konen-Waisman, and I.R. Cohen. 1997. Hsp60 peptide therapy of NOD mouse diabetes induces a Th2 cytokine burst and downregulates autoimmunity to various beta-cell antigens. Diabetes 46:758-764.

164. Cohen, I.R. 1997. The Th1/Th2 dichotomy, hsp60 autoimmunity, and type I diabetes. Clin Immunol Immunopathol 84:103-106.

165. Tian, J., M. Clare-Salzler, A. Herschenfeld, B. Middleton, D. Newman, R. Mueller, S. Arita, C. Evans, M.A. Atkinson, Y. Mullen, N. Sarvetnick, A.J. Tobin, P.V. Lehmann, and D.L. Kaufman. 1996. Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. Nat Med 2:1348-1353.

166. Qin, H.Y., M.W. Sadelain, C. Hitchon, J. Lauzon, and B. Singh. 1993. Complete Freund's adjuvant-induced T cells prevent the development and adoptive transfer of diabetes in nonobese diabetic mice. J Immunol 150:2072-2080.

167. Sadelain, M.W., H.Y. Qin, J. Lauzon, and B. Singh. 1990. Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. Diabetes 39:583-589.

168. Weaver, D.J., Jr., B. Liu, and R. Tisch. 2001. Plasmid DNAs encoding insulin and glutamic acid decarboxylase 65 have distinct effects on the progression of autoimmune diabetes in nonobese diabetic mice. J Immunol 167:586-592.

169. Pop, S.M., C.P. Wong, Q. He, Y. Wang, M.A. Wallet, K.S. Goudy, and R. Tisch. 2007. The type and frequency of immunoregulatory CD4+ T-cells govern the efficacy of antigen-specific immunotherapy in nonobese diabetic mice. Diabetes 56:1395-1402.

170. Harrison, L.C., and D.A. Hafler. 2000. Antigen-specific therapy for autoimmune disease. Curr Opin Immunol 12:704-711.

171. Gong, Z., Y. Jin, and Y. Zhang. 2007. Suppression of diabetes in non-obese diabetic (NOD) mice by oral administration of a cholera toxin B subunit-insulin B chain fusion protein vaccine produced in silkworm. Vaccine 25:1444-1451.

172. Ma, S., Y. Huang, Z. Yin, R. Menassa, J.E. Brandle, and A.M. Jevnikar. 2004. Induction of oral tolerance to prevent diabetes with transgenic plants requires glutamic acid decarboxylase (GAD) and IL-4. Proc Natl Acad Sci U S A 101:5680-5685.

173. Ma, S.W., D.L. Zhao, Z.Q. Yin, R. Mukherjee, B. Singh, H.Y. Qin, C.R. Stiller, and A.M. Jevnikar. 1997. Transgenic plants expressing autoantigens fed to mice to induce oral immune tolerance. Nat Med 3:793-796.

174. 2002. Effects of insulin in relatives of patients with type 1 diabetes mellitus. N Engl J Med 346:1685-1691.

175. Skyler, J.S., J.P. Krischer, J. Wolfsdorf, C. Cowie, J.P. Palmer, C. Greenbaum, D. Cuthbertson, L.E. Rafkin-Mervis, H.P. Chase, and E. Leschek. 2005. Effects of oral insulin in relatives of patients with type 1 diabetes: The Diabetes Prevention Trial--Type 1. Diabetes Care 28:1068-1076.

176. Seifarth, C., S. Pop, B. Liu, C.P. Wong, and R. Tisch. 2003. More stringent conditions of plasmid DNA vaccination are required to protect grafted versus endogenous islets in nonobese diabetic mice. J Immunol 171:469-476.

CHAPTER 2

GENE GUN-MEDIATED DNA VACCINATOIN ENHANCES ANTIGEN-SPECIFIC IMMUNOTHERAPY AT A LATE PRECLINICAL STAGE OF TYPE 1 DIABETES IN NONOBESE DIAEBTIC MICE

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2.1 ABSTRACT

Type 1 diabetes (T1D) is characterized by the T cell mediated destruction of the insulin producing β cells. Antigen-specific immunotherapies are used to selectively tolerize β cell-specific pathogenic T cells either directly, or indirectly through the induction of immunoregulatory T cells. A key concern of antigen-specific immunotherapy is exacerbating autoimmunity. We compared the T cell reactivity and efficacy induced by plasmid DNA (pDNA) encoding glutamic acid decarboxylase 65 (GAD65) administered via intramuscular versus gene gun vaccination in NOD mice at a late preclinical stage of T1D. Whereas intramuscular injection of pGAD65 promoted a predominant type 1 $CD4^+$ T cell response and failed to suppress ongoing β cell autoimmunity, gene gun vaccination preferentially induced IL-4 secreting $CD4^+$ T cells and significantly delayed the onset of diabetes. These findings demonstrate that gene gun delivery of autoantigen-encoding pDNA preferentially elicits immunoregulatory T cells and offers a safe, effective strategy of pDNA vaccination for the treatment of T1D and other autoimmune diseases.

2.2 INTRODUCTION

Type 1 diabetes (T1D) is caused by the autoimmune-mediated destruction of the insulin-producing β cells of the islets of Langerhans . Based on studies in the nonobese diabetic (NOD) mouse, a spontaneous model of T1D, the primary effectors of β cell destruction are $CD4^+$ and $CD8^+$ T cells (1). Pathogenic effector T cells typically exhibit a type 1 phenotype, and target a number of β cell autoantigens including proinsulin, insulin, islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP), and glutamic acid decarboxylase 65 (GAD65) . The critical events associated with the development and expansion of these type 1 β cell-specific effector T cells remain ill-defined, although aberrant peripheral tolerance is believed to contribute. For example, insufficient numbers of IL-4 secreting type 2 T cells or IL-10 secreting Tr1 cells, and defective development and/or function of FoxP3-expressing immunoregulatory T cells ($FoxP3^+$Treg) have been reported in NOD mice and diabetic patients .

A variety of immunotherapies have been developed to reestablish the functional balance between pathogenic and immunoregulatory T cells and prevent and/or treat T1D. Antigen-specific immunotherapy is one such strategy to induce β cell-specific immunoregulatory T cells and selectively block the initiation and/or progression of β cell autoimmunity . The efficacy of the approach is largely dependent on the frequency of immunoregulatory T cells induced upon administration of a given β cell autoantigen versus induction and/or expansion of pathogenic T cells. The conditions to preferentially elicit immunoregulatory T cells in an antigen-specific manner become more stringent at

late preclinical or clinical stages of T1D when β cell autoimmunity is well established (3, 4).

We and others have successfully used plasmid DNA (pDNA)-based vaccines to induce β cell-specific immunoregulatory $CD4^+$ T cells, and in turn prevent and suppress β cell autoimmunity in NOD mice (5-14). In general, pDNA offer: i) a relatively facile approach for vaccination, overcoming the need to produce and store antigen, and ii) an opportunity to readily manipulate the nature of the induced immune response (15). Studies using NOD mice have delivered β cell autoantigen-encoding pDNA prepared in saline via intramuscular (i.m.) (9, 11-13) and intradermal (i.d.) needle injection (8), or via oral administration (8, 14). Typically, effective prevention of diabetes has been dependent on co-injection of pDNA encoding anti-inflammatory cytokines such as IL-4 and/or IL-10, especially once β cell autoimmunity is well established (9, 11-14). Indeed, i.m. injection of pDNA encoding antigen-only has had either no immunotherapeutic effect or worse, elicited β cell-specific type 1 $CD4^+$ T effectors (12, 13). The current study was initiated to determine how the mode of delivery affects the efficacy of pDNA to prevent diabetes at a late preclinical stage in NOD mice. Specifically, the nature of the T cell response and immunotherapeutic efficacy of pDNA encoding a fragment of murine GAD65 fused to human immunoglobulin Fc (pGAD65) administered by i.m. versus gene gun vaccination were compared. Gene gun vaccination entails bombardment of the upper most layers of the skin with pDNA-coated particles(15, 16). Notably, studies have shown that gene gun immunization preferentially induces type 2 T effectors (17, 18). With this in mind, we tested the hypothesis that gene gun vaccination of pGAD65-only is more

effective at eliciting GAD65-specific immunoregulatory T cells and suppressing ongoing β cell autoimmunity than i.m. injection of pGAD65 prepared in saline.

2.3 MATERIALS AND METHODS

Mice

NOD/LtJ, NOD.*il4* ^{tm1Cgn} (NOD.IL-4^{null}), and NOD.*scid* mice were purchased from Jackson Laboratory (Bar Harbor, MA) and bred under specific pathogen-free conditions. All experiments with mice were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

pDNA vaccination

pDNA encoding human immunoglobulin Fc (IgFc) fusion molecules consisting of a fragment of murine GAD65 (pGAD65) or hen egg lysozyme (pHEL) spanning nucleotides 656-1070 and 175-270, respectively, have previously been described [22]. pDNA was prepared from DH5α *Escherichia coli* using a Qiagen endotoxin-free kit (Qiagen, Valencia, CA). For i.m. injection, pDNA was resuspended at 1.0 mg/ml in PBS, and NOD mice received four i.m. injections of 50 ul (50 μg) of pDNA in each hind quadricep muscle over 28 days with an insulin syringe. Biolistic delivery of pDNA was carried out using a Helios gene gun (Bio-Rad, Hercules, CA) and 1.6 μM gold particles (Bio-Rad). Gold particles (0.5 mg) were coated with 1 μg of pDNA using 1 mM $CaCl_2$ and 0.05 mM spermidine (Sigma, St. Louis, MO). The efficiency of coating was assessed by eluting pDNA off the gold particles in Tris-EDTA buffer and then quantitating pDNA via agarose gel electrophoresis and spectrophotometry (Beckman Coulter, Fullerton, CA). NOD mice with shaven abdomens received four shots at 1-week intervals at a helium pulse of 425 pounds per square inch.

In other experiments pDNA encoding a luciferase gene (pLUC) driven by a CMV/CB promoter was administered via gene gun or i.m. injection as described above. *In vivo* bioluminescence was measured by injecting mice intraperitoneally (i.p.) with 5 mg of luciferin (Promega, Madison, WI) and using a two-photon Xenogen imaging system (Xenogen, Alameda, CA) while mice were under isofluorane anesthesia. The intensity of bioluminescence was measured at the site of injection as the number of photons per second per centimeter squared per steradian as determined by the Xenogen software and reperesented as relative optic intensity (ROI) units.

GAD65-IgFc ELISA

Serum levels of GAD65-IgFc were measured using a human IgG ELISA kit as per the manufacturer's recommendations (Bethyl Laboratories, Montgomery, TX). Briefly, the capture antibody was incubated for 45 minutes in PBS on a 96-well high-binding plate (Corning, Lowell, MA) at room temperature. The plate was blocked with 1% bovine serum albumin (BSA)/PBS, washed, and 25 ul of serum diluted 1:3 with 1% bovine serum albumin/PBS was added to each well in duplicate. Samples were incubated at room temperature for 2 hours followed by washing three times. Detection antibody was then added and incubated at room temperature for 1 hour. The plate was washed and developed using substrate A and substrate B (BD Pharmingen, San Francisco, CA); the reaction was stopped using 4N phosphoric acid (Sigma, St. Louis, MO). Optical density values were determined at 450 nm wavelength using an ELISA plate reader (Molecular Devices, Sunnyvale, CA.)

Assessment of diabetes and insulitis

NOD mice were monitored weekly for the development of glycosuria via Diastix (Ames, Elkhart, IN). Diabetes was diagnosed by 2 consecutive positive measurements over 48 hours. Insulitis was assessed by histology. Pancreata were prepared for histology by fixation in neutral buffered formalin followed by paraffin embedding. A minimum of five sections, 90 µm apart, were cut from each block, stained with hematoxylin and eosin (H&E), and viewed by light microscopy. A minimum of 30 islets were scored for each animal. The severity of insulitis was scored as either peri-insulitis (islets surrounded by an infiltrate) or intra-insulitis (< or >50% infiltration of the islets).

Flow cytometry

Antibodies specific for murine CD3 (2c11), CD4 (GK1.5), and CD25 (PC61) (eBiosience, San Diego, CA) were used to stain splenocytes, inguinal lymph nodes and pancreatic lymph nodes at 1:500 dilution. Staining with anti-FoxP3 (FJK-16s) and isotype control (IgG2a) antibody (eBioscience, San Diego, CA) was done according to manufacturer's recommendations at a 1:400 dilution. Acquisition of samples was performed on a 3-laser Cyan and analyzed using Summit software build 4.3 (DAKO, Ft. Collins, CO).

Peptides

Peptides were synthesized using standard F-moc chemistry on a Rainin Symphony (Rainin Instruments, Woburn, MA) at the peptide synthesis facility of University of North Carolina. The purity of the peptides was verified by reverse phase HPLC and mass spectroscopy.

ELISPOT

ELISPOT was carried out as previously described [19]. Briefly, ImmunoSpot M200 plates (Millipore, Billerica, MA) were coated overnight at 4°C with either 2 µg/ml anti-IL-5, -IL-10, or -IFNγ and 1 µg/ml anti-IL-4 (BD PharMingen, San Jose, CA) prepared in PBS, and then blocked with 1% BSA-PBS for a minimum of 1 hour at room temperature. Splenocytes suspensions prepared from individual mice were plated at $1x10^6$ cells/well. Inguinal and pancreatic lymph nodes of 5 mice from each treatment group were harvested, pooled and cultured overnight in the presence of 4 ng/ml of mIL-2 (Peprotech, Rocky Hill, NJ) then washed and plated at $2x10^5$ with an equal number of irradiated splenocytes. Peptide was added in duplicate to wells at a final concentration of 40 µg/ml. The plates were incubated for 48 hours at 37°C in 5.5% CO_2 and then washed. Biotinylated anti-IL-4, -IL-5, -IL-10 or -IFNγ (BD PharMingen, San Jose, CA) were added at 2 µg/ml, in 1% BSA-PBS and plates incubated overnight at 4°C. Plates were washed and incubated with streptavidin-HRP (BD PharMingen; 1/1000) for 2 hours at room temperature. This was followed by three washes with 0.025% Tween 20-PBS and three washes with PBS only. Development solution consisted of 0.8 ml of 3-amino-9-ethyl-carbazole (Sigma, St. Louis, MO) added to 24 ml of 0.1 M sodium acetate (pH 5.0), plus 0.12 ml of 3.0% hydrogen peroxide; 0.2 ml was added per well.

Adoptive transfer

$CD4^+$ T cells were isolated from splenocytes of gene gun treated NOD and NOD.IL-4^{null} female mice using a CD4 enrichment kit (BD Pharmingen, San Jose, CA), and resuspended at $20x10^6$/ml in PBS. In addition, splenocytes were harvested from diabetic

NOD female mice, red blood cells lysed and the cells resuspended in PBS at $20x10^6$/ml. NOD.*scid* male mice then received i.p. injections of $8x10^6$ diabetogenic splenocytes alone or in combination with $2x10^6$ $CD4^+$ T cells isolated from treated NOD or NOD.IL-4^{null} donor mice.

2.4 RESULTS

2.4.1 Gene gun or i.m. delivery of pGAD65 yield high systemic levels of transgene expression.

The type of immune response induced by pDNA vaccination is dependent on both the site and mode of delivery (10, 15, 16). The first set of experiments was aimed to determine the levels and localization of transgene expression of pDNA delivered to the skin via gene gun and the muscle via needle injection. Under the gene gun parameters employed, the 1.6 μm gold particles were delivered to the dermal layer of the skin (Fig. 1A). To initially evaluate the duration and level of transgene expression, pLUC which encodes luciferase was delivered once to the abdomen (1 μg) or quadriceps (50 μg) of NOD mice via gene gun or i.m. injection, respectively, and bioluminescence measured at the site of delivery over a 2 week period. The kinetics of bioluminescence after a single vaccination were similar between the 2 treatment groups; luciferase expression was readily detected after 24 hours and persisted up to 14 days post-treatment (Fig. 1B). However, despite a 50-fold difference in the amount of pLUC administered, up to an ~3-fold increase in the intensity of bioluminescence was detected in NOD mice receiving pLUC delivered via gene gun versus i.m. injection (Fig. 1B).

The serum level of GAD65-IgFc protein in NOD mice receiving pGAD65 via gene gun versus i.m. injection was compared. Here, pGAD65 was administered under treatment conditions used to prevent diabetes (see below). Namely, NOD mice received a total of 4 injections over 4 weeks of: i) 50 μg of pGAD65 delivered i.m. to each quadricep, or ii) gold-particles coated with a total of 1 μg of pGAD65 to the abdomen.

Note that the treatment protocol for i.m. injection of pGAD65 was established in a previous study [22].The concentration of GAD65-IgFc in serum was then measured over time by ELISA specific for human IgFc. Significant levels of GAD65-IgFc (e.g. 2-3 ng/ml) were detected 7 days after the initial treatment in both experimental groups (Fig. 1C). Notably, an ~3-fold increase in GAD65-IgFc was detected at later time points in NOD mice receiving pGAD65 via gene gun versus i.m. injection (Fig. 1C). Together these findings demonstrate that although robust and persistent transgene expression is induced by i.m. injected pDNA, gene gun delivered pDNA results in enhanced and more efficient transgene expression.

2.4.2 Gene gun but not i.m. delivered pGAD65 prevents diabetes in NOD mice with ongoing β cell autoimmunity.

The immunotherapeutic efficacy of gene gun versus i.m. vaccination of pGAD65 was assessed. Ten week-old NOD female mice were treated as above and diabetes monitored up to 36 weeks of age. At 10 weeks of age, NOD female mice are typically euglycemic yet β cell autoimmunity is well established. The majority of NOD female mice receiving control pHEL via gene gun (8/10) and i.m. injection (7/10) or left untreated (9/10) developed overt diabetes with no significant difference in the time of diabetes onset (Fig. 2A). Furthermore, all NOD female mice treated with pGAD65 via i.m. injection (10/10) developed diabetes (Fig. 2A). On the other hand, significant protection was detected in NOD female mice receiving gene gun delivered pGAD65 versus i.m. injected pGAD65 or the respective control groups ($P \leq 0.005$; Kaplan-Meier Log Rank Test). In addition, a significant reduction in the frequency of diabetes was detected in NOD female mice

receiving gene gun (5/10) versus i.m. (10/10) delivered pGAD65 (P=0.039; Chi Square) (Fig. 2A). Histological analysis of the nondiabetic 36 week-old NOD female mice demonstrated that the majority of islets were infiltrated in the pGAD65 gene gun delivered group, but that the frequency of "severe" intra-insulitis (e.g. >50% intra-insulitis) was reduced compared to pHEL gene gun vaccinated or untreated NOD mice (Fig. 2B). No correlation was observed between the serum levels of GAD65-IgFc measured over time and protection against diabetes in the NOD female mice treated with gene gun delivered pGAD65 (Fig. 2C,D). In summary, these results demonstrate that gene gun but not i.m. vaccination of pGAD65 prevents diabetes at a late preclinical stage of T1D, and that protection is independent of systemic levels of GAD65-IgFc.

2.4.3 Protection mediated by gene gun delivery of pGAD65 correlates with the induction of type 2 GAD65-specific T cells.

The magnitude and nature of GAD65-specific T cell reactivity elicited by gene gun versus i.m. injection of pGAD65 were compared. Groups of five 10-week old NOD female mice were vaccinated with pGAD65 as described above or left untreated, and 3 weeks after the final treatment the frequency of IL-4, IL-5, IL-10 and IFNγ secreting T cells in response to the IA^{g7}-restricted GAD65 peptides spanning amino acid residues 206-220 (p206), p217-236 (p217) and p290-309 (p290) was measured via ELISPOT. These three peptides are the immunodominant epitopes found within the fragment of GAD65 encoded by pGAD65. T cell reactivity was assessed in the: i) pancreatic lymph nodes (PLN) which drain the pancreas, ii) inguinal lymph nodes (ILN) which drain the abdominal site of gene gun delivered pGAD65, and iii) the spleen. A typical type 1-like

response was detected in NOD female mice treated i.m. with pGAD65 (Fig. 3). For example, a significant increase in the frequency of IFNγ secreting T cells in response to the GAD65-specific peptides was detected in the PLN, ILN and spleen of NOD mice vaccinated i.m. with pGAD65 versus untreated animals (Fig. 3). However, no significant increase in the frequency of IL-4 and IL-5 secreting T cells in the PLN or ILN was detected in the pGAD65 i.m. group compared to untreated animals (Fig. 3). Interestingly, a small but significant frequency of IL-4 secreting GAD65 peptide-specific T cells was also detected in the spleen of the i.m. pGAD65 vaccinated NOD mice; this response, however, was markedly reduced compared to NOD mice treated with gene gun delivered pGAD65 (Fig. 3). A predominate type 2-like GAD65 peptide-specific T cell response was detected in NOD mice receiving gene gun delivered pGAD65. The frequency of IL-4 and IL-5 secreting T cells was significantly increased in the PLN, ILN and spleen of the pGAD65 gene gun treated group versus pGAD65 i.m. vaccinated and untreated NOD mice (Fig. 3). GAD65-specific T cells secreting IFNγ were also induced via gene gun delivery of pGAD65, however, the frequency of these type 1 effectors was reduced compared to the frequency of IL-4 (and to a lesser extent IL-5) secreting T cells detected within a given tissue (Fig. 3). No significant increase was detected in the frequency of GAD65 peptide-specific IL-10 secreting T cells relative to medium-only controls in either of the pGAD65 treatment groups or in untreated NOD mice, regardless of the tissue examined. Furthermore, GAD65-specific T cell reactivity was similar between untreated NOD female mice and animals receiving pHEL by gene gun or i.m. delivery (data not shown).

No significant increase was detected in the frequency of FoxP3-expressing $CD4^+CD25^+$ T cells residing in the PLN, ILN and spleen of the two pGAD65 treatment groups or in untreated NOD mice (Fig. 4). In conclusion, these results demonstrate that whereas i.m. injection of pGAD65 induces a type 1-like T cell response, gene gun delivery of pGAD65 elicits a predominately type 2-like response marked by a high frequency of GAD65 peptide-specific IL-4 secreting T cells.

2.4.4 GAD65-specific IL-4 secreting $CD4^+$ T cells are necessary to mediate the protective effect of gene gun delivered pGAD65.

The above findings suggested that suppression of β cell autoimmunity via gene gun delivered pGAD65 was mediated by GAD65-specific $CD4^+$ T cells secreting IL-4. To directly examine this possibility, a co-adoptive transfer model of T1D was employed exploiting the use of NOD mice lacking IL-4 expression (NOD.$IL4^{null}$). Here 10 week-old wild-type NOD or NOD.$IL4^{null}$ female mice received pGAD65 via gene gun. Three weeks after the final treatment, $CD4^+$ T cells were isolated from the pGAD65 treatment groups, and co-adoptively transferred with diabetogenic splenocytes into groups of 5 NOD.*scid* mice. As demonstrated in Fig. 5, the transfer of diabetes was significantly blocked by $CD4^+$ T cells prepared from pGAD65 gene gun vaccinated wild-type NOD mice compared to mice receiving diabetogenic splenocytes-only ($P<0.0053$; Kaplan Meier Log Rank Test) (Fig. 5). In contrast, $CD4^+$ T cells prepared from pGAD65 gene gun vaccinated NOD.$IL4^{null}$ failed to block the transfer of diabetes; no significant difference in the frequency and time of onset of diabetes was detected in this group compared to NOD.*scid* mice receiving diabetogenic splenocytes-only (Fig. 5). These data

demonstrate that suppression of β cell autoimmunity by gene gun delivered pGAD65 is dependent on GAD65-specific IL-4 secreting $CD4^+$ T cells.

2.5 DISCUSSION

The key finding made in this study is that despite ongoing β cell autoimmunity, gene gun delivered pGAD65 preferentially promotes a robust type 2 $CD4^+$ T cell response that significantly prevents the development of diabetes in NOD mice. This is in marked contrast to i.m. injection of pGAD65 which induced a dominant type 1 GAD65-specific $CD4^+$ T cell response and failed to prevent diabetes in 10 week-old NOD female mice.

Our findings are consistent with previous studies demonstrating that antigen-encoding pDNA delivered via gene gun typically induces type 2 immunity, whereas i.m. needle injection of soluble pDNA promotes type 1 immunity (17, 18). Currently, the mechanisms which drive these "default" T cell responses remain ill-defined. Interestingly, both gene gun and i.m. vaccination of pGAD65 elicited high systemic levels of GAD65-IgFc protein based on serum levels in treated NOD mice (Fig. 1C). Although serum levels of GAD65Ig were greater in gene gun treated NOD mice, protection did not correlate with systemic GAD65-IgFc concentrations (Fig. 2C,D). These observations argue that the nature of the T cell response and suppression of β cell autoimmunity are largely dependent on the site and mode of pDNA delivery. Indeed, work by Escher and colleagues demonstrated that the onset of diabetes was delayed when 4 week-old NOD female mice received i.d. versus i.m. needle injections of pDNA encoding human GAD65 (8). In this study, however, i.d. needle injection failed to induce a significant increase in GAD65-specific IL-4 secreting T cells despite the relatively non-stringent conditions (e.g. lack of ongoing β cell autoimmunity) (8). Our results in which a significant frequency of GAD65-specific type 2 T cell reactivity was induced by gene

gun delivery at a late preclinical stage of T1D further underscores the potent effects of particle-delivery of pDNA to the skin. The latter in part may be explained by the tolergenic properties of epidermal Langerhans cells that have been transfected by pDNA or which have endocytosed the transgene encoded protein.

The increased efficiency of transgene expression detected with gene gun versus i.m. vaccination was another parameter favoring the use of particle-mediated delivery of pDNA (Fig. 1B,C). Levels of protein expressed by pLUC or pGAD65 were increased by gene gun delivery, despite using 50- to 100-fold less pDNA compared to i.m. injection (Fig. 1B,C). This difference is likely attributed to the fact that pDNA coated on particles is directly delivered to the cytoplasm and/or nucleus of cells residing in the skin (16). Intramuscular vaccination of pDNA in solution results in transfection of cells found at the site of injection as well as systemically, but this process is nevertheless relatively inefficient.

Our results contrast findings made by Joussemet *et al*. (19) in which no significant induction of type 2 T effectors or protection was observed when 3 week-old NOD female mice were treated with gene gun delivered pDNA encoding full-length human GAD65. The major difference between the respective studies is the cellular localization of the pDNA encoded GAD65 protein. Full-length GAD65 is intracellularly expressed and presentation of GAD65 epitopes would be largely limited to professional antigen presenting cells (APC) residing in the dermal region that have been directly transfected. Furthermore, intracellular GAD65 would be

preferentially processed and presented via the MHC class I pathway, which would reduce the efficiency of $CD4^+$ T cell activation. On the other hand, GAD65-IgFc is secreted by the transfected cells so that an increased number of resident APC can endocytose the antigen, and preferentially present the GAD65-specific epitopes via the MHC class II pathway and efficiently stimulate $CD4^+$ T cells. Indeed, other studies have demonstrated that the magnitude and nature of T cell immunity induced by pDNA vaccination is in part determined by the cellular localization of the protein (e.g. secreted versus intracellular) (20).

Although gene gun delivered pGAD65 proved to be more effective than i.m. injection, a significant number of NOD female mice nevertheless developed diabetes (Fig. 2A). This is likely due to the frequency and/or type of GAD65-specific immunoregulatory T cells induced following pGAD65 vaccination. For example, effective and long-term suppression of ongoing β cell autoimmunity requires a relatively high frequency of diverse subsets of immunoregulatory T effectors . Protection mediated by gene gun delivered pGAD65 was dependent and limited to the induction of IL-4 secreting GAD65-specific $CD4^+$ T cells (Figs. 3, 5). Other types of immunoregulatory T cells such as IL-10 secreting Tr1 cells or FoxP3-expressing $CD4^+CD25^+$ T cells (Fig. 4) were not significantly increased following pGAD65 gene gun vaccination. Accordingly, the magnitude and diversity of immunoregulatory T effectors can be effectively enhanced by co-immunization with pDNA encoding other β cell autoantigens (e.g. insulin B chain) and/or anti-inflammatory cytokines such as IL-4, IL-10 and TGFβ.

In summary, this study demonstrates that the route and mode of antigen-specific pDNA vaccination are critical parameters for blocking β cell autoimmunity at a late preclinical stage of T1D. Importantly, particle-delivered pDNA offers a promising approach to enhance the therapeutic efficacy and safety of autoantigen-specific vaccination.

2.6 ACKNOWLEDGEMENTS

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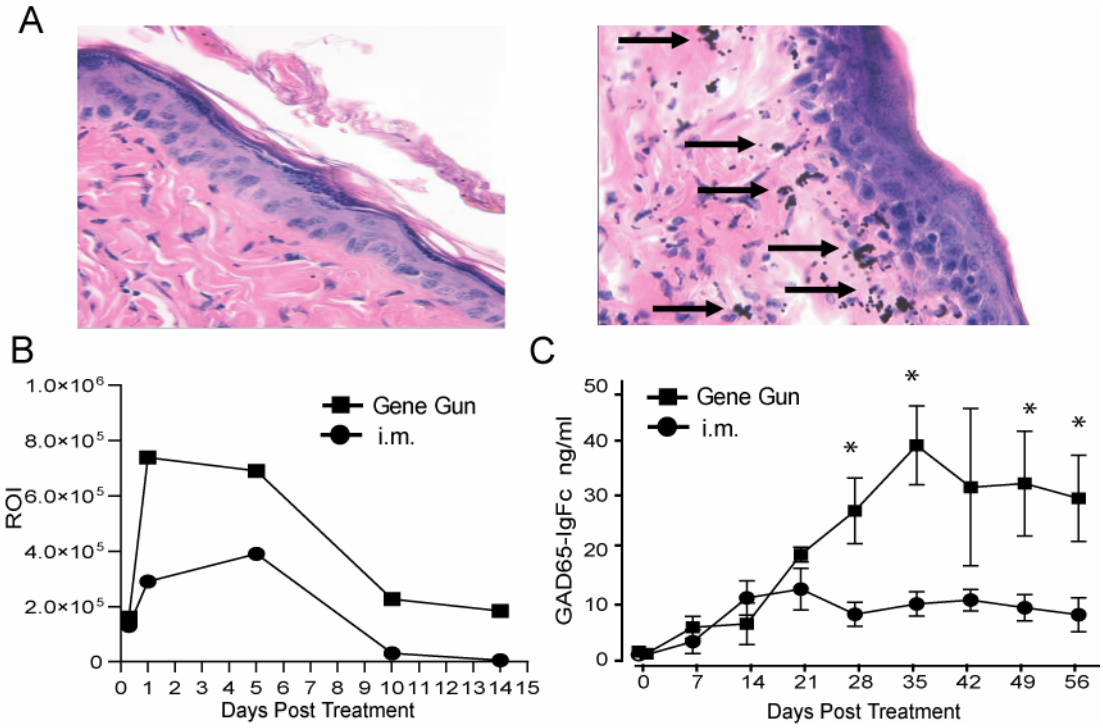

Figure 2.1 Increased transgene expression detected with gene gun versus i.m. delivery of pDNA. (A) Abdominal skin from untreated (left panel) or gene gun-treated (right panel) mice was harvested, sectioned and stained with H&E. Arrows indicate the site of 1.6 µM gold particles delivered by the gene gun. (B) Bioluminescence of NOD mice treated with pLUC by gene gun or i.m. was measured at the site of injection. (*; $P \leq 0.05$ comparing i.m. to gene gun using Student's T test, n=5 per group). (C) Kinetics of systemic expression of GAD65-IgFc transgene was assessed in 10 week-old NOD female mice treated at days 0, 7, 14, and 21 using gene gun or i.m. delivery; data represents the average $\pm$ SD of n=5 per group.

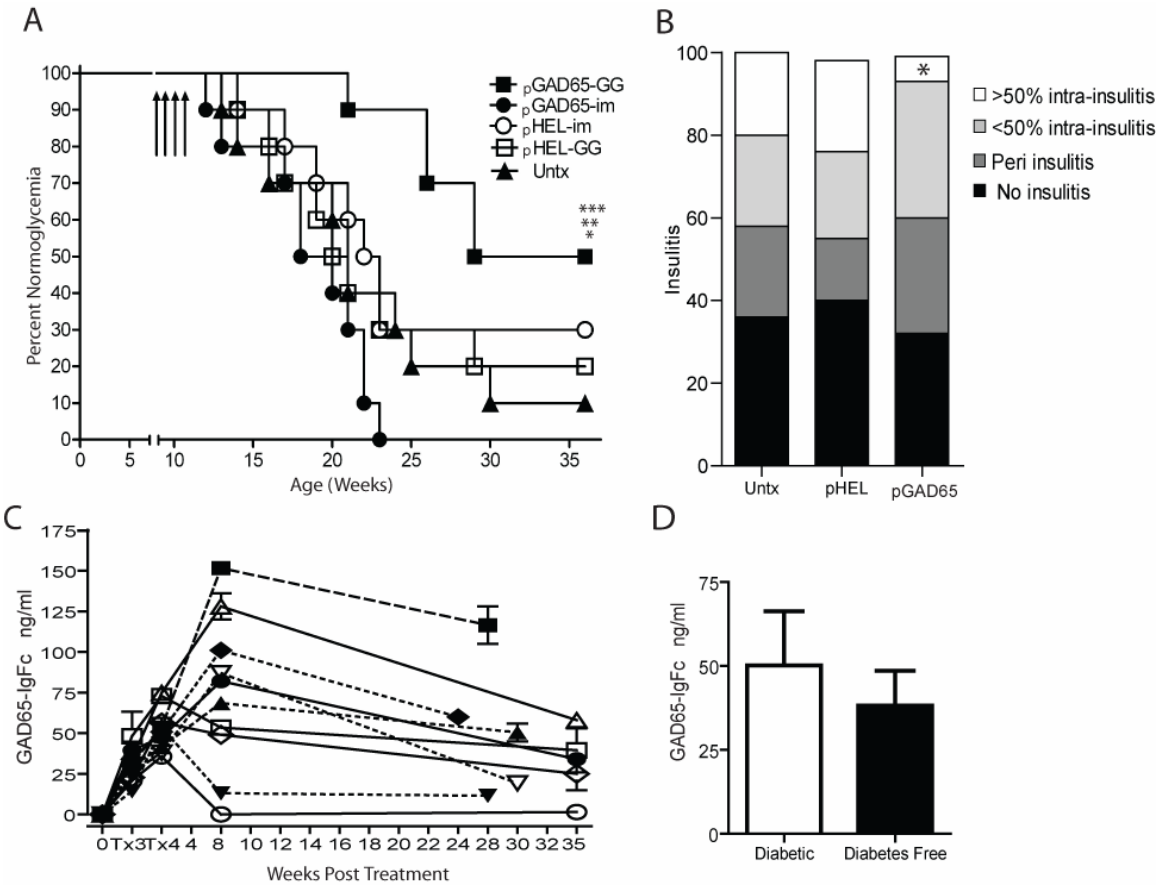

Figure 2.2 p**GAD65 delivery via gene gun protects against diabetes.** (A) Groups of 10 female NOD mice 10 weeks of age received 4 gene gun (GG) treatments of pGAD65 or pHEL, or 4 i.m. injections of pGAD65 or pHEL or were left untreated. The treatment groups were monitored for diabetes on a weekly basis. ***P=10^{-4}, pGAD65 gene gun versus pGAD65 i.m.; **P=0.0053, pGAD65 gene gun versus untreated; *P=0.02, pGAD65 gene gun versus pHEL gene gun (Kaplan-Meier Log Rank Test). (B) The frequency of insulitis was assessed via H&E staining of pancreatic sections prepared from nondiabetic 36 week-old NOD female mice receiving via gene gun pGAD65 (n=5), or pHEL (n=2), or left untreated (n=1); a minimum of 30 islets was examined per mouse. (C) Serum levels of GAD65-IgFc were measured from pGAD65 gene gun treated NOD female mice in the above experiment. Dashed and solid lines indicate NOD female mice that became diabetic or remained diabetes-free during the course of the experiment, respectively. (D) The average serum levels of GAD65-IgFc in pGAD65 gene gun treated mice depicted in panel C.

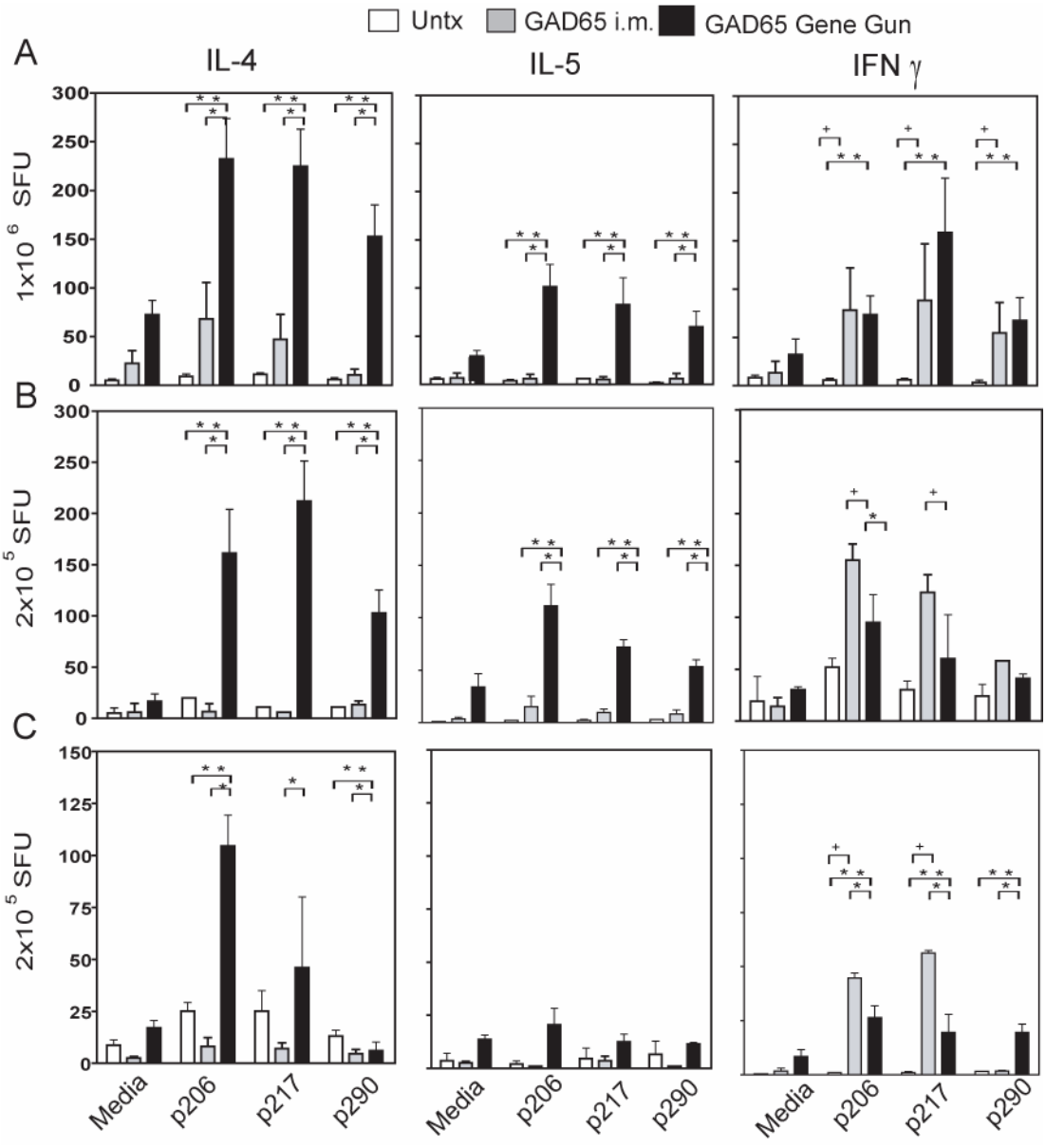

Figure 2.3 Gene gun induces GAD65-specific type 2 effector cells. Splenocytes (A), ILN (B), and PLN (C) were prepared from NOD female mice treated with pGAD65 delivered via gene gun (black bar) or i.m. injection (gray bar) or from untreated NOD mice (open bar) and stimulated with 40 μg/ml peptide. The frequency of GAD65 peptide-specific IL-4, IL-5, and IFN-γ, secreting T cells were measured via ELISPOT and statistical significance determined using a one-way ANOVA (*P≤0.05 pGAD65 gene gun versus pGAD65 i.m.; **P≤0.05 pGAD65 gene gun versus untreated; +P≤0.05 pGAD65 i.m. versus pGAD65 gene gun).

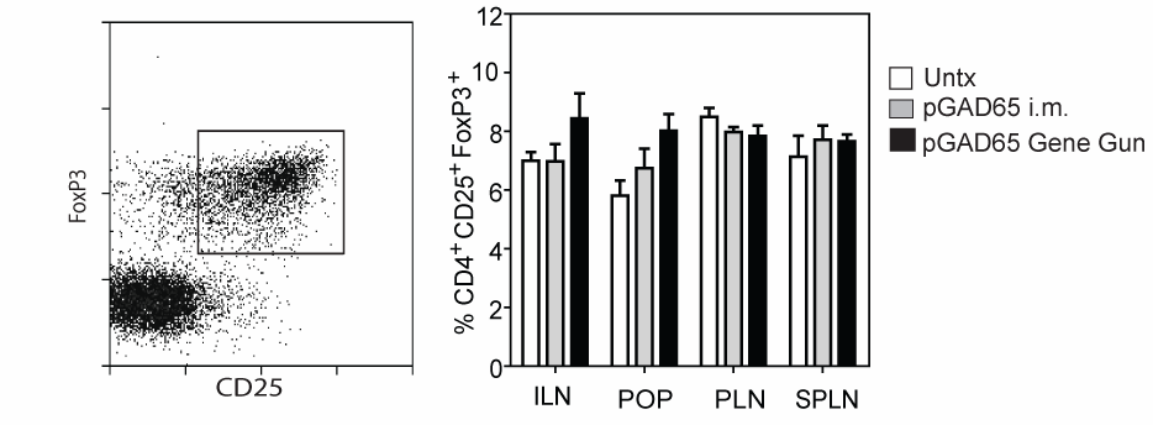

Figure 2.4 Gene gun and i.m. pGAD65 delivery had no effect on the frequency of FoxP3-expressing CD4$^+$CD25$^+$ T cells. The frequency of FoxP3-expressing CD25$^+$ T cells was determined by gating on CD3$^+$ and CD4$^+$ T cells found in ILN, popliteal lymph nodes (POP), PLN and spleen (SPLN) of NOD female mice treated at 10 weeks of age with pGAD65 delivered via gene gun or i.m. injection or left untreated.

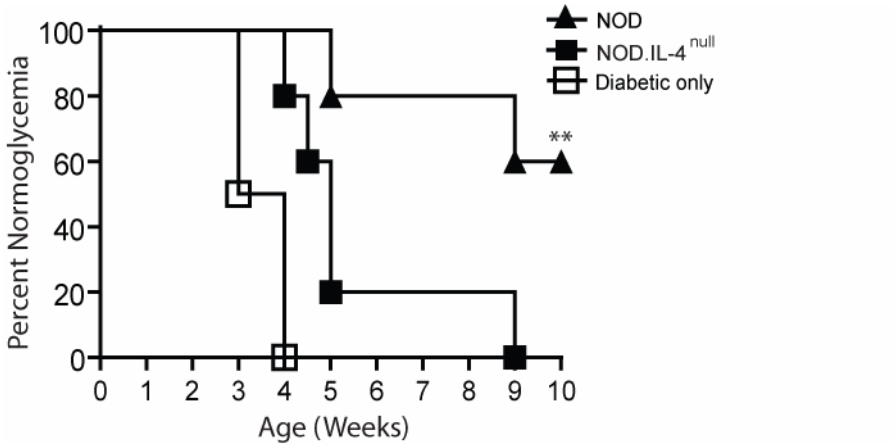

Figure 2.5 CD4$^+$ T cells from pGAD65 gene gun treated NOD mice prevent diabetes in an IL-4 dependent manner. A mixture of diabetogenic splenocytes and CD4$^+$ T cells purified from the spleens of wild type NOD or NOD.IL-4^{null} (■) female mice treated with gene gun delivered pGAD65 were transferred into groups of 5 NOD.*scid* mice (**P< 0.0053, Kaplan Meier Log Rank Test comparing NOD group to the NOD.IL-4^{null} group).

2.7 REFERENCES

1. Wang, Y., O. Pontesilli, R.G. Gill, F.G. La Rosa, and K.J. Lafferty. 1991. The role of CD4+ and CD8+ T cells in the destruction of islet grafts by spontaneously diabetic mice. *Proc Natl Acad Sci U S A* 88:527-531.

2. Pop, S.M., C.P. Wong, D.A. Culton, S.H. Clarke, and R. Tisch. 2005. Single cell analysis shows decreasing FoxP3 and TGFbeta1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes. *J Exp Med* 201:1333-1346.

3. Tisch, R., B. Wang, and D.V. Serreze. 1999. Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent. *J Immunol* 163:1178-1187.

4. Olcott, A.P., J. Tian, V. Walker, H. Dang, B. Middleton, L. Adorini, L. Washburn, and D.L. Kaufman. 2005. Antigen-based therapies using ignored determinants of beta cell antigens can more effectively inhibit late-stage autoimmune disease in diabetes-prone mice. *J Immunol* 175:1991-1999.

5. Cameron, M.J., C.A. Strathdee, K.D. Holmes, G.A. Arreaza, G.A. Dekaban, and T.L. Delovitch. 2000. Biolistic-mediated interleukin 4 gene transfer prevents the onset of type 1 diabetes. *Hum Gene Ther* 11:1647-1656.

6. Filippova, M., J. Liu, and A. Escher. 2001. Effects of plasmid DNA injection on cyclophosphamide-accelerated diabetes in NOD mice. *DNA Cell Biol* 20:175-181.

7. Gauvrit, A., M. Debailleul, A.T. Vu, P. Sai, and J.M. Bach. 2004. DNA vaccination encoding glutamic acid decarboxylase can enhance insulitis and diabetes in correlation with a specific Th2/3 CD4 T cell response in non-obese diabetic mice. *Clin Exp Immunol* 137:253-262.

8. Li, A.F., and A. Escher. 2003. Intradermal or oral delivery of GAD-encoding genetic vaccines suppresses type 1 diabetes. *DNA Cell Biol* 22:227-232.

9. Pop, S.M., C.P. Wong, Q. He, Y. Wang, M.A. Wallet, K.S. Goudy, and R. Tisch. 2007. The type and frequency of immunoregulatory CD4+ T-cells govern the efficacy of antigen-specific immunotherapy in nonobese diabetic mice. *Diabetes* 56:1395-1402.

10. Prud'homme, G.J., R. Draghia-Akli, and Q. Wang. 2007. Plasmid-based gene therapy of diabetes mellitus. *Gene Ther* 14:553-564.

11. Seifarth, C., S. Pop, B. Liu, C.P. Wong, and R. Tisch. 2003. More stringent conditions of plasmid DNA vaccination are required to protect grafted versus endogenous islets in nonobese diabetic mice. *J Immunol* 171:469-476.

12. Tisch, R., B. Wang, D.J. Weaver, B. Liu, T. Bui, J. Arthos, and D.V. Serreze. 2001. Antigen-specific mediated suppression of beta cell autoimmunity by plasmid DNA vaccination. *J Immunol* 166:2122-2132.

13. Weaver, D.J., Jr., B. Liu, and R. Tisch. 2001. Plasmid DNAs encoding insulin and glutamic acid decarboxylase 65 have distinct effects on the progression of autoimmune diabetes in nonobese diabetic mice. *J Immunol* 167:586-592.

14. Wolfe, T., A. Bot, A. Hughes, U. Mohrle, E. Rodrigo, J.C. Jaume, S. Baekkeskov, and M. von Herrath. 2002. Endogenous expression levels of autoantigens influence success or failure of DNA immunizations to prevent type 1 diabetes: addition of IL-4 increases safety. *Eur J Immunol* 32:113-121.

15. Ulmer, J.B., B. Wahren, and M.A. Liu. 2006. Gene-based vaccines: recent technical and clinical advances. *Trends Mol Med* 12:216-222.

16. Fuller, D.H., P. Loudon, and C. Schmaljohn. 2006. Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. *Methods* 40:86-97.

17. Feltquate, D.M., S. Heaney, R.G. Webster, and H.L. Robinson. 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol* 158:2278-2284.

18. Weiss, R., S. Scheiblhofer, J. Freund, F. Ferreira, I. Livey, and J. Thalhamer. 2002. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that over-rules the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. *Vaccine* 20:3148-3154.

19. Joussemet, B., A.T. Vu, P. Sai, and J.M. Bach. 2005. Gene-gun biolistic immunization encoding glutamic acid decarboxylase: a model for studying Langerhans cell abnormalities and mimicry in the nonobese diabetic mouse. *Ann N Y Acad Sci* 1051:613-625.

20. Morel, P.A., D. Falkner, J. Plowey, A.T. Larregina, and L.D. Falo. 2004. DNA immunisation: altering the cellular localisation of expressed protein and the immunisation route allows manipulation of the immune response. *Vaccine* 22:447-456.

CHAPTER 3

IL-2 AND IL-21 CONTRIBUTE TO IMPAIRED IMMUNOEREGULATOIN OF AUTOIMMUNE DIABETES

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3.1 ABSTRACT

Type 1 diabetes (T1D) occurs in the non-obese diabetic (NOD) mouse as a result of the functional imbalance between FoxP3$^+$ expressing regulatory T cells (FoxP3$^+$Treg) and pathogenic type 1 effector T cells specific for β-cell antigens. The insulin-dependent diabetes locus 3 (*Idd3*) of the NOD mouse encodes two key cytokines involved the development and maintenance of FoxP3$^+$Treg: interleukin-2 (IL-2) and interleukin-21 (IL-21). The current study was initiated to determine the relative role of these two cytokines in the pathogenesis of T1D by comparing NOD mice congenic for a disease resistant *Idd3* interval (NOD.Idd3) with wild-type NOD mice. Relative to NOD.Idd3 mice, the frequency and absolute numbers of CD62L^{high}FoxP3$^+$Treg were significantly reduced in the pancreatic lymph nodes and islets of NOD female mice. Notably, administration of IL-2 to NOD mice elevated the frequency of CD62L^{high}FoxP3$^+$Treg *in vivo*. Furthermore, the capacity of NOD CD4$^+$ T cells to upregulate FoxP3-expression and differentiate into adaptive FoxP3$^+$Treg *in vitro* was markedly reduced compared NOD.Idd3 CD4$^+$ T cells. This limited efficacy to differentiate into adaptive FoxP3$^+$Treg by NOD CD4$^+$ T cells was due to reduced IL-2 secretion, in addition to an increased sensitivity to the inhibitory effects of IL-21. Taken together these results demonstrate that dysregulation of IL-2 production coupled with altered sensitivity to IL-21 by CD4$^+$ T cells impair the development and maintenance of FoxP3$^+$Treg in NOD mice.

3.2 INTRODUCTION

The hallmark of Type 1 diabetes (T1D) is the T cell mediated destruction of the insulin-producing β cells in the pancreatic islets (1-3). Recent studies in humans as well as the nonobese diabetic mouse (NOD), a spontaneous model of T1D, implicate the loss of function and/or frequency of immunoregulatory T cells as a factor contributing to the expansion of β-cell specific, pathogenic type 1 effector T cells (4-6). The primary function of regulatory T cells is to inhibit the function of $CD4^+$ and $CD8^+$ T cells independent of their activation, proliferative and effector status (7, 8). Several subsets of immunoregulatory T cells with distinct phenotypes and effector mechanisms have been identified to play a role in controlling T1D (9). These subsets include: i) type 2 T effectors which dominantly secrete IL-4, ii) Th3 cells, which primarily secrete IL-4 and transforming growth factor-β (TGFβ) (10), iii) Tr1 cells, which secrete high levels of IL-10 (11), and iv) natural and adaptive $CD4^+CD25^+$ T cells which express the transcription factor Forkhead box P3 ($FoxP3^+$Treg), and that exhibit suppressor function mediated by cell-cell contact and contact-independent mechanisms (12).

FoxP3 $^+$Treg are widely-considered to be the most potent subset of immunoregulatory T cells, and as such play a pivotal role in establishing and maintaining self-tolerance (13). For example, humans and mice lacking functional FoxP3 protein develop a highly aggressive, systemic form of T cell autoimmunity (14-16). One mode that $FoxP3^+$Treg suppress T cells is through their constitutive expression of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and the glucocorticoid-induced TNF receptor (GITR) which block necessary co-stimulatory signals needed for T cell activation (17).

Additionally, FoxP3^{+}Treg elicit suppression through a bystander effect via TGFβ and to a lesser extent IL-10 expression (13, 18). TGFβ and IL-10 block the effector function of antigen presenting cells (APC) by inhibiting upregulation of costimulatory molecules and pro-inflammatory cytokine secretion, and by directly inhibiting IFNγ and TNFα production by type 1 effector T cells (19).

The phenotype of FoxP3^{+}Treg can be further defined based on CD62L expression. For instance, the suppressor function of CD62L^{high}FoxP3^{+}Treg is superior *in vitro* and *in vivo* compared to CD62L^{low}FoxP3^{+}Treg (6, 20, 21). The potent suppressor function of CD62L^{high}FoxP3^{+}Treg is in part due to the secretion of high levels of TGFβ (6). Notably, our group previously showed that the function and frequency of CD62L^{hi}FoxP3^{+}Treg decline with age in NOD female mice which in turn is thought to promote β cell autoimmunity (6). Work done by Szanya *et al.* also indicated that CD62L^{high}FoxP3^{+}Treg from the spleen of NOD mice exhibit an enhanced capacity to prevent diabetes in an adoptive transfer model compared to CD62L^{low}FoxP3^{+}Treg (20).

The critical events that induce and maintain the frequency of CD62L^{high}FoxP3^{+}Treg are currently ill-defined. However, recent studies have demonstrated that IL-2 plays a key role in the maintenance of FoxP3^{+}Treg in the periphery (22). For example, mice lacking or having reduced expression of the *il2* gene develop severe, systemic autoimmunity due to the reduction/absence of FoxP3^{+}Treg (23, 24). Furthermore, Sakaguchi *et al.* showed that diabetes is exacerbated in NOD mice when treated with a neutralizing antibody specific for IL-2 at an early age (25). Also, IL-

2 in combination with TGFβ has been shown to be important for the differentiation of naïve conventional $CD4^+$ T cells in to adaptive $FoxP3^+$Treg *in vitro* (26, 27).

Similar to IL-2, IL-21 is a survival factor for resting and activated T cells. The IL-21 receptor (IL-21R) shares the common γ of the IL-2 receptor (IL-2Rγ) (28-32). Furthermore, IL-21 signaling leads to Stat3 phosphorylation that has important implications on the differentiation of the proinflammatory T helper 17 (Th17) and T follicular cells (Tfh) T cells (33, 34), and enhances IgG production by B cells and the class switching from IgG1 to IgG3 (35). Importantly, IL-21 blocks up-regulation of FoxP3 expression (36).

Greater than 20 chromosal loci, termed insulin-dependent diabetes (*idd*) regions, have been identified where many candidate genes are known to affect T cell responses (37, 38). While no one gene is sufficient for the development of diabetes, the combined effects of susceptibility genes influence the progression of β cell autoimmunity (39). One of the major gene determinants affecting tolerance in NOD mice is located in the *idd3* locus. For example, mice congenic for an *idd3* interval derived from a diabetes resistant strain of mice exhibit a markedly reduced incidence and delayed onset of T1D (40, 41). Mapping of *idd3* demonstrated that the interval spans 780 kilobases (kb) and contains genes that encode a variety of immunoregulatory molecules including the cytokines IL-2 and IL-21 (42, 43). The NOD *Idd3* locus has been associated with reduced IL-2 expression by T cells due to a SNP found -1010 bp upstream of the *il2* affecting the binding of the transcription factor AP-1 (44). Furthermore, attenuated IL-2 production in

NOD mice has been proposed to limit the frequency and function of FoxP3^{+}Treg (45, 46). Moreover, NOD mice have also been reported to have increased expression of IL-21 which may promote the expansion of β cell specific pathogenic effector T cells (40). Together, these findings suggest that diabetes is regulated impart by an imbalance of IL-2 and IL-21 expression, which leads to attenuated FoxP3^{+}Treg frequency and/or function found in NOD mice. The current study was initiated to further define the role of IL-2 and IL-21 in mediating β cell autoimmunity in the NOD mouse.

3.3 MATERIAL & METHODS

Mice

NOD/LtJ and NOD.*scid* mice were obtained from The Jackson Laboratory. NOD.B6c3D (NOD.Idd3) mice were a kind gift from Dr. Ed Leiter of The Jackson Laboratory. NOD.Idd3 mice were established by introgression of a 0.05 centimorgan (cM) region of the *Idd3* interval derived from C57BL/6 (B6) mice. All mice were bred and housed under pathogen-free conditions. All mouse experiments used in this study were approved by the University of North Carolina Animal Use and Care Committee.

Diabetes monitoring and insulitis scoring

Diabetes was diagnosed by two elevated urine glucose readings (Diastix) over 2 days. Pancreata were harvested and treated with formalin for 24 hrs. After fixation, pancreata were serial sectioned (90 uM apart) and stained with hemotoxylin and eosin (H&E). Severity of insulitis was scored based upon the level of infiltration of the islets (0=no infiltration; 1=peri-insulitis, minor infiltration; 2=insulitis <50%, infiltration of islet is less than 50%; 3=peri-insulitis, severe infiltration greater than 50%). More than 100 islets were scored per group.

Cell harvesting

Single cell suspensions were prepared from the thymus, pancreatic lymph node (PLN), and spleen, and filtered with a 70 um cell strainer. Peripheral blood lymphocytes (PBL) were obtained via submandibular puncture (Goldenrod) and collected in Gibson solution followed by RBC lysis. Islet infiltrating cells were isolated by digesting the pancreas

with collagenase D (Roche) for 30 mins and culture for 3 hrs in RPMI 1640 (Gibco) complete medium containing 10% heat-inactivated fetal-calf serum (FCS), 100 U/ml penicillin/streptomycin (Gibco), 50 uM 2-ME (Sigma). After three rounds of pipeting to disrupt islets, cells were washed with PBS and prepared for staining as described below.

Flow cytometric analysis

Total cells from the respective tissues were stained with a variety of fluorochrome-conjugated monoclonal antibodies (mAbs) including: anti-CD3 (2C11), anti-CD4 (L3T4), anti-CD8 (Ly-2), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-CD62L (MEL14), anti-FoxP3 (FJK.16 kit) (eBioscience). Fc receptors were blocked with a 1/200 dilution of rat Ig prior to staining. Stained cells were acquired on a Cyan flow cytometer (Beckman-Coulter) and analyzed using Summit software (Dako).

T cell cultures

For general cell culture, $2x10^5$ cells were resuspended in RPMI complete medium. Stimulation of T cells for cytokine secretion was done using 96-well plates coated with varying concentrations of purified anti-CD3 (2C11, eBioscience) and soluble, functional-grade anti-CD28 at 2 ug/ml (37.51, eBioscience). To assess IL-2 production after 24 hr treatment with IL-21, 200 ng/ml or a titration thereof of murine recombinant IL-21 (PeproTech) was added to culture.

***In vitro* suppression assays**

PLN and spleen $CD4^+CD25^+$ T cells were isolated from 16 week-old mice using FACS sorting. Treg were sorted into two groups based on their CD62L expression level (high vs. low). Different ratios of Treg were cultured with naïve $5x10^4$ $CD4^+$ T cells from NOD.Idd3 mice in 96-well round bottom plates (Costar). The T cells were cultured with $5x10^4$ irradiated T cell depleted spleen cells NOD.Idd3 and 5 μg/ml anti-CD3 for 3 days. Proliferation was measured in triplicate by the incorporation of [^{3}H]thymidine during the last 10-12 hr of the co-culture. Plates were harvested on a Beta counter and data is presented in counts per million (CPM).

Cell adoptive transfers

NOD diabetogenic splenocytes ($2.5x10^6$) were suspended in PBS and injected i.p. into 8 week-old NOD.*scid* male mice alone or in combination with FACS sorted $CD4^+CD25^+$ T cells ($1x10^5$) isolated from the spleen or PLN of NOD or NOD.Idd3 mice. Mice were monitored bi-weekly post-transfer for diabetes as described above.

Isolation of naïve T cells

Naïve $CD4^+$ T ells were isolated by negative selection to enrich $CD4^+$ cells followed by positive selection for $CD62L^+$ cells (Miltnyi). Briefly, total lymphocytes were incubated with biotin-labeled antibody cocktail enriching for $CD4^+$ T cells but depleting $CD4^+CD25^+$ cells. Enriched $CD4^+$ T cells were then incubated with CD62L-conjugated micro-beads and isolated using a magnetic column.

ELISA

Supernatant was collected, diluted 1:3 in 1% BSA PBS, and assessed for IL-2 24 hrs post stimulation. The anti-IL-2 antibody set (JES6-1 capture antibody and biotinylated JES6-5 detection antibody; eBioscience) was used at 2 ug/ml on a high-binding ELISA plate (Costar). IL-21 detection in culture supernatant or diluted serum (1:4) was done using ELISA (R&D).

In vitro induction of FOXP3^{+}Treg

Purified, naïve CD4^{+} T cells (see above for purification description) from NOD or NOD.Idd3 mice were plated at 1x10^{6} on a 96-well tissue-culture treated, flat-bottom plate (Costar) coated with 2ug/ml of anti-CD3 (2C11, eBioscience) and anti-CD28 (37.51, eBioscience) in HL-1 media (Bio-Whittaker). Medium was supplemented with 100 U/ml penicillin/streptomycin (Gibco). Medium was supplemented with human TGF-β1 (5 ng/ml) (R&D), recombinant murine IL-2 (20 ng/ml) and/or IL-21 (100 ng/ml) (PeproTech). In some experiments functional grade anti-IL-2 (JES6-1, eBioscience) or isotype control (eBioscience) was added to the medium.

NOD IL-2 gene cloning

Using the forward primer 5'-gaagcttcaggcatgtacagcatgcagctc-3' that includes a HindIII restriction site and the reverse primer 5'-gtcgactagttattgagggcttgttgagat-3' that contains an EcoRV restriction site, the *il2* gene was amplified from mRNA (Qiagen) of ConA (Sigma) stimulated lymphocytes. After 35 rounds of PCR using 56^{0}C as the annealing temperature and PFU Turbo (Promega) as the high-fidelity polymerase, the PCR product was cloned into the topo-TA vector (Invitrogen). The *il2* gene was sequenced and then

cloned into an andeno-assocaited virus (AAV)-Tet-on vector using Sal 1 and EcoRV sites. Transgene expression was verified by measuring IL-2 secretion by transfected HEK 293 cells via ELISA (see methods above).

AAV induction of FoxP3^{+}Treg

NOD female mice were either vaccinated with $5x10^{10}$ viral particles of AAV-Tet-on IL-2 virus serotype 1 (AAV1-Tet-on-IL-2) in contra-lateral hind limb muscles using an insulin syringe (BD) or left untreated. After injection, mice were fed chow containing 200 mg/kg doxycycline (BioServ) for two weeks. Mice were bled weekly for the identification of FoxP3-expressing Treg until the end of the experiment.

3.4 RESULTS

3.4.1 Reduced Diabetes and IFNγ secreting T cells in NOD.Idd3 Mice

To investigate the role of IL-2 and IL-21 in β cell autoimmunity, NOD.Idd3 mice, congenic for a 0.05 cM interval derived from the B6 genotype were employed (41). Initially, the frequency of diabetes was compared between NOD and NOD.Idd3 female mice. As demonstrated in Fig. 1A, a significant reduction in the incidence of diabetes was detected in the cohort of NOD.Idd3 versus NOD female mice ($p<0.0001$). The frequency of insulitis was also markedly reduced in pancreata from 16 week-old NOD.Idd3 female mice relative to age matched NOD female mice (Fig. 1B&C). These findings demonstrate that the frequency of both insulitis and diabetes is reduced in NOD.Idd3 mice.

3.4.2 An Increased Frequency of FoxP3$^+$Treg is Detected in NOD.Idd3 Mice

FoxP3$^+$Treg play a critical role in maintaining self-tolerance in the periphery. Since IL-2 is a key regulatory molecule for the induction and maintenance of FoxP3$^+$Treg, the effect of the B6 *Idd3* interval on the frequency and absolute number of FoxP3$^+$Treg in the thymus, blood, spleen and PLN were compared to NOD mice in a temporal manner. Interestingly, levels of expression of CD25 by FoxP3$^+$Treg T cells increased with age in NOD.Idd3 but not NOD mice (Fig. 2B). Furthermore, a subtle but nevertheless significant increase in the frequency of FoxP3$^+$Treg T cells was detected in the blood, spleen, and PLN but not the thymus of NOD.Idd3 mice at all ages examined relative to NOD mice (Fig 2C). Most importantly, we found an approximate two-fold increase in the frequency of FoxP3$^+$Treg in the islets of NOD.Idd3 versus NOD mice (Fig. 2D).

3.4.3 NOD.Idd3 Mice Contain An Increased Frequency and Number of CD62L^{high}FoxP3^{+}Treg

We and others have shown that CD62L is a marker for the suppressor "status" of FoxP3^{+}Treg (6, 20). Namely CD62L^{high}FoxP3^{+}Treg exhibit an increased *in vitro* suppressor function compared to CD62L^{low}FoxP3^{+}Treg. Accordingly, the frequency and number of CD62L^{high}FoxP3^{+}Treg in the PLN of NOD.Idd3 and NOD female mice were assessed. As demonstrated in Fig. 3B the frequency of CD4^{+}CD25^{+}FoxP3^{+}CD62L^{high} T cells was increased in the PLN of 16 week-old NOD.Idd3 versus NOD female mice. Furthermore, an ~2-fold increase in the absolute number of CD4^{+}CD25^{+}FoxP3^{+}CD62L^{high} T cells was detected in the spleen and PLN but not the thymus of NOD.Idd3 versus NOD female mice regardless of age (Fig. 3C). Similarly, the frequency of CD62L^{high}FoxP3^{+}Treg was increased in the islets of 16 week-old NOD.Idd3 mice (Fig. 3D). These results indicate that the frequency and number of CD62L^{high}FoxP3^{+}Treg are increased NOD.Idd3 female mice.

Elevated numbers of CD62L^{high}FoxP3^{+}Treg in NOD.Idd3 mice would be expected to enhance suppression of effector T cells in the respective tissues. To test this possibility, an adoptive transfer experiment was carried out. CD4^{+}CD25^{+} T cells were prepared from PLN of 16 week-old NOD.Idd3 or NOD female mice, co-injected with splenocytes from diabetic NOD donors into NOD.*scid* mice, and diabetes monitored. All NOD.*scid* mice receiving diabetogenic splenocytes-alone or a mixture of diabetogenic splenocytes plus NOD CD4^{+}CD25^{+} T cells developed diabetes (Fig. 4A). In contrast, the frequency of diabetes was significantly reduced in NOD.*scid* mice receiving NOD.Idd3

$CD4^+CD25^+$ T cells plus diabetogenic splenocytes (Fig. 4A). Therefore the pool of $CD4^+CD25^+$ T cells from NOD.Idd3 mice exhibited an increased suppressor function compared to NOD $CD4^+CD25^+$ T cells.

To verify that the increased suppressor function of NOD.Idd3 $CD4^+CD25^+$ T cells was due to quantitative and not qualitative differences within the pool of $CD62L^{high}FoxP3^+$Treg, the suppressor activity of these immunoregulatory effectors was tested *in vitro*. $CD4^+CD25^+CD62L^{low}$ and $CD4^+CD25^+CD62L^{high}$ T cells were sorted via flow cytometry from the PLN of 16 week-old NOD.Idd3 and NOD female mice, and then cultured at various ratios with naïve $CD4^+$ T cells from the spleen of NOD mice. As expected, $CD4^+CD25^+CD62L^{low}$ T cells from either NOD.Idd3 or NOD female mice failed to suppress proliferation of the stimulated $CD4^+$ T cells (Fig. 4B). On the other hand, $CD4^+CD25^+CD62L^{high}$ T cells effectively suppressed proliferation of the responder $CD4^+$ T cells. Importantly, no significant difference in suppressor activity was detected between NOD.Idd3 and NOD $CD4^+CD25^+CD62L^{high}$ T cells (Fig. 4B). Therefore, the enhanced suppressor activity in NOD.Idd3 mice is due to an increased number of $CD4^+CD25^+CD62L^{high}$ T cells.

Finally, to assess the impact of increased numbers of $FoxP3^+$Treg on β cell autoimmunity, the frequency of $CD4^+CD25^+Foxp3^+CD62L^{high}$ T cells versus IFN-γ secreting $CD4^+$ and $CD8^+$ T cells in the islets and PLN of 16 week-old NOD.Idd3 and NOD female mice was determined. The $CD62L^{high}$ $Foxp3^+$Treg ratio was increased in the PLN and most notably the islets of NOD.Idd3 versus NOD mice (Fig. 5). These

results indicate that in NOD.Idd3 mice the balance between Treg and effector T cells is skewed towards CD62L^{high}FoxP3^{+}Treg.

3.4.4 Impaired IL-2 Production Attenuates NOD FoxP3^{+}Treg Induction

As noted above IL-2 has been reported to play a key role in the induction and maintenance of FoxP3^{+}Treg (25, 47). With this in mind, the level of IL-2 secretion by CD4^{+} T cells was assessed in NOD.Idd3 and NOD mice in a temporal manner. As demonstrated in Fig. 6A a 2- to 3-fold reduction in IL-2 secretion was detected in stimulated CD4^{+} T cells isolated from the spleen of NOD versus NOD.Idd3 mice, regardless of age. Similar results were obtained for CD4^{+} T cells from other lymph organs including the PLN (data not shown). These results demonstrate that the NOD *Idd3* interval results in decreased IL-2 secretion by CD4^{+} T cells.

To gain insight into how reduced IL-2 secretion may affect the number of FoxP3^{+}Treg in NOD mice, the efficacy of naïve CD4^{+} T cells from NOD and NOD.Idd3 mice to induce FoxP3-expression *in vitro* was compared. Using culture conditions established by the Shevach group (27), naïve CD4^{+} T cells purified from NOD and NOD.Idd3 mice were stimulated with anti-CD3 and -CD28 antibodies and cultured with or without TGFβ1. The frequency of FoxP3-expressing T cells was increased in NOD.Idd3 versus NOD CD4^{+} T cells (Fig. 6B). Increased expression of FoxP3 in NOD.Idd3 CD4^{+} T cells was detected over varying doses of anti-CD3 antibody (Fig. 6C) and TGFβ1 (Fig. 6D) stimulation. Notably, an equivalent frequency of FoxP3-expressing T cells was observed in NOD.Idd3 and NOD cultures following the addition of exogenous IL-2 (Fig. 6B). The latter finding suggested that endogenous IL-2 secretion by

NOD $CD4^+$ T cells was limiting the induction of FoxP3 expression. Indeed, addition of a neutralizing anti-IL-2 antibody to cultures containing TGFβ1 effectively blocked the induction of FoxP3 expression by both NOD.Idd3 and NOD $CD4^+$ T cells (Fig. 6E). These observations demonstrate that reduced IL-2 secretion by NOD $CD4^+$ T cells limits the induction of adaptive $FoxP3^+$Treg.

3.4.5 The frequency of $CD62L^{high}FoxP3^+$Treg is Increased *In Vivo* in NOD mice Treated with IL-2

Since IL-2 secretion is limited in NOD mice, then increasing the level of "endogenous" IL-2 would be expected to enhance the frequency of $FoxP3^+$Treg *in vivo*. To test this hypothesis 10 week-old NOD female mice were injected intramuscularly with a doxycycline inducible AAV recombinant encoding IL-2 (AAV-Tet-On-IL-2). No difference was detected in the frequency of $CD4^+CD25^+Foxp3^+$ T cells in AAV-Tet-On-IL-2 treated but uninduced NOD mice or animals left untreated (Fig. 7A & B). In contrast, NOD mice treated with AAV-Tet-On-IL-2 and in which IL-2 transgene expression was induced exhibited an increased frequency of $CD4^+CD25^+Foxp3^+$ in all organs tested, and showed a dramatic increase in $CD4^+CD25^+Foxp3^+CD62L^{high}$ T cells in the PLN (Fig. 7). These results indicate that IL-2 availability *in vivo* is a key factor regulating the frequency of $FoxP3^+$Treg.

3.4.6 NOD $CD4^+$ T cells Exhibit an Increased Sensitivity to the Inhibitory Effects of IL-21

The gene encoding IL-21 also resides within the *Idd3* interval (28). Furthermore, recent studies have shown that IL-21 inhibits the induction and/or effector function of FoxP3$^+$Treg (36, 48). Supplementing cultures with recombinant IL-21 blocked *in vitro* induction of FoxP3 expression by naïve CD4$^+$ T cells prepared from NOD or NOD.Idd3 mice (Fig. 8A). Accordingly, whether IL-21 contributed to the limited numbers of FoxP3$^+$Treg in NOD mice was investigated. Initially, IL-21 production by naïve CD4$^+$ T cells from NOD and NOD.Idd3 mice was compared in a temporal manner. No difference in the level of IL-21 secretion was detected in cultures established from age matched NOD and NOD.Idd3 female mice (Fig. 8B). Furthermore, serum levels of IL-21 (49, 50) were similar in 16 week-old NOD and NOD.Idd3 female mice (Fig. 8C). These findings indicate that production of IL-21 *per se* did not differ significantly between NOD and NOD.Idd3 mice.

Based on our above findings the level of endogenous IL-2 secretion is critical for the induction of adaptive FoxP3$^+$Treg (Figs. 6,7). With this in mind the effect of IL-21 on IL-2 secretion by NOD versus NOD.Idd3 naïve CD4$^+$ T cells was investigated. IL-2 secretion by NOD.Idd3 CD4$^+$ T cells was inhibited by ~60% at the maximum concentration of IL-21 tested (200 ng/ml) (Fig. 7D). Strikingly, IL-2 secretion by NOD CD4$^+$ T cells was inhibited ~70% with only 50 ng/ml, and ~92% with 200 ng/ml of IL-21 (Fig. 7D & E). Furthermore, IL-21 reduced the frequency of FoxP3-expressing CD4$^+$ T cells *in vitro* in a dose-dependent manner (Fig. 7F). Taken together these results indicate that the level of IL-21 secretion on a per cell basis is not significantly different between NOD and NOD.Idd3 CD4$^+$ T cells. However, NOD CD4$^+$ T cells are more sensitive to

the inhibitory effects of IL-21, characterized by markedly reduced levels of IL-2 secretion and a limited capacity to up-regulate FoxP3 expression relative to NOD.Idd3 $CD4^+$ T cells.

3.5 DISCUSION

FoxP3^{+}Treg have been cited as the most potent immunoregulatory cell capable of controlling the induction and expansion of type 1 effector cells that lead to T1D (12). Considering the critical role IL-2 plays in induction, expansion, and function of FoxP3^{+}Treg, we hypothesized that the NOD.Idd3 protective alleles that encode *il2* aides in increasing induction and maintenance of the proper balance of immunoregulation. We found that the protective *Idd3* allele, relative to the NOD allele, enhances the production of IL-2 by activated naïve CD4^{+} T cells that, in turn, induces a greater frequency of Foxp3^{+}Treg in the periphery including the islets. Here we show that T1D susceptibility correlates with the reduced frequency of the highly suppressive CD62L^{high}FoxP3^{+}Treg in NOD mice. The protective role this has was evident by drastic reduction in diabetes incidence in the NOD.Idd3 mice. We also determined that that increased IL-2 whether by allelic production or introduced by gene therapy is sufficient to induce/expand the CD62L^{high}FoxP3^{+}Treg.

The significance of the impaired ability of NOD mice to transcribe IL-2 maybe partly attributed to regulatory cytokines such as IL-21. Here we show that NOD naïve CD4^{+} T cells are more sensitive to IL-21 thus suppressing their ability to produce IL-2 compared CD4^{+} T cells of NOD.Idd3 mice. The functional consequence of reduced IL-2 production in the presence of IL-21 is noted as FoxP3 induction is reduced contingent upon the IL-21 levels in the culture. We also demonstrated that IL-21 inhibition of IL-2 can be rescued by the addition of rIL-2 reulting in the increase of FoxP3^{+}Treg. Collectively, we show that elevated IL-2 expression either from a protective *Idd3* allele

or by a gene therapy approach is important for maintaining the functional homeostasis of FoxP3^{+}Treg required to suppress autoimmune reactions.

One surprising discovery in this study was the finding that Treg from NOD and NOD.Idd3 mice have similar suppressive capabilities. This finding contests a previous report showing that NOD.Idd3 CD4^{+}CD25^{+} Treg have enhanced suppressive ability compared to NOD Treg due to IL-2 expression (51). The major difference in this study is that we evaluated the suppressive ability of 62L^{high} and 62L^{low} FoxP3^{+}Treg as we discovered a major difference in the composition of these populations in PLN. Once separated by expression profiles and cultured at identical ratios the respective strains have similar suppressive abilities. We previously noted the difference in suppressor function of 62L^{high} and 62L^{low} Treg from NOD mice is primarily due to the expression of TGF-β (6).

One of the original hypotheses that attribute diabetes susceptibility to the NOD *Idd3* locus was the multiple SNPs and gylcosylation pattern of the NOD versus C57B/6 *il2* region (42). Conceivably, differential gylcosylation could lead to attenuated signaling and action due to stereohinderance, among other possibilities, that result in reduced Treg frequency. Despite the 9 amino acid difference (located within the first 90bp of 5' region of il2) resulting in higher N-glysocylation of NOD IL-2 there was no functional differences noted between these two proteins. Additionally, the α, β, & γ IL-2 receptor binding domains on IL-2 lye outside of the region affected by the SNPs suggesting that NOD and C57B/6 have similar functional ability despite their gylcosylation. Even

though SNPs and protein modifications are not likely the cause of the reduced Treg formation in this scenario that is not to say that there could be human T1D or other automimmne cases where either IL-2 or CD25 proteins are modified at key residues resulting similar reduced Treg numbers.

The most logical explanation for the difference in IL-2 levels between NOD and NOD.Idd3 is more likely attributable to the upstream binding transcriptional binding domains of the IL-2 gene. Promoter studies have ruled out the possibility that differential IL-2 expression is due to the differences attributed to promoters from the respective strains (43). The most likely candidate explaining the difference between the NOD and C57B/6 *Idd3* region is the finding of multipible SNPs upstream of the IL-2 promoter that effect an AP-1 binding site that is (44). In this study, when the SNPs in the NOD AP-1 site were changed to C57B/6 sequence, similar levels of expression were restored.

In summary, our results suggest that the effect of impaired expression of IL-2 governs the size of the FoxP3^{+}Treg pool and ultimately autoimmune diabetes. By identifying IL-2 deficiency as a root cause of diabetes development in the NOD mouse we ultimately would like to transfer this knowledge to clinical directed therapy. Given the fact that we were able to overcome the NOD Treg deficiency by both introducing the *Idd3* locus as well as expression of IL-2 using gene therapy gives us hope that by the exclusive use of IL-2 to alter the Treg population we will be able to prevent T1D. However, before we move to the clinic we must determine the optimal IL-2 regimen as it pertains to the duration, amount, route, and side effects associated with such a treatment.

Are attention is now focused on elucidating these measures as well as identify the mechanisms by which Tregs in elevated IL-2 environments elicit their effects.

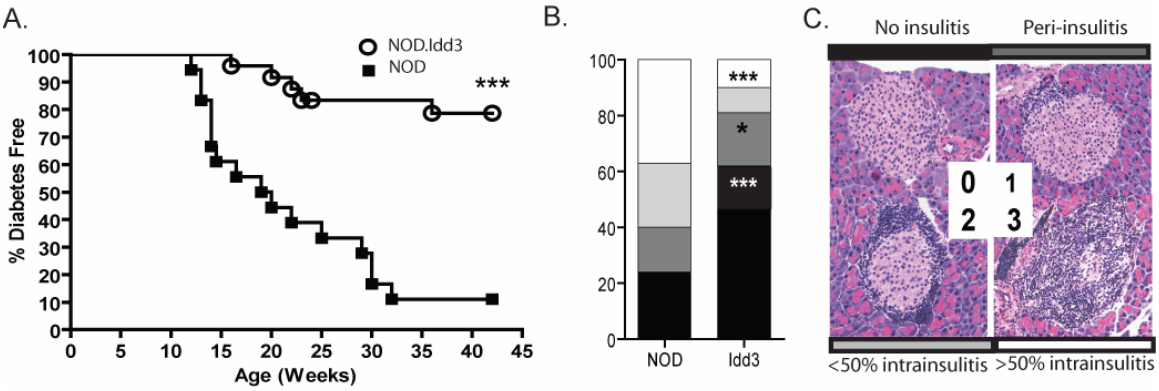

Figure 3.1 NOD.Idd3 mice have reduced diabetes and insulitis. A.) Female NOD (■, n=20) and NOD.Idd3 (○, n=19) were followed for diabetes incidence, (Kaplan-Meier Log Rank Test of NOD to NOD.Idd3; ***, $p<0.001$). B.) Islets from NOD (n=7) and NOD.Idd3 (n=6) mice were scored from H&E stained pancreata (NOD to NOD.Idd3 Chi Square analysis; * $p<0.05$, *** $p<0.001$). C.) The severity of insulitis was scored based upon the level of islet infiltration: 0= no infiltration, 1= peri-insulitis, 2= <50% intra-insulitis, 3=>50% intra-insulitis.

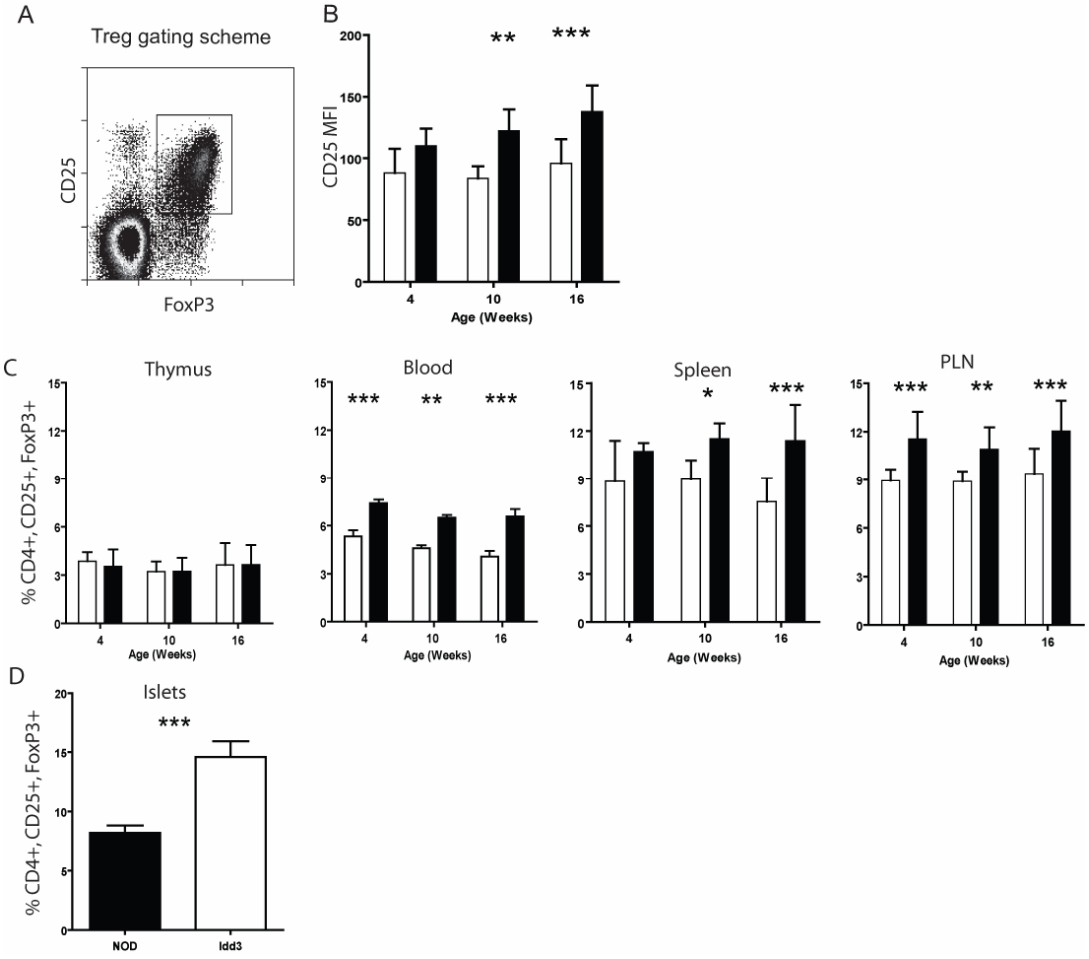

Figure 3.2 NOD.Idd3 mice have a higher frequency of FoxP3$^+$Tregs. A.) FoxP3$^+$Treg were determined by gating on CD3$^+$, CD4$^+$, CD25$^+$ and FoxP3$^+$ using flow cytometery B.) The CD25 MFI was determined on FoxP3$^+$Treg from the PLN of 16 week-old NOD (open bars, n=10) and NOD.Idd3 females mice (closed bars, n=9) by gating on CD3$^+$CD4$^+$FoxP3$^+$ cells (statistical significance of NOD to NOD.Idd3 mice was determined using two-way ANOVA; *, $p<0.05$, **, $p<0.01$). C.) FoxP3$^+$Treg frequency was measured in female NOD (open bars, n=9-15) and NOD.Idd3 mice (closed bars, n=7-16) at 4, 10 and 16 weeks of age for the thymus, spleen and PLN (statistical significance of NOD to NOD.Idd3 mice was determined using two-way ANOVA; *, $p<0.05$, **, $p<0.01$ ***, $p<0.001$). D.) The frequency of FoxP3$^+$Treg in islets isolated from NOD (n=7) and NOD.Idd3 (n=8) 16 week old female mice (statistical significance was determined using Students T test).

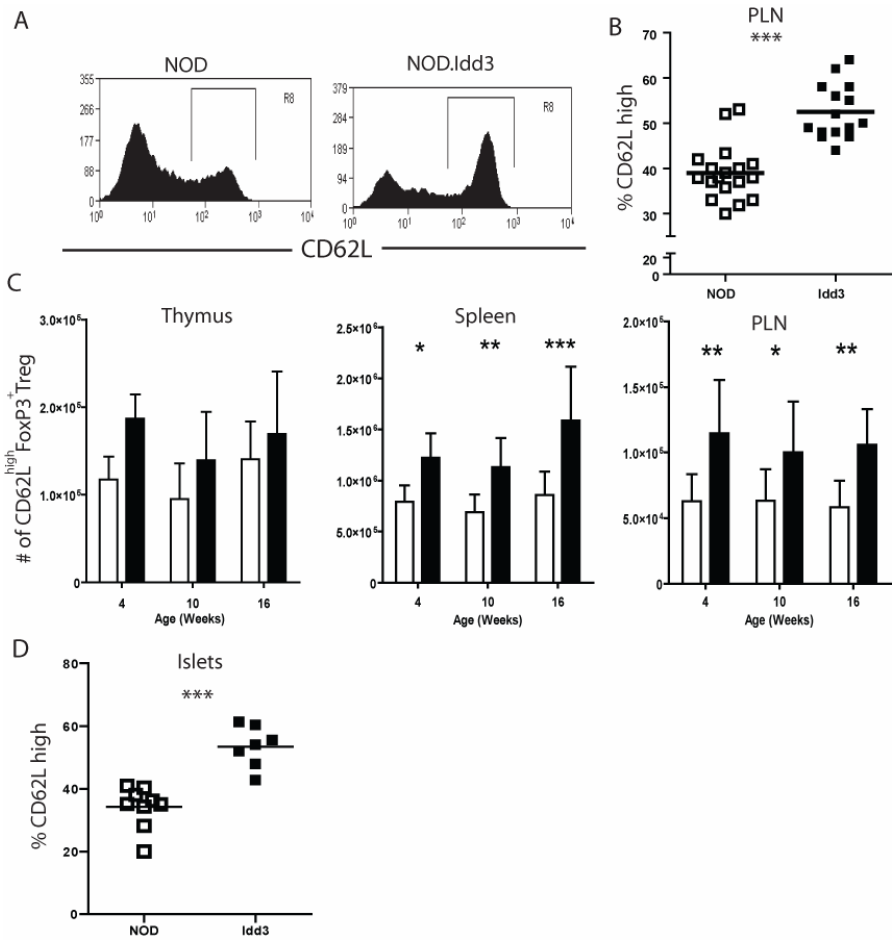

Figure 3.3 Similar Suppressive Capabilities of NOD and NOD.Idd3 Treg *In Vitro*. A.) CD62L expression on FoxP3^{+}Treg was classified into high versus low expression for NOD.Idd3 and NOD mice. B.) The percentage of CD62L^{high}FoxP3^{+}Treg from the PLN of 16 week-old NOD (n=17) and NOD.Idd3 (n=14) mice was preformed using the gating scheme described in A (statistical significances was determined using Student's t Test (***, $p<0.0001$)). C.) Absolute number of FoxP3^{+}Treg was determined by multiplying the frequency of CD62L^{high}FoxP3^{+}Treg by the overall cellularity of the organ. The thymus, spleen and PLN of NOD (open bar, n=9-15) and NOD.Idd3 female mice (closed bar, n=7-16) at 4, 10 and 16 weeks of age were evaluated (statistical significance of absolute number of NOD to NOD.Idd3 mice was done using two-way ANOVA, (*, $p<0.05$, **, $p<0.001$, ***, $p<0.0005$)). D.) The frequency of CD62L^{high}FoxP3^{+}Treg found in the islets of NOD and NOD.Idd3 mice 16 weeks of age (Student's t Test (***, $p<0.0005$)).

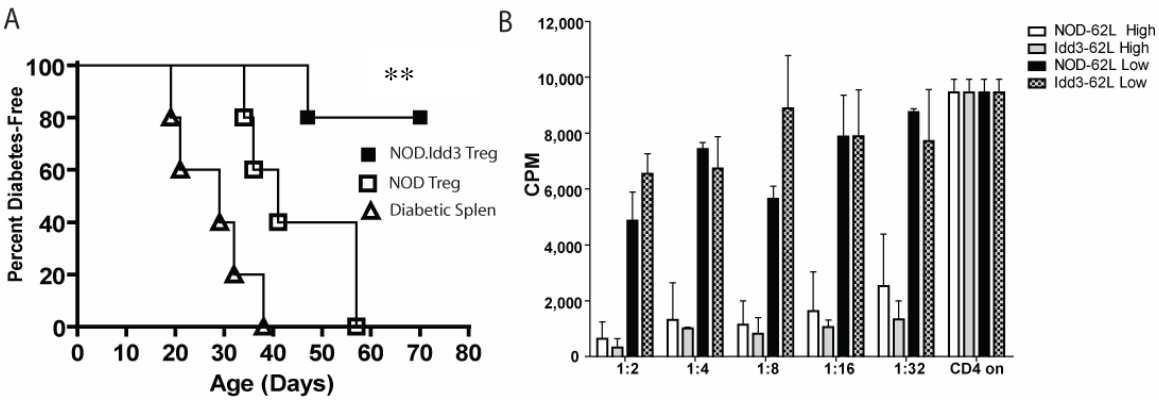

Figure 3.4 Comparison of the Suppressor Function of NOD and NOD.Idd3 FoxP3^{+}Treg.

A.) Pooled PLN CD4^{+}CD25^{+} T cells sorted from NOD or NOD.Idd3 16 week-old female mice were transferred with diabetogenic splenocytes in to NOD.*scid* mice; as a control diabetogenic splenocytes alone were also transferred. NOD.scid recipients were followed for diabetes (statistical significance of diabetes development between NOD and NOD.Idd3 PLN Treg was done by Kaplan-Meier Log Rank Test, (**, $p<0.001$). B.) Tregs were sorted from the PLN based upon their CD62L profile (open bar, NOD CD62L^{high}; gray bar, NOD.Idd3 CD62L^{high}; black bar, NOD CD62L^{low}; NOD.Idd3 CD62L^{low} thatched bar) and cultured with APC, 2µg/ml anti-CD3 and naïve CD4^{+} T cells. This is a representative graph of three individual experiments.

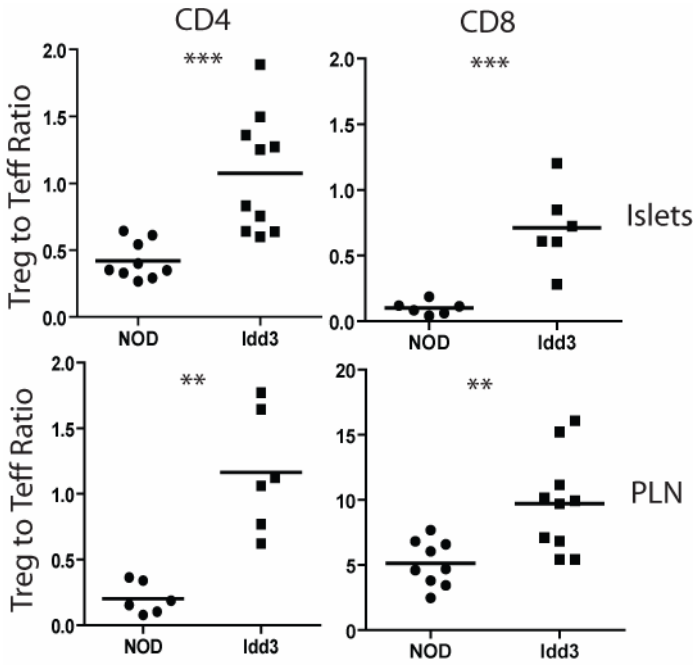

Figure 3.5 An Increased Ratio of FoxP3$^+$Treg to effector T cells is Detected in NOD.Idd3 mice. The ratio of FoxP3$^+$Treg to CD4$^+$ and CD8$^+$ IFN-γ positive type 1 effectors (Teff) was determined by dividing the frequency of FoxP3$^+$Treg by the frequency of IFNγ producing T cells from 16 week-old NOD (n=6) and NOD.Idd3 (n=5) female mice. This ratio was determined for the islets, and PLN (statistical significance was determined using Student's t Test (**, $p<0.01$; ***, $p<0.001$)).

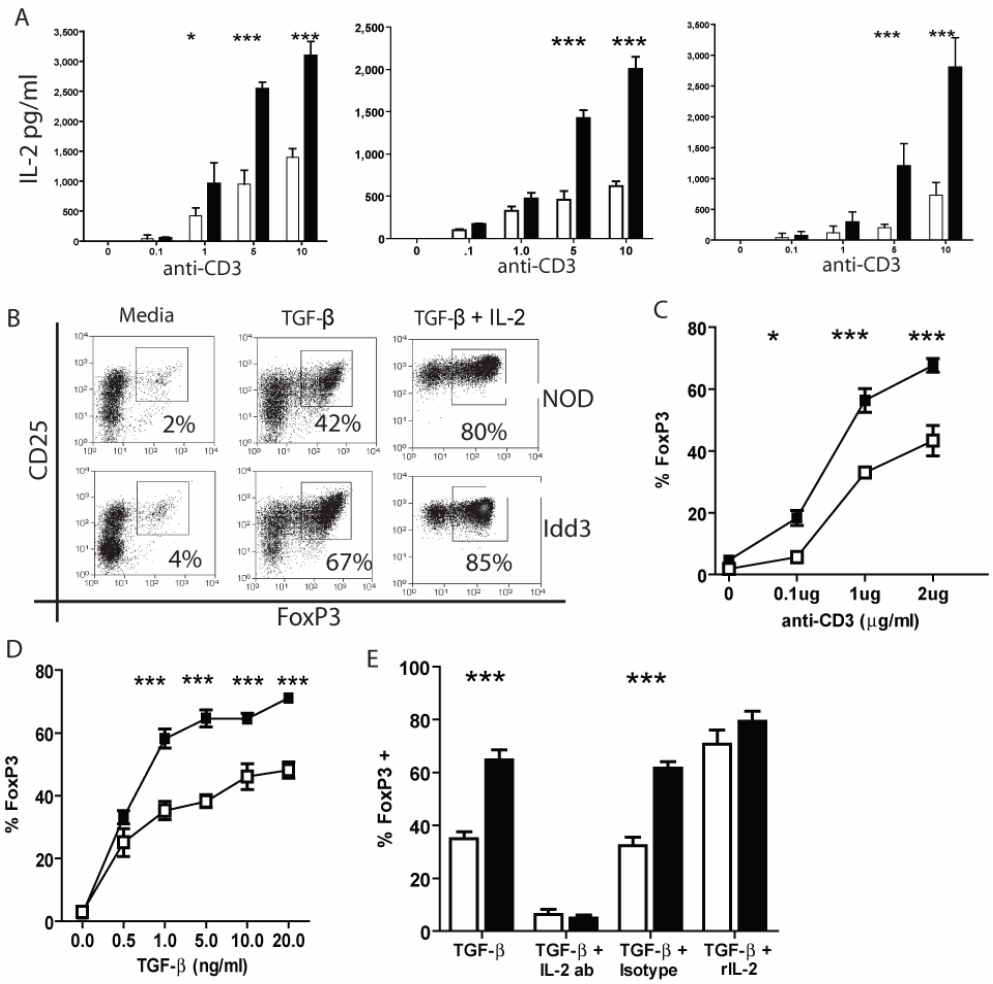

Figure 3.6 Impaired IL-2 production accounts for reduced FoxP3 expression in NOD CD4$^+$ T cells. A.) Naïve CD4$^+$ T cells from 4, 10 or 16 week old female mice were stimulated with varying concentrations of plate-bound anti-CD3 (10, 5, 1, 0.01 μg/ml anti-CD3) and soluble anti-CD28. IL-2 was measured 24 hrs post-stimulation by ELISA (statistical significance of NOD IL-2 levels to NOD.Idd3 was done using two-way ANOVA, (*, $p<0.05$; ***, $p<0.001$)). B.) Naïve NOD and NOD.Idd3 CD4$^+$ T cells were stimulated in the presence of medium alone, TGFβ1, or TGFβ1 and IL-2. The dot plots are representative of greater than five independent experiments; anti-IL-2, isotype control or rIL-2 at 20 ng/ml (statistical significance of NOD CD4$^+$ T cells to NOD.Idd3 was done using two-way ANOVA, (**, $p<0.01$; ***, $p<0.001$)). C.) Purified naïve CD4$^+$ T cells from NOD or NOD.Idd3 mice were stimulated with varying concentrations of plate-bound anti-CD3 in the presence of 5 ng/ml of hTGF-β1 for 3 days (statistical analysis of NOD to NOD.Idd3 mice was determined using two-ANOVA; *, $p<0.05$; **, $p<0.01$). D.) Naïve CD4$^+$ T cells from NOD or NOD.Idd3 mice were stimulated with varying concentrations of TGF-β1 in the presence of 2 μg/ml anti-CD3 and anti-CD28 for 3 days (statistical analysis of NOD to NOD.Idd3 mice was determined using two-Anova; *, $p<0.05$; **, $p<0.01$). E.) Naïve CD4$^+$ T cells were stimulated in the presence of anti-IL-2, isotype control or rIL-2 at 20 ng/ml (statistical significance of NOD CD4$^+$ T cells to NOD.Idd3 was done using two-way ANOVA, (**, $p<0.01$; ***, $p<0.001$).

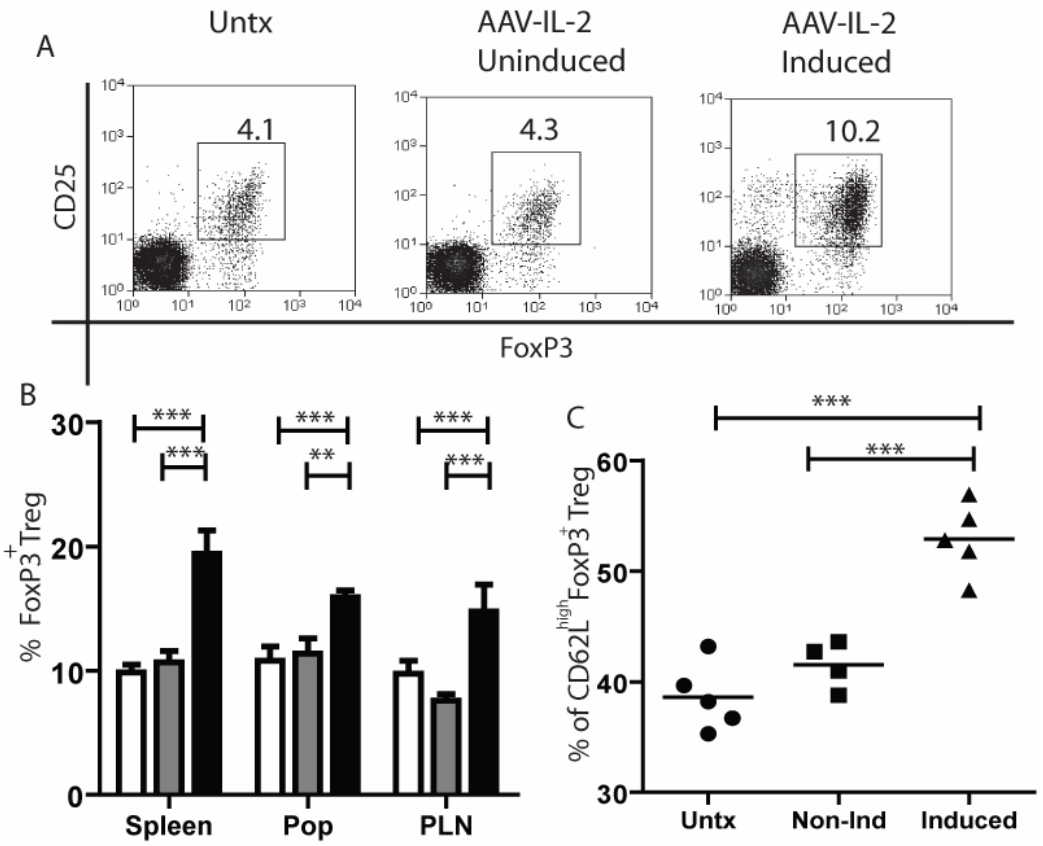

Figure 3.7 Over-expression of IL-2 induces FoxP3^{+}Treg. A.) NOD mice were left untreated (n=5) or injected with AAV-Tet-on-IL-2 (n=5) and either given doxycycline containing chow for two weeks (induced) or not (uninduced). Two weeks post-induction all mice were bled and cells were stained for CD3, CD4, CD25, and FoxP3. A representative flow cytometric plot for each group is shown and the numbers in each dot plot represent the average of five mice. B.) After three weeks post-induction the spleen, popliteal (6) and PLN of non-injected (open bars), AAV-Tet-on-IL-2 uninduced (gray bars) and AAV-Tet-on-IL-2 induced for three weeks (black bars) were evaluated for CD3, CD4, CD25, and FoxP3 (statistical significance was done using two-way ANOVA, (**, $p<0.01$; ***, $p<0.001$). C.) The percentage of CD62L^{high} FoxP3^{+}Treg was determined in the PLN of the respective groups (non-injected (●), AAV-Tet-on-IL-2 not induced (■) and AAV-Tet-on-IL-2 induced for three weeks (▲) by staining for CD3, CD4, CD25, and FoxP3 (statistical significance was done using two-way ANOVA (***, $p<0.001$)).

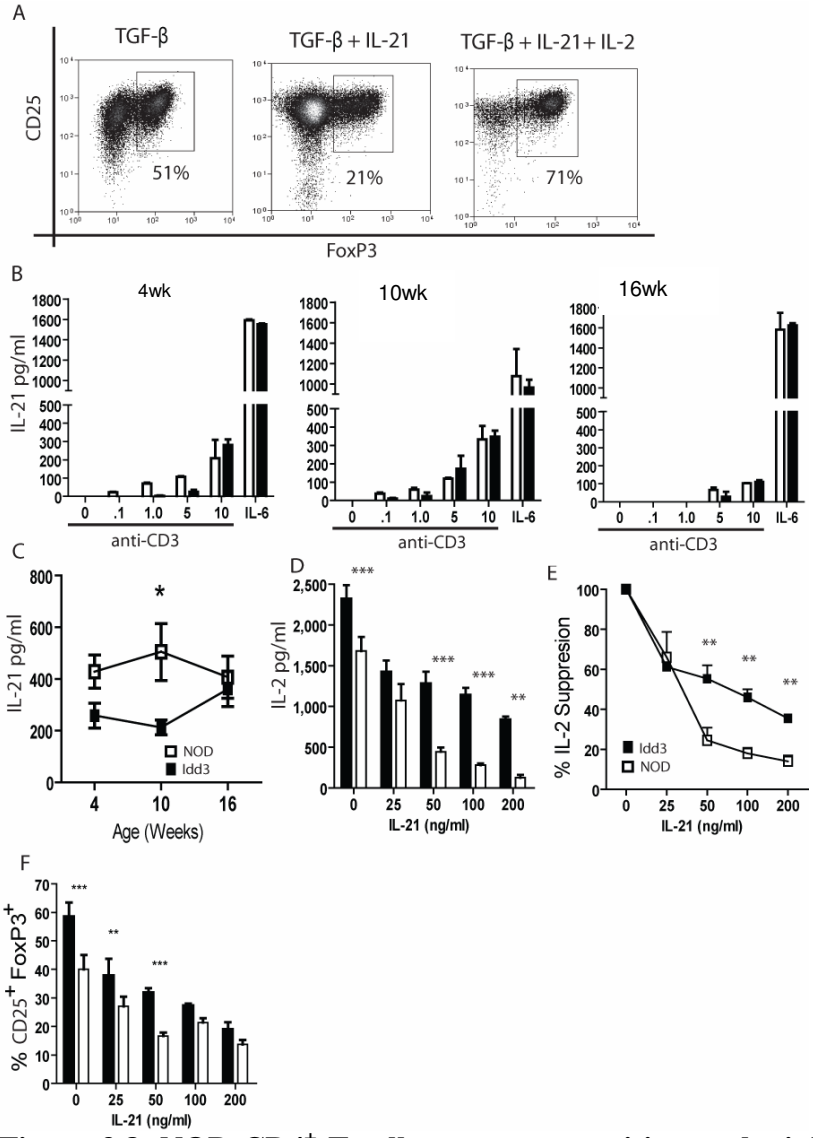

Figure 3.8 NOD CD4$^+$ T cells are more sensitive to the inhibitory effects of IL-21. A.) Induction of FoxP3 was assessed for NOD and NOD.Idd3 naïve CD4$^+$ T cells cultured with TGF-β alone, TGF-β and IL-21 (100 ng/ml) or TGF-β + IL-21 (100 ng/ml) + IL-2 (20 ng/ml) and stimulated with plate bound anti-CD3 and anti-CD28. The dot plots are representative of 3 separate experiments. B.) Naïve CD4$^+$ T cells from 4, 10 or 16 week old female mice were stimulated with varying concentrations of plate-bound anti-CD3 (10, 5, 1, 0.01 µg/ml anti-CD3) and 2 µg/ml soluble anti-CD28, and IL-21 measured in supernatants 4 days post stimulation. C.) Systemic IL-21 levels were determined by measuring serum from female NOD (□, n=7-10) and NOD.Idd3 (■, n=6-9) at 4, 10 and 16 weeks of age by ELISA (statistical significance was determined using two-way ANOVA, (*, $p<0.05$)). D.) Naïve CD4$^+$ T cells were cultured with plate-bound anti-CD3 and varying concentrations of rIL-21, and IL-2 measured from the culture supernatants after 24 hrs. E.) The percent inhibition of IL-2 secretion by IL-21 was determined by dividing the levels of IL-2 in the presence of IL-21 by IL-2 secretion in the absence of IL-21. F.) The frequency of FoxP3 induction after three days of culture in the presence of IL-21.

3.6 REFERENCES

1. Bach, J.F. 1994. Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr Rev* 15:516-542.

2. Chatenoud, L., E. Thervet, J. Primo, and J.F. Bach. 1994. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 91:123-127.

3. Chatenoud, L., J. Primo, and J.F. Bach. 1997. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. *J Immunol* 158:2947-2954.

4. You, S., M. Belghith, S. Cobbold, M.A. Alyanakian, C. Gouarin, S. Barriot, C. Garcia, H. Waldmann, J.F. Bach, and L. Chatenoud. 2005. Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. *Diabetes* 54:1415-1422.

5. Brusko, T.M., C.H. Wasserfall, M.J. Clare-Salzler, D.A. Schatz, and M.A. Atkinson. 2005. Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes. *Diabetes* 54:1407-1414.

6. Pop, S.M., C.P. Wong, D.A. Culton, S.H. Clarke, and R. Tisch. 2005. Single cell analysis shows decreasing FoxP3 and TGFbeta1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes. *J Exp Med* 201:1333-1346.

7. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101:455-458.

8. Shevach, E.M. 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2:389-400.

9. Ramsdell, F. 2003. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity* 19:165-168.

10. Chen, Y., V.K. Kuchroo, J. Inobe, D.A. Hafler, and H.L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265:1237-1240.

11. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J.E. de Vries, and M.G. Roncarolo. 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389:737-742.

12. Chen, Z., A.E. Herman, M. Matos, D. Mathis, and C. Benoist. 2005. Where CD4+CD25+ T reg cells impinge on autoimmune diabetes. *J Exp Med* 202:1387-1397.

13. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.

14. Bennett, C.L., J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20-21.

15. Brunkow, M.E., E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27:68-73.

16. Chatila, T.A., F. Blaeser, N. Ho, H.M. Lederman, C. Voulgaropoulos, C. Helms, and A.M. Bowcock. 2000. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J Clin Invest* 106:R75-81.

17. Bluestone, J.A., and H. Boehmer. 2006. Regulatory T cells. *Semin Immunol* 18:77.

18. Zheng, S.G., J. Wang, P. Wang, J.D. Gray, and D.A. Horwitz. 2007. IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. *J Immunol* 178:2018-2027.

19. Conti, P., D. Kempuraj, S. Frydas, K. Kandere, W. Boucher, R. Letourneau, B. Madhappan, K. Sagimoto, S. Christodoulou, and T.C. Theoharides. 2003. IL-10 subfamily members: IL-19, IL-20, IL-22, IL-24 and IL-26. *Immunol Lett* 88:171-174.

20. Szanya, V., J. Ermann, C. Taylor, C. Holness, and C.G. Fathman. 2002. The subpopulation of CD4+CD25+ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. *J Immunol* 169:2461-2465.

21. Ermann, J., P. Hoffmann, M. Edinger, S. Dutt, F.G. Blankenberg, J.P. Higgins, R.S. Negrin, C.G. Fathman, and S. Strober. 2005. Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. *Blood* 105:2220-2226.

22. Antony, P.A., C.M. Paulos, M. Ahmadzadeh, A. Akpinarli, D.C. Palmer, N. Sato, A. Kaiser, C.S. Hinrichs, C.A. Klebanoff, Y. Tagaya, and N.P. Restifo. 2006. Interleukin-2-dependent mechanisms of tolerance and immunity in vivo. *J Immunol* 176:5255-5266.

23. Furtado, G.C., M.A. Curotto de Lafaille, N. Kutchukhidze, and J.J. Lafaille. 2002. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* 196:851-857.

24. Malek, T.R., A. Yu, V. Vincek, P. Scibelli, and L. Kong. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 17:167-178.

25. Setoguchi, R., S. Hori, T. Takahashi, and S. Sakaguchi. 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* 201:723-735.

26. Thornton, A.M., E.E. Donovan, C.A. Piccirillo, and E.M. Shevach. 2004. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. *J Immunol* 172:6519-6523.

27. Davidson, T.S., R.J. DiPaolo, J. Andersson, and E.M. Shevach. 2007. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J Immunol* 178:4022-4026.

28. Spolski, R., M. Kashyap, C. Robinson, Z. Yu, and W.J. Leonard. 2008. IL-21 signaling is critical for the development of type I diabetes in the NOD mouse. *Proc Natl Acad Sci U S A* 105:14028-14033.

29. Hinrichs, C.S., R. Spolski, C.M. Paulos, L. Gattinoni, K.W. Kerstann, D.C. Palmer, C.A. Klebanoff, S.A. Rosenberg, W.J. Leonard, and N.P. Restifo. 2008. IL-2 and IL-21 confer opposing differentiation programs to CD8+ T cells for adoptive immunotherapy. *Blood* 111:5326-5333.

30. Zhou, L., Ivanov, II, R. Spolski, R. Min, K. Shenderov, T. Egawa, D.E. Levy, W.J. Leonard, and D.R. Littman. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 8:967-974.

31. Zeng, R., R. Spolski, E. Casas, W. Zhu, D.E. Levy, and W.J. Leonard. 2007. The molecular basis of IL-21-mediated proliferation. *Blood* 109:4135-4142.

32. Ozaki, K., R. Spolski, C.G. Feng, C.F. Qi, J. Cheng, A. Sher, H.C. Morse, 3rd, C. Liu, P.L. Schwartzberg, and W.J. Leonard. 2002. A critical role for IL-21 in regulating immunoglobulin production. *Science* 298:1630-1634.

33. King, C. 2008. T helper cell differentiation: IL-21 and T helper cell differentiation: Jack of all trades? *Immunol Cell Biol* 86:554-556.

34. Sonderegger, I., J. Kisielow, R. Meier, C. King, and M. Kopf. 2008. IL-21 and IL-21R are not required for development of Th17 cells and autoimmunity in vivo. *Eur J Immunol* 38:1833-1838.

35. Herber, D., T.P. Brown, S. Liang, D.A. Young, M. Collins, and K. Dunussi-Joannopoulos. 2007. IL-21 has a pathogenic role in a lupus-prone mouse model and its blockade with IL-21R.Fc reduces disease progression. *J Immunol* 178:3822-3830.

36. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-238.

37. Fox, C.J., A.D. Paterson, S.M. Mortin-Toth, and J.S. Danska. 2000. Two genetic loci regulate T cell-dependent islet inflammation and drive autoimmune diabetes pathogenesis. *Am J Hum Genet* 67:67-81.

38. Atkinson, M.A., and N.K. Maclaren. 1993. Islet cell autoantigens in insulin-dependent diabetes. *J Clin Invest* 92:1608-1616.

39. Redondo, M.J., M. Rewers, L. Yu, S. Garg, C.C. Pilcher, R.B. Elliott, and G.S. Eisenbarth. 1999. Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study. *Bmj* 318:698-702.

40. King, C., A. Ilic, K. Koelsch, and N. Sarvetnick. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 117:265-277.

41. Encinas, J.A., L.S. Wicker, L.B. Peterson, A. Mukasa, C. Teuscher, R. Sobel, H.L. Weiner, C.E. Seidman, J.G. Seidman, and V.K. Kuchroo. 1999. QTL influencing autoimmune diabetes and encephalomyelitis map to a 0.15-cM region containing Il2. *Nat Genet* 21:158-160.

42. Podolin, P.L., M.B. Wilusz, R.M. Cubbon, U. Pajvani, C.J. Lord, J.A. Todd, L.B. Peterson, L.S. Wicker, and P.A. Lyons. 2000. Differential glycosylation of interleukin 2, the molecular basis for the NOD Idd3 type 1 diabetes gene? *Cytokine* 12:477-482.

43. Lyons, P.A., N. Armitage, F. Argentina, P. Denny, N.J. Hill, C.J. Lord, M.B. Wilusz, L.B. Peterson, L.S. Wicker, and J.A. Todd. 2000. Congenic mapping of the type 1 diabetes locus, Idd3, to a 780-kb region of mouse chromosome 3: identification of a candidate segment of ancestral DNA by haplotype mapping. *Genome Res* 10:446-453.

44. del Rio, R., R. Noubade, M. Subramanian, N. Saligrama, S. Diehl, M. Rincon, and C. Teuscher. 2008. SNPs upstream of the minimal promoter control IL-2

expression and are candidates for the autoimmune disease-susceptibility locus Aod2/Idd3/Eae3. *Genes Immun* 9:115-121.

45. Schimpl, A., I. Berberich, B. Kneitz, S. Kramer, B. Santner-Nanan, S. Wagner, M. Wolf, and T. Hunig. 2002. IL-2 and autoimmune disease. *Cytokine Growth Factor Rev* 13:369-378.

46. Schoenberger, S.P., and E.M. Janssen. 2006. IL-2 gets with the program. *Nat Immunol* 7:798-800.

47. Malek, T.R. 2003. The main function of IL-2 is to promote the development of T regulatory cells. *J Leukoc Biol* 74:961-965.

48. Clough, L.E., C.J. Wang, E.M. Schmidt, G. Booth, T.Z. Hou, G.A. Ryan, and L.S. Walker. 2008. Release from regulatory T cell-mediated suppression during the onset of tissue-specific autoimmunity is associated with elevated IL-21. *J Immunol* 180:5393-5401.

49. Sen, P., M.A. Wallet, Z. Yi, Y. Huang, M. Henderson, C.E. Mathews, H.S. Earp, G. Matsushima, A.S. Baldwin, Jr., and R.M. Tisch. 2007. Apoptotic cells induce Mer tyrosine kinase-dependent blockade of NF-kappaB activation in dendritic cells. *Blood* 109:653-660.

50. Poligone, B., D.J. Weaver, Jr., P. Sen, A.S. Baldwin, Jr., and R. Tisch. 2002. Elevated NF-kappaB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. *J Immunol* 168:188-196.

51. Yamanouchi, J., D. Rainbow, P. Serra, S. Howlett, K. Hunter, V.E. Garner, A. Gonzalez-Munoz, J. Clark, R. Veijola, R. Cubbon, S.L. Chen, R. Rosa, A.M. Cumiskey, D.V. Serreze, S. Gregory, J. Rogers, P.A. Lyons, B. Healy, L.J. Smink, J.A. Todd, L.B. Peterson, L.S. Wicker, and P. Santamaria. 2007. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat Genet* 39:329-337.

CHAPTER 4

FUTURE PERSPECTIVES

4.1 Gene Gun Delivered pDNA Induces Type 2 Effectors: How?

pDNA vaccination is one approach of antigen-specific immunotherapy that has proven to be an effective strategy for preventing autoimmunity in experimental models including the NOD mouse. From our studies and those of others, the success of pDNA vaccination is highly contingent upon the modality by which pDNA is delivered, the β cell autoantigen(s) targeted, and the use of "helper" plasmids to assist in skewing the immune reaction. Here, we established an approach in which pDNA encoding a fragment of GAD65 was injected into the epidermal and dermal layers via gene gun, resulting in the induction of type 2 immunoregulatory T cells capable of preventing diabetes in NOD mice. Given the fact that not all NOD mice were protected from diabetes underscores the necessity for improved treatment conditions before this therapy can be applied to the clinic.

A key issue that was not addressed in our study is the mechanism by which gene gun delivery of pGAD65 preferentially induces IL-4 secreting immunoregulatory effector T cells. IL-4 is essential for the differentiation of naïve $CD4^+$ T cells into type 2 effector T cells *in vivo*. How IL-4 induction might occur upon biolistic delivery of pDNA is not entirely clear but other studies have noted similar findings to ours (1, 2). The cellular composition of the skin and draining lymph nodes is likely to be a key factor. For instance, skin is known to possess "tolerogenic" properties which may in part be due to Langerhan DC (3, 4). Another possibility is that T cell differentiation is in part dependent on the quality and/or magnitude of the TCR signaling events. As described in

Chapter 2, GAD65-Ig serum levels are significantly higher in NOD mice treated with pGAD65 delivered via gene gun versus i.m. injection. Thus, it is possible that the "strength" of TCR stimulation due to increased antigen availability at the site of pDNA injection may also promote a predominant type 2 T cell phenotype.

Additional experiments are needed to address the possible effect of the Ig scaffold of the GAD65 recombinant on type 2 T effector cell differentiation. This is of particular interest in view of a study by Bach *et al.* in which gene gun delivery of pDNA encoding full-length GAD65 failed to delay or prevent diabetes in NOD mice. The immunotherapeutic effect of GAD65-Ig may in part be attributed to Ig binding by Fc receptors (FcR) on APC. There are four types of FcR (I-IV) that can either activate or suppress APC. GAD65-Ig binding of inhibitory FcRIIγ receptors may "condition" APC to preferentially induce type 2 T effectors.

Defining the key events that promote immunoregulatory T cells will provide important insight needed to improve the efficacy of gene gun delivery of pDNA vaccination and its possible clinical application.

4.2 A Move Towards IL-2 Therapy?

A significant effort has gone into identifying the gene(s) residing in *Idd3* that is associated with diabetes susceptibility. Of particular interest is the *il2* gene. For instance, several observations suggest that deficient IL-2 expression leads to ineffective induction

and maintenance of "natural" and "adaptive" FoxP3^{+}Treg (5, 6). However, work by King *et al.* indicates IL-21, another gene residing in the *Idd3* locus, enhances homeostatic expansion of β cell-specific pathogenic T cells (7). Our findings indicate that both IL-2 and IL-21 play integral roles in regulating the balance between FoxP3^{+}Treg and pathogenic effector T cells. These results also highlight the complexity associated with the control of immunoregulatory "networks" in the periphery. Subtle changes in the level of cytokine expression (e.g. IL-2) and/or responses to a given cytokine (e.g. IL-21) appear to have marked effects on the progression of autoimmunity. Key questions that still need to be addressed include the relative contribution of IL-2 and IL-21 in driving β cell autoimmunity, and the "physiological" levels of IL-2 required for "appropriate" maintenance and/or induction of FoxP3^{+}Treg. Of particular interest is defining the role of IL-2 in the dichotomy that exists between CD62L^{high}FoxP3^{+}Treg and CD62L^{low}FoxP3^{+}Treg in NOD.Idd3 and NOD mice, respectively. Finally, the effect of IL-2 on FoxP3^{+}Treg can be exploited for the purpose of immunotherapy. Recent studies ongoing in the laboratory suggest that β cell autoimmunity can be effectively suppressed in AAV-Tet-On-IL-2 treated NOD mice after a short course of IL-2 induction.

4.3 References

1. Weiss, R., S. Scheiblhofer, J. Freund, F. Ferreira, I. Livey, and J. Thalhamer. 2002. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that over-rules the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. *Vaccine* 20:3148-3154.

2. Scheiblhofer, S., A. Stoecklinger, C. Gruber, C. Hauser-Kronberger, B. Alinger, P. Hammerl, J. Thalhamer, and R. Weiss. 2007. Gene gun immunization with clinically relevant allergens aggravates allergen induced pathology and is contraindicated for allergen immunotherapy. *Mol Immunol* 44:1879-1887.

3. Kaplan, D.H., M.O. Li, M.C. Jenison, W.D. Shlomchik, R.A. Flavell, and M.J. Shlomchik. 2007. Autocrine/paracrine TGFbeta1 is required for the development of epidermal Langerhans cells. *J Exp Med* 204:2545-2552.

4. Kaplan, D.H., M.C. Jenison, S. Saeland, W.D. Shlomchik, and M.J. Shlomchik. 2005. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* 23:611-620.

5. Yamanouchi, J., D. Rainbow, P. Serra, S. Howlett, K. Hunter, V.E. Garner, A. Gonzalez-Munoz, J. Clark, R. Veijola, R. Cubbon, S.L. Chen, R. Rosa, A.M. Cumiskey, D.V. Serreze, S. Gregory, J. Rogers, P.A. Lyons, B. Healy, L.J. Smink, J.A. Todd, L.B. Peterson, L.S. Wicker, and P. Santamaria. 2007. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat Genet* 39:329-337.

6. Feuerer, M., W. Jiang, P.D. Holler, A. Satpathy, C. Campbell, M. Bogue, D. Mathis, and C. Benoist. 2007. Enhanced thymic selection of FoxP3+ regulatory T cells in the NOD mouse model of autoimmune diabetes. *Proc Natl Acad Sci U S A* 104:18181-18186.

7. King, C., A. Ilic, K. Koelsch, and N. Sarvetnick. 2004. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 117:265-277.