Studying the role of naturally-occurring regulatory T cells in a model of type 1 diabetes

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ABSTRACT

T_{reg} cells counter-balance autoreactive immune cells in healthy individuals. In the absence of T_{reg} cells, multi-organ immune diseases manifest. Thus, T_{reg} cell defects are suspected to contribute to T1D. Currently, it remains unclear whether T_{reg} cell defects are responsible for the manifestation of T1D. Thus, we hypothesized that T_{reg} cell defects results in T1D. We observed that T_{reg} cells are present in normal frequencies in the thymus and peripheral immune system of diabetes-prone mice. Furthermore, thymic and peripheral T_{reg} cells are operative and mediate regulation by suppressing effector T (T_{eff}) cells in the pancreatic lymph nodes and pancreas. Specifically, regulation corresponded with increased frequencies of T_{reg} cells and reduced in diabetogenic T cells in both sites, and controlled insulitis. However, as the immune response against β cells of the pancreatic islets progresses in prediabetic mice, peripheral T_{reg} cells wane in function and cannot sustain sufficient T_{eff} cell regulation in the pancreatic lymph nodes and pancreas. In summary, this study characterizes areas of T_{reg} cell immunoregulation of T1D; and highlights a T_{reg} cell dysfunction, not a quantitative T_{reg} cell defect, manifesting in the peripheral immune system prior to diabetes onset but following T cell activation, which contributes to the escape of autoreactive T cells.

Résumé

Le rôle des cellules régulatrices T (T_{reg}) naïve dans le diabète de type 1 (T1D) est mal compris. Nous avons émis comme hypothèse que le développement et la fonction des cellules T_{reg} sont normals avant l'apparition du diabète, mais que l'évolution de la maladie est le résultat de la perte de l'immuno-régulation assurée par les cellules T_{reg}. Nous avons observé une tolérance dominante par les cellules régulatrices T_{reg} dans les ganglions pancréatiques ainsi que dans le pancréas. Dans ces deux sites, nous démontrons que la régulation effectuée par les cellules T_{reg} corrèle avec une fréquence accrue de ces dernières et une réduction du nombre de cellules diabétogeniques en plus du contrôle de l'insulite. Par contre, le mécanisme de régulation observé chez les T_{reg} devient inefficace lorsque les souris sont pré-diabétique. Ainsi, notre étude a permis de caractériser de manière rigoureuse plusieurs domaines de l'immuno-régulation dans le modèle T1D et ainsi démontrer qu'une mauvaise régulation de la part des cellules T_{reg} peut modifier le fragile équilibre de la discrimination soi et non-soi et ainsi permettre la survie des cellules T auto-réactive.

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ABBREVIATIONS

T1D: type 1a diabetes

T_{reg} cell: naturally-occurring CD4+Foxp3+ regulatory T cell

IPEX: immunodysregulation polyendrocrinopathy enteropathy X-linked syndrome

FOXP3: (human) *forkhead* box protein 3

Foxp3: (murine) *forkhead* box protein 3

TF: transcription factor

T_{eff} cell: effector T cell

iT_{reg}: induced CD4+ T_{reg} cell

NOD: non-obese diabetic (mouse/mice)

IDD: (man) insulin-dependent diabetes (loci)

Idd: (mouse) insulin-dependent diabetes (loci)

I-A^{g7}: H-2^{g7} (MHC II, NOD variant)

I-A^{b}: H-2^{b} (MHC II, C57/B6 variant)

IL-2: interleukin-2

OTII: OVA-specific CD4 TCR transgenic

p.t.: post transfer

$CD4^{SP}$: $CD4^{+}CD8^{-}$

CFSE: carboxyfluorescin diacetate succinimidyl ester

H/E: hematoxylin/eosin

CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1. Type 1a Diabetes

i. Background

Type 1a diabetes (T1D) is the result of a deficiency in insulin, a hormone involved in blood glucose homeostasis, which is caused by immune-mediated destruction of the insulin producing β-cells located in the islets of Langerhans of the pancreas. T1D represents approximately 10% of all diabetic individuals. Other forms of diabetes manifest as a result of insulin deficiency with no evidence of autoimmunity (Type 1b), insulin resistance (Type 2; over 85% of individuals with diabetes), genetic defects related to insulin resistance (Type 3b) or the pancreatic β-cells (Type 3a), diseases of the pancreas (Type 3c), hormonal defects (Type 3d), chemicals or drugs (Type 3e), and pregnancy (Type 4). Nonetheless, it is estimated that T1D affects 10-20 million individuals worldwide, annually.

The etiology of T1D is unknown. However, studies have demonstrated that the distribution of T1D across a population varies as a result of geographical location, race and ethnic background [1], age [2-4], and genetic susceptibility [5, 6]. The incidence of T1D is higher in developed countries relative to undeveloped countries [7]. Furthermore, additional variables include dietary factors [8], seasonal variability [9, 10], and exposure to viruses [11].

In the pancreas of individuals developing T1D, the destruction of a critical number of insulin-producing β-cells results in hyperglycemia (>200mg/dL or 11mmol/L). Subsequently, glucosuria, excessive urination, thirst, dehydration, diabetic ketoacidosis, and weight loss follow. In the absence of insulin therapy, T1D is a lethal disorder. Treatment of T1D involves subcutaneous administration of insulin in order to maintain adequate glucose homeostasis. Recombinant human insulin is administered 2-4 times daily. The specific insulin requirement is dependent on daily blood glucose monitoring and the formula of the recombinant human insulin (short-acting, intermediate-acting, and/or long-acting).

Follow-ups with medical professionals are also required to ensure adequate control of glucose homeostasis. Furthermore, it is critical that healthy lifestyle factors (such as proper dietary intake, exercise, and abstinence from cigarette smoking and alcohol) be exercised. Thus, a lot is necessary to minimize the manifestations of any acute and long-term complications. However, all diabetic individuals inevitably encounter episodes of hypoglycemia, hyperglycemia, diabetic ketoacidosis, and increased risk of infection. Aside from these acute complications, long-term microvascular (i.e. diabetic retinopathy) or macrovascular (i.e. coronary disease) complications may manifest. The costs associated with medical care for T1D individuals is 5$ billion per year, 10 times

more than individuals without diabetes [3]. Consequently, insulin is not a cure, although it is currently the best method to manage diabetes.

To provide more effective treatment, other therapies, albeit experimental, have emerged. The most popular is allogeneic islet transplantation [12-14], but is limited by the availability of donor pancreata. Furthermore, this method does not restore β islet function indefinitely [13, 14] due to graft rejection and a memory anti-β islet cell immune response. Similar approaches with different limitations include xenotransplantation [15], cell (gene) therapy [16], and allogeneic or syngeneic stem cell therapy [16]. To complement therapies that restore the β islet cell population, pharmaceuticals and/or cellular therapy are being developed for suppressing or regulating the immune nature of T1D [16-21]. Simultaneously, researchers are clarifying the mechanism of this immune disorder to provide additional therapeutic targets.

ii. Models of T1D: nontransgenic and transgenic NOD mice

The development of the non-obese diabetic (NOD) mouse by Makino et al. [22] occurred serendipitously during an attempt to develop a cataract model. By random mutagen-induced mutations of the mouse genome, they introduced sufficient subtle immune defects to predispose the manifestation of spontaneous autoimmune diabetes. Afterwards, genetic analyses would demonstrate similarities between susceptibility genes in individuals with T1D and NOD mice

[5]. Furthermore, these similar diabetes susceptibility genes in man [23] and mouse [24] contribute to immune system dysregulation. Thus, the NOD mouse provides an excellent platform for the analysis of T1D.

Already, NOD mice have contributed significantly in dissecting the role of each immune cell, and assembling plausible immunological mechanism and various tolerance mechanisms in T1D [24, 25]; and identifying lifestyle and environmental factors [25], as well as hundreds of immunotherapeutics [19]. However, the mechanism of diabetogenesis is still ill-defined as reflected by the outcomes of these immunotherapies, which produce mixed results [19].

Recent studies have involved the use of transgenics to rigorously elucidate additional contributors of T1D [26, 27]. Of particular significance are the T cell receptor (TCR) transgenic mice which express a TCR transgene derived from diabetogenic effector T (T_{eff}) cells located in the pancreatic infiltrate of diabetic NOD mice (for example, the $CD4^+$ TCR transgenic BDC2.5 mouse [28] and the $CD8^+$ TCR transgenic 8.3 mouse [29]). These mice have contributed significantly to resolving T-cell associated questions, such as the relevance of $CD4^+$ or $CD8^+$ T cells in the initial phases of diabetes, and identifying autoantigens that contribute to T1D pathogenesis [27, 30].

T cells in the BDC2.5 mice are restricted to a $V\alpha1$, $V\beta4$ TCR repertoire specific for an unknown β-islet antigen recognized by $CD4^+$ T cells.

Correspondingly, the CD4+ T cell population in BDC2.5 mice is diabetogenic. Thus, unlike wild-type NOD mice that develop diabetes and/or other spontaneous organ-specific autoimmune diseases (i.e. sialoadenitis, thyroiditis, and autoimmune peripheral polyneuropathy [24]), BDC2.5 mice specifically develop autoimmune diabetes. Regarding the kinetics of diabetes development, BDC2.5 mice exhibit a delayed diabetes incidence relative to wild-type NOD mice [28]. However, rapid-onset diabetes manifests in BDC2.5 when crossed to $RAG^{-/-}$ mice, which eliminates endogenous recombination of the TCRα gene during T cell development and produces a monoclonal T_{eff} cell population [31]. Therefore, BDC2.5 mice model a mechanism of T cell pathogenesis, as well as a mechanism of T cell regulation of T1D.

iii. Susceptibility to T1D

A partial list of T1D susceptibility loci, designated by the prefix IDD/idd (insulin-dependent diabetes), and genes is associated with this complex polygenic autoimmune disease. IDD1/idd1 corresponds to the class II major histocompatibility complex (MHC II) genes. IDD2 locus is associated with the insulin gene. In addition, CTLA-4 and PTPN22 are also associated with T1D susceptibility. Other susceptibility loci (more than a dozen) and their corresponding candidate gene(s) remain unknown because of difficulties differentiating between true and false associations; however, more extensive and

larger linkage and association studies may lead to the identification of these genetic contributors [5, 6, 32]. Notably, immunological studies have identified other significant contributors in T1D, such as costimulatory molecules, cytokines, and qualitative changes

MHC II, associated with IDD1/idd1, is a heterodimeric peptide binding protein that functions in multiple immunological processes, as it is essential for antigen presenting cells to display (self and non-self) processed antigens to $CD4^+$ T cells. MHC II polymorphisms are localized to the antigen binding pocket. Thus, these polymorphisms impact the repertoire of antigens present in the antigen binding pocket, which are presented to T cells. In T1D, the susceptibility MHC II genes causes characteristic peptide-binding problem(s) [33]. Therefore, MHC II susceptibility alleles may reflect defective autoantigen-MHC II interactions. The MHC II alleles associated with the IDD1/idd1 locus are HLA-DQ/DR variants in man, and H-2^{g7} in mouse. Meanwhile, other MHC II alleles exhibit varying degrees of susceptibility/protection to T1D [34]. Furthermore, MHC II alleles (including the former and the latter) can impact the development of other autoimmune diseases [35]. However, the physiological significance of multiple MHC II molecular variants in a host has yet to be evaluated. Nevertheless, it is the highest genetic contributor of T1D susceptibility and is necessary, but not sufficient, for the development of T1D [36].

The insulin gene, associated with the IDD2/idd2 locus, does not affect the pancreatic β cells. Instead, these polymorphisms reduce thymic expression of insulin [5], which is necessary for central tolerance deletion of insulin-specific T cells. Furthermore, the significance of insulin is observed by the presence of insulin-specific autoantibodies in susceptible individuals prior to T1D development. Notably, insulin is the only autoantigen specifically produced by β cells. Thus, it may seem to be the only target capable of producing an immune response leading to T1D. However, other β cell autoantigens are also believed to contribute to diabetes development: GAD, insulinoma-associated protein 2, and heat shock protein 60. All autoantigens have been implicated in T cell activation; however, the relative significance of each antigen is currently unknown. Therefore, an immune response against β cells may be triggered by a single autoantigen; alternatively, a number of theories (epitope spreading, molecular mimicry, and defective tolerogenic processes [24]) propose mechanisms that may include numerous autoantigens.

Costimulatory molecules have numerous functions in the immune system, but are classically thought as essential to activation requirements for T cells. The absence of T cell costimulation by B7 molecules and CD40-CD40L interaction, in B7-2$^{-/-}$ NOD mice and CD40L$^{-/-}$ NOD mice, increased the population of autoreactive T cells [37]. Blockade of the CTLA-4 molecule, a negative regulator

of T cell activation, induced autoimmune diabetes in neonates[38]. PD-1, another co stimulatory molecule, is expressed on inflamed β islet cells, and PD-1 antibody blockade induced autoimmune diabetes [38]. Similarly, ICOS antibody blockade also induced diabetes [24]. Costimulatory molecules can also drive the T cell differentiation into either a T_{H1} or T_{H2} immune response, which exacerbates or prevents T1D, respectively [39]. Thus, the mechanisms of costimulatory molecules in T1D development are widespread.

Cytokines have similar widespread implications on the immune system, and correspondingly on the development of T1D. Besides its role in T_{H1} or T_{H2} immune responses, IL-10 and TGFβ are observed in the pancreatic lesion, and can inhibit T1D onset [24]. However, the roles of these molecules are inter-related and complex and the significance of cytokine molecules are not entirely known. For example, a paradoxical observation with IL-12, a T_{H1} cytokine, has been made: IL-12 has been shown to accelerate diabetes in NOD mice [40]; while IL-12 knockout NOD mice develop diabetes normally [41].

Qualitative changes to T cells may also contribute to diabetes development. In normal healthy individuals, autoreactive T cells can escape thymic negative selection and are present in the peripheral immune system. Furthermore, these autoreactive T cells can become more pathogenic through a process that promotes the survival of autoreactive T cells with increasingly higher

TCR avidity for a specific autoantigen, this mechanism is known as avidity maturation. Thus far, this mechanism has only been observed in the $CD8^+$ T cell population [42, 43]. Other qualitative changes have been characterized in the $CD4^+$ T cell population. Pathogenic $CD4^+$ T_{eff} cells in NOD mice become progressively less sensitive to immunoregulation, show higher proliferative capacity, higher IFN-γ production, and less IL-4 and IL-10 cytokine production [44]. Qualitative defects have also been assessed at the genetic level: the idd3 locus, associated with the IL-21 (and IL-2) cytokine, may cause aberrant qualitative changes in T cell autoreactivity through an IL-21 dependent mechanism [45].

iv. A mechanism of T1D

The etiology or trigger responsible for the onset of T1D is currently unknown. Nonetheless, a number of hypotheses have surfaced to explain the etiology of T1D and other autoimmune disorders. The most prevalent theories include: molecular mimicry [11], the hygiene hypothesis [7], beneficial or protective autoimmunity [46, 47], lymphopenia-induced autoimmunity [48, 49], and defects in tolerance [24, 50-52]. Notably, these theories are not necessarily mutually exclusive; and thus, they highlight the complex nature of T1D/autoimmunity. Therefore, the T1D-trigger may arise for numerous reasons,

through the interplay of multiple factors: genetic, lifestyle (dietary factors), and environmental (infection).

The immune response against insulin-producing β islet pancreatic cells resulting in T1D is often described using the checkpoint theory. It is described by two checkpoints (or intervals), but can include up to four distinct intervals prior to T1D onset: healthy, peri-insulitis, controlled-insulitis (checkpoint 1), and destructive-insulitis (checkpoint 2). These sequential intervals are localized near or at the β islets and are increasingly immunoreactive. Initially in a healthy state, immune cells do not localize to the pancreas. However, the trigger results in the accumulation of an immune infiltrate to surround the β islets. The immune cells are composed of a nonpathogenic population of monocytes such as dendritic cells, macrophages and NK cells – and characterized by the absence of $CD4^+$ and $CD8^+$ T cells. This stage is known as peri-insulitis. This interval underscores the relevance of the innate immune population. Depletion of macrophages and dendritic cells in already established (peri-)insulitis, reverses autoimmunity [20]. Similarly, NK cell depletion inhibits diabetes development [53]. The next stage is known as insulitis (and transitions through checkpoint 1), which is characterized by the invasion and subsequent destruction of β-islets. This stage is further characterized by the accumulation of T cells, which follow a classical pathway of activation in the pancreatic-draining lymph nodes prior to trafficking and

expanding in the site of inflammation (Figure 1) [54]. Of note, both β-cell specific $CD4^+$ T cells and $CD8^+$ T cells are necessary for β-cell destruction. Considerable importance is given to the $CD4^+$ T cell population in the induction of T1D - as observed, $CD4^+$ T cells from prediabetic and diabetic donors transferred diabetes [55]. The final stage involves the transition from a controlled-insulitic state to full-blown destructive insulitis (checkpoint 2), resulting in the destruction of above 90% of the β islets and onset of diabetes.

The kinetics of this response has been mapped in NOD mice. Following a physiological wave of β cell death occurring at 15-18 days of age, T cells become activated in the pancreatic lymph nodes [56]. Insulitis appears at 4 weeks of age, and is well-established by 6-8 weeks. Thereafter, onset of diabetes occurs between 12 and 30 weeks, and reaches a maximal incidence of 80% in mice colonies. Notably, the kinetics of T1D in man is not as predictable. The distribution of T1D onset across a population of individuals exhibits a scattered frequency across one year to forty-nine years of age [57]. Environmental factors are thought to account for this distribution in man, which can also influence diabetes onset in NOD mice.

2. Regulatory T cells in T1D

i. Immunoregulation of autoreactivity

Any individual, whether they are healthy or prone to autoimmune disorders contain autoreactive immune cells. Thus, the threat of autoimmunity is presence in every individual. However, regulatory or tolerance mechanisms function in the immune system to circumvent this threat. These mechanisms are present during development in the thymus and, afterwards, in the periphery.

In the thymus, tolerance mechanisms are essential for the deletion of autoreactive T cells through a process called negative selection. Negative selection of autoreactive T cells occurs as a result of high avidity interactions with MHC molecules. Furthermore, negative selection can specifically delete autoantigen-specific T cells, like insulin-specific T cells, through the AIRE transcription factor mechanism of thymic autoantigen expression [5]. In the absence of this regulatory mechanism, $AIRE^{-/-}$ mice ultimately develop multi-organ specific autoimmunity [58].

In the periphery, regulatory mechanisms are necessary because autoreactive T cells can escape the thymus and enter the periphery. For example, the IL-2 cytokine plays a crucial role in peripheral dominant tolerance mechanisms and activation-induced cell death of T cells [59]; and the TGFβ cytokine has anti-inflammatory effects on the immune system and may be

involved in development or function of cellular immunoregulation [60]. The cell surface Fas-FasL interaction is an important mediator of cell death during activation or in a target tissue [61]. Transcription factors, such as Foxp3, are responsible for the development and function of dominant tolerance mechanisms in the periphery [52]. Thus, cytokines, cell-surface molecules, and transcription factors have been identified as molecular signals responsible for controlling autoreactivity. Furthermore, peripheral mechanisms of regulation also include immune cells whose function is to regulate autoreactive cells, and are composed of a heterogeneous population of immune cells. Immunoregulatory capacities are believed to comprise subpopulations of immune cells: NK cells [62], B cells [63], DCs [64, 65], $CD4^+$ T cells [66], $CD8^+$ T cells [67, 68], NKT cells [69], and $\gamma\delta$ T cells [70]. Therefore, the immune system contains a broad network of mechanisms that protects against autoreactivity.

In the prevention of T1D, numerous mechanisms of immunoregulation have been characterized. IL-10 producing cells are protective and function in the pancreatic infiltrate [71, 72]. Other mechanisms include: decreasing pathogenicity in the T cell population with age [73], the two TCR hypothesis [74], and regulation mediated by $CD62L^+$ T cells [31], NKT cells [75], $CD4^+Dx5^+$ T cells [76], induced T_{reg} cells [77], DCs [78], NKT cells [75], $\gamma\delta$ T cells [79], double negative ($CD4^-CD8^-$) regulatory T cells, and $CD8^+$ regulatory T cells [80]. In

addition, considerable interactions likely exist between these subsets [80]. However, naturally-occurring regulatory T (T_{reg}) cells are fundamentally regarded as the suppressors of autoimmunity.

ii. Naturally-occurring T_{reg} cells: development, homeostasis, and function

T_{reg} cells develop in the thymus, and their developmental requirements are similar to classical $CD4^+$ T_{eff} cells, since $TCR^{-/-}$, $\gamma c^{-/-}$, or MHC $II^{-/-}$ mice, are each characterized by an absence in $CD4^+$ T_{reg} cells [81, 82] and $CD4^+$ T_{eff} cells. However, T_{reg} cells ontogeny is delayed compared to T_{eff} cells, as observed by 3 days thymectomy, which results in multi-organ autoimmune diseases; but thymectomy at a later time point does not result in autoimmunity because sufficient T_{reg} cells have already established themselves in the periphery [83]. Other unique features of T_{reg} cell ontogeny include their developmental (and functional) requirements for the forkhead transcription factor Foxp3 [84]. Thymic development of T_{reg} cells occurs through distinct interactions with the Hassal corpuscles, DCs and/or cortical epithelial cells [85, 86]. TCRs on T_{reg} cells are distinct: the ligand-TCR affinity is higher on T_{reg} cells than T_{eff} cells, but not sufficient to induce negative selection [87]. Furthermore, the TCR repertoire of T_{reg} cells is polyclonal; however, it is skewed toward self-antigens as a result of auto-antigen expression in the thymus [88].

In the periphery, T_{reg} cells comprise 5-10% of $CD4^+$ T cells in a healthy individual. T_{reg} cells require IL-2 for homeostasis, as observed in IL-$2^{-/-}$ knockout mice, which are T_{reg} cell deficient [82]. IL-2 is also required by T_{eff} cells for growth, differentiation and survival during an immune response [89]. Interestingly, T_{reg} cells are incapable of IL-2 production, while T_{eff} cells produce this cytokine. Therefore, T_{reg} cells receive their IL-2 requirement from other cells (i.e. activated T_{eff} cells). Other T_{reg} cell survival requirements are derived from peripheral antigen expression and interaction with particular cell subsets (i.e. B cells and DCs) [90].

The suppressive function of T_{reg} cells, as observed through *in vitro* experiments, is contact-dependent, cytokine-independent. They are anergic to *in vitro* stimulation unless exogenous IL-2 is added to the cultures. *In vivo*, these T_{reg} cells suppress in a cytokine-dependent (IL-10 and TGF-β) manner; however, evidence has recently identified *in vivo* contact dependent mechanisms[66, 91].

a. Markers of T_{reg} cells: Functional assays, CD25 and FOXP3/Foxp3

Through *in vitro* analysis, T_{reg} cells are known to mediate suppression via a contact-dependent and cytokine-independent mechanism. This feature of T_{reg} cell function is helpful to distinguish themselves from other regulatory T cells which may reside within the T cell population. Several types of regulatory T cells exist within the $CD4^+$ T cell population. Most are comprised by the term induced

regulatory T (iT_{reg}) cells, which develop their function during an immune response, unlike (naturally-occurring) T_{reg} cells that develop regulatory function in the thymus (Figure 2 and 3). Both T_{reg} cells and iT_{reg} cells are significant suppressors of autoimmunity, and regulators of immune responses. Their depletion leads to enhanced immunity against parasites, bacteria, viruses, allografts, cancer-antigens, and self-antigens. However, the mechanism of suppression differs between these $CD4^+$ T cell subsets, as iT_{reg} cells function via a contact-independent and cytokine-dependent mechanism. Consequently, *in vitro* assays provide insight concerning the mechanism of suppression, which is a useful marker for T_{reg} cells and iT_{reg} cells. In addition, cellular markers can identify T_{reg} cells from iT_{reg} cells (and other T cells).

The classical surface marker of (naturally-occurring) T_{reg} cells is CD25, the α chain of the IL-2 receptor complex, which is constitutively expressed on T_{reg} cells. Other laboratories have demonstrated that other markers ($CD45Rb^{lo}$, CD5, CTLA-4, GITR, and CD62L) can define the same $CD4^+$ T_{reg} cell population [31, 92-95]. Thus, the use of marker combinations might conceivably identify a single T_{reg} cell population. However, combinations of cell surface markers did not, but did identify functional heterogeneity with the T_{reg} cell population: $CD25^+CD62L^-$ T cells are selectively protective against gastritis and $CD25^+CD62L^+$ T cells are selectively protective against diabetes [96]. Therefore, cell surface markers are

limited in their capacity to identify T_{reg} cells. Further limitations exist with CD25 and other markers because they are upregulated upon activation on $CD4^+$ T_{eff} cells. Thus, the use of cell-surface markers is useful for T_{reg} cell identification in a naïve healthy individual, but become inadequate in activated, inflamed environments [97].

Recently, a more restrictive marker capable of identifying T_{reg} cells in a healthy and activated environment has been identified; FOXP3/Foxp3 (in man and mouse, respectively), *forkhead* box protein 3, belonging to the *forkhead* winged-helix family of transcription factors (TFs). Foxp3 TF consists of a zinc finger domain, leucine zipper domain, FKH (forkhead) domain, and repressor domain [98]. The zinc finger domain and the leucine zipper domain may be essential for forming homodimers and/or heterodimers. The FKH domain is common to the *forkhead* family and is necessary for DNA binding and nuclear localization. The repressor domain is thought to be responsible for suppressing other transcription factors, such as NFAT and $NF\text{-}_kB$ subunit P65 [99], resulting in the down-regulation of cytokine genes, such as IL-2, IL-4, and IFN-γ.

Foxp3 is a convincing T_{reg} cell marker since genetic and molecular FOXP3 gene defects in man and *scurfy* mice lack T_{reg} cells, resulting in severe wasting disease characterized by extensive mononuclear cell infiltration in the lungs, liver, thyroid, stomach, and pancreas known as immunodysregulation

polyendocrinopathy enteropathy X-linked syndrome (IPEX) [100-102]. Correspondingly, the administration of T_{reg} cells temporarily resolves this defect [81, 103]. Furthermore, immunotherapies involving retroviral Foxp3 gene transduction transforms T_{eff} cells into effective suppressors T_{reg} cells, as observed by *in vitro* assays and *in vivo* regulation of autoimmunity [84, 104, 105].

The implication of Foxp3 as a T_{reg} cell marker is best observed in studies conducted with Foxp3-GFP (green fluorescent protein) [81] fusion or –RFP (red fluorescent protein) bicistronic[103] knock-in mice, which can identify a cell based on their intranuclear Foxp3 expression. For example, Foxp3 was compared to the classical marker, CD25, and was shown through *in vitro* functional assays to be a better T_{reg} cell maker. Furthermore, Foxp3 was proved to be essential for T_{reg} cells in the thymus for their development, and in the periphery for their function [98]. As a side note, these studies determined the genes that are upregulated in T_{reg} cells, and also revealed another regulatory T cell subpopulation: the $CD8^{+}Foxp3^{+}$ T_{reg} cell compartment. Therefore, Foxp3, as well as these mice, are excellent tools for the isolation and study of T_{reg} cells.

Certain precautions with Foxp3 as a T_{reg} cell marker are important to consider, particularly during study in man. In man, not mouse, FOXP3 is complicated by the presence of two isoforms [98]. It is currently unknown whether both isoforms exist within the same T_{reg} cell; however, they are thought

to have similar functions. Nonetheless, this highlights a difference between the immune systems of man and mouse [106]. In addition, *in vitro* activated human T cells can upregulate FOXP3, which produces a phenotype and function that is indistinguishable from naturally-occurring FOXP3$^+$ T_{reg} cells [98]. In contrast, significant difficulty is encountered in mice to induce *in vitro* generation of Foxp3+ T_{reg} cells. Studies have demonstrated that TGFβ can induce T_{reg} cell generation [98, 107], but CTLA-4 may also be required for this process: TGFβ could not induce peripheral generation of Foxp3$^+$ T_{reg} cell in CTLA-4$^{-/-}$ mice or in the presence of CTLA-4 antibody blockade [107]. Meanwhile, other studies using very convincing systems that accurately follow Foxp3$^+$ T_{reg} cells, have been unable to induce *de novo* or TGFβ generation of T_{eff} cells into Foxp3$^+$ T_{reg} cells [81, 103]. Therefore, extra-thymic generation of Foxp3$^+$ T_{reg} cells is controversial, but Foxp3 remains essential for the analysis of T_{reg} cell biology in mouse and man.

iii. The mechanism of T_{reg} cells in T1D

CD4$^+$Foxp3$^+$ T_{reg} cells have significant immunotherapeutic potential resulting in suppression of diabetes in NOD mice [108-110]. Recently, several studies have confirmed this directly in BDC2.5 mice. For example, unlike BDC2.5 mice have a diabetes incidence that is delayed compared to NOD mice; Foxp3$^{-/-}$ BDC2.5 mice develop rapid-onset diabetes at 2-3 weeks-old. Furthermore,

BDC2.5 RAG$^{-/-}$ mice lack BDC2.5 T_{reg} cells and contain monoclonal BDC2.5 T_{eff} cells since TCR chain recombination cannot occur in the thymus during T cell development; consequently, these mice develop autoimmune diabetes with a similar incidence signature as the Foxp3$^{-/-}$ BDC2.5 and RAG$^{-/-}$ Foxp3$^{-/-}$ BDC2.5 mice[31]. Furthermore, the administration of BDC2.5 T_{reg} cells in these mice temporarily protects from autoimmunity. Given that Foxp3^{+} T_{reg} cells are necessary for regulating autoimmunity in the diabetes-specific BDC2.5 mouse model of T1D, this highlights the potency of T_{reg} cells to control diabetes [111].

The site of immunoregulation by CD4^{+}Foxp3^{+} T_{reg} cells is believed to occur at multiple locations (Figure 1). β-islet autoantigens are available early during life (by day 14 in mice) and result in diabetogenic T cell activation [56]. However, activation of CD4^{+} and CD8^{+} diabetogenic T cells in the pancreatic lymph node may be suppressed by T_{reg} cells [112, 113], and simultaneous regulation may occur in the systemic circulation [114] and directly in the pancreatic environment [111]. The mechanism of pancreatic T_{reg} cells [111] involves ICOS-ICOSL interaction, which induces an IL-10 dependent mechanism of suppression [72]. For peripheral CD4^{+}Foxp3^{+} T_{reg} cells in this system, their role is unknown. However, CTLA-4-antibody blockade was shown to exacerbate diabetes in young and not old BDC2.5 mice [115]. In young mice, CTLA-4 is only expressed on naïve peripheral CD4^{+}Foxp3^{+} T_{reg} cells. Notably, CTLA-4

expression on T_{eff} cells is required to increase their activation threshold. Thus, CTLA-4 antibody blockade may exacerbate the disease by activated T_{eff} cells. However, T_{reg} cells can still regulate CTLA-4$^{-/-}$ T_{eff} cells [116]. Therefore, it is more likely that CTLA-4 antibody blockade is affecting peripheral T_{reg} cells, and not T_{eff} cells, in young NOD.BDC2.5 mice. Meanwhile, older mice are not affected by this blockade, possibly because they also contain activated pancreatic T_{reg} cells, which are known to behave differently.

The mode of regulation mediated by T_{reg} cells is believed to function through multiple context-dependent mechanisms in T1D. This is supported by microarray analysis demonstrating that pancreatic-derived or activated CD4^{+}Foxp3^{+} T_{reg} cells have distinct gene profiles from their naïve counterparts [111]. Using 2-photon microscopy in the pancreatic lymph node, β-islet antigen-specific T_{reg} cells were suggested to suppress through a contact-dependent mechanism involving stable interactions between T_{reg} cells and DCs, which disables swarming and arrest of T_{eff} cell with autoantigen-bearing DCs [112]. In contrast, in the site of inflammation (the pancreatic lesion), cytokine-dependent mechanisms of T_{reg} cells are required to prevent T1D, in a TGF-β [117, 118] and/or IL-10 [72] dependent manner. Additionally, T_{reg} cells may have other function, and are thought to be responsible for the generation of induced regulatory T cell subsets (TGF-β dependent and/or IL-10 dependent) [80]. Thus,

T_{reg} cell immunoregulation may vary according to intrinsic or extrinsic differences: TCR affinity [108], a restricted vs. polyclonal repertoire of T_{reg} cells[108-110], activation status [109-111], developmental differences (i.e. potential encounter with thymic antigens during development [88, 111, 119]), environmental triggers (autoantigen vs. molecular mimicry by viral antigen), and host genetic defects [50, 120]. Alternatively, the T_{reg} cell mechanism may simply change based on the type of cell-cell interactions: T_{reg} cells has been observed both *in vitro* and *in vivo*, to interact with T_{eff} cells [121], iT_{reg} cells [122, 123], and DCs [112]. Therefore, careful and rigorous examination of T_{reg} cells using simplified analysis in TCR-transgenic models provides an excellent platform to examine autoimmunity.

iv. T_{reg} cell defects contribute to T1D

Although present, T_{reg} cells in T1D demonstrate aberrant activity [23], which is characterized by a reduction in T_{reg} cell potency [44, 124]. This was demonstrated by functional studies performed using T_{reg} cells derived from older diabetes-prone or overtly diabetic donors, which exhibited a reduction in their capacity to inhibit *in vitro* proliferation and *in vivo* diabetes incidence [44]. A corresponding reduction in anti-inflammatory cytokines produced was also suggested to contribute to the waning suppressive activity [124]. Furthermore, temporal quantitative defects resulting in a decline of T_{reg} cells in the peripheral lymph nodes, pancreatic lymph nodes, and pancreatic immune infiltrates of NOD

mice, were observed by single-cell Foxp3/TGFβ mRNA analysis of T cell cohorts [125], suggesting that quantitative defects are also observed. Significantly, reversal of T_{reg} cell changes has been associated with particular susceptibility loci, such as Idd6, Idd3/Idd5, and Idd9 [113, 120]. Interestingly, congenic studies have revealed that reversing the genetic contributor of T1D can specifically restore checkpoints in $CD8^+$ T cell tolerance. For example, the Idd3/Idd5 loci derived from C57BL/6 (autoimmune-resistant) mice provided NOD congenics with sufficient genetic resistance to suppress proliferation in the pancreatic lymph node, and the Idd9 loci from C57BL/6 mice provided NOD congenics with regulation in the pancreas [113, 120].

3. RATIONALE AND OBJECTIVES

T_{reg} cells counter-balance autoreactive immune cells in healthy individuals. In the absence of T_{reg} cells, multi-organ immune diseases manifest. Thus, many have suspected T_{reg} cell defects contribute to T1D. In immunotherapy studies, T_{reg} cell administrations were shown to suppress diabetogenic T_{eff} cells and T1D development in mice. Furthermore, an aberrant T_{reg} cell activity *in vitro* has been identified in T1D. However, it remains unclear whether T_{reg} cell defects are responsible for the manifestation of T1D. Thus, we hypothesize that T_{reg} cell defects results in T1D.

To examine T_{reg} cells for defects that contribute to spontaneous development of T1D, we assessed the population and function of T_{reg} cells during development and homeostasis in pre-diabetic mice. Simultaneously, these experiments provide insight into the mechanism of T_{reg} cells in T1D suppression, which is unclear. We determined the clinical impact of T_{reg} cells on diabetes onset, incidence, and severity; and we determined their effect on the population, priming, and differentiation of self-reactive $CD4^+$ T cells in the pancreatic lesion and the pancreatic lymph nodes. Notably, Foxp3 is used in our study to specifically identify T_{reg} cells. Thus, we are able to distinguish regulatory and effector T cell subsets, even in an activated-inflamed environment. Previous studies using Foxp3 in healthy mice revealed considerable insights into T_{reg} cell development, homeostasis, and function. Now, we use this system in order to accurately quantify T_{reg} cells in T1D-prone environments and during anti-islet β cell immune responses. Therefore, fundamental and novel questions regarding the role of T_{reg} cells in tolerance induction and prevention of autoimmunity were thoroughly evaluated.

Chapter II. Materials and Methods

Mice. NOD, C57BL/6, NOD.TCR$\alpha^{-/-}$ and NOD.BDC2.5 TCR mice were bred and maintained under pathogen-free conditions at the animal facility of McGill University. NOD.TCR$\alpha^{-/-}$ and NOD.BDC2.5 TCR mice were kind gifts from Dr. Christophe Benoist (Harvard Univ., Boston, USA). NOD mice exhibit mild insulitis as early as 4 wk of age and become diabetic starting at 12 wk of age. BDC2.5 mice develop diabetes after 12 wks of age.

Phenotypic analysis of T lymphocytes. For surface staining experiments, cells were initially stained with blocking anti-Fcγ-receptor mAb 2.4G2 at 4°C in PBS containing 1% BSA and 0.1% azide, and subsequently stained. Staining was performed with the following fluorochrome-conjugated or biotinylated mAbs: anti-CD4 (clone RM5), anti-Vb4 (clone CTVB4), anti-CD25 (clone PC61 or 7D4), anti-CD69 (clone H1.2F3), anti-CD44 (clone IM7), and anti-CD62L (clone MEL14) and anti-Foxp3 (all purchased from BD Bioscience, San Diego, CA). Intranuclear FoxP3 stainings were performed as in manufacturer's protocol (ebioscience).

Purification of CD4$^+$ T cell subsets. CD4$^+$ T cell subsets were isolated from spleen, thymus or lymph nodes. Fresh lymph nodes (axillary, inguinal, brachial and mesenteric), spleens or thymus were collected and single-cell suspensions were prepared by passing cells through a sterile wire mesh. Cells were

resuspended in complete RPMI, and the resulting cell preparation was stained with PE-conjugated anti-CD25 (8 μg/10^8 cells) and FITC-CD4 (8 μg/10^8 cells) in PBS/2% FCS for 20 minutes at 4°C. For thymocyte suspensions, $CD8^+$ T cells were depleted by successive treatments of anti-CD8α (5.367 culture supernatant) and low-toxicity rabbit complement (Cedarlane, Hornby, Ont.) for 45 minutes at 37°C. Cells were washed and resuspended in complete RPMI, gently layered on a Lympholyte-M gradient (Cederlane) to remove dead cells, according to the manufacturer's specifications. Cells suspensions were then depleted of $CD25^+$ cells by negative selection using anti-PE Microbeads (Miltenyi Biotech, San Diego, CA). Purity was assessed on a FACSCalibur flow cytometer (BD Bioscience, Mountain View, CA). The purity of the final $CD4^+CD25^+$ preparation was routinely over 98%.

Adoptive cell transfers. Thymocytes and/or lymphocytes were prepared as described in the previously paragraph. $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were transferred intravenously, either alone or in combination, into $TCR\alpha^{-/-}$ NOD recipient mice (1–3x10^5/mouse). Mice receiving PBS were included as controls. At either 14 or 30 days post-transfer, the lymphoid organs and the pancreas of the diabetic mice were harvested, and single-cell suspensions were prepared. Pancreata were divided in two, one half being fixed in 10% buffered formalin, the other half was diced, digested with collagenase-2 (Invitrogen, Burlington, Ont.),

resuspended in non-enzymatic dissociation buffer (Invitrogen, Burlington, Ont.) and washed extensively. T cells were then separated from the digested tissue by centrifugation on a Lympholyte-M gradient (Cederlane) according to the manufacturer's specifications. Cell suspensions were then stained with anti-Vβ4 TCR and anti-CD4 mAbs, and analyzed by FACS, as previously described. In some experiments, donor T cells were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) to assess cell proliferation *in vivo*. In these instances, labeled cells were recovered 72h post-transfer from pancreatic and mesenteric lymph nodes, and then stained as aforementioned.

Diabetes Diagnosis. Recipient mice were tested every 1-3 days for diabetes. Overt diabetes was defined as two positive glucosuria tests and glycemia > 250 mg/dl. Glukotest and Haemoglukotest kits were kindly provided by Roche Diagnostics (Canada).

Chapter III. Results

1. The thymic T_{reg} cell compartment of healthy and T1D-prone mice

A deficiency in the population of T_{reg} cells may exist in the thymus of susceptible hosts, prior to diabetes onset. Thus, we quantified thymic T_{reg} cells from prediabetic NOD mice, in comparison to (autoimmune resistant) C57BL/6 mice. T_{reg} cells were identified using multi-parametric FACS analysis of intranuclear Foxp3$^+$ cells in the CD4$^+$CD8$^-$ (CD4^{SP}) population. We demonstrated that T_{reg} cells in the thymus are stable in prediabetic NOD mice, representing 5% of the CD4^{SP} T cell population, and are not significantly different from age-matched C57BL/6 mice (figure 4).

We continued our quantitative analysis into BDC2.5 mice to, which contain a restricted β cell specific CD4 TCR repertoire. BDC2.5 mice only develop autoimmune diabetes; in contrast, the polyclonal T cell population of NOD mice can trigger other autoimmune disease can occur. We demonstrated that thymic T_{reg} cells in the BDC2.5 are present and stable over time in prediabetic BDC2.5 mice (figure 4). Although thymic T_{reg} cells represents approximately 1% of CD4^{SP} thymocytes, TCR transgenic mice are known to contain reduced T_{reg} cells as a result of a restricted TCR repertoire [126]. Therefore, no quantitative defect was observed to hallmark spontaneous diabetes in prediabetic NOD mice.

2. Thymic T_{reg} cell regulation of diabetogenicity

Thymic T_{reg} cells are present, but they may be functionally defective in prediabetic NOD mice. Thus, we assessed thymic T_{reg} cell function in T1D, specifically, by adoptively transferring $CD4^{SP}$ BDC2.5 thymocytes: $2.5x10^5$ $CD4^{SP}$ T cells were transferred into mature 6-8 week old $TCR\alpha^{-/-}$ NOD mice. The transferred cells were either $CD4^{SP}$ cells containing a normal, physiological population of T_{reg} cells or $CD4^{SP}CD25^-$ cells containing a T_{reg} cell-deficient population. The classical CD25 marker was used to deplete T_{reg} cells because we could not separate cells on the basis of intranuclear expression of Foxp3. Via intranuclear analysis, we observed 0.28% $Foxp3^+$ cells in $CD4^{SP}CD25^-$ donor thymocytes (figure 5); this represents 23.3% of the original thymic T_{reg} cell compartment. Therefore, T_{reg} cell regulation mediated by a normal T_{reg} cell population was compared to a T_{reg} cell deficient (but not depleted) population.

As observed, the depletion of $CD4^{SP}CD25^+$ cells always increased incidences of autoimmune diabetes compared to recipients of $CD4^{SP}$ T cells. Onset was observed at day 10, and peaked at day 26 with an incidence of approximately 70%. In stark contrast, recipients of $CD4^{SP}$ thymocytes did not develop diabetes (figure 6). Therefore, thymic T_{reg} cells derived from prediabetic NOD mice are functional and prevent autoimmune diabetes. Consequently, T1D predisposing defects do not affect T_{reg} thymocytes.

3. The peripheral T_{reg} cell compartment of healthy and T1D-prone mice

Next, we suspected that a defect could manifest on T_{reg} cells in the periphery of prediabetic NOD mice. In order to identify any quantitative defects, we measured T_{reg} cell compartments, as previously described. Secondary lymphoid organs were analyzed. This included the pancreatic lymph nodes because a defect may localize to this site where diabetogenic T cells are activated. We also quantified $CD4^+Foxp3^+$ T_{reg} cells before and after 4 weeks (15-28 days) of age in prediabetic NOD mice, to determine whether a deficiency manifests before or after insulitis development (at 3-4 weeks of age).

In prediabetic NOD mice, T_{reg} cell populations in the pancreatic lymph nodes and spleen were stable over time. T_{reg} cells represented approximately 10% of the total T cell population in the pancreatic lymph nodes, and approximately 15% in the spleen. In C57BL/6 mice, T_{reg} cell populations were present in similar quantities (figure 7). Therefore, no apparent quantitative defect in T_{reg} cells is present in the peripheral immune system of prediabetic NOD mice. Similarly, no quantitative defect was observed in the periphery of BDC2.5 mice (figure 7). Therefore, a quantitative decline of T_{reg} cells is not present in prediabetic NOD mice to indicate spontaneous diabetes development.

4. Peripheral T_{reg} cell regulation of diabetogenicity

To identify whether a peripheral T_{reg} cell dysfunction is present in prediabetic mice, we utilized the previously described adoptive transfer system. Notably, a previous study demonstrated that (3-4 week old) BDC2.5-derived peripheral T_{reg} cells suppress diabetes in Foxp3$^{-/-}$ mice [111]; however, other studies have demonstrated that (6-8 week old) BDC2.5 T cells can transfer diabetes [71, 72]. However, the presence and contribution of a T_{reg} cell dysfunction in this model is unknown. Therefore, we evaluated the role of T_{reg} cells derived from prediabetic BDC2.5 mice aged 3-4 weeks and 6-8 weeks.

Initially, we examined activation marker expression of T cells derived from the spleen and pancreatic lymph nodes. This was necessary to determine whether the T_{eff} cell populations differ after a 3-4 week interval. Comparative analysis demonstrated identical activation levels between age groups (figure 8C). Activation in the pancreatic lymph nodes was not significantly different, as demonstrated by CD69 and CD44 activation profiles. In addition, comparative analysis in the spleen also demonstrated no significant difference (figure 8C). This suggests that T_{eff} cells in prediabetic mice have similar activation status and are not more diabetogenic. Thereafter, we evaluated donor T cells derived from 3-4 and 6-8 week old mice in our adoptive transfer model. Onset and synchronism of diabetes observed in recipients of 3-4 week old and 6-8 week old

$CD4^+CD25^-$ T cells were similar (figure 8A-B). Therefore, this further suggests that 3-4 week T_{eff} cells and 6-8 week T_{eff} cells have a similar activation and potency.

Regarding the T_{reg} cells of these mice, T_{reg} cell immunoregulation was present and functional in the 3-4 week old T cell compartment. Incidence of diabetes was completely reduced in the presence of their T_{reg} cells (figure 8B). In contrast, recipients of a peripheral T cell population derived from 6-8 week old BDC2.5 mice were unaffected by CD25-depletion. However, diabetes suppression was observed in the presence of additional, 40% $CD25^+$ T cells (derived from 6-8 week old mice) (figure 8B). As a result, our data suggests that T_{reg} cell potency is reduced but still present in 6-8 week old mice. Therefore, T_{reg} cells become impaired in the periphery of prediabetic mice, and hallmarks diabetes development.

5. Early events in the T_{reg} cell regulation of diabetes.

In our adoptive transfer model of diabetes, we demonstrated that T_{reg} cell regulation can prevent diabetes development. However, the mechanism of this regulation is poorly understood. Previous studies have demonstrated that the BDC2.5 T cell homes to the pancreatic lymph nodes as early as 66 hours post transfer [56]. As a result, we evaluated several features that might affect

diabetogenic T cells during early events in the pancreatic lymph nodes (prior to T cell infiltration in the pancreas), including trafficking, activation, and proliferation. Donor T cells were harvested from thymi of 3-4 week old mice, CFSE-labelled, and then transferred. In the pancreatic lymph nodes receiving BDC T_{eff} or BDC T_{eff} + BDC T_{reg} cells, $CD4^+V\beta4^+$ T cells were present at 0.49% and 0.55%, respectively; the CD69 activation T cell profile was 23.8% and 26.2%, respectively; and the CFSE proliferation T cell profile was 88.8% and 81.6%, respectively (figure 9). In nondraining sites, we also detected activated BDC2.5 T_{eff} cells: in recipients of BDC T_{eff} or BDC T_{eff} + BDC T_{reg} cells, the T cell proliferation/CFSE profile was 30.4% and 28.5%, respectively. These T cells became activated in the pancreatic lymph nodes and are circulating in the periphery to the site of inflammation. In addition, they were not affected by the presence or absence of T_{reg} cells. Thus, T_{reg} cells do not affect homing, activation, and proliferation in the pancreatic lymph nodes at early events (prior to infiltration in the pancreatic lesion).

6. Late events in T_{reg} cell regulation/dysregulation of diabetes.

Previous studies, using different models of diabetes, have examined the role of BDC2.5 T_{reg} cells in regulation and have identified immunoregulation in the pancreatic lymph nodes [112] or the pancreatic lesion [111]. Interestingly,

neither study has observed simultaneous regulation in both compartments. Therefore, we examined T_{reg} cell immunoregulation in both the pancreatic lymph nodes and pancreas of recipients of donor thymocytes or peripheral lymphocytes derived from 3-4 week old mice. In addition, we also examined T_{reg} cells in adoptive transfers using T cells derived from 6-8 week old mice to evaluate the dysfunction to the T_{reg} cell population. We assessed regulation or dysregulation in our adoptive transfer system by evaluating mice following onset of diabetes. T_{reg} cells were differentiated by multi-parametric FACS analysis.

Initially, we assessed regulation by examining the frequency of diabetogenic $CD4^+V\beta4^+$ cells in the pancreatic lymph node and pancreas. Non-diabetic mice contained reduced pancreas-infiltrating $CD4^+V\beta4^+$ T cells compared to diabetic recipients (figure 10). Furthermore, regulation in each compartment was evaluated by comparing the T_{eff} cell: T_{reg} cell ratio. It was observed that protected mice contained 1 T_{reg} cell for every 5-10 T_{eff} cells, and diabetic recipients contained 2-3 fold more T_{eff} cells per T_{reg} cells (figure 10). Therefore, T_{reg} cells suppress diabetes during late events (following T cell infiltration into the pancreas) by maintaining reduced diabetogenic T cells in the pancreatic lymph nodes and pancreatic lesion.

CD25-depletion did not induce diabetes development in all recipients. At day 30, we compared three recipient groups: nondiabetic recipients of $CD4^{SP}$ T

cell, diabetic recipients of $CD4^{SP}CD25^-$ T cells, and nondiabetic recipients of $CD4^{SP}CD25^-$ T cells. In nondiabetic recipients of $CD4^{SP}CD25^-$ T cells, proportions of infiltrating T cells were significantly reduced compared to diabetic recipients of $CD4^{SP}CD25^-$ T cells. Furthermore, these nondiabetic recipients of $CD4^{SP}CD25^-$ cells contained a reduced T_{eff} cell: T_{reg} cell ratio profile compared to the diabetic recipients (figure 10). Therefore, the $CD4^{SP}CD25^-$ T cells, which contained a reduced T_{reg} cell population compared to $CD4^{SP}$ recipients, were capable of T_{eff} cell regulation, and this regulation was similar to nondiabetic recipients of $CD4^{SP}$ cells. This further highlights the significance of T_{reg} cell mediated late-regulatory events on infiltrating T cells in both the pancreatic lymph nodes and pancreatic lesion.

In the adoptive transfers of T cells derived from 6-8 week old BDC2.5 mice, we evaluated three recipient groups: diabetic mice that received either $CD4^+CD25^-$ T cells or $CD4^+$, as well as protected mice that received $CD4^+$ T cells. In general, the presence of T_{reg} cells was not sufficient to control diabetes, as demonstrated by Foxp3 analysis (figure 11). Recipients that did not develop diabetes contained an increased population of T_{reg} cells in their pancreatic lesion compared to the majority of diabetic recipients of $CD4^+$ T cells, which contained a reduced T_{reg} cell population. Although T_{reg} cells were present in these recipients, we demonstrate that diabetes occurs because diabetogenic T_{eff} cells are not

regulated. Thus, the environment of diabetic mice (containing $CD4^+$ T cells) resembled the population in diabetic recipients of $CD4^+CD25^-$ T cells. Therefore, T_{reg} cells become dysfunctional and incompetent, insulitis is not controlled: T_{eff} cells can potentially escape checkpoint 2, which can predispose the development of diabetes in prediabetic mice.

Chapter IV. Discussion and Conclusion

We hypothesized that spontaneous diabetes results from a defect in T_{reg} cells, which contributes to the escape of T_{eff} cells. Thus, T_{reg} cells were assessed in the thymus and in the periphery to assess whether T_{reg} cells are inherently or progressively defective. We demonstrated that NOD T_{reg} thymocytes were present and replete in the thymus. In addition, no functional T_{reg} thymocyte defect was observed. In the peripheral immune system, the spleen and pancreatic lymph nodes contained normal T_{reg} cell numbers (compared to autoimmune resistant C57BL/6 mice). As a result, a quantitative T_{reg} cell defect in either primary or secondary lymphoid organs does not predispose or hallmark susceptibility to T1D. However, a T_{reg} cell defect was identified and correlates with decreased function of T_{reg} cells, not increased diabetogenicity of T_{eff} cells. Simultaneously, we analyzed the mechanism of T_{reg} cell T1D-regulation, and observed that T_{reg} cells do not affect T_{eff} cell homing, activation, or proliferation during early events (prior to T cell infiltration in the pancreas) in the pancreatic lymph nodes. However, regulation of T_{eff} cells does occur in both the pancreatic lymph nodes and pancreas at later events – following T_{eff} cell infiltration of the pancreas. T_{reg} cell prevention of diabetes corresponded with the maintenance of reduced T cell infiltrates and increased proportions of T_{reg} cells per T_{eff} cells in both sites. Therefore, T_{reg} cells do not control the initial activation of T cells,

known as checkpoint 1, which results in the appearance of insulitis; however, T_{reg} cells prevent the development of destructive insulitis, known as checkpoint 2. Meanwhile, a dysfunction in peripheral T_{reg} cells at this stage is observed and correlated with a waned ability to control insulitis, and hallmarks diabetes in prediabetic mice.

Previously, studies used CD25 marker to examine T_{reg} cells in NOD mice, and they have suggested that individuals with diabetes contain reduced T_{reg} cells. An additional study demonstrated by single-cell analysis of Foxp3 mRNA that a temporal quantitative decline in T_{reg} cells from the pancreatic lymph nodes, popliteal lymph nodes, and pancreas can be observed in 16 wk old female NOD mice [23, 45]. Consequently, a quantitative deficiency in T_{reg} cells is claimed to contribute to T1D susceptibility. In our analysis, we did not observe a defect correlating with a reduced T_{reg} cell population in the thymus or the peripheral immune system. However, a decline in T_{reg} cells was observed in mice of our adoptive transfer model that developed diabetes compared to protected nondiabetic mice. As a result, both observations are not necessarily mutually exclusive. We suggest that the dysfunction in T_{reg} cells represents the original T_{reg} cell-associated defect which then contributes to a decline in the normal population of T_{reg} cells. The association between the quantitative T_{reg} cell defects resulting from a functional T_{reg} cell defect is unclear, but either T_{eff} cells are

expanding because T_{reg} cell functional potency is waning and/or T_{reg} cell function depends on their own expansion. *In vitro* studies have already suggested that the suppressive capacity of T_{reg} cells decreases [127, 128]. Our study provides additional insight by demonstrating that waning T_{reg} cell function occurs after checkpoint 1, but before checkpoint 2, from an inability to control the population of T_{eff} cells in both the pancreatic lymph nodes and pancreas. Additional insight will likely require the isolation of viable Foxp3$^+$ T_{reg} cells from a pool of T cells in an activated immune system.

Prior to the T_{reg} cell dysfunction, we observed that T_{reg} cells are present, functional, and are capable of mediating diabetes prevention by suppressing T_{eff} cells in both the pancreatic lymph nodes and the pancreas. Specifically, the mechanism of the suppression in both sites was observed at the onset of diabetes. However, the initial T_{eff} cell activation and proliferation prior to T cell infiltration into the pancreas (or early events) was uninhibited.

The delay in pancreatic lymph node regulation may highlight a defect to the T_{reg} cell mechanism in NOD mice. A previous study demonstrated that early events of T cell regulation in the pancreatic lymph was restored in NOD congenic mice bearing the protective C57BL/6 idd3/5 loci [113]. However, this study evaluated T_{reg} cell regulation of diabetogenic CD8$^+$ T_{eff} cells. Meanwhile, different mechanisms may exist for regulating CD4$^+$ T_{eff} cells. Furthermore, it is unclear

what triggers the activation of this T_{reg} cell mechanism. A previous study demonstrated *in vivo* T_{reg} cell-mediated regulation of T_{eff} cells in the pancreatic lymph nodes during early events, but they depended on *in vitro* activation of T_{reg} cells [112]. Thus, an unknown activation signal either in the pancreatic lymph nodes or within the pancreas occurs in our system triggers the pancreatic lymph node mechanism of T_{reg} cells. Furthermore, *in vitro* activated T_{reg} cells were observed using two-photon laser-scanning microscopy to interact with autoantigen-bearing DCs and inhibit T_{eff} cell activation, proliferation, and effector function [112]. However, it is unknown whether T_{reg} cells in our adoptive transfer system mediate the same mechanism of regulation as *in vitro*-activated T_{reg} cells.

The mechanism of T_{reg} cells in the pancreas was shown, in a previous study, to depend on ICOS-LICOS interaction for IL-10 mediated regulation [72]. This contradicts the classical T_{reg} cell mechanism of contact-dependent, cytokine-independent, suppression. However, T_{reg} cell function may be context-dependent. A previous study has reported that different gene-array profiles are observed between pancreas-derived T_{reg} cells and periphery or pancreatic lymph node derived T_{reg} cells [111]. Thus, T_{reg} cells may function through a contact dependent mechanism in the pancreatic lymph nodes and a cytokine dependent mechanism in the pancreas. Notably, these studies have only reported T_{reg} cell regulation in the pancreas, but did not report regulation in the pancreatic lymph

nodes. Thus, additional gene arrays are required to properly assess T_{reg} cell regulation of T1D. Meanwhile, our study is the first to demonstrate regulation in both sites, simultaneously. Interestingly, we observed similarities between regulation in the pancreas and pancreatic lymph nodes, which may suggest a similar mechanism of regulation in both sites. However, our results do not exclude the possibility of context-dependent regulation. Therefore, we suggest that the overall effect of T_{reg} cell-mediated regulation of T_{eff} cells results in a reduced population of T_{eff} cells and a similar T_{reg} cell: T_{eff} cell ratio.

In order to assess the role of T_{reg} cells in our study, we relied on the classical CD25-depletion method, which removes approximately 75% of T_{reg} cells. This was sufficient to induce diabetes in most cases. However, the protected recipients of CD25 depleted T cells, contained residual T_{reg} cells capable of T_{eff} cell and T1D regulation. Furthermore, the mechanism of T_{eff} cell regulation was nearly identical to a normal T_{reg} cell population, as observed by the similar T_{eff} cell: T_{reg} cell ratios. Thus, T_{reg} cells can potentially restore themselves, and respond during a decline in T_{reg} cells. This suggests that a reduction in T_{reg} cell numbers is not necessarily sufficient to abrogate regulation and induce diabetes. Therefore, this further highlights the contribution of T_{reg} cell dysfunction to the development of diabetes.

Interestingly, this residual Foxp3^{+}CD25^{-} T cell compartment had a more substantial effect in thymocytes than peripheral lymphocytes: some recipients of CD4^{SP}CD25^{-} thymocytes could suppress diabetes (figure 6) and all recipients of CD4^{+}CD25^{-} lymphocytes developed diabetes (figure 8). A possible explanation is that the specific function of CD25 (the α-chain for the receptor of the IL-2 cytokine) on T_{reg} cells in the thymus versus the periphery may be different. Previous studies have demonstrated that CD25 is necessary for T_{reg} cell survival-homeostasis in the periphery, and is required to a lesser extent in the thymus [82, 129, 130]. Consequently, CD25 depletion had a corresponding effect, by depleting fit T_{reg} cells found in the periphery; whereas the fitness of thymic T_{reg} cells does not depend exclusively on this cytokine. Therefore, we suggest that differences are present between T_{reg} cells from the thymus versus the periphery. Nonetheless, each T_{reg} cell population is capable of regulating diabetes.

Lymphopaenia, a reduction in lymphocyte number, has been suggested to drive autoimmunity [45]. However, it should be noted that lymphopaenia does not indiscriminately drive diabetes; as observed in our study, the lymphopenic recipients of donor BDC2.5 thymocytes and 3-4 week donor T cells did not develop diabetes. Notably, lymphopaenia can occur in every individual as a result of stress or illness, but autoimmunity is not a threat because normal physiological mechanisms regulate lymphopaenia. For example, T_{reg} cell

immunoregulation during lymphopaenia is increased [131]. Thus, lymphopaenia-induced autoimmunity is dependent on other factors, such as the potency of the peripheral T_{reg} compartment.

T_{reg} cell immunoregulation in a non-lymphopaenic environment is thought to be necessary in order to observe a real, physiological dissection of the T_{reg} cell immune mechanism. Thus, some T_{reg} cell studies used T_{reg} cell deficient Foxp3$^{-/-}$ or B7$^{-/-}$ recipients and suggested that they are non-lymphopaenic models [111, 112]; however, these mice contain excessive autoreactivity and so are not adequate models. For example, Foxp3$^{-/-}$ mice suffer from T_{reg} cell extrinsic defects, since Foxp3 influences differentiation of T_{eff} cells during an immune response [132], and Foxp3 is required during thymopoeisis for normal T_{eff} cell behavior [107]. Furthermore, the absence of a T_{reg} cell niche in these models provides additional peripheral cytokines (i.e. IL-2) and homeostatic signals (i.e. autoantigens) for the expansion of T_{eff} cells. Interestingly, the latter are features characteristic of lymphopaenia. Therefore, these models may have the adverse affect of also hampering proper T_{reg} cell evaluation. In stark contrast, our diabetes study utilized T_{eff} cells and T_{reg} cells from the physiological environment of diabetes-prone wild-type NOD mice. And although lymphopaenia is present, it was useful to evaluate the role of T_{reg} cells in diabetes-prone prediabetic mice.

Nonetheless, T_{reg} cell immunoregulation has yet to be evaluated in a non-lymphopaenic environment.

In itself, autoreactivity in the immune system is not an indicator of susceptibility to autoimmunity. In healthy individuals, autoreactive T cells are present and circulate in the periphery. However, physiological levels of T_{reg} cells in healthy hosts regulate autoreactivity [133, 134]. T_{reg} cell regulation of diabetes is also observed in diabetes-prone mice, but is predisposed to develop diabetes during T_{reg} cell incompetence. Thus, our study demonstrates that the ability to regulate autoreactivity is present following T cell ontogeny but wanes with age in NOD mice. Furthermore, this study is critically important to fully elucidate the role of T_{reg} cells in the control of spontaneous autoimmunity in the NOD mouse. Until recently, limitations in science prevented proper identification of $Foxp3^+$ T_{reg} cells; as a result, our study is the first to accurately demonstrate that prediabetic NOD mice do not contain a reduced T_{reg} cell population in the thymus, periphery, or pancreatic lymph nodes. A qualitative temporal change(s) to the T_{reg} cell compartment is instead contributing to diabetes development in NOD mice. This study provides insight into the cellular basis of disease susceptibility and may lead to the development of novel approaches to potentiate T_{reg} cell activity.

More Future Directions:

The GFP-Foxp3 knock-in NOD mice provides a powerful and sensitive way to track autoreactive T cells and T_{reg} cells independently. This system has already demonstrated its capacity to clarify several areas of T_{reg} cell development, homeostasis, and function [81, 82, 103, 135]. In contrast, identification of T_{reg} cells using surface markers is restricted in the presence of activated T_{eff} cells, because they are also known to express the same markers, which leads to inconclusive results. In this system, we will specifically examine regulation mediated by BDC2.5 T_{reg} cells, since BDC2.5 mice provide an antigen specific system for the analysis of T cells responses in diabetes. We will examine the regulatory mechanism present in pancreatic lymph nodes and pancreas, as well as monitor the dynamics of each population relative to the other, and allow for the fine analysis of individual T cell responses through a kinetic study. Therefore, in the presence or absence of T_{reg} cells, an examination of the changes in the diversity of the TCR repertoire, degree of proliferation and death, expression of chemokines, inflammatory and anti-inflammatory cytokines, and other effector molecules will be compiled. In addition, we will evaluate other mechanisms of regulation in the BDC2.5, such as the influence of T_{reg} cells in mediating regulation in older (10-12 week) BDC2.5 mice, which are known to regulate diabetes through an unknown IL-10 mediated mechanism.

Figure Legends

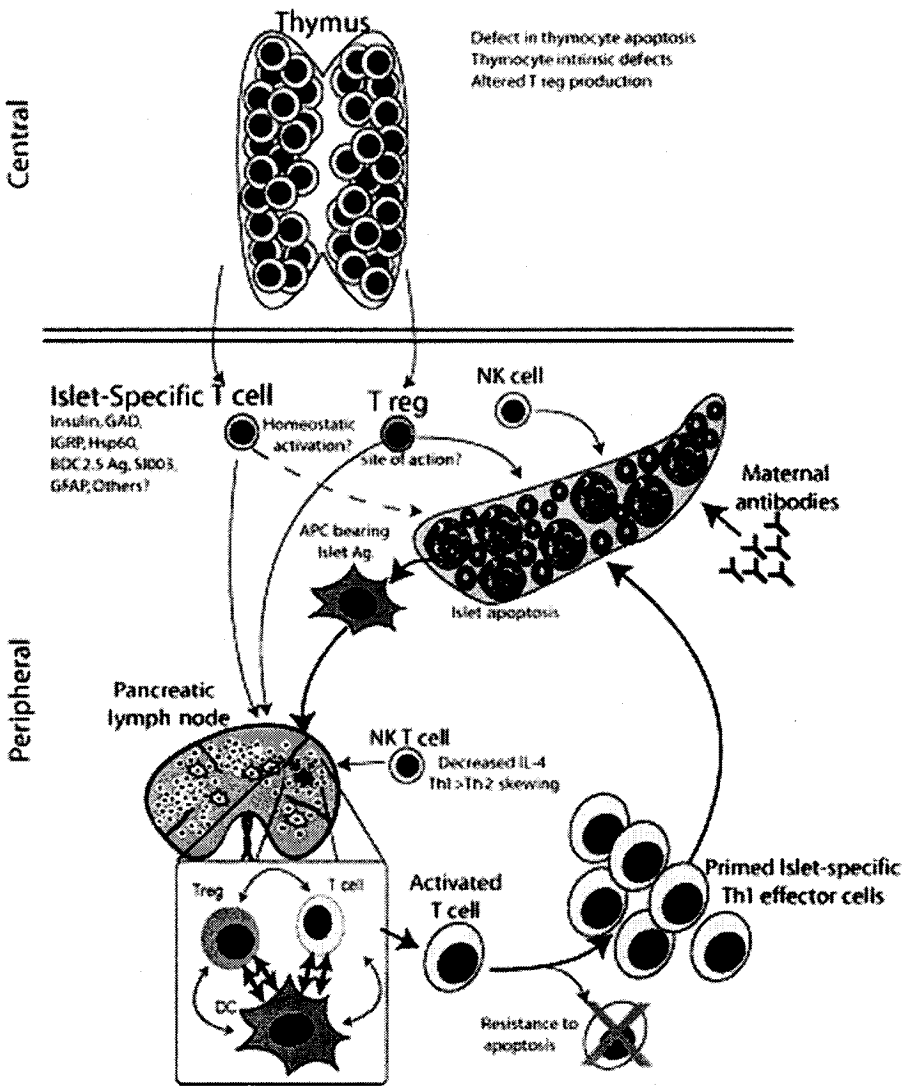

Figure 1. Central and peripheral tolerance defects in NOD mice. This figure depicts the general understanding of potential defects in various tolerogenic processes that might be responsible for the development of diabetes in NOD mice. The figure emphasizes the potential roles of various T cell and other lymphocyte subsets in disease development and progression". The figure and legend were taken from [24].

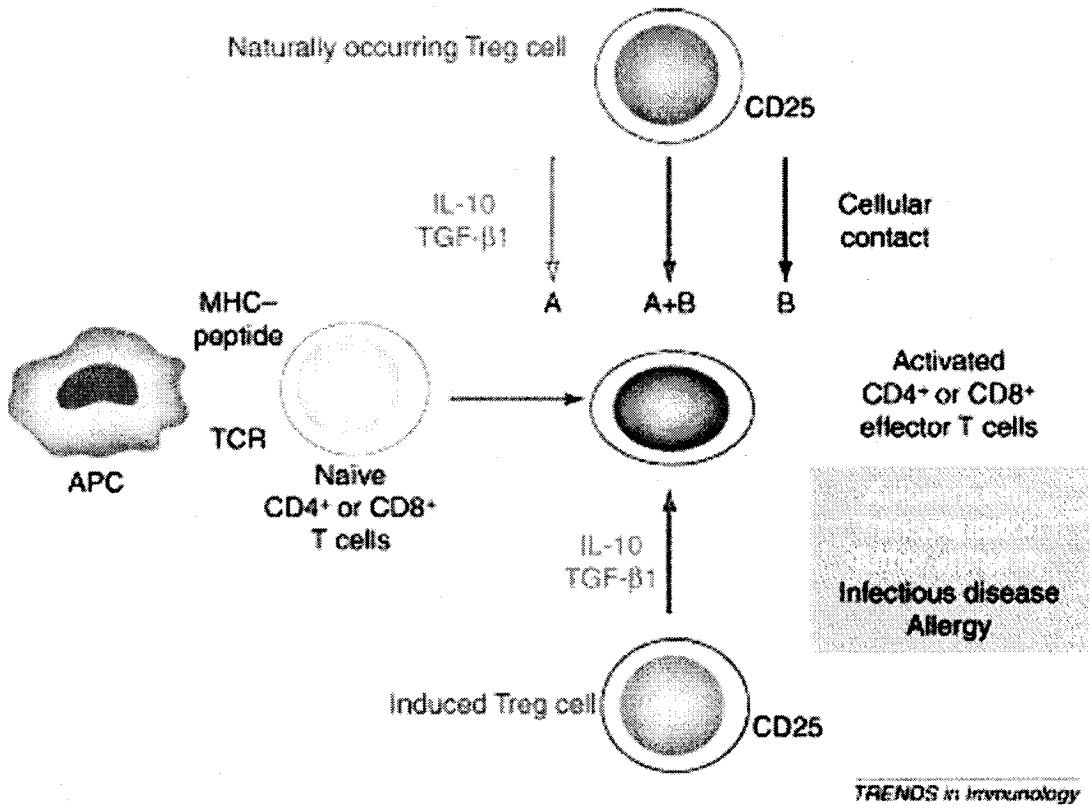

Figure 2. Possible mechanisms of action of CD4+ regulatory T cells. T_{reg} cells have crucial roles in suppressing the functions of activated $CD4_+$ or $CD8_+$ effector T cells in various types of immune responses (purple box), and the mechanisms for achieving this regulation are diverse. Whereas i T_{reg} cells operate via the secretion of immunosuppressive cytokines, including IL-10 and TGF-β1, T_{reg} cells might opt for either cytokine dependent (A), cell contact dependent (B) or cytokine/cellular contact dependent (A + B) modes of action to control similar T-cell responses". The figure and legend was taken from [136].

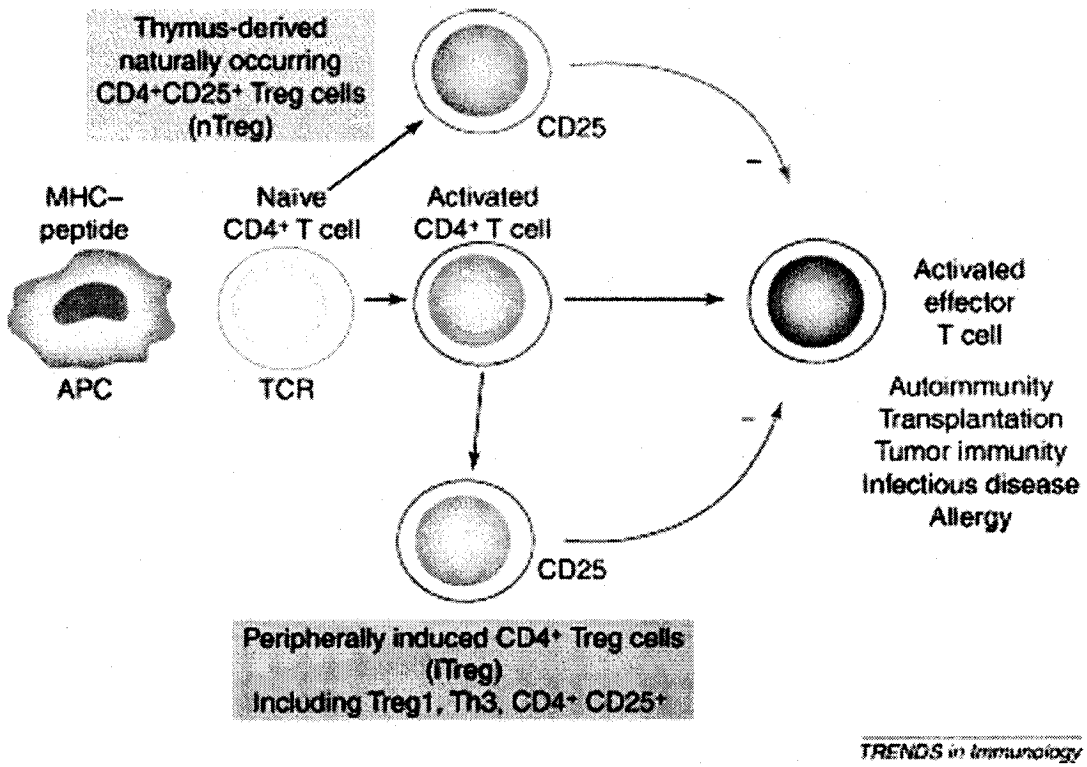

Figure 3. Naturally occurring and induced CD4+ regulatory T cells control T cell responses. Both T_{reg} and iT_{reg} CD4+ regulatory T cell populations potentially downregulate the function of activated T_{eff} cells in several immunological settings (yellow box). Whereas CD4+CD25+ T_{reg} cells differentiate in the thymus and are found in the normal, naive CD4+ T-cell repertoire, multiple iT_{reg} cell subsets, possibly expressing CD25, emerge from conventional CD4+ T cells, which are activated and differentiated in the periphery under unique stimulatory conditions. The relative contribution of each population in the overall regulation of immune responses is unclear but both conceivably can cooperate to achieve this goal". The figure and legend was taken from [136]

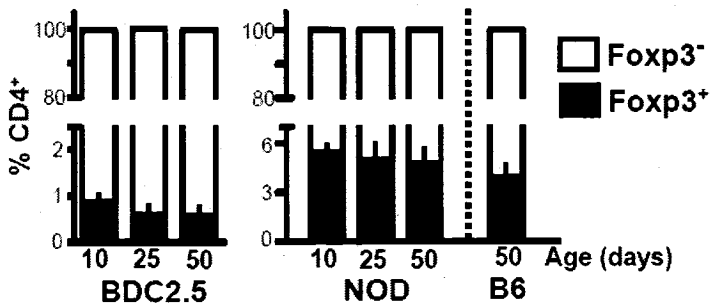

Figure 4. Normal thymic development of T_{reg} cells in NOD mice. The cellular frequency of $CD4^{SP}$ T cells expressing Foxp3 was determined in thymocytes from 10, 25, and 50 day old adult NOD and BDC2.5 mice. Values for 50 day old C57BL/6 mice are shown as a reference. The bars represent percentages of the T_{reg} cell ($Foxp3^+$, dark region) and T_{eff} cell ($Foxp3^-$, light region) compartment within the $CD4^+CD8^-$ T compartment. Data represents pooled results of three separate experiments, with 3-5 mice analyzed per age group.

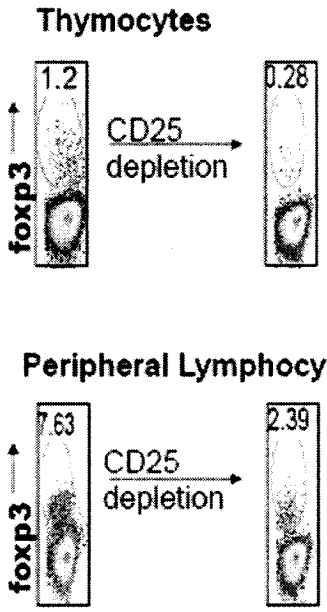

Figure 5. Foxp3 analysis of donor T cell populations used in adoptive transfers. Foxp3 analyses of the T_{reg} cell population in thymocytes, or peripheral lymphocytes are observed on the left-hand, and the $CD25^{-}$ T_{reg} cell deficient T cells are observed on the right-hand. Data represent pooled results of three separate experiments with similar results.

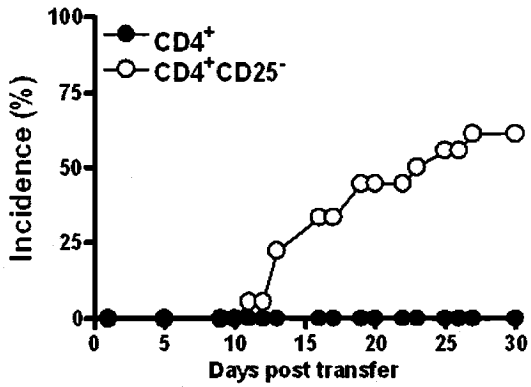

Figure 6. Thymic T_{reg} cells maintain tolerance to β-islet antigens in pre-diabetic BDC2.5 mice. Donor T cells, $CD4^{SP}$ (closed circles) or $CD4^{SP}CD25^{-}$ (open circles), were isolated by FACS from thymocytes of BDC2.5 mice. Each cell subsets was transferred i.v. (2.5×10^5) into NOD.TCR$\alpha^{-/-}$ recipient mice, and the onset and incidence of diabetes development was assessed by blood glucose measurments every 24-48h. Data represent pooled results of three separate experiments with similar results.

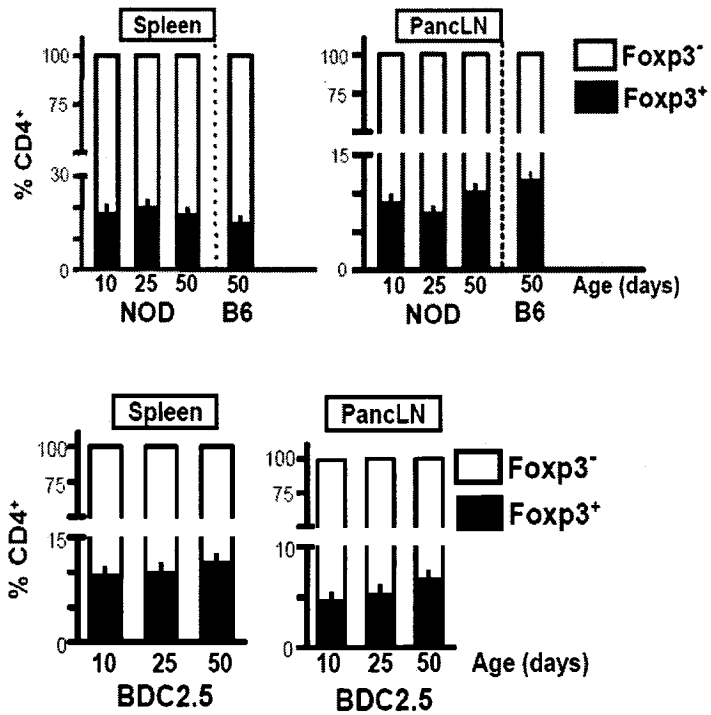

Figure 7. A stable pool of Foxp3-expressing CD4⁺ T_{reg} cells in the peripheral immune system of NOD mice. The cellular frequency of CD4⁺ T cells expressing Foxp3 was determined in CD4⁺ T cells from the spleen (left) or the pancreatic lymph node (PancLN; right). The latter was carried out on NOD or BDC2.5 mice aged 10, 25 or 50 days. Values for 50 day old B6 mice are shown as a reference. The bars represent percentages of the T_{reg} cell (Foxp3⁺, dark region) and T_{eff} cell (Foxp3⁻, light region) compartment within the CD4⁺ T compartment. Data represents pooled results of three separate experiments, with 3-5 mice analyzed per age group.

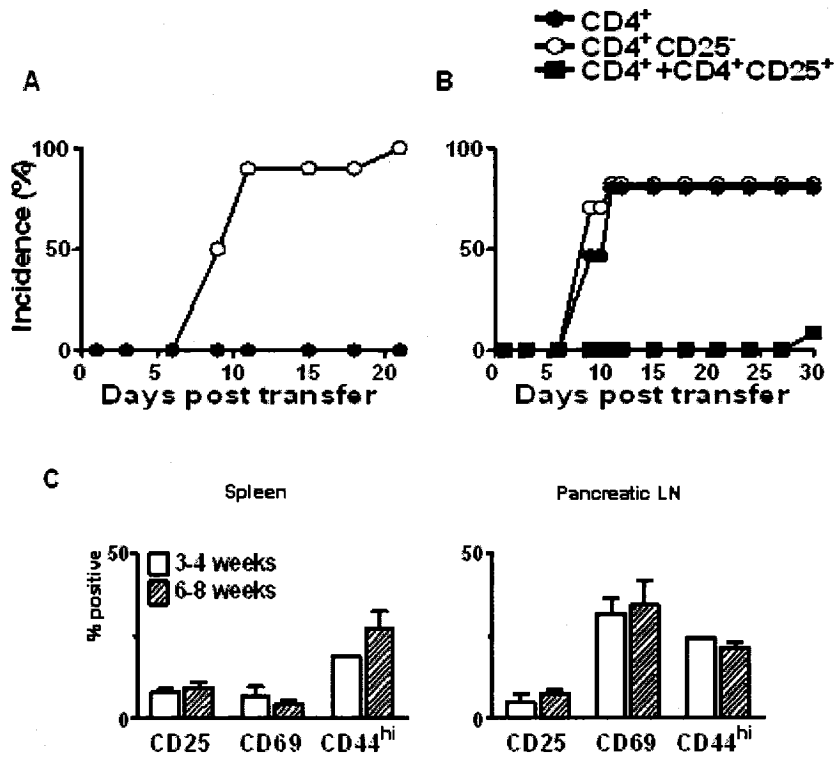

Figure 8. Peripheral T_{reg} cell immunoregulation is progressively dysregulated in pre-diabetic BDC2.5 mice. Donor T cells, $CD4^+$ (closed circles), $CD4^+CD25^-$ (open circles) or $CD4^+$ with $CD4^+CD25^+$ (closed squares), were isolated by FACS from peripheral T cells of 3-4 week old (panel A) or 6-8 week old (panel B) BDC2.5 mice. Each cell subsets was transferred i.v. (2.5×10^5) into NOD.TCR$\alpha^{-/-}$ recipient mice, and the onset and incidence of diabetes development was assessed by blood glucose measurments every 24-48h. In panel C, the cellular frequency of $CD4^+V\beta4^+$ T cells expressing the CD25, CD69, or CD44 activation markers was determined in $CD4^+$ T cells from spleen (left) or pancreatic lymph nodes (PancLN; right) of 3-4 week old (white bars) and 6-8 week old (dashed

bars) BDC2.5 mice. Data represents pooled results of three separate experiments, with 3-5 mice analyzed per age group.

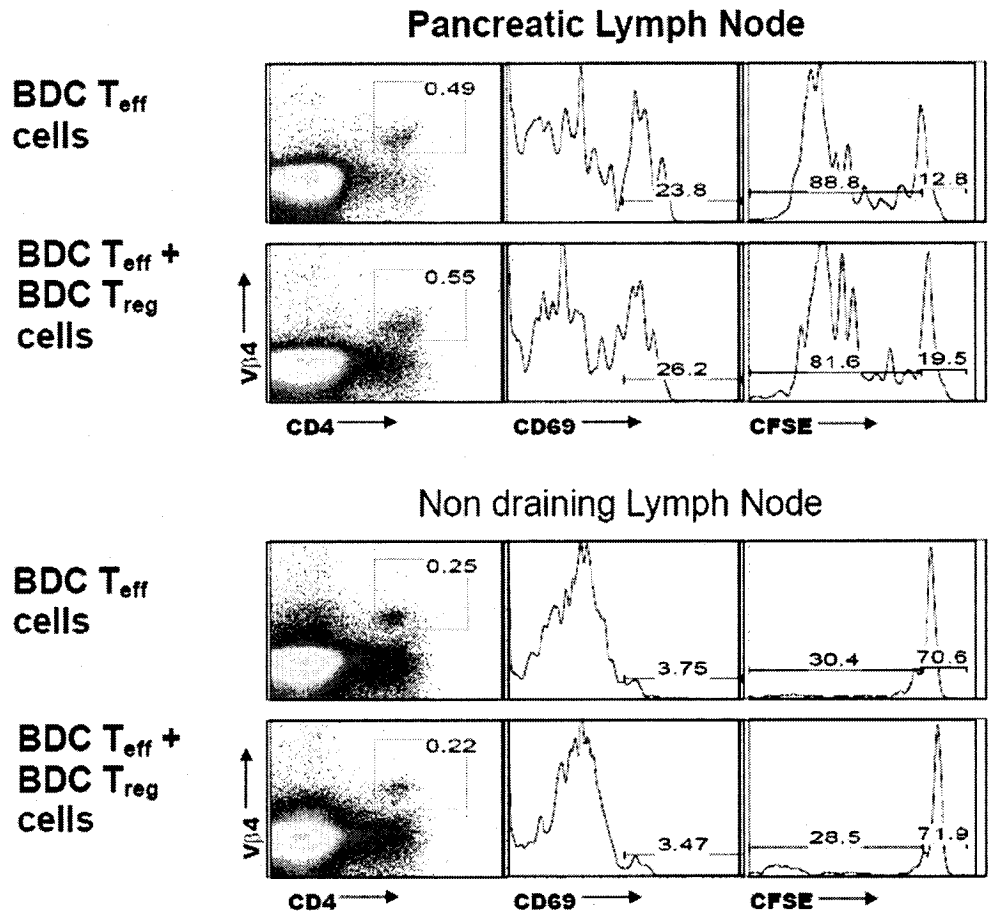

Figure 9. T_{reg} cells do not affect antigen-induced priming and proliferation of diabetogenic CD4⁺ T cells. T cells (BDC T_{eff} + BDC T_{reg}) or CD25 depleted T cells (BDC T_{eff}) were isolated by FACS from thymocytes or peripheral lymphocytes of 3-4 week old BDC2.5 mice, CFSE-labelled, and injected separately i.v. (1x 10^6) into NOD.TCR$\alpha^{-/-}$ mice. Pancreatic and non-draining lymph nodes of recipient mice were harvested on day 3 post-T cell transfer, and the percentages of BDC2.5 CD4⁺Vβ4⁺ T cells, as well as their activation status (CD69 expression) and proliferative capacity (CFSE dilution profile) in pancreatic (top) and non-draining lymph nodes (bottom) are indicated, either in the presence or absence

of BDC2.5 Treg cells. In all instances, cytometric analysis was done on gated $CD4^+V\beta4^+$ T cells. Similar results were obtained in three independent experiments.

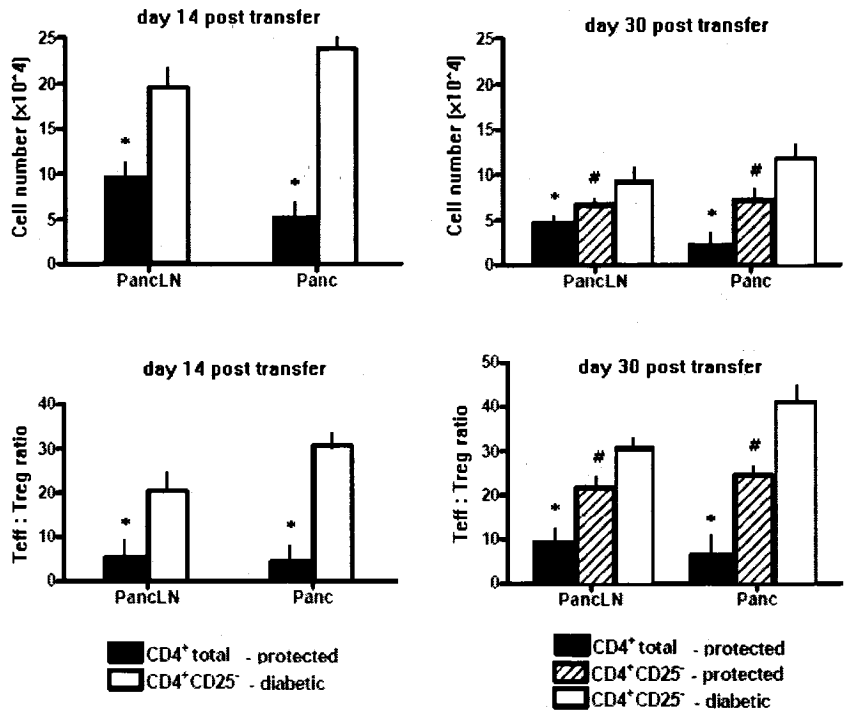

Figure 10. Resistance to T1D correlates with an increased proportion of Foxp3$^+$ CD4$^+$ T_{reg} cells in pancreatic environments. CD4^{SP} or CD4^{SP}CD25$^-$ T cells were isolated by FACS sorting from thymocytes or (3-4 week old) peripheral lymphocytes of BDC2.5 mice, and injected separately i.v. (2.5 x 10^5) into NOD.TCRα-/- mice. The onset of diabetes was assessed by blood glucose measurements every 24-48h. 14 and 30 days post-T cell transfer, pancreatic lymph nodes and pancreata were harvested from diabetic (white bars) and nondiabetic (hashed bars) recipients of CD4$^+$CD25$^-$ donor T cells and non-diabetic recipients of CD4$^+$ donor T cells (black bars). The population of infiltrating CD4$^+$Vβ4$^+$ T cells was quantified and the ratio of T_{eff} :T_{reg} cells (CD4$^+$Vβ4$^+$Foxp3$^-$:CD4$^+$Vβ4$^+$Foxp3$^+$) in these sites was determined by

intranuclear Foxp3 staining in $CD4^+V\beta4^+$ T cells. Data represents 3-5 mice analyzed. Error bars represent the mean ± SD.

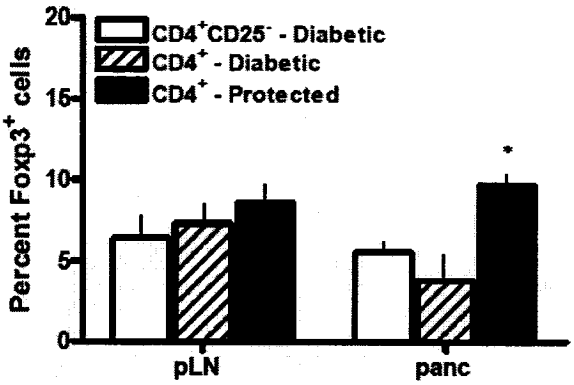

Figure 11. Temporal decline in the function of Foxp3-expressing CD4+ T_{reg} cells in NOD mice. CD4+ or CD4+CD25- T cells were isolated by FACS sorting from peripheral lymphocytes of 6-8 week old mice of BDC2.5 mice, and injected separately i.v. (2.5×10^5) into NOD.TCRα-/- mice. The onset of diabetes was assessed by blood glucose measurements every 24-48h. 14 days post-T cell transfer, pancreatic lymph nodes and pancreata were harvested. The proportion of Foxp3+ T_{reg} cells within the CD4+Vβ4+ T cell subset in pancreatic lymph nodes and pancreas of diabetic (white bars) recipients of CD4+CD25- donor T cells and diabetic (hashed bars) and non-diabetic (black bars) recipients of CD4+ donor T cells was analyzed. Data represents 3-5 mice analyzed. Error bars represent the mean ± SD.

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