Functional impact of the protective Idd3 allele on regulatory T cells and protection from type-1 diabetes

by

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Table of Contents

Abstract

NOD.B6 *Idd3* congenic mice, whose *Idd3* locus originates from the autoimmune resistant C57BL/6 mouse, demonstrate a drastic reduction in diabetes onset. In this study we demonstrate that NOD.B6 *Idd3* mice show halted disease progression and also show reduced levels of T_{H1} cells in their peripheral immune system. *In vitro*, $CD4^+$ $CD25^-$ effector T (T_{EFF}) cells from congenic mice display increased proliferation and IL-2 production compared to wild type NOD mice. However, unfractionated NOD.B6 *Idd3* $CD4^+$ T cells maintain a higher T_{REG}:T_{EFF} ratio and proliferate less than their NOD counterparts, suggesting increased immunoregulation. *In vivo*, the *Idd3* locus does not appear to reduce the number of islet-reactive cells or increase the systemic frequency of T_{REG} cells in the immune system of pre-diabetic mice. Instead, reduced infiltration of T_{EFF} cells in the pancreas of NOD.B6 *Idd3* mice suggests increased suppression of autoimmune cells at the site of inflammation *in vivo*. Consistent with this hypothesis, tolerance mechanisms in the NOD.B6 *Idd3* mouse are much better at suppressing the proliferation of islet-reactive T_{EFF} cells in the pLN. Furthermore, using a novel strain of BDC.Idd3 mice, which contain islet-reactive TCR transgenic $CD4^+$ T cells and which are homozygous for the B6-derived *Idd3* allele, we demonstrate that T_{EFF} cells containing the protective *Idd3* allele do not possess reduced diabetogenic potential *in vivo*. Using an adoptive transfer model for type-1 diabetes, we demonstrate that $CD4^+CD25^+$ T_{REG} containing the protective *Idd3* allele are intrinsically better suppressors than T_{REG} cells of NOD genotype. Collectively, our data show that the protective *Idd3* allele favours immunoregulation in the pancreatic lesion by enhancing T_{REG} cell function. Although the precise mechanism of this protection remains unknown, these findings may ultimately help develop therapeutic strategies to reduce or prevent the autoimmune destruction of pancreatic β islet cells in type-1 diabetes patients.

Résumé

Les souris congéniques NOD.B6 *Idd3*, dont le locus *Idd3* provient de la lignée C57BL/6 résistante à l'auto-immunité, présentent une faible incidence de diabète. Dans cette étude, nous montrons que dans les souris NOD.B6 *Idd3* la progression de la pathologie est arrêtée et que le nombre de cellules T_{H1} dans le système immunitaire périphérique y est réduit. *In vitro*, les cellules T effectrices $CD4^+CD25^-$ (T_{EFF}) issues de ces souris congéniques présentent une plus grandes capacités de prolifération et de production d'IL-2 que des souris NOD normales. Cependant, les cellules T $CD4^+$ NOD.B6 *Idd3* conservent une proportion de T_{REG} plus élevée et prolifèrent moins que leur équivalent NOD, suggérant un niveau plus élevé d'immuno-régulation. *In vivo*, le locus *Idd3* ne semble ni réduire le nombre de cellules auto-réactives, ni augmenter la fréquence des cellules T_{REG} dans le système immunitaire des souris pré-diabétiques. Au contraire, la diminution de l'infiltration des cellules T_{EFF} dans le pancréas des souris NOD.B6 *Idd3* suggère une meilleure suppression des cellules auto-immunes au site d'inflammation *in vivo*. En accord avec cette hypothèse, les mécanismes de tolérance dans la souris NOD.B6 *Idd3* permettent une plus forte suppression de la prolifération des cellules T_{EFF} diabétogènes dans les ganglions lymphatiques pancréatiques. De plus, grâce a l'utilisation d'une nouvelle lignée de souris BDC.Idd3, dont les cellules T $CD4^+$ expriment un TCR transgénique dirigé contre les îlots de Langherans, et sont homozygotes pour l'allèle *Idd3* provenant de la lignée C57BL/6, nous démontrons que le locus *Idd3* n'inhibe pas le potentiel diabétogène des cellules T_{EFF} *in vivo*. Grâce à un modèle de transfert adoptif du diabète de type 1, nous montrons que les cellules T_{REG} $CD4^+CD25^+$ portant l'allèle *Idd3* protecteur sont intrinsèquement meilleurs suppresseurs que les cellules T_{REG} issues de souris NOD. Ainsi, nos données démontrent que l'allèle protecteur *Idd3* favorise l'immuno-régulation au sein des lésions pancréatiques par le biais d'une amélioration fonctionnelle des cellules T_{REG}.

Abbreviations

AICD: activation-induced cell death

AIRE: autoimmune regulator gene

APC: antigen presenting cell (dendritic cells, macrophages, B-cells, etc.)

BrdU: 5-bromo-2-deoxyuridine

CFSE: carboxyfluorescin diacetate succinimidyl ester

CTLA-4: cytotoxic T-lymphocyte associated antigen-4

CY: cyclophosphamide

FACS: fluorescent-activated cell sorting

FasL: Fas ligand

Fgf-2: fibroblast growth factor

Foxp3: forkhead box protein 3

$Foxp3^{gfp}$ mice: express Foxp3-GFP fusion protein

GAD: glutamic acid dcarboxylase

GFP: green fluorescent protein

GITR: glucocorticoid-induced TNFR family related gene

ICOS: inducible costimulator

Idd: insulin-dependent diabetes (loci)

IFN-γ: interferon γ

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

IL-x: interleukin-x

IL-2R: interleukin-2 receptor

iNOS: inducible nitric oxide synthatse

IPEX: immunodysregulation polyendrocrinopathy enteropathy X-linked syndrome

LN: peripheral lymph nodes (always excludes the pancreatic lymph nodes)

MHC: major histocompatibility complex

NK: natural killer

NO: nitric oxide

NOD: non-obese diabetic (mouse)

pLN: pancreatic lymph node

RT-PCR: reverse transcription polymerase chain reaction

T1D: type-1 diabetes

TCR: T cell receptor

T_{EFF}: $CD4^{+}Foxp3^{-}$ non-regulatory T cells

TGF-β: tranforming growth factor β

T_{H1}: $CD4^{+}$ T helper-1 cells

T_{H2}: $CD4^{+}$ T helper-2 cells

TNF-α: tumor necrosis factor α

T_{REG}: $CD4^{+}Foxp3^{+}$ regulatory T cell

Introduction and Literature Review

I – Autoimmune type-1 Diabetes and the NOD mouse

A) Type-1 Diabetes Characteristics

Autoimmune type-1 diabetes (T1D) is a disease resulting from the targeted destruction of insulin-producing β islet cells in the pancreas by the immune system (1, 2). Clinical symptoms become apparent when patients can no longer produce sufficient levels of insulin and blood glucose levels rise above physiological levels leading to hyperglycaemia. If insulin levels are not therapeutically maintained by various methods such as daily injections, prolonged hyperglycaemia can lead to several complications including blindness, kidney failure and heart disease (1). Originally called juvenile diabetes, T1D is usually diagnosed before the age of 30, mostly in children and teenagers, and is thought to constitute at least 10% of all Canadian diabetics (2). The remaining diabetics are mostly afflicted with type-2 diabetes which is caused by either the reduced sensitivity to insulin or its reduced production and has a strong correlation with obesity and aging (1). T1D can be subdivided into two categories: autoimmune diabetes (type-1A) which occurs when the patient's immune system is responsible for the destruction of the β islet cells and idiopathic diabetes (type-1B) which is not immune-related and whose etiology is currently unknown (3). Since type-1B diabetes only represents a small fraction of all T1D cases and its existence remains controversial, the remainder of this text will focus solely on type 1A (4). A recent study looking at the worldwide incidence of T1D in young children revealed that disease frequency varies according to geographical location, race and ethnic background (5). Generally, T1D is more common in developed countries relative to underdeveloped countries with Finland, Sweden and Canada showing the highest incidence rates, at more than 25 cases per 100,000 people (5). Although genetic susceptibility appears to be a prerequisite for T1D (Table 1), the

concordance rate in monozygotic twins is only 30-50% (6). This suggests an important role for environmental factors on T1D onset such as pathogen infections and diet (7-9). The current model of T1D proposes that autoimmunity is triggered by an environmental factor in genetically susceptible individuals (Fig. 1).

Animal models have been an important tool for understanding the mechanism of autoimmune diabetes pathogenesis and for identifying genes involved in disease susceptibility. The non-obese diabetic (NOD) mouse, developed by Makino and colleagues in 1980, is the most frequently studied animal model for T1D (10, 11). These mice, when raised under conventional pathogen-free conditions, are known to develop spontaneous diabetes by 15-20 weeks of age in 70-80% of females and 20-30% of males (12). In recent years, it was found that type-1 diabetes patients and NOD mice share several immunogenetic and immunopathophysiological features (Table 2), thereby validating this animal model (10). A striking similarity for both mice and patients is the importance of the major histocompatibility complex (MHC) class II region as a genetic determinant for T1D. Additionally, multiple similarities between NOD and human disease progression suggest complex polygenetic dysregulations in both central and peripheral tolerance, leading to uncontrolled T cell survival, activation and function (10, 13). Ultimately, defective immunoregulation within the peripheral immune system will lead to the T cell-driven infiltration of the pancreas and destruction of β islet cells.

B) Pathogenesis of T1D

Since the pancreas and pancreatic draining lymph nodes (pLNs) are not readily accessible in humans, the NOD mouse has helped a great deal in elucidating disease progression. In fact, the T cell-mediated destruction of β islet cells in NOD mice is extremely synchronous with two important checkpoints: peri-insulitis and overt diabetes. At birth, all NOD mice show minimal immune

cell infiltration in the pancreas, nonetheless by 3 to 4 weeks of age, mononuclear immune cells suddenly begin surrounding the β islet cells: this constitutes checkpoint 1. Since the immune cells accumulate around the edges of the pancreatic islets without infiltrating them, this phenomenon is referred to as peri-insulitis. The infiltrate consists mostly of $CD4^+$ T cells, although cytotoxic $CD8^+$ T cells, natural killer (NK) cells and various antigen presenting cells (APCs) such as B-cells, macrophage and dendritic cells are also present (11, 14). Although multiple cell types are present in the pancreatic lesion, disease is dependent on $CD4^+$ T cells which recognize peptides presented by professional APCs via MHC class II. Diabetes progression is also dependent on $CD8^+$ T cells which recognize peptides displayed by MHC class I, expressed on all cells (15, 16). At the onset of peri-insulitis, the immune response is T_{H2} dominated and non-destructive to islet cells. However, by 10 weeks of age, the immune response has already shifted towards a pro-inflammatory T_{H1} response and immune cells infiltrate the pancreatic islet, causing severe insulitis (17). Despite the aggressive insulitis, some β islet cells still persist in the pancreas and checkpoint 2, or overt diabetes, only occurs when over 90% of these insulin-producing cells are destroyed and physiological blood glucose levels cannot be maintained.

In NOD mice, raised under pathogen-free conditions and on a specific diet, it is thought that the delay between birth and peri-insulitis simply constitutes the time required for self-reactive $CD4^+$ T cell activation and recruitment from the peripheral immune system into the pancreas (17). Interestingly, a wave of β-cell death has been observed in young 14-17 day old rodents and may be the initial trigger for diabetes progression (18-20). This phenomenon, which kills roughly one third of all β cells, is thought to be a consequence of pancreatic tissue remodelling and may lead to the uptake of antigen from the necrotic β cells by dendritic cells and its subsequent presentation to self-reactive $CD4^+$ T cells in the pLNs. This model is supported by a study showing that removal of the pLNs in 3 week old but not 10 week old NOD mice leads to diabetes resistance (21). This suggests that initiation of the autoimmune response occurs in the pLNs of 3-4

week old NOD mice and removal of the pLNs probably prevents the dendritic cell/ T cell encounter. This hypothesis is supported by another study, which determined that the CD86 co-stimulatory molecule, expressed predominantly on dendritic cells, is important for the activation of self-reactive T cells (22). The interaction between CD86 and CD28, which is expressed on T cells, was shown to be important in the early activation and proliferation of islet-reactive $CD4^+$ T cells, but not for their effector function which causes destruction of the pancreatic tissue. Taken together, these studies suggest that three factors are important for initiation of T1D in NOD mice: a wave of β cell death, the expression of CD86 on dendritic cells and the activation of $CD4^+$ T cells in the pLNs.

Based on various studies, the current model of autoimmune β cell destruction (23) proposes that macrophages and dendritic cells are the first immune cells present in the islets, where they uptake β cell auto-antigens and produce IL-12. The antigen and IL-12 will activate $CD4^+$ T cells which will, in turn, produce IL-2 and proliferate. The exact role of $CD8^+$ T cells is still not clearly understood, one model proposes that the $CD4^+$ T cell-derived IL-2 will activate the $CD8^+$ T cells and induce their differentiation into cytotoxic T cells. In this model, the migration of $CD4^+$ T cells into the pancreatic islets is followed immediately by the cytotoxic $CD8^+$ T cells (24). The $CD8^+$ T cells, which recognize antigen displayed by β cells via MHC class I, will target these cells for apoptosis, thus promoting a more aggressive $CD4^+$ T cell response in the later stages of insulitis (25). A second model suggests that $CD8^+$ T cell-mediated killing of β cells is the initiator of diabetes and leads to antigen uptake by APCs and subsequent activation of self-reactive $CD4^+$ T cells (23). Although, the precise role of $CD4^+$ and $CD8^+$ T cell subsets in diabetes progression remain controversial, it is accepted that both subsets contribute synergistically to the destruction of β cells. Islet-reactive cytotoxic $CD8^+$ T cells will induce apoptosis in β cells either by the perforin/granzyme pathway or by expression of Fas ligand (FasL) which binds to its receptor, Fas, on β cells (12, 26). Although activated $CD4^+$ T cells also upregulate FasL, they produce several pro-inflammatory

cytokines such as interferon (IFN)-γ, tumour necrosis factor (TNF)-α, IL-1 and IL-6 which have all been shown to induce β cell death (12, 26). In NOD mice, high levels of these cytokines correlate with cell destruction in Langerhans islets (27), however the majority of β cell death was shown to be caused by the synergistic effect of both IFN-γ and TNF-α (28). The TNF-α, IFN-γ and IL-1 cytokines can also cause destruction of islet cells indirectly by increasing the expression of inducible nitric oxide synthase (iNOS) in macrophages or endogenously in β-cells (26). This enzyme is responsible for production of nitric oxide free radicals, which can damage genomic DNA and lead to β cell apoptosis (29). *In vivo*, increased iNOS concentrations in the pancreatic islets correlate with decreasing insulin levels and eventual diabetes onset (30).

Given these findings, it is widely accepted that T_{H1} cells which produce TNF-α and IFN-γ cytokines are involved in disease progression. Inversely, T_{H2} cells, which produce IL-4 and IL-10 help regulate the T_{H1} response and can control the progression from peri-insulitis to destructive insulitis (16). However this appears to be a simplified view since experimental data is not so clear cut (Table 3). The APC-derived IL-12 is thought to be important in the activation of $CD4^+$ T cells and their differentiation into T_{H1} cells. Therefore, it is not surprising that intravenous (i.v.) administration of IL-12 accelerates diabetes progression (31). Unexpectedly however, IL-12-deficient and wild-type NOD mice were found to develop diabetes at similar frequency (32). Other studies have shown that administration of an anti- IFN-γ neutralizing antibody into mice prevents diabetes, whereas IFN-γ knockout NOD mice only show delayed disease (33, 34). Data regarding TNF-α also suggest a complex link between this cytokine and diabetes since administration of anti-TNF-α into 2 week old NOD mice is protective, whereas antibody treatment of 4 week old mice simply delays diabetes (35). Surprisingly, transgenic NOD mice which constitutively express TNF-α in their islets are also protected (36). Fortunately, both studies were reconciled when TNF-α was shown to have a dual role in diabetes progression (37). In early stages, TNF-α is important for disease progression and in late stages, it can abrogate the

immune response and reduce the number and function of self-reactive T cells in the islets. The precise role of T_{H2} cytokines is also unclear since administration of IL-4 or IL-10 prevents diabetes (38, 39) nevertheless IL-4-deficient or IL-10-deficient NOD mice do not show accelerated onset (40, 41). Furthermore, whereas transgenic expression of IL-4 in the β cells prevents T1D, the transgenic expression of IL-10 in these cells accelerates disease (42, 43). Thus, in general, favouring T_{H2} responses by cytokine injection or by blocking T_{H1} cytokines can prevent T1D. However, knockout studies have made apparent that the multiple cytokines involved in an autoimmune response have overlapping functions (12).

C) Defects in Central Tolerance

In NOD mice, some of the antigens recognized by self reactive $CD4^+$ and $CD8^+$ T cells include insulin, glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 and heat shock protein 60 (12, 44). Therefore, given the multitude of self reactive T cells in the NOD mouse's immune system, a dysregulation in central tolerance (during thymic selection) is likely to play a key role in disease susceptibility. In support of this hypothesis, the intra-thymic grafting of syngeneic or allogeneic islets prevent diabetes onset in NOD mice (45-48). In fact, three major defects in thymocyte selection have been reported to date by various independent groups. First, a global defect in apoptosis during the negative selection of semi-mature thymocytes was observed (49). Second, data from the protein crystal structure of the NOD MHC class II molecule ($I\text{-}A^{g7}$), the major genetic determinant of T1D, revealed that its structure may be intrinsically unstable, making it a poor peptide binder and thus hindering self-peptide-mediated negative selection (50, 51). Third, studies using the insulin 2 (*ins2*) knockout NOD mice, have revealed that abrogating insulin expression in the thymus greatly exacerbates disease (52, 53). Specifically, abrogating expression of the *Ins2* gene only affected thymic levels of insulin, not the pancreatic expression of the *Ins1* gene and this caused earlier onset of disease. This finding indirectly supports the observed defect in the NOD thymus regarding expression

of the Autoimmune Regulator (AIRE) gene, which is responsible for the thymic expression of numerous self-proteins and is important for the thymic deletion of numerous self-reactive T cells(54). Consistent with these findings, T1D patients show reduced expression of insulin mRNA in the thymus, suggesting that improper deletion of insulin-reactive T cells may be an important determinant for disease susceptibility (55). Therefore, multiple defects in the thymic selection process lead to increased numbers of islet-reactive T cells in the periphery favouring autoimmunity (Fig. 2).

Since islet-reactive cells are central in the pathogenesis in T1D, several groups have successfully isolated and cloned T cells from inflamed islets and spleens of NOD mice. Some of these T cell clones were used to produce T cell receptor (TCR) transgenic mice which recognize pancreas-derived antigens. The most frequently used strains of transgenic NOD mice are the 8.3 and BDC2.5 mice which possess islet-reactive $CD8^+$ and $CD4^+$ T cells, respectively. The 8.3 transgenic $CD8^+$ T cells were found to recognize islet-specific specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), which localizes to the endoplasmic reticulum and has no known function (12, 56). The specificity of BDC2.5 transgenic $CD4^+$ T cells, on the other hand, is still unknown, however a recent report suggests a GAD65-derived peptide may in fact be the targeted antigen (57). Both transgenic mice have proven to be a valuable tool for elucidating the various mechanisms involved in the progression to T1D, since they represent a monoclonal population of diabetogenic T cells which arise normally, albeit at small frequencies, in the wild-type NOD mouse. After onset of insulitis, NOD mice show a high percentage of $CD8^+$ T cells with the 8.3 transgenic TCR specificity in the peripheral immune system (58). Similarly, 0.1-0.2% of all mature $CD4^+$ T cells in the NOD mouse show the same specificity as the BDC2.5 transgenic $CD4^+$ T cells (59).

D) Defects in Peripheral Tolerance

Although self-reactive cells are present in T1D patients and NOD mice, they can also be found in all individuals and autoimmune-resistant mouse strains (e.g. BALB/c, C57BL/6 and C57BL/10). Under normal circumstances, these auto-reactive cells which escape negative selection in the thymus are kept under control by the various mechanisms of peripheral tolerance. Therefore, since NOD mice and autoimmune patients are capable of mounting a significant immune response to self-antigens this suggests a dysregulation in peripheral tolerance mechanisms (Fig 2). One mechanism of T cell regulation in the periphery is T cell co-stimulation. Activation of naïve T cells requires two separate signals to induce proliferation: antigen recognition via the TCR and recognition of co-stimulatory molecules CD80 or CD86 expressed on APCs by CD28 (16). If a T cell receives no co-stimulation after antigen recognition, it will be rendered unresponsive or anergic to subsequent TCR stimulation or it can undergo apoptosis (16). The NOD mouse possesses several defects in T cell co-stimulation, which are thought to be responsible for the initiation and progression of the autoimmune response (12). A well studied example is the CTLA-4 molecule, which is expressed on activated T cells and also binds CD80 and CD86. Unlike CD28, CTLA-4 is a down-regulator of the T cell response, inhibiting cell cycle progression, proliferation and IL-2 production (60). NOD mice show reduced surface expression of CTLA-4 on T cells compared to other autoimmune resistant strains of mice, which suggests that activated NOD T cells may be more resistant to this mechanism of peripheral tolerance (61). This is further supported by studies in which the administration of anti-CTLA-4 antibody before the onset of insulitis exacerbated disease (62, 63). These studies demonstrate that CTLA-4 signalling in pancreas-infiltrating T cells can control progression to overt diabetes, favouring the delay between checkpoints 1 and 2.

In addition, dendritic cells, which are the main initiators of the immune response, can express the CD80/CD86 co-stimulatory molecules and are therefore

important mediators of peripheral tolerance. One study showed that transfer of dendritic cells derived from the pLN, but not any other LN, protected recipient NOD mice from diabetes (64). This suggests a dysfunction in dendritic cell function since proper peripheral tolerance requires the addition of extra cells. Also, dendritic cells from both human diabetics and NOD mice show reduced growth in response to various growth factors compared to non-diabetic controls, suggesting a dysregulation in their development (65, 66). In NOD mice, developing dendritic cells show reduced proliferation and increased cell death *in vitro* (67). Once mature they show reduced expression of MHC class II and CD80/CD86 molecules compared to other strains of mice (68). Since $CD4^+$ T cell production of IFN-γ does not require costimulation whereas IL-4 does, this could account for, at least partially, the NOD mouse's predisposition for an islet-destructiveT_{H1} response (69, 70).

Another mechanism of peripheral tolerance is activation-induced cell death (AICD), which is similar to negative selection of T cells in the thymus. When T cells proliferate in response to TCR activation and costimulation, their sensitivity to apoptosis is greatly increased compared to naïve T cells (71, 72). AICD occurs when the TCR of a proliferating T cell is re-engaged causing apoptosis. AICD is important for controlling self-reactive T cells since these cells will bind abundant antigen, undergo apoptosis and will not cause damage to the host (73). Several studies have shown that both $CD4^+$ and $CD8^+$ T cells in the NOD mouse are more resistant to AICD when compared to C57BL/6 *in vitro* and *in vivo* (74-76). These studies imply that the self-reactive T cells present in NOD mice are capable of escaping AICD and initiating an autoimmune response.

Regulatory T (T_{REG}) cells are also important mediators of peripheral tolerance and crucial in the prevention of autoimmunity. Multiple studies have linked defects in this population with T1D in mice and humans. Early studies focusing on immunoregulation employed a model of diabetes transfer where splenocytes originating from diabetic NOD mice were injected into irradiated

NOD recipients, causing diabetes within 3 weeks (77). The transfer of diabetic splenocytes into non-irradiated 6 week old NOD recipients was unable to induce diabetes, suggesting efficient immunoregulation in pre-diabetic mice. The improper T_{REG} cell function in NOD mice was first shown when the co-transfer of diabetic splenocytes with splenocytes from young non-diabetic NOD mice could delay diabetes onset in irradiated recipients (78-80). Depletion experiments revealed that $CD4^+$ T cells were responsible for the immunoregulatory properties of the non-diabetic splenocytes. Furthermore, thymocytes were also shown to delay disease transferred by splenocytes of diabetic donors, and thymectomy of non-diabetic females abrogated the immunosuppressive properties of their splenocytes, suggesting that a population of $CD4^+$ T cells originating from the thymus are capable of regulating diabetes (78). Additional thymectomy experiments in NOD mice revealed that removal of the thymus in 3 week old mice but not 6-7 week old mice was capable of accelerating diabetes onset in NOD females (81). These findings thus suggest that a functional thymus-derived $CD4^+$ T_{REG} population is present in young NOD mice and is somehow defective in diabetic mice.

Additional evidence for the importance of T_{REG} cell function comes from the cyclophosphamide (CY) treatment of NOD mice which accelerates diabetes onset (82). It was demonstrated that CY accelerates the progression from peri-insulitis to diabetes and that the effect of this treatment affected immune cells rather than the β cells in the pancreas (82, 83). Consistent with these findings, the transfer of splenocytes from nondiabetic NOD donors into CY-treated mice prevented diabetes, suggesting that the treatment was affecting immunoregulatory mechanisms (82). Although the exact effects of CY on the immune system remain unknown, multiple studies have demonstrated increased IFN-γ production subsequent to treatment, which may reflect the reduced suppression of self-reactive T cells differentiating into T_{H1} (84, 85).

The acceleration of diabetes in CY-treated NOD mice can also be prevented by administration of anti-CD3 antibody (86). Surprisingly, anti-CD3 therapy was also capable of reversing established diabetes in NOD mice, reversing insulitis to peri-insulitis for at least 4 months post-treatment (86, 87). It was determined that anti-CD3 administration did not restore self-tolerance by depleting diabetogenic T cells or favouring a T_{H2} response. Instead, it appears that the antibody increases the frequency of specific T cell subsets with suppressive properties in the pLNs (88). This increased frequency is not due to expansion of endogenous T_{REG} cells but rather the induction of suppressive T cells from non-suppressive precursors (88). Thus, taken together these various models of diabetes enhancement or protection clearly highlight the need for additional suppressive T_{REG} cells in NOD mice to compensate for defective immunoregulation.

II - Naturally occurring regulatory T cells

A) Characteristics and Phenotype

In addition to T cells that mediate the immune response, classes of regulatory/suppressor T cells circulate in the periphery, controlling immunity and limiting the activation of T cells, B cells and certain cells of the innate immune system. A variety of T_{REG} cells can be induced in the peripheral immune system by various conditions such as continuous IL-10 exposure or antigen feeding which can give rise to T_{R1} and T_{H3} cells, respectively (89). However, the immune system also contains a population of naturally occurring $CD4^+CD25^+$ T_{REG} cells which originate from the thymus and represent roughly 10% of peripheral $CD4^+$ T cells in mice. These cells are capable of suppressing the proliferation of both $CD4^+$ and $CD8^+$ T cells *in vitro* in a dose dependent fashion (90, 91). Suppressor function was shown to necessitate the TCR activation of T_{REG} cells *in vitro*, however, once activated, suppression was independent of the target T cell's antigen specificity (92). *In vitro*, T_{REG} cell suppression is cell contact dependent and cytokine independent (90). Conversely, various *in vivo* models have suggested a role for

IL-10 and TGF-β, two immunosuppressive cytokines, in T_{REG} cell-mediated suppression (88, 93-95). Furthermore, T_{REG} cells can also directly inhibit B-cell responses or modulate dendritic cell activity, suggesting that multiple mechanisms underlie T_{REG} cell suppression *in vivo* (96, 97).

The existence of "suppressor T cells" was first hypothesized in 1972 by Gershon *et al.*, however it was only in 1995 that Sakaguchi *et al.* convincingly demonstrated their existence, renaming them "regulatory" T cells in the process (98, 99). It was observed that a small population of $CD4^+$ T cells expressed CD25 in normal non-immunized mice. Sakaguchi and colleagues noticed that transfer of $CD4^+$ T cells, when depleted of the $CD25^+$ subset, into immunodeficient mice induced various autoimmune diseases such as thyroiditis, gastritis, insulitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis, and polyarthritis (99). The transfer of $CD4^+CD25^+$ T cells prevented autoimmunity in a dose dependent fashion by inhibiting the expansion of self-reactive $CD4^+CD25^-$ T cells. These findings clearly demonstrate that pathogenic T cells are present in the peripheral immune system of healthy mice and that they are kept in check by a population of suppressive cells.

These naturally occurring T_{REG} cells were identified by the expression of the CD25 cell surface marker which is also termed the α-chain of the high affinity heterotrimeric IL-2 receptor (IL-2R) (99). It should be noted that all T cells constitutively express the β (CD122) and common γ (CD132) chains, which form a low affinity IL-2 receptor that is incapable of binding ligand and, thus, incapable of initiating a signalling cascade (100). The CD25 molecule is expressed on activated non-regulatory T cells subsequent to activation and, prior to 1995, was considered exclusively as an activation marker for $CD4^+$ T cells (100). Signalling through the fully assembled IL-2 receptor activates the STAT-5 transcription factor and leads to a positive feedback loop of CD25 expression, which assures the presence of the high affinity heterotrimeric IL-2 receptor (100). Although CD25 can be expressed by non-regulatory T cells, simply activating $CD4^+$ or

$CD8^+$ T cells to render them $CD25^+$ does not endow them with immunoregulatory properties either *in vitro* or *in vivo* (90, 101). These findings establish the $CD4^+CD25^+$ T_{REG} cells as a functionally and phenotypically distinct population of T cells. However, since CD25 expression is not exclusive to T_{REG} cells, this marker is only reliable within a naïve CD4 T cell population.

Since their discovery in 1995, numerous cell surface markers have been used to further distinguish T_{REG} cells such as GITR, CTLA-4, neuropilin-1, CCR4, CCR8, CD62L, LAG-3, CD103 or $CD45RB^{low}$(89). These various markers strongly suggest a heterogeneous population of regulatory T cell and, interestingly, when several subsets were compared, they displayed different suppressor properties (102). It was observed that specific autoimmune diseases, targeting different organs, could be induced when $CD4^+$ T cells from NOD mice were depleted of various regulatory subsets and transferred into immunodeficient hosts. Adoptive transfer of CD25-depleted $CD4^+$ T cells induced gastritis and mild diabetes whereas CD62L-depleted $CD4^+$ T cells induced fulminant diabetes and $CD45RB^{high}$ $CD4^+$ T cells induced major colitis with wasting disease. Although these three regulatory populations show some overlap with each other, they clearly demonstrate their inhibition of distinct organ-specific autoimmune diseases.

No precise combination of surface markers was established as a "strict" definition for naturally occurring regulatory T cells until 2003, when several groups reported that expression of the Foxp3 transcription factor correlated with $CD4^+CD25^+$ regulatory T cells (103-106). Furthermore, transduction of $CD4^+CD25^-$ T_{EFF} cells with a Foxp3-expressing retrovirus converted these cells into suppressive regulatory T cells (104). Inversely, a mutation in the gene encoding Foxp3 was identified as the cause of a fatal autoimmune lymphoproliferative disease in *scurfy* mice and patients with IPEX (immune dysfunction/ polyendocrinopathy/ enteropathy/ X-linked) syndrome (107). It was originally reported that $CD4^+$ T cells in *scurfy* mice were hyperresponsive to TCR

stimulation and produced increased levels of cytokines both *in vivo* and *in vitro* (108). However, later studies determined that disease is due to a $CD4^+CD25^+$ regulatory T cell deficiency and not an intrinsic $CD4^+CD25^-$ T cell defect (103). Several studies have now shown that Foxp3 is required for the development and function of regulatory T cells, thus establishing $CD4^+CD25^+Foxp3^+$ cells as the definition for naturally occurring T_{REG} cells. In 2005, Fontenot *et al.* generated mice expressing a Foxp3-GFP (green fluorescent protein) fusion protein, allowing the detection of this intra-nuclear marker using flow cytometry (109). This $Foxp3^{gfp}$ mouse revealed a new population of suppressive $CD4^+Foxp3^+$ cells which did not express CD25 and whose frequency varied in each lymphoid organ. These $CD4^+CD25^-Foxp3^+$ cells were also found to increase in aging mice, contributing to the characteristic hyporesponsiveness of $CD4^+CD25^-$ T cells in very old mice (110). It was proposed that the $CD4^+CD25^-Foxp3^+$cells are a reservoir of regulatory cells, since they can regain CD25 expression upon activation or homeostatic expansion (111). Therefore, Foxp3, unlike CD25, correlates perfectly with suppressive function and has become the most reliable marker for identifying T_{REG} cells in the murine immune system.

A population of T_{REG} cells has also been identified in human peripheral blood, which express CD25 and Foxp3, however, unlike mice, Foxp3 can be expressed by activated T_{EFF} cells (106). Thus, much like CD25, expression of Foxp3 can induced by activation of human $CD4^+$ T cells, demonstrating a different regulation of Foxp3 expression (107). Nonetheless, transduction of human lymphocytes with a Foxp3-expressing retrovirus did render them suppressive albeit less than in the murine studies (112). Therefore, although differences are already apparent between murine and human T_{REG} cells, Foxp3 is currently the best marker for this T cell subset.

B) Thymic development of T_{REG} cells

Unlike inducible regulatory T cells, the naturally occurring T_{REG} cells develop in the thymus alongside $CD4^+$ and $CD8^+$ T cells (113-115). An early study showed that $CD25^+$ T cells only appear in the murine peripheral immune system at 3 days of age at low levels and progressively increase until they represent 10% of all $CD4^+$ T cells by 3 weeks of age (115). Furthermore, thymectomy of day 3 neonatal BALB/c mice, before the appearance of the $CD25^+$ T cells, leads to various organ-specific autoimmune diseases (113, 115). Pathology can be prevented by the transfer of adult $CD4^+CD25^+$ T_{REG} cells into thymectomized mice, suggesting a delayed output of T_{REG} cells compared to T_{EFF} cells. A more recent study, using the $Foxp3^{gfp}$ mouse, showed delayed Foxp3 induction in thymocytes of neonatal mice compared to the generation of T_{EFF} cells (116). Analysis of adult $Foxp3^{gfp}$ mice revealed that the majority of T_{REG} cells reside in the thymic medulla (109). Interestingly, neonate mice were found to have an underdeveloped thymic medulla whose area tripled between day1 and day 4 after birth, suggesting that the appearance of T_{REG} cells may be limited by the delayed formation of their physiological niche in the thymus (116). When the thymus is fully matured, $CD25^+Foxp3^+$ cells are found mostly within the CD4 single-positive thymocyte compartment. Earlier stages of thymocyte maturation such as $CD4^+CD8^+$ and $CD4^-CD8^-$ cells, accounted for less than 10% of all Foxp3 expressing cells in the thymus, suggesting that Foxp3 expression is induced at the later stages of thymocyte development. The presence of $Foxp3^+$ cells in the thymic medulla also supports this theory since this region typically harbours the most mature thymocyte population (109).

Analysis of $Foxp3^{gfp}$ mice also suggests that T_{REG} cell development is not hard-wired into cells, instead, it requires TCR/MHC interactions in the thymus (109). Although T_{REG} cells are a polyclonal population, several studies have suggested that T_{REG} cells preferentially express TCRs which recognize self-antigens (117-119). The current view is that high affinity TCR/self-peptide

interactions direct the selection of T_{REG} thymocytes in a process distinct from positive selection and clonal deletion (118). However, efficient generation of T_{REG} cells requires another important signal: CD28 co-stimulation. The engagement of CD28 on developing $CD4^+CD8^+$ thymocytes was shown to induce Foxp3 expression and thus initiate the T_{REG} cell development program (120). In fact, abrogation of the CD28 signalling pathway, by knocking out CD28 or both of its ligands (CD80 and CD86), drastically reduces thymic T_{REG} cell development in various strains of mice (109, 120). These findings also explain the strong presence of $Foxp3^+$ cells in the thymic medulla since the expression of CD86 in the thymus is largely restricted to this region.

The CD28 co-stimulatory signal is known to promote IL-2 production in activated T cells by at least two distinct mechanisms. First, co-stimulation induces histone acetylation and chromatin remodelling of the IL-2 promoter region, rendering it highly accessible to DNA binding proteins and thus, transcription (121). Second, the half-life of IL-2 mRNA was shown to increase subsequent to CD28 signalling (122). Therefore, not surprisingly, CD28 signalling is responsible for the production of IL-2 in the thymus in quantities sufficient to support efficient T_{REG} cell development (120). However, CD28-mediated induction of Foxp3 was shown to be independent of IL-2, suggesting that this cytokine is favourable but not essential for T_{REG} development (120). Indeed, IL-2 knockout mice contain $Foxp3^+$ cells in the thymus, albeit at reduced levels, supporting the IL-2 independent development of T_{REG} cells (123, 124). However, IL-2 belongs to the family of common γ-chain (CD132) cytokines which includes IL-4, 7, 9, 15 and 21, and at least one of these cytokines is essential for T_{REG} cell generation (124, 125). This was shown when γ-chain-deficient $Foxp3^{gfp}$ mice demonstrated a complete absence of Foxp3 cells in the thymus (124). This suggests that other common γ-chain cytokines may compensate for an IL-2 deficit during T_{REG} cell development. Thus, to date, three signals have been identified as essential to induce Foxp3 expression and hence influence T_{REG} cell development in the thymic medulla: a high affinity TCR/MHC interaction, presumably with

self-antigen, a CD28 co-stimulatory signals and a common γ-chain cytokine-derived signal.

C) T_{REG} cell homeostasis and IL-2

Although IL-2 is not essential for T_{REG} cell development in the thymus, multiple studies have shown the necessity of this cytokine for the survival and proper function of T_{REG} cells in the peripheral immune system. Early *in vitro* studies described the T_{REG} cells as anergic since they do not proliferate in response to TCR activation regardless of the concentration of anti-CD3 or concanavalin A used (90). The addition of IL-2 to cell cultures did restore T_{REG} cell responsiveness to TCR signalling, where proliferation was comparable to T_{EFF} cells. Unlike activated T_{EFF} cells, T_{REG} cells are incapable of producing IL-2. The absence of IL-2, subsequent to T_{REG} cell activation is due to a lack of chromatin remodelling at the promoter region of the IL-2 gene, rendering it inaccessible to transcription factors (126). Also, several studies have shown that the Foxp3 transcription factor directly suppresses production of IL-2, IL-4 and IFN-γ following TCR-mediated stimulation (107). Thus, this inability to produce IL-2 entails the requirement for exogenous sources to support T_{REG} cell survival both *in vivo* and *in vitro*. The primary source for IL-2 *in vivo* is antigen-stimulated mature T cells and, recently, a small pool of activated T_{EFF} cells was identified in the peripheral immune system which are $CD25^{low}Foxp3^{-}$ (109, 127). These cells probably represent a small pool of self-reactive T_{EFF} cells in the unmanipulated immune system, which remain non-pathogenic and are essential for T_{REG} cell homeostasis. Although controversial, another possible source for this cytokine, are dendritic cells which can produce IL-2 early after their activation (128).

The importance of IL-2 in immunological tolerance is clearly illustrated in *Il2*$^{-/-}$, *Il2ra*$^{-/-}$ and *Il2rb*$^{-/-}$mice which fail to express IL-2, CD25 and CD122 respectively. Even though disrupting IL-2 signalling impairs T cell proliferation and effector functions, the knockout mice paradoxically develop a fatal

lymphoproliferative disease (129). Disease reflects immune-dysregulation of both $CD4^+$ and $CD8^+$ T cells since either subset is sufficient to cause disease (129). The *Il2*$^{-/-}$, *Il2ra*$^{-/-}$, *Il2rb*$^{-/-}$ and *STAT5*$^{-/-}$ mice were all shown to have reduced numbers of T_{REG} cells in the periphery and this appears to be the main reason for the observed uncontrolled proliferation of T_{EFF} cells (123, 124, 129, 130). Although the transfer of wild-type $CD4^+CD25^+$ T_{REG} cells into neonatal IL-2-deficient mice could not rescue disease, their transfer into neonatal *Il2rb*$^{-/-}$ mice completely prevented disease (131). These *Il2rb*$^{-/-}$ mice, which can produce IL-2, are capable of expanding small number of transferred T_{REG} cells effectively suppressing disease. The transferred T_{REG} cells were shown to seed the lymph nodes (LN) of recipient mice and undergo extensive IL-2-mediated proliferation (132). This IL-2 driven expansion surely explains the failure to rescue *Il2*$^{-/-}$ mice by T_{REG} cell transfer. Surprisingly, T_{REG} cells only migrated into the spleen three weeks after their transfer, suggesting that the majority of homeostatic T_{REG} cell expansion occurs in the LN. Furthermore, blocking IL-2 *in vivo* by administration of a neutralizing antibody was also capable of reducing peripheral T_{REG} cell numbers in BALB/c mice, which lead to autoimmune gastritis (127). IL-2 neutralization blocked physiological proliferation of $CD4^+CD25^+$ T_{REG} cells and reduced overall cell frequency by 80%. Inversely, mice expressing a constitutively active form of STAT-5, which mimics constant IL-2 signalling, show a 6-fold increase of T_{REG} cell numbers (133). Taken together, these findings suggest a direct role for IL-2 in T_{REG} cell expansion in neonatal mice and in the maintenance of appropriate T_{REG} cell numbers in the periphery, thereby preventing the proliferation of self-reactive T_{EFF} cells.

The downstream effects of IL-2 signalling which promote T_{REG} cell survival were elucidated by two independent studies using bone marrow reconstitution experiments. When chimeras were generated by mixing *Il2ra*$^{-/-}$and *Il2ra*$^{+/+}$ bone marrow, the absence of IL-2 signalling did not prevent the thymic development of $Foxp3^+$ cells (123). However, in the peripheral immune system, the majority of $Foxp3^+$ cells were of *Il2ra*$^{+/+}$ origin, suggesting that IL-2

signalling provides mature T_{REG} cells with a competitive advantage (123, 124). DNA micro arrays determined that the various genes affected by IL-2 signalling in T_{REG} cells involved the maintenance of homeostatic proliferation and metabolic fitness (124). Furthermore, the administration of recombinant IL-2 or IL-2 neutralizing antibody into recipient mice was shown to increase or decrease Foxp3 expression on a per cell basis, suggesting it can directly promote the expression of the "master switch" gene (124). Paradoxically, although IL-2 is essential for T_{REG} cell survival, the current belief is that they inhibit T_{EFF} cell proliferation by suppressing IL-2 mRNA (90, 134). This probably reflects a negative feedback loop where an overly suppressed immune system cannot support T_{REG} cell survival.

Although controversial, IL-2 is thought to play an important role in T_{REG} cell function since it can modulate Foxp3 expression. One study has demonstrated that IL-2 is essential for initiating the suppressive activity of T_{REG} cells *in vitro* since addition of IL-2 neutralizing antibody to T_{EFF}/T_{REG} co-cultures inhibited T_{REG}-mediated suppression of *Il-2* transcription in T_{EFF} cells (134). However, when IL-4 was added simultaneously with IL-2 antibody, suppression of IL-2 mRNA was observed, suggesting that IL-4 can substitute for IL-2 in the activation of the T_{REG} cell suppressor mechanism. *In vivo*, IL-2 signalling also appears to be essential for the efficient suppressive function of T_{REG} cells, as demonstrated in an animal model for immunity to tumor/self-antigen (135). This study shows that *Il2ra*$^{-/-}$ T_{REG} cells cannot suppress immune responses *in vivo*, whereas wild type T_{REG} cells can. In contrast, other studies have shown that thymus-derived *Il2ra*$^{-/-}$ T_{REG} cells are less suppressive than their wild-type counterparts, nonetheless, even in the absence of IL-2 signalling, T_{REG} cells did show suppressive activity (123). This suggests that although IL-2 signalling favours T_{REG} cell-mediated suppression, it may be dispensable. Surprisingly, one study even reported no discernable difference in the suppression mediated by $CD4^+Foxp3^+$ cells isolated from the LNs of *Il2ra*$^{-/-}$ or wild-type mice (124). Unfortunately, the authors did not address whether IL-4 signalling was compensating for the lack of CD25 in

Il2ra$^{-/-}$cells. Additionally, CD25 is able to regulate high-affinity interactions in trans, therefore another unaddressed possibility is that CD25 expressed on activated T_{EFF} cells may augment IL-2 signalling in the *Il2ra*$^{-/-}$ T_{REG} cells which still express the β and γ chains of the IL-2 receptor (136). Therefore since T_{EFF}-derived IL-2 may still be providing signals to T_{REG} cells in this system, the effects of this cytokine on T_{REG} cell suppressor activity cannot be fully discounted. Taken together, these studies support a model of suppression where IL-2 or IL-4, produced initially by antigen-stimulated T_{EFF} cells, activates T_{REG} cell suppressor function and is then shut down.

This complex relationship between IL-2 and T_{REG} cells reveal an underlying feedback loop where an excess of activated T_{EFF}-derived cytokines will favour expansion and function of T_{REG} cells. This system also limits the over-accumulation of T_{REG} cells given the scarce amounts of IL-2 produced in a highly suppressed peripheral immune system. Any disruption of this balance can thus favour autoimmunity or prevent the establishment of a proper immune response.

D) T_{REG} cells and costimulation

Since IL-2 production requires co-stimulation of T cells, the CD28 signalling pathway predictably favours T_{REG} homeostasis in the peripheral immune system. The importance of CD28 was demonstrated when the administration of anti-CD80 and anti-CD86 antibodies in several strains of mice consistently reduced the physiological proliferation and survival of T_{REG} cells (137). This CD28 signalling blockade ultimately leads to a substantial decrease of T_{REG} cell numbers. Although CD28 signalling is undoubtedly responsible for *in vivo* production of T_{EFF} cell-derived IL-2, it was also shown that CD28 signalling directly affects T_{REG} cells. The transfer of CFSE-labelled T_{REG} cells into untreated and anti-CD80/CD86-treated recipients revealed that CD28 signalling is important for their steady state homeostatic proliferation in the immune system. Additionally, since wild-type T_{REG} cells fail to survive when transferred into

CD28-deficient recipients, this suggests that the effect of CD28 may be indirect. Given the reduced levels of IL-2 detected in CD28-deficient mice, it is likely that CD28 signalling may simply be important for the production of IL-2 by T_{EFF} cells. However, T_{REG} cells pre-cultured in IL-2 prior to their transfer into CD28-deficient recipients are capable of surviving for at least 30 days, suggesting a direct role for CD28 on T_{REG} cell homeostasis as well. Since blocking CD80/CD86 signalling also correlates with reduced expression of CD25 on T_{REG} cells, it was suggested that the role of CD28 is to maintain expression of CD25 on this population, allowing for their survival. This is also demonstrated by the maintenance of CD25 expression on wild-type T_{REG} cells pre-cultured in IL-2 and transferred into CD28-deficient hosts. However evidence is insufficient to determine whether CD28 signalling directly affects T_{REG} cells. This hypothesis is further contradicted by the flow cytometric analysis of T_{REG} cells in CD28-deficient mice, which, although reduced in numbers, express equivalent CD25 as their wild-type counterparts (138).

In vitro, CD28 mediated co-stimulation, unlike IL-2 or IL-4 signalling, does not appear to be required for the activation of T_{REG} cell suppressive activity (139). However, T_{REG} cell proliferation readily occurs in the presence of activated dendritic cells without the addition of IL-2 to cell cultures (140). Interestingly, when $CD86^{high}$ dendritic cells were sorted out and used to activate T_{REG} cells, this yielded the highest proliferation, suggesting that CD28-mediated signalling may replace IL-2 for breaking the characteristic anergy of these cells. Nonetheless, it was shown that CD86 and TCR stimulation are not sufficient to drive T_{REG} cell division, since activated $CD86^{+}$ B-cells are unable to induce proliferation of T_{REG} cells (141). Taken together, these findings show that CD86 is important but not sufficient for T_{REG} cell proliferation and demonstrate the importance of CD28 signalling in the homeostasis of peripheral T_{REG} cells.

III – The role of T_{REG} cells in Diabetes

A) Role of T_{REG} cells in diabetes suppression

In the NOD mouse, the delay between checkpoints 1 and 2 is strongly suggestive of either a gradual loss of T_{REG} cell function or accumulation of activated T_{EFF} cells which overwhelm the T_{REG} cell compartment. Although peri-insulitis begins at 3-4 weeks of age, an additional 5-6 weeks are required for the immune infiltrate to actually enter the pancreatic islets. As previously mentioned, early experiments demonstrated that $CD4^+$ T cells from young pre-diabetic NOD mice could suppress disease induced by lymphocytes from diabetic NOD mice (78, 80). These experiments reveal the presence of an immuno-regulatory population and the age-dependent decrease in their number or function. Since then, various studies have identified $CD4^+CD62L^{hi}$ and $CD4^+CD25^+$ T cells as the suppressors of diabetes, with $CD4^+CD25^+CD62L^{hi}$ cells being the most suppressive (102, 142-144). Some studies have also suggested that TCR specificity of the T_{REG} cell population may influence suppressor function since islet-specific T_{REG} cells from the BDC2.5 TCR transgenic NOD mouse are more effective than polyclonal NOD T_{REG} cells at controlling diabetes progression (59, 145, 146). It was shown that *ex vivo* expanded BDC2.5 T_{REG} cells were highly efficient at suppressing diabetes when co-transferred with diabetogenic splenocytes into immunodeficient recipients. Remarkably, these expanded T_{REG} cells were also capable of reversing established diabetes in regular NOD mice (145). Although the BDC2.5 T_{REG} cells represent a monoclonal pool of transgenic T cells, wild-type NOD mice contain a rare population of polyclonal T_{REG} cells with the same specificity and which are also quite efficient at suppressing diabetes (59).

Studies using the BDC2.5 T_{REG} cells have been invaluable for elucidating the precise mechanism of T_{REG} cells in the suppression of diabetes. First, although BDC2.5 mice show no immune cell infiltration in the pancreas the first two weeks

of life and very few activated $CD4^+CD69^+$ T cells in the pLN, T cells abruptly invade the pancreatic islets by 15 to 18 days (147). This infiltration rapidly progresses into insulitis and by 3-4 weeks of age all of the islets in the pancreas of BDC2.5 mice are heavily infiltrated. These observations demonstrate that the BDC2.5 transgenic $CD4^+$ T cell response to self-antigen is highly synchronous and thus a good model for elucidating the various steps involved in the loss of self tolerance in the context of T1D. Second, paradoxically, only 10-15% of BDC2.5 female mice develop diabetes by 30 weeks of age which is significantly less than the ~80% incidence observed in non-transgenic/wild-type NOD mice. Therefore, even though insulitis occurs earlier and is seemingly more aggressive in BDC2.5 mice, immunoregulatory mechanisms are able to control autoimmunity. This may reflect a more suppressible monoclonal T_{EFF} population possessing a single specificity or alternatively, the efficiency of a highly-specific T_{REG} cell population.

Recent studies have been successful at elucidating the mechanism of diabetes suppression in BDC2.5 mice and have established a working model (148, 149). It was observed that a population of $CD4^+CD25^+CD69^-$ T_{REG} cells are in fact present in the pancreas along with T_{EFF} cells in 3-4 week old BDC2.5 mice and that both subsets are actively proliferating (148). Analysis of these pancreas-infiltrating cells suggests that they represent a functionally different and more "specialized" population of T_{REG} or T_{EFF} cells than their counterparts in the pLN and other non-draining LNs. The pancreatic T_{EFF} cells were very potent inducers of diabetes compared to spleen-derived T_{EFF} cells in adoptive transfer experiments and, conversely, pancreatic T_{REG} cells were very efficient suppressors of disease. Microarray analysis confirmed their increased suppressor function since most conventional T_{REG} cell marker-genes were upregulated in pancreas-derived cells compared to non-draining LNs and even pLN. Once in the pancreas T_{REG} cells increase expression of immunosuppressive cytokine IL-10, inducible costimulator (ICOS) and GITR (148). Consequently, the suppression of diabetes may be linked to T_{REG}-derived IL-10 since this cytokine is a well documented

suppressor of T_{H1} immune responses. Amazingly, the administration of anti-ICOS blocking antibodies into neonate BDC2.5 mice induced diabetes by 25 days of age, shortly after the onset of insulitis. Therefore, blocking ICOS prevented immunoregulation typically seen after pancreas infiltration in BDC2.5 mice, suggesting loss of T_{REG} cell function. Similarly, Foxp3-deficient BDC2.5 mice, which contain no T_{REG} cells, develop diabetes by day 21 (149). However, contrary to the systemic deficiency of T_{REG} cells in the Foxp3 knockout mice, anti-ICOS antibody administration only decreased T_{REG} cell frequency locally in the pancreas. Therefore, ICOS-derived signals are important in the pancreas for increased T_{REG} cell function which leads to diabetes protection in BDC2.5 mice. However, one problem with this study is the use of CD25 to identify T_{REG} cells and not Foxp3, implying that the observed effects of anti-ICOS treatment may simply reflect a reduction of CD25 expression in $CD4^+Foxp3^+$ T_{REG} cells without necessarily reducing their numbers in the pancreas. Also, the microarray data, which is based on the identification of T_{REG} cells by CD25 expression, may not be very relevant since each organ analyzed may contain varying frequencies of contaminating $Foxp3^-$ T_{EFF} cells within the $CD4^+CD25^+$ population.

Once "primed", pancreatic T_{REG} cells are thought to suppress the progression of insulitis, not its initiation (142, 149). It was observed comparing wild-type and Foxp3-deficient BDC2.5 mice that T_{REG} cells do not influence the activation or proliferation of T_{EFF} cells, as assessed by CD69 expression and 5-bromo-2-deoxyuridine (BrdU) incorporation, respectively (149). Differentiation of T_{EFF} cells into T_{H1} cells was not affected by T_{REG} cells either, since no differences were observed in IFN-γ mRNA levels. The main impact of T_{REG} cell suppression is the delay of insulitis severity, resulting in reduced T_{EFF} cell frequency in the pancreas. This is highlighted in Foxp3-deficient or anti-ICOS treated BDC2.5 mice where insulitis is immediately aggressive. Additionally, since pancreatic T_{REG} cells adoptively transferred into immuno-deficient recipients do not migrate to the pancreas, this suggests a model where T_{EFF} cell-derived signals attract T_{REG} cells into the pancreas. Once there, T_{REG} cells receive

costimulatory signals via the ICOS molecule and this increases signature T_{REG} genes such as *Il-10* which mediate the suppression of insulitis progression. Unfortunately, in non-transgenic NOD mice, the precise role of IL-10 remains controversial since the administration of a neutralizing antibody did not abrogate $CD4^+CD62L^{high}$-mediated suppression in adoptive transfer experiments (142). Also, single cell analysis of pancreas-derived $CD4^+CD25^+CD62L^{high}$ T_{REG} cells revealed no expression of IL-10 mRNA. However, since BDC2.5 mice represent an exaggerated autoimmune reaction, this may simply reflect a system of multiple overlapping immunosuppressive mechanisms mediated by T_{REG} cells in the pancreatic islet cells.

In addition, TGF-β has also been linked to diabetes protection in NOD mice in at least two separate models. One group showed that a transient pulse of TGF-β in the pancreatic islets during checkpoint 1 (peri-insulitis) is sufficient to inhibit disease by increasing the frequency of intra-islet $CD4^+CD25^+Foxp3^+$ T_{REG} cells (150). This increase may be due to the expansion of naturally occurring T_{REG} cells or possibly by the TGF-β-mediated induction of Foxp3 in T_{EFF} cells (151). Since TGF-β can cause non-regulatory cells to become suppressive, this population may represent induced T_{REG} cells. Similarly, the anti-CD3 treatment of diabetic NOD mice was shown to induce a population of T_{REG} cells from non-T_{REG} cell precursors (88). This was demonstrated when anti-CD3 injection into CD28-deficient NOD mice (which contain very few T_{REG} cells) lead to the appearance of immunosuppressive $CD4^+CD25^+$ T cells and the remission of diabetes. Disease remission was abrogated by co-administration of a TGF-β neutralizing antibody, supporting the theory of a TGF-β-induced population of T_{REG} cells. Unfortunately, since the exact source of TGF-β remains uncertain and the origin of this T_{REG} population is unknown (naturally occurring or induced), these findings remain controversial. Also, since CD25 was used as a T_{REG} cell marker in this study, it is unclear whether TGF-β merely expands the population of $CD25^-Foxp3^+$ T_{REG} cells or whether it is capable of inducing *de novo* Foxp3 expression.

Although several studies have focused on the mechanism of diabetes suppression by T_{REG} cells, the exact reason for loss of tolerance remains unclear. Since diabetes still occurs in a small number of BDC2.5 mice which contain functional T_{REG} cells, this suggests that the NOD genotype is responsible for the age-related loss of immunoregulation and the possible decrease of T_{REG} cell numbers or function (152, 153). However, compared to other non-autoimmune strains, NOD mice do not show decreased frequency of T_{REG} cells in either the thymus or peripheral immune system (153). Instead, several studies point to an age-related decrease in T_{REG} cell suppressor function and expression of *Foxp3*, *Il-10* and *Tgfb1* genes (152, 154). However, once again these studies did not use Foxp3 to identify T_{REG} cells and these findings may simply reflect that the $CD4^+CD25^+$ cells are progressively contaminated with activated non-regulatory T_{EFF} cells. Therefore, an age-dependent loss of T_{REG} cell numbers or function has yet to be clearly demonstrated. Additionally, it appears as though T_{EFF} cells become "less suppressible" in older NOD mice, displaying resistance to TGFβ-dependent mediated inhibition, which leads to increased cell proliferation and IFN-γ production (152, 155). Therefore, it appears as though loss of immunoregulation in NOD mice is due to qualitative age-dependent changes in both T_{REG} and T_{EFF} populations.

B) Role of IL-2 in diabetes protection

In addition to these age-related changes in cell function, both $CD4^+$ and $CD8^+$ T cells in NOD mice are hyporesponsive to TCR activation (156). Surprisingly, T cells respond normally to TCR activation until 4 weeks of age, at which point T cells begin to demonstrate an intrinsic defect in their response to TCR stimulus, coinciding with the onset of insulitis. A subsequent study revealed that T cell anergy was caused by the drastic reduction of IL-2 produced by T cells and the complete absence of IL-4 (157). Addition of IL-2 or IL-4 to cell cultures rescues T cells from this hyporesponsiveness phenotype (157). Given the

importance of IL-2 on T_{REG} cell homeostasis, reduced levels of this cytokine may explain, at least partially, the loss of self tolerance which leads to peri-insulitis (checkpoint 1) and, ultimately, overt diabetes (checkpoint 2) in NOD mice.

At the onset of peri-insulitis, these reduced IL-2 levels may be responsible for the age-related decrease in T_{REG} cell function observed in NOD mice. This hypothesis is supported by IL-2 neutralization studies carried out in the NOD mouse in which administration of anti-IL-2 exacerbated diabetes by promoting earlier disease onset and more aggressive insulitis (127). Furthermore, the majority of NOD mice receiving antibody also developed a wide spectrum of additional organ specific autoimmune diseases, including gastritis and thyroiditis (127). These findings demonstrate that perturbation of the IL-2 pathway accentuates the NOD phenotype and highlights the importance of this cytokine in the maintenance of self-tolerance. Certain lines of evidence also identify IL-2 as one of several susceptibility genes for T1D.

C) The NOD.B6 *Idd3* congenic mouse

To date, over 20 genetic loci (termed *Idd* loci, for insulin-dependent diabetes) have been mapped that contribute to the T1D susceptibility in NOD mice (Table 4). These loci were identified mostly through the use of congenic mice which are generated by crossing NOD mice with autoimmune resistant strains such as C57BL/6 or C3H/HeJ mice, followed by the successive backcrossing of progeny onto the NOD background (158). After multiple backcrosses, strains of NOD mice are generated which containing the complete NOD genome at all but one genetic region which originates from diabetes-resistant mice. Most of the *Idd* loci have been mapped to well defined regions of the mouse genome and were found to contain multiple genes. Therefore, each *Idd* locus may contain several candidate genes, illustrating the complex genetic control of disease susceptibility. Certain strains, such as the NOD mouse containing a protective *Idd5* allele (termed NOD.B6 *Idd5*), show slightly reduced

diabetes onset (60% of females by 30 weeks (158)) whereas others show a more dramatic decrease in disease.

The *Idd3* locus seems to be one of the most important determinants of diabetes susceptibility. Female NOD mice containing a protective *Idd3* locus from C57BL/6 mice (termed NOD.B6 *Idd3* mice) show less than 20% incidence of diabetes by 30 weeks of age (158, 159). The *Idd3* locus was eventually mapped to a 780 kb region of mouse chromosome 3 and contains three genes: *Il-2*, *Il-21* and fibroblast growth factor-2 (*Fgf-2*) (160, 161). The main candidate genes for diabetes protection by the *Idd3* locus are *Il-2* and *Il-21*. Thus far, only one study has suggested a role for IL-21 whereby expression of the protective *Idd3* allele results in reduced levels of this cytokine (161). In fact, in wild-type NOD mice the higher expression of IL-21 leads to the reduced survival of T cells and the onset of lymphopenia, a known catalyst for autoimmunity (162). Contrary to other common γ-chain cytokines, IL-21 does not support the survival of T cells. Instead, it promotes their proliferation and consequential down-regulation of anti-apoptotic genes, which causes lymphopenia in NOD mice.

Given the importance of IL-2 in the maintenance of tolerance, this cytokine is also a strong candidate for protection in NOD.B6 *Idd3* mice. One study identified different glycosylation patterns between NOD- and NOD.B6 *Idd3*-derived IL-2 (163). Whereas NOD mice produce a single glycosylated species of IL-2, NOD.B6 *Idd3* mice produce both unglycosylated and glycosylated species of this cytokine. It is thought that the difference in glycosylation may affect the half-life, binding affinity or signalling of the IL-2 molecule although none of these theories have been directly proven. Several studies, in which allogeneic islet transplantations were performed in NOD and NOD.B6 *Idd3* mice, show a generalized defect in tolerance induction in wild-type NOD mice (164, 165). It was determined that one or more genes within the protective *Idd3* locus lead to increased survival of the transplanted allogeneic islets in NOD.B6 *Idd3* mice compared to wild type NOD mice (165). Given these

findings it is tempting to speculate that the same gene(s) involved in the augmented tolerance induction to allograft may be at play increasing tolerance to self.

Although, thus far, the *IL-2* gene is not considered a candidate gene for susceptibility to human T1D, the *IL-2RA* gene, which encodes CD25, is thought to be a genetic determinant for disease (166). Therefore, several lines of evidence support the role for IL-2-mediated signalling in the maintenance of self tolerance which promotes homeostasis and, most likely, function of T_{REG} cells. A dysregulation in either IL-2 or IL-2R signalling is thought to occur in both NOD mice and T1D patients which may impact the T_{REG} population and ultimately leads to a breakdown of self-tolerance leading to autoimmunity.

Rationale and Objectives

The increased frequency of T_{REG} cells in transgenic mice which either continuously express IL-2 in their serum or which contain a constitutively-active form of STAT-5 clearly reflects the importance of IL-2 on T_{REG} cell homeostasis (133, 135). Therefore, given the presence of the *Il-2* gene in the *Idd3* locus, we hypothesize that NOD.B6 *Idd3* congenic mice either produce a more potent differentially glycosylated IL-2, a larger amount of this cytokine or differences in its expression kinetics which endows these mice with a quantitative or qualitative advantage in their T_{REG} population compared to wild type NOD mice. The goal of our study was to elucidate the various effects of a protective *Idd3* locus on both T_{EFF} and T_{REG} subsets. For this purpose, various *in vitro* assays were performed on total $CD4^+$ or $CD4^+CD25^-$ (T_{EFF}) cells purified from NOD and NOD.B6 *Idd3* mice. Also, given the discovery of Foxp3 as a reliable marker for T_{REG} cells, the identification of this subset in autoimmune mice which may express $CD25^+$ T_{EFF} cells is no longer problematic. Thus, using a novel intra-nuclear staining protocol for Foxp3 coupled with multi-parametric flow cytometric analysis, we screened the thymus, various peripheral lymphoid organs and pancreas in order to identify

whether any changes in T_{REG} cell frequency were responsible for diabetes resistance in NOD.B6 *Idd3* mice. Finally, it was important to determine whether the *Idd3* locus could affect the ability of T_{REG} cells to suppress diabetes *in vivo*.

Material and Methods

Mice. NOD and NOD.B6 *Idd3* mice were purchased from Taconic Farms (Germantown, NY). NOD.TCR$\alpha^{-/-}$ and BDC2.5 TCR transgenic NOD mice (henceforth termed BDC2.5) were initially a kind gift from D. Mathis and C. Benoist (Joslin Diabetes Center, Boston, MA) and maintained by breeding in our animal facilities. BDC2.5 TCR transgenic NOD mice containing the B6 *Idd3* locus (termed BDC.Idd3) were generated by crossing male BDC2.5 transgenic NOD mice with NOD.B6 *Idd3* females. The F_1 litters which were transgenic for the BDC2.5 TCR were backcrossed to NOD.B6 *Idd3* mice. Transgenic F_2 litters were screened for homozygous presence of the B6 *Idd3* locus by PCR using the two distal microsatellite markers that differentiate NOD and B6 genomic segments: D3Nds36 and D3Nds34. NOD.B6 *Idd3* homozygote BDC2.5 transgenic mice were subsequently used as breeders. The BDC2.5 TCR transgenic mice were screened for the expression of Vβ4 on $CD4^+$ peripheral blood leukocytes. All mice were bred and maintained under specific pathogen-free conditions in the Lyman Duff animal facility at McGill University. All experiments were performed using cells from age-matched female mice. Fasting glycemia was monitored using Accu-check advantage colorimetric strips, a kind gift from Roche Diagnostics (Laval, Quebec). Overt diabetes was defined as glycemia >300 mg/dl.

Flow Cytometry. For all flow cytometry experiments, single cell suspensions were prepared by glass-slide disruption of thymus, spleen, pancreatic lymph nodes (pLN) and pooled peripheral lymph nodes (inguinal, brachial and axillary lymph nodes) (LN). Red blood cells were removed from splenocyte preparations by hypotonic lysis and viable cells were counted in all samples using the Trypan Blue exclusion assay. Cells were subsequently stained at 4°C in PBS containing 2% FBS and 0.1% sodium azide. Staining was performed with fluorochrome-conjugated antibodies to CD4 (GK1.5 or RM4-5), CD25 (PC61), CD69 (H1.2F3) or Vβ4 (CTVB4) (eBioscience, San Diego, CA). Intra-nuclear staining for Foxp3

(FJK-16s) was performed according to manufacturer's protocol (eBioscience). In certain experiments, intracellular staining for IFN-γ (XMG1.2) and IL-2 (JES6-5H4) was performed following PMA/Ionomycin stimulation in the presence of monesin for the final 3h. Cells were stained for surface markers, fixed for 10min in 4% paraformaldehyde, subsequently permeabilized with 0.5% saponin (Sigma-Aldrich, St-Louis, MO), stained for cytokines at room temperature for 30 min and washed twice with 0.5% saponin. Samples were acquired on FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Isolation of islet-infiltrating lymphocytes. Once all pLNs were removed from the mice, the remaining pancreas tissue was cut into small pieces using dissection scissors and digested in HBSS (Invitrogen, Burlington, Ont) with 1mg/ml collagenase V (Invitrogen) and 1 μg/ml DNase I (Sigma-Aldrich) by slow shaking at 37°C for 30 min. The digestion was stopped by adding cold FBS and pancreatic tissue was centrifuged for 1 min at 200g. Tissue was washed twice with HBSS-FBS 10% and islets were dissociated in non-enzymatic dissociation buffer (Invitrogen) for 10 min at 37°C. Cells were passed through a 200 μm mesh nylon screen and left on ice for 5 min to allow debris to sediment. The supernatant containing the cells was collected, stained for surface and intracellular markers and gated based on the conventional lymphocyte FSC/SSC profile.

Cell cultures. All T cells were cultured in cRPMI: 1640 RPMI supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 10 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate (all from Invitrogen) and 50 μM 2-mercaptoethanol (Sigma-Aldrich). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

RT-PCR. High purity (>99.7%) $CD4^+CD25^-$ T cells were isolated from the inguinal, axillary, brachial and mesenteric lymph nodes of NOD and NOD.B6 *Idd3* mice using the FACSAria flow cytometer (BD Bioscience). Cells were

activated in 24-well plates coated with 10 μg/ml purified anti-CD3 (2C11) and harvested at various time points where total RNA was extracted using TRIzol (Invitrogen) and reverse transcribed using Superscript II Kit following the manufacturer's recommendations (Invitrogen). PCR amplification was performed on all samples (5 min at 94°C followed by 28 cycles (45 s at 96°C, 45 s at 72°C and 1 min at 55°C) followed by a final extension of 10min at 72°C) using the following primers: interleukin-2 (IL-2) TTCAAGCTCCACTTCAAGCT-CTACAGCGGAAG (forward) and GACAGAAGGCTATCCATCTCCTCAGA-AAGTCC (reverse); glyceraldehyde-3-phosphate dehydrogenase (G3PDH) CACTCACGGCAAAT-TCAACGGC (forward) and ATCACAAACATGGG GGCATC-GG (reverse). RNA integrity and cDNA synthesis were verified by amplifying GAPDH cDNA. Amplified fragments were size-separated by electrophoresis in 1.8% agarose gels and visualized using ethidium bromide.

In vitro functional assays. Proliferation assays were performed by culturing 5 x 10^4 FACSAria-sorted $CD4^+$ and $CD4^+CD25^-$ T cells in 96 well flat-bottom microtiter plates with purified soluble anti-CD3 and $2x10^5$ irradiated (3000 rads) splenocytes in a total volume of 200 μl. Cells were cultured in cRPMI for 72h in a humidified atmosphere containing 5% CO_2 and pulsed with 0.5 μCi of [^{3}H] thymidine for the last 8h of culture. Cells were then harvested and thymidine incorporation was measured using a Microbeta Trilux plate reader (Wallac-PerkinElmer, Wellesley, MA).

Preparation and adoptive transfer of CFSE-labelled lymphocytes. Single cell suspensions were prepared from the LN and spleen of 8-10 week old BDC2.5 or BDC.Idd3 mice using a steel-wire mesh. Cells were subsequently labelled with PE-conjugated anti-CD25 for 20 min at 4°C. Cells were then labeled with anti-PE microbeads (Miltenyi Biotech, San Diego, CA) and lymphocytes were depleted of $CD25^+$ using autoMACS cell sorting. The CD25-depleted lymphocytes were then stained in 1.25 μm carboxy-succinimidyl-fluorescein-ester (CFSE) (Molecular Probes-Invitrogen) at room temperature for 5 min. Flow cytometry was used to

determine the total percentage of $CD4^+CD25^-$ T cells in cell preparations and a total of 2-3 x 10^6 $CD4^+CD25^+$ T cells were injected i.v. in the tail vein of 6-8 wk old NOD or NOD.B6 *Idd3* recipients. Four days after adoptive transfer (90-92h), recipient mice were sacrificed. The pLN and mLN were excised and processed separately to obtain single cell suspensions, counted and stained with fluorochrome-conjugated antibodies. Data shown is gated on CFSE-labelled $CD4^+V\beta4^+$ lymphocytes.

Preparation and adoptive transfer of purified T cells. $CD4^+$ T cell subsets were isolated from the spleen, inguinal, brachial, axillary and mesenteric LNs of 6-8 wk BDC2.5 and BDC.Idd3 mice. Single cell suspensions were prepared from pooled organs using a steel wire mesh. Cells were resuspended and stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 for 20 min at 4°C. Cells were then labelled with anti-PE microbeads (Miltenyi Biotech, San Diego, CA) and $CD25^+$ cells were sorted using autoMACS. The purity of the $CD25^+$ fraction was assessed by flow cytometry and was routinely ~85% $CD4^+CD25^+$. The $CD25^-$cell fraction was then labeled with anti-FITC microbeads (Miltenyi) and purified using autoMACS cell sorting (>96 % $CD4^+CD25^-$). For total $CD4^+$ T cell purification, only FITC anti-CD4 antibody was used, coupled with anti-FITC beads (purity >96%). Cells (0.5 x 10^6) were injected i.v. in the tail vein of 8-12 wk old NOD.TCR$\alpha^{-/-}$ recipients and blood glucose was monitored every 2-3 days starting on day 5 post-transfer. At day 14, recipients were sacrificed and various organs were excised, single cell suspensions prepared and stained for various markers. Data shown is gated on $CD4^+V\beta4^+$ cells.

Data and statistical analysis. Data always represent averaged values with one standard deviation. All statistical analysis was performed using the Student's t test (two-tailed). Only values of $p<0.05$ were considered significant.

Results

NOD.B6 Idd3 mice show a reduced incidence of T1D and decreased accumulation of pathogenic T_{H1} cells in vivo.

NOD.B6 *Idd3* mice were initially reported to have reduced diabetes incidence compared to wild-type NOD mice (159). In our colony, female NOD mice start to develop overt diabetes by ~14 wk of age and incidence reaches 85% by 28 weeks. In contrast, only 10% of NOD.B6 *Idd3* female mice were diabetic by 28 wk of age, with the earliest onset occurring at 25 wk of age (Fig. 3A). Since diabetes is caused by the T_{H1}-mediated destruction of the pancreatic β islet-cells, various lymphoid organs of pre-diabetic NOD and NOD.B6 *Idd3* mice were screened in order to determine whether the protective *Idd3* locus affects the accumulation of pro-inflammatory T_{H1} cells *in vivo*. In order to screen for T_{H1} cells, single cell suspensions were prepared from the pLNs, spleen and pooled peripheral lymph nodes (LNs) of individual mice, activated for 4 hours with PMA/ionomycin and stained for intracellular IFN-γ. The frequency of IFN-γ^+ $CD4^+$ T cells was assessed in the aforementioned organs of each mouse by flow cytometric analysis. IFN-γ^+CD4^+ T cells were readily detected in the pLNs, LNs and spleen of 12 wk old pre-diabetic NOD mice (Fig. 3B). In the pLNs of NOD mice 3.86 ± 0.09% of $CD4^+$ T cells were IFN-γ^+ compared to only 1.81 ± 0.32% for NOD.B6 *Idd3* mice. Similarly, 3.16 ± 0.23 % of $CD4^+$ T cells from NOD LNs produced IFN-γ subsequent to *in vitro* activation, compared to a mere 1.62 ± 0.21% in NOD.B6 *Idd3* LNs. In the spleen, NOD and NOD.B6 *Idd3* mice both show the highest frequency of T_{H1} differentiated cells at 12.02 ± 2.18 % and 7.46 ± 0.27 % of total $CD4^+$ T cells, respectively. Thus these findings demonstrate that T_{H1} cells are systemically present in both NOD and NOD.B6 *Idd3* pre-diabetic mice. However, the protective *Idd3* allele is capable of reducing by at least 40% the frequency of pro-inflammatory potentially pathogenic T_{H1} cells throughout the peripheral immune system. Therefore, diminished accumulation of T_{H1} cells

correlates with reduced diabetes incidence in NOD.B6 *Idd3* mice, suggesting that the *Idd3* locus halts the progression to clinical autoimmunity in this model. Our data also suggest that NOD.B6 *Idd3* mice restrict the optimal priming, expansion or effector functions of potentially diabetogenic cells.

The protective Idd3 allele increases IL-2 gene expression and production by activated effector T cells.

Since the IFN-γ gene is not found within the protective *Idd3* locus, our findings exclude the possibility that protection in the NOD.B6 *Idd3* mice is simply due to a decrease in IFN-γ expression. Instead, it is more likely that reduced T_{H1} cell frequency is a downstream effect of the protection conferred by the B6-derived *Idd3* allele. Present within the *Idd3* locus, is the gene encoding IL-2, a well described T cell growth factor and important promoter of immunological tolerance (129). Given its importance in the survival and function of immunosuppressive $CD4^+CD25^+$ T_{REG} cells, the *Il-2* gene has long been proposed as a candidate for diabetes protection in NOD.B6 *Idd3* mice (159, 160, 163, 167). Interestingly, the *Il-2* promoter from the protective *Idd3* allele does possess some sequence variants compared to the NOD *Idd3* allele and this may influence IL-2 expression (160, 163). Therefore, monitoring IL-2 production in both mouse strains was a good starting point to elucidate the mechanism of increased tolerance in NOD.B6 *Idd3* mice. *In vivo*, IL-2 is produced primarily by activated $CD4^+$ T_{EFF} cells (127). Therefore, $CD4^+CD25^-$ T_{EFF} cells from young pre-diabetic 3-4 wk old NOD and NOD.B6 *Idd3* mice were isolated by FACS and activated with plate-bound anti-CD3 for 48 hours and *Il-2* gene expression was assessed at various time points using semi-quantitative RT-PCR. For this experiment $CD4^+CD25^+$ T_{REG} cells were purposely excluded from cultures since they have been shown to suppress IL-2 mRNA expression in T_{EFF} cells (90, 134). Subsequent to activation, NOD.B6 *Idd3* T_{EFF} cells expressed higher levels of IL-2 mRNA than NOD T_{EFF} cells (Fig. 4A). Although IL-2 mRNA levels were comparable at 12h in both T_{EFF} populations, only NOD.B6 *Idd3* T_{EFF} cells

maintained high expression levels until 48h compared to NOD T_{EFF} cells which peaked at 12h and quickly diminished (Fig 4A). In order to correlate these findings with the production of IL-2 protein, $CD4^+CD25^-$ T_{EFF} cells from 3-4 wk old mice were activated with PMA/ionomycin for 24h and cells were subsequently stained for intracellular IL-2. Consistent with the RT-PCR results, higher frequencies of IL-2-producing NOD.B6 *Idd3* T_{EFF} cells were detected compared to NOD T_{EFF} cells (Fig 4B). Thus, these findings demonstrate that the protective *Idd3* allele favours increased IL-2 gene expression and protein production in activated T_{EFF} cells.

The protective Idd3 allele bolsters T_{REG} function in activated $CD4^+$ T cells

T_{REG} cells fail to make IL-2, suppress T_{EFF} cells by down-regulating IL-2 synthesis, yet paradoxically require IL-2 for their development (124, 127, 132, 134, 139). As T_{EFF} cells are the major source in IL-2, we hypothesized that the increased IL-2 production by NOD.B6 *Idd3* T_{EFF} cells potentiated T_{REG} cell suppressive function. To determine if this was the case, we compared the proliferation of $CD4^+CD25^-$ T_{EFF} cells and total $CD4^+$ T cells from both strains. Since T_{REG} cells constitute approximately 10% of all $CD4^+$ T cells, this assay allowed us to determine the suppressive potential of T_{REG} cells in NOD and NOD.B6 *Idd3* mice at their physiological frequencies. When we compared the proliferation of $CD4^+CD25^-$ T_{EFF} cells (in the absence of T_{REG} cells), NOD.B6 *Idd3* T_{EFF} cells proliferated more vigorously than NOD T_{EFF} cells in response to anti-CD3 stimulation, as assessed by thymidine incorporation (Fig. 5A). Interestingly, this corroborates our IL-2 gene/protein expression data since IL-2 is a T cell growth factor and higher cytokine production by activated NOD.B6 *Idd3* T_{EFF} cells would lead to increased proliferation relative to NOD T_{EFF} cells. Surprisingly, when $CD4^+$ T cell proliferation was assessed, NOD cells proliferated significantly more than the NOD.B6 *Idd3* cells (Fig. 5A). To adequately compare the suppressor potency of $CD4^+CD25^+$ T_{REG} cells within the unfractionated $CD4^+$ T cells, we calculated the reduction of T_{EFF} proliferation in the presence of T_{REG}

cells [100% – ($CD4^+$ prolif. ÷ $CD4^+CD25^-$ prolif.). Whereas the presence of $CD4^+CD25^+$ T_{REG} cells only reduced the proliferation of T_{EFF} cells by ~7% in NOD cells, NOD.B6 *Idd3* T_{REG} cells were capable of suppressing proliferation of T_{EFF} cells by ~55% at physiological ratios. These findings demonstrate that the protective *Idd3* allele reduces the proliferation of total $CD4^+$ T cells *in vitro* and this may account for reduced T_{H1} differentiation and diabetes *in vivo*. Importantly, these data also reveal that a difference in the $CD4^+CD25^+$ population is responsible for this reduced proliferation since removal of these cells unleashes the proliferative capacity of the NOD.B6 *Idd3* $CD4^+CD25^-$ T_{EFF} cells.

To gain better insight on how the protective *Idd3* allele favoured the suppressive function of T_{REG} cells, the proliferative capacity of $CD4^+$ T cells was determined by CFSE dilution analysis. Since CFSE-labelled cells will lose half their fluorescence at each round of cell division, it is possible to determine the total number of cell divisions within a labelled population. Thus, $CD4^+$ T cells were isolated from both strains of mice, CFSE-labelled and activated for 72h with soluble anti-CD3 in the presence of irradiated splenocytes. In order to monitor T_{REG} cells frequency in this assay, CD25 could not be used since it is only a good marker for T_{REG} cells within a naïve $CD4^+$ T cell population. Therefore, we made use of an intra-nuclear Foxp3 staining kit in order to adequately identify $Foxp3^+$ T_{REG} cells within the CFSE-labelled $CD4^+$ T cells. For our analysis, to evaluate the dominance of T_{REG} cells within the $CD4^+$ population, the T_{REG}:T_{EFF} ratios were calculated and compared for both strains at various time points subsequent to activation. Interestingly, freshly isolated $CD4^+$ T cells from both mice strains had comparable frequencies of $CD4^+Foxp3^+$ T_{REG} cells, typically between 8-10%. However, subsequent to anti-CD3 stimulation, the protective *Idd3* locus promoted an increased accumulation of $Foxp3^+$ cells throughout the 72h period observed, leading to increased T_{REG}:T_{EFF} ratios (Fig. 5B). Surprisingly, CFSE dilution-based cell cycle analysis at 72h revealed that the increased T_{REG}:T_{EFF} ratio in activated NOD.B6 *Idd3* $CD4^+$ T cells did not correlate with higher proliferation of T_{REG} cells (Table 5). In contrast, when we compared the proliferation of $CD4^+Foxp3^-$

T_{EFF} cells, NOD cells showed a greater frequency of cells having undergone at least 5 rounds of division compared to NOD.B6 *Idd3* cells (32.5% vs. 28.4%; Table 5). The overall proliferation of NOD $CD4^+$ T cells also shows a greater proportion of cells having undergone at least 5 rounds of division compared to NOD.B6 *Idd3* $CD4^+$ T cells (31.7% vs. 26.4%; Table 5). Therefore, higher T_{REG}:T_{EFF} ratios do not reflect the increased proliferation of T_{REG} cells in NOD.B6 *Idd3* cells but instead coincide with a reduction in the proliferative capacity of T_{EFF} cells. Interestingly, differences in $Foxp3^+$ T_{REG} cell frequencies were apparent as early as 24h, before any detectable cell division, suggesting that the survival of T_{REG} cells may also be favoured in the presence of the protective *Idd3* allele. Nonetheless, our data demonstrate that NOD.B6 *Idd3* $CD4^+$ T cells proliferate less than NOD $CD4^+$T cells and this correlates with increased T_{REG}:T_{EFF} ratios subsequent to activation.

The protective Idd3 allele does not increase the systemic frequency of T_{REG} cells in vivo

Given the impact of the protective *Idd3* allele on T_{REG} frequency within an activated $CD4^+$ T cell population, it was important to determine whether reduced diabetes in NOD.B6 *Idd3* mice may simply be due to the favoured development and consequently increased numbers of T_{REG} cells in their immune system. Ultimately, if an increase in T_{REG} cell frequency was caused by the B6-derived *Idd3* allele, this could potentially increase tolerance in NOD.B6 *Idd3* mice and protect from T1D. It was also possible that reduced diabetes in NOD.B6 *Idd3* mice was due to the decreased number of islet-reactive T_{EFF} cells present in the immune system, leading to a less aggressive autoimmune response. Thus, it was important to survey the various lymphoid organs in NOD and NOD.B6 *Idd3* mice for any differences in T_{EFF} cell and T_{REG} cell populations which would reflect diabetes susceptibility or protection. For our analysis, we compared 12 week old pre-diabetic animals since NOD mice at this age have established insulitis yet are still several weeks away from overt diabetes (17). Various lymphoid organs were

harvested from age-matched female mice, prepared into single-cell suspensions, counted, stained for various cell markers and analyzed by flow cytometry. Since Foxp3 expression correlates highly with suppressive function (109, 111), we compared the total frequency and absolute numbers of T_{EFF} ($CD4^+Foxp3^-$) and T_{REG} ($CD4^+Foxp3^+$) cells in both strains of mice.

We first looked at thymic CD4 single-positive ($CD4^+CD8^-$) T cells, in order to elucidate any effects of the *Idd3* locus on T cell development in the thymus. Since no discernable differences were observed in the frequency or absolute numbers of thymic T_{EFF} and T_{REG} cells, it was concluded that the *Idd3* locus does not affect the development of these $CD4^+$ T subsets in the thymus (Fig. 6). In the peripheral immune system, we first looked at whether reduced numbers of T_{EFF} cells correlated with diabetes protection in NOD.B6 *Idd3* mice. Surprisingly, in pLNs, spleen and pooled peripheral LNs there were no detectable differences in either the frequency (Fig. 6A) or absolute number (Fig. 6B) of T_{EFF} cells between NOD and NOD.B6 *Idd3* mice. When we compared the frequency of recently activated T_{EFF} cells expressing either the CD69 or CD25 early activation markers we could detect no differences in the pLN (NOD=10.5 ± 0.7% vs. NOD.B6 *Idd3*=12.1 ± 1.3%). Since these markers of early activation are likely to reflect the initiation of a diabetogenic response in the pLNs, our findings suggest that a reduction in pancreas-specific T_{EFF} cells is not a likely cause for disease resistance in NOD.B6 *Idd3* mice. Thus, these data demonstrate that NOD.B6 *Idd3* mice do not possess reduced numbers or overall frequency of T_{EFF} cells and most likely do not contain less islet-reactive cells in their $CD4^+$ T cell repertoire than NOD mice. When we screened these same peripheral organs for differences in the $CD4^+Foxp3^+$ T_{REG} cell population, NOD.B6 *Idd3* mice did not show increased frequencies (Fig. 6C) or absolute numbers (Fig. 6D) of these immunosuppressive cells. Surprisingly, no discernable differences could be detected in the pLN, the site of diabetes initiation. Thus, these findings exclude the possibility that a systemic or localized (pLN) increase of T_{REG} cell numbers in the immune system are responsible for diabetes protection in NOD.B6 *Idd3* mice.

Since recent studies have shown that T_{REG} cell-mediated suppression of diabetes takes place inside the pancreatic islets and not in the peripheral immune system (148, 149), the next step was to assess the frequency of T_{EFF} and T_{REG} cells present within the pancreas. For this purpose, pancreas infiltrating lymphocytes were isolated and analyzed by flow cytometry. As expected, $CD4^+$ T cells were readily detected in the pancreas of NOD mice, consistent with islet-infiltration of the immune system and progression towards T1D. Surprisingly, NOD.B6 *Idd3* mice also contained $CD4^+$ T cells in the pancreas however at much lower frequencies than NOD mice. Whereas $CD4^+$ T cells represented 9.02 ± 1.13 % of the pancreatic infiltrate in the NOD mice, they only represented 4.49 ± 0.39 % of the infiltrate in NOD.B6 *Idd3* mice (p=0.0064). When the $CD4^+$ T cells were subdivided according to Foxp3 expression, we found that NOD mice possessed a greater frequency of T_{EFF} cells in the pancreas compared to NOD.B6 *Idd3* mice (Fig. 6A). Interestingly, this increase was specific to $CD4^+Foxp3^-$ T cells since the frequency of pancreas-infiltrating $CD8^+$ T cells appeared to be unaffected by the *Idd3* allele, consisting of 3.02 ± 0.50 % and 2.42 ± 0.44 % of the pancreatic infiltrate for NOD and NOD.B6 *Idd3* mice, respectively. Additional flow cytometric analysis of the pancreas revealed that the overall frequency of T_{REG} cells was identical in the pancreas of both strains (Fig. 6C), suggesting that increased numbers of suppressive cells is not the cause of diabetes protection in NOD.B6 *Idd3*. However, consistent with our *in vitro* data (Fig. 5B), activated NOD.B6 *Idd3* $CD4^+$ T cells in the pancreas maintain higher T_{REG}:T_{EFF} ratios than their NOD counterparts (0.21 ± 0.01 vs. 0.13 ± 0.01, p=0.018; Fig. 6C). As a whole, these data demonstrate that diabetes resistance is not due to an increase in T_{REG} cell numbers in the thymus, peripheral immune system or pancreas of NOD.B6 *Idd3* mice. Instead, the protective *Idd3* allele decreases the accumulation of T_{EFF} cells in the pancreas which correlates with an increased T_{REG}:T_{EFF} ratio.

The protective Idd3 allele delays the accumulation of islet-reactive T_{EFF} cells in the pLN and pancreas

Our data demonstrate that the protective *Idd3* allele favours high T_{REG}:T_{EFF} ratios within *in vitro* activated $CD4^+$ T cells. The requirement for T cell activation in the *Idd3*-mediated control of $CD4^+$ T cell function, would explain why the pancreas, which contains exclusively islet-reactive cells, is the only organ with a difference in the T_{REG}:T_{EFF} ratio when comparing NOD and NOD.B6 *Idd3* mice. Thus, it is likely that the protective *Idd3* allele will predominantly influence the behaviour of activated cells *in vivo*. Therefore, in order to determine the effects of the B6-derived *Idd3* locus on a diabetogenic population of $CD4^+$ T cells, we generated BDC.Idd3 mice by crossing BDC2.5 and NOD.B6 *Idd3* mice. This novel strain of mice is homozygous for the protective B6-derived *Idd3* allele and possesses a monoclonal population of TCR transgenic islet-specific $CD4^+$ T cells. Since BDC2.5 and BDC.Idd3 mice contain high frequencies of islet-reactive T cells in their immune system, we wanted to determine the effect of the *Idd3* locus on the accumulation of T_{EFF} cells in the pLN and pancreas of these TCR transgenic mice. Islet-reactive $CD4^+$ T cells in BDC2.5 mice were shown to become activated in the pLN and start to infiltrate into the pancreas at approximately 18 days after birth (147). Therefore, we looked at 3-4 week old weanlings which were presumably in the early stages of insulitis. As a reference point, we also looked at 8 week old adult BDC2.5 and BDC.Idd3 mice to determine any differences caused by prolonged insulitis. Thus, mice were sacrificed and various organs were harvested in order to assess the frequency of transgenic T_{EFF} and T_{REG} cells by flow cytometry. Much like our observations in non-transgenic mice, no differences were detected in the thymus, spleen and peripheral lymph nodes of BDC2.5 and BDC.Idd3 mice (data not shown). Interestingly, in the pLNs of 4 wk old BDC.Idd3 mice, T_{EFF} cells were present at higher frequencies (54.4 ± 4.0 % vs. 63.6 ± 2.0 %, p=0.003; Fig. 7A) and total numbers (3.24 ± 0.44 x 10^6 vs. 2.02 ± 0.41 x 10^6, p=0.013) compared to BDC2.5 mice. Although this did not correlate with increased T_{REG} frequencies, BDC.Idd3 mice did show a statistically significant increase in the T_{REG}:T_{EFF} ratio (Fig. 7A). It should be noted that this trend did not persist, since by 8 weeks of age,

BDC.Idd3 mice show similar levels of total T_{EFF} cells in the pLN (Fig. 7A). Nevertheless, slightly increased T_{REG} cell frequencies in the pLN of 8 week old BDC.Idd3 mice lead to a higher T_{REG}:T_{EFF} ratio compared to BDC2.5 mice.

In the pancreas, a similar trend was observed regarding T_{EFF} cells since 4 week old BDC.Idd3 mice show lower levels of infiltrating T_{EFF} cells compared to BDC2.5 mice, correlating with an elevated T_{REG}:T_{EFF} ratio but not the increased frequency of T_{REG} cells (Fig. 7B). Similar to the pLN, older BDC2.5 and BDC.Idd3 mice show comparable levels of T_{EFF} cells in the pancreas. Nevertheless the T_{REG}:T_{EFF} ratio, albeit reduced in older mice, remained significantly higher in BDC.Idd3 pancreata (Fig. 7B far right). Interestingly, even though the pancreatic infiltrate is comparable between the two mouse strains at 8 weeks of age, the BDC.Idd3 mice show very low onset of diabetes at only 5% by 26 wk of age (n=20). Thus, these findings suggest that the protective *Idd3* allele maintains the increased T_{REG}:T_{EFF} ratios within a population of islet-reactive $CD4^+$ T cells in the pLN , correlating with reduced accumulation of T_{EFF} cells. This, in turn, leads to the delayed pancreatic infiltration of T_{EFF} cells in BDC.Idd3 mice. Therefore, within a monoclonal islet-specific population of $CD4^+$ T cells, the *Idd3* allele appears to modulate the kinetics of the autoimmune response.

Presence of the protective Idd3 locus decreases proliferation of diabetogenic T_{EFF} cells in the pLNs.

Since adult NOD.B6 *Idd3* and young BDC.Idd3 mice show reduced numbers of T_{EFF} cells in the pancreas, this intimated one of two possibilities: either impaired T_{EFF} function or enhanced immunoregulation. To address this first possibility and evaluate the impact of a protective *Idd3* locus on the pathogenic potential of a T_{EFF} cell *in vivo*, lymphocytes were prepared from peripheral LNs and spleens of 8-10 wk old BDC2.5 and BDC.Idd3 mice and depleted of $CD25^+$ cells via magnetic bead cell sorting. The CD25-depleted cells were then CFSE-labelled and transferred into 8 wk old NOD or NOD.B6 *Idd3* recipients (22, 168).

Depletion of $CD25^+$ cells was important to ensure the absence of immunoregulation amongst the transferred cells. When recipients were sacrificed 4 days after adoptive transfer of CFSE-labelled cells, we observed the abundant proliferation of BDC2.5 T_{EFF} cells in the pLN of NOD recipients (Fig. 8A) but not in other peripheral LNs which do not drain the pancreas (data not shown). Since the BDC2.5 $CD4^+$ T cells recognize an islet-derived antigen, localized cell division in the pLN confirms that proliferation is antigen-specific. Similarly, the BDC.Idd3 T_{EFF} cells also proliferated abundantly in the pLN but not other LNs of NOD recipients. In fact, both proliferation (Fig. 8A) and accumulation (Fig. 8B) of BDC.Idd3 T_{EFF} cells in the pLNs were similar to BDC2.5 T_{EFF} cells when transferred into NOD mice. Hence, these findings clearly illustrate the intact function of BDC.Idd3 T_{EFF} cells and demonstrate that the protective *Idd3* allele does not impede the pathogenic potential of autoreactive T_{EFF} cells.

To determine whether the *Idd3* allele of recipient mice influenced immunoregulatory mechanisms *in vivo,* BDC2.5 or BDC.Idd3 T_{EFF} cells were transferred into NOD.B6 *Idd3* recipient mice. Our results demonstrate a significant reduction in the antigen-driven proliferation of both BDC2.5 ($p=0.024$) and BDC.Idd3 ($p=0.033$) T_{EFF} cells in the pLNs of NOD.B6 *Idd3* mice (Fig. 8A). Additionally, NOD.B6 *Idd3* mice were more efficient at reducing the total accumulation of pathogenic CFSE-labelled T_{EFF} cells present in the pLN (Fig. 8B). Our results also show that BDC.Idd3 T_{EFF} cell proliferation was not more efficiently suppressed than BDC2.5 T_{EFF} cells in NOD.B6 *Idd3* recipients. This suggests that presence of the B6-derived *Idd3* allele in the transferred T_{EFF} cells, which can promote higher IL-2 expression, is not capable of increasing immunoregulatory mechanisms in the recipients. Instead, suppression of islet-reactive T_{EFF} cells is strictly dependent on the presence of the protective *Idd3* allele in recipient mice.

The protective Idd3 allele increases T_{REG} suppressive function in vivo, which is responsible for reduced diabetes onset

Since NOD.B6 *Idd3* mice possess an environment which can limit the control of diabetogenic T cells more efficiently than wild-type NOD mice, it is likely that that T_{REG} cells are key mediators of disease protection. Therefore, it was important to determine whether T_{REG} cells originating from mice containing the protective *Idd3* allele were better suppressors of diabetogenic T_{EFF} cells *in vivo*. To accomplish this, $CD4^+$ total and $CD4^+CD25^-$ (T_{EFF}) cells were purified from 6-8 week old BDC2.5 or BDC.Idd3 mice and transferred into T cell-deficient NOD.TCR$\alpha^{-/-}$ recipients. When comparing diabetes frequency in the recipients of BDC2.5 and BDC.Idd3 T_{EFF} cells (Fig. 9A), it was observed that the *Idd3* allele did not affect diabetogenic potential of the T_{EFF} cells *in vivo*. Our data show that onset occurred simultaneously between day 7 and 9 and with similar incidence in both groups of recipient mice (Fig. 9A). When various lymphoid organs and pancreas were harvested from diabetic recipients at day 15 post-transfer, flow cytometric analysis revealed similar frequencies and total numbers of transferred BDC2.5 and BDC.Idd3 T_{EFF} cells in recipient mice (data not shown). These findings once again highlight the inability of the *Idd3* allele to directly dampen the diabetogenic potential of T_{EFF} cells *in vivo*. When total $CD4^+$ T cells were transferred into NOD.TCR$\alpha^{-/-}$ recipients, 71% of the recipients receiving BDC2.5 cells developed diabetes compared to only 43% for BDC.Idd3. Whereas the BDC.Idd3 $CD4^+CD25^+$ T_{REG} cells within the $CD4^+$ population were capable of reducing diabetes incidence by ~50% (82% vs. 43%), the presence of BDC2.5 $CD4^+CD25^+$ T_{REG} cells barely reduced diabetes frequency (88% vs. 71%). Since the frequency of $Foxp3^+$ cells within the BDC2.5 and BDC.Idd3 $CD4^+$T cells was similar prior to transfer (data not shown), these findings suggests a functional difference in the T_{REG} population. However, it was essential to compare the ability of BDC2.5 and BDC.Idd3 T_{REG} cells to suppress the same T_{EFF} cell population in this model of T1D. Thus, BDC2.5 $CD4^+CD25^+$ T_{REG} cells were purified and transferred at physiological ratios (1T_{REG} for 10 T_{EFF} cells) with T_{EFF} cells from

BDC.Idd3 mice. Similarly, another group of recipient mice received BDC2.5 T_{EFF} cells transferred with BDC.Idd3 T_{REG} cells. Interestingly, diabetes onset revealed that the origin of the T_{REG} cell influences disease progression rather than the origin of the T_{EFF} cell since BDC2.5 T_{EFF} cells co-transferred with BDC.Idd3 T_{REG} cells induced diabetes in only 30% of recipients. Conversely, BDC.Idd3 T_{EFF} cells co-transferred with BDC2.5 T_{REG} cells induced diabetes in 71% of recipient mice, similar to what we observed for BDC2.5 $CD4^+$cells. Thus, taken together, these findings indicate that BDC.Idd3 T_{REG} cells are intrinsically better than their BDC2.5 counterparts, due to their maturation in the presence of the protective *Idd3* locus.

To better elucidate differences in T_{REG} cell suppressor function, recipients were sacrificed at day 15 and various lymphoid organs were analyzed by flow cytometry to monitor $CD4^+$ T cell frequency and T_{REG}:T_{EFF} ratios in the pLN and pancreas. Surprisingly, the adoptively transferred T_{EFF} cells from both strains, which were depleted of $CD4^+CD25^+$ cells, developed a population of $CD4^+Foxp3^+$ cells by day 15, representing 7-15% of total $CD4^+V\beta4^+$ T cells. These $Foxp3^+$ cells, which probably originate from the undepleted $CD4^+CD25^-$ $Foxp3^+$ population (109, 111), expanded in the immunodeficient recipients but ultimately were unable to control disease progression. Since the diabetic recipients of BDC2.5 and BDC.Idd3 T_{EFF} cells showed virtually no differences in $CD4^+$ T cell frequency or T_{REG}:T_{EFF} ratios, values were pooled and used as a reference when comparing all other groups of recipient mice having received T_{REG} cells (Fig. 9B). When we examined the pLN of all recipient mice, we could not detect any significant differences in the overall frequency (Fig. 9B, top left) or numbers (data not shown) of $CD4^+V\beta4^+$ T cells. In the pancreas, our data show that diabetes resistance correlates with a 50% decrease in the frequency of infiltrating $CD4^+V\beta4^+$ T cells (Fig. 9B, top right). Interestingly, whereas BDC2.5 T_{REG} cells only reduced $CD4^+$ T cell infiltration in the pancreas of diabetes-resistant mice, BDC.Idd3 T_{REG} cells reduced $CD4^+$ T cell infiltration in all recipients (Fig. 9B, top right). In addition, when comparing the T_{REG}:T_{EFF} ratios in

diabetes resistant mice, recipients of BDC.Idd3 T_{REG} cells always show higher ratios than recipients of BDC2.5 T_{REG} cells in both the pLN and pancreas, suggesting increased T_{REG} cell suppression of the T_{EFF} cells (Fig.9, bottom). Thus, these findings demonstrate the increased suppressor function of BDC.Idd3 T_{REG} cells in the pLN and pancreas, resulting in elevated T_{REG}:T_{EFF} ratios and increased protection from diabetes.

Discussion and Future Directions

T1D is a polygenetic disease and several *Idd* genetic loci have been shown to predispose the NOD mice to autoimmunity. The most influential of these is the I-A^{g7} MHC class II locus, however several others have been identified to date (169). Thus far, the *Idd3* locus is clearly one of the most influential genetic regions for diabetes susceptibility. This is apparent in the NOD.B6 *Idd3* mice which contain a protective *Idd3* allele and show a marked reduction in diabetes onset compared to wild type NOD mice (Fig. 3A, (159)). The *Il-2* gene is present within the *Idd3* locus and is a strong candidate for diabetes resistance (160, 167). Since IL-2 is known to promote T_{REG} cell homeostasis and suppressive function (123, 124, 127, 133, 134) and since T_{REG} cells have been directly linked to diabetes suppression (148, 149, 170), we hypothesized that increased diabetes protection in NOD.B6 *Idd3* mice is T_{REG} cell-dependent. The involvement of IL-2 in the *Idd3* allele-mediated resistance to diabetes was proposed but never shown. Therefore, the first part of this study focused on differences in IL-2 production between NOD and NOD.B6 *Idd3* mice. The second portion of this study focused on the effect of the protective *Idd3* allele on T_{REG} cell development, homeostasis and function. To date, at least one other study has demonstrated increased immunological tolerance in NOD.B6 *Idd3* mice compared to wild-type NOD mice, however the underlying mechanisms for enhanced immunotolerance were not investigated (165).

Since T_{EFF} cells were identified as the main producers of IL-2 *in vivo* (127), it was important to determine whether the *Idd3* locus could directly influence the expression of this cytokine in activated T_{EFF} cells. Our findings demonstrate that highly purified $CD4^+CD25^-$ T_{EFF} cells containing the B6 *Idd3* allele, once activated, consistently expressed augmented levels of IL-2 mRNA and protein. Since IL-2 is a well described T cell growth factor, the increased proliferation of NOD.B6 *Idd3* T_{EFF} cells compared to their NOD counterparts, is

consistent with increased levels of this cytokine. However, our results are in contradiction with another study showing no difference in IL-2 expression between NOD and NOD.B6 *Idd3* mice (160) although several reasons may explain this apparent discrepancy. First, IL-2 mRNA was quantified within unfractionated splenocytes and since $CD4^+$ T cells only represent ~20% of spleen cells, this undoubtedly decreases the sensitivity of their assay. Second, in this previous study, splenocytes were activated using PMA and ionomycin, independently of the TCR, which renders these findings irrelevant in a physiological context. Third, the authors of this study only looked at 4h post activation and we have shown that differences are only apparent starting 24h after T cell activation. Finally, this study did not look at differences in IL-2 protein production in activated splenocytes. In support of our findings, other published data indirectly suggest a possible difference in IL-2 expression between protective and susceptible *Idd3* alleles. First, the *Il-2* promoter from the protective *Idd3* allele does possess some sequence variants, which may directly influence IL-2 synthetic rates at the transcriptional and translational levels (160, 163). Second, differences in NOD and C56BL/6 *Il-2* genes ultimately lead to differential glycosylation of the IL-2 molecule which can affect protein folding and may increase the cytokine's half-life or affinity for its receptor (163). Therefore, in our experiments, increased IL-2 mRNA expression by NOD.B6 *Idd3* T_{EFF} cells after 24h may be the consequence of increased IL-2R signalling by a more "potent" IL-2. This would fuel a positive feedback loop, leading to increased *Il-2* gene expression. Thus, our findings show differential IL-2 gene expression and protein production by activated T_{EFF} cells of NOD and NOD.B6 *Idd3* origin however the exact cause for this difference still needs to be determined.

Since IL-2 is important for T_{REG} cell homeostasis and function, higher levels of this cytokine *in vivo* may be the direct cause for diabetes protection in NOD.B6 *Idd3* mice. In fact, T cells in the peripheral immune system of NOD.B6 *Idd3* mice were shown to have higher expression of the β- (CD122) and γ- (CD132) chains of the IL-2 receptor, reflecting possible differences in IL-2

activity *in vivo* (161). However, if NOD.B6 *Idd3* mice produce increased levels of IL-2, our data demonstrate that this does not translate into increased numbers of T_{REG} cells in the peripheral immune system. Nonetheless, flow cytometric analysis of central and peripheral lymphoid organs revealed reduced progression of diabetes in the presence of the B6-derived *Idd3* allele. First, the NOD.B6 *Idd3* mice show a significant reduction in the frequency of T_{EFF} cells in the pancreatic islets. Also, these mice appear to have a reduced accumulation of T_{H1}-differentiated cells in the pLN, LNs and spleen. This decrease in T_{H1} cells did not correlate with a reduction in the frequency of activated T_{EFF} cells expressing either CD25 or CD69 in the pLNs, suggesting that self-reactive T_{EFF} cells are still present in NOD.B6 *Idd3* mice but are restrained in their pathogenic potential. This excludes the possibility of increased clonal deletion in the thymus as a mechanism for diabetes protection in these mice. Also, since the protective *Idd3* allele did not completely prevent islet-infiltration of T_{EFF} cells or inhibit T_{H1} cell accumulation, it appears as though NOD.B6 *Idd3* mice are protected from the late stages of the autoimmune response. When applying our findings to the two checkpoint model of diabetes progression, the protective *Idd3* allele does not appear to affect checkpoint 1 or the establishment of peri-insulitis. Instead, the *Idd3* locus most likely influences the transition from checkpoint1 to checkpoint 2 (overt diabetes).

A hallmark of reduced diabetes progression in NOD.B6 *Idd3* mice is the high T_{REG}:T_{EFF} ratio in the pancreas. Interestingly, the increased ratios in the pancreas are not due to augmented T_{REG} cell frequencies. Rather, the high proportion of $Foxp3^+$ cells within the $CD4^+$ population appears to be the consequence of either reduced infiltration or decreased proliferation of T_{EFF} cells in the pancreatic islets. Similarly, our *in vitro* findings demonstrate higher T_{REG}:T_{EFF} ratios in NOD.B6 *Idd3* $CD4^+$ T cells than NOD $CD4^+$ T cells subsequent to TCR activation. Our CFSE dilution analysis of these i*n vitro* activated cells indicates that higher T_{REG}:T_{EFF} ratios are not due to increased proliferation of NOD.B6 *Idd3* T_{REG} cells. Instead, T_{EFF} cell proliferation is reduced due to superior immunosuppression within the NOD.B6 *Idd3* $CD4^+$ T

cells. Thus, the most likely cause for the pancreas-specific increase in T_{REG}:T_{EFF} ratio is the presence of activated islet-reactive NOD.B6 *Idd3* T_{EFF} cells in the insulitic lesion producing high levels of IL-2 which favour T_{REG} cell function and reduced T_{EFF} cell accumulation. Since the T_{EFF} cells present in the LNs and spleen of mice are most likely not producing IL-2 to the magnitude of islet-reactive T cells in the pancreas, this would justify the lack of increased T_{REG}:T_{EFF} ratios throughout the peripheral immune system. Surprisingly, the pLNs of NOD.B6 *Idd3* mice did not show higher frequencies of T_{REG} cells. However, this may be due to the low prevalence of self-reactive T cells within total lymphocytes, rendering any differences undetectable by our means of analysis. When we compared a larger population of self-reactive $CD4^+$ T cells in the BDC2.5 and BDC.Idd3 mice, the protective *Idd3* allele did increase T_{REG}:T_{EFF} ratios in both the pLN and pancreas. Thus, these findings suggest the B6-derived *Idd3* allele increases the ability of T_{REG} cells to dominate an activated population of T_{EFF} cells at the site of antigen encounter. However, this localized dominance of T_{REG} cells suggests two possibilities for diabetes resistance in NOD.B6 *Idd3* mice: the decreased pathogenic potential of T_{EFF} cells or increased immunoregulation.

We were able to determine that the protective *Idd3* allele does not directly impede T_{EFF} cell function or pathogenic potential. The first evidence of this comes from the similar proliferation of CFSE-labelled BDC2.5 and BDC.Idd3 T_{EFF} cells transferred into NOD mice. Since no difference could be detected, this demonstrates the inability of the *Idd3* locus to influence the antigen-driven proliferation of T_{EFF} cells. Moreover, the adoptive transfer of BDC.Idd3 T_{EFF} cells into $TCR\alpha^{-/-}$ NOD mice induced diabetes at frequencies comparable to BDC2.5 T_{EFF} cells. This suggests that the *Idd3* locus does not reduce the pathogenic potential of self-reactive T cells. Additional *in vitro* assays also disprove the possibility of reduced T_{EFF} cell function since NOD.B6 *Idd3*T_{EFF} cells proliferate more than their NOD counterparts. Similarly, our preliminary data suggest that BDC.Idd3 T_{EFF} cells stimulated with peptide mimicking their natural antigen proliferate more abundantly than BDC2.5 T_{EFF} cells (data not shown). Thus,

diabetes protection in NOD.B6 *Idd3* mice is unlikely to be caused by the reduced pathogenic potential of T_{EFF} cells.

Our findings clearly show the NOD.B6 *Idd3* mice possess increased immunoregulatory mechanisms compared to NOD mice. *In vitro*, the $CD25^+$ T_{REG} cells present at physiological ratios within pre-diabetic NOD.B6 *Idd3* $CD4^+$ T cells reduced the proliferation of T_{EFF} cells by at least 50%. In stark contrast, T_{REG} cells present within NOD $CD4^+$ T cells only suppressed proliferation of T_{EFF} cells by 6-7%, demonstrating inefficient function of the T_{REG} cells at physiological ratios. Evidence of poor immunoregulation was also apparent *in vivo*, where CFSE-labelled BDC2.5 T_{EFF} cells proliferated abundantly in the pLNs of NOD mice. NOD.B6 *Idd3* mice, on the other hand, were much more efficient at limiting the antigen-driven proliferation of these BDC2.5 T_{EFF} cells in the pLNs. Our findings support previously published data assessing the proliferation of CFSE-labelled 8.3 transgenic islet-reactive $CD8^+$ T cells transferred into NOD and NOD.B6 *Idd3* mice (161). Similar to the BDC2.5 T_{EFF} cells, the 8.3 $CD8^+$ T cells showed little proliferation in the pLN of NOD.B6 *Idd3* recipients compared to wild-type NOD recipients. Similarly, a second study showed that transfer of islet-reactive $CD8^+$ T cells into double congenic NOD.B6 *Idd3/Idd5* mice failed to proliferate in the pLN (168). Interestingly, when anti-CD25 blocking antibody was used to deplete $CD4^+CD25^+$ T_{REG} cells in recipient mice prior to transfer of islet reactive $CD8^+$ T cells, NOD and NOD.B6 *Idd3/Idd5* showed similar accumulation and proliferation of $CD8^+$ T cells in the pLN. This study demonstrated that T_{REG} cells were responsible for enhanced immunoregulation in the NOD.B6 *Idd3/Idd5* mice. Although our findings do not directly implicate T_{REG} cells for the increased suppression of BDC2.5 T_{EFF} cells in the pLN of NOD.B6 *Idd3* mice, their involvement is quite probable.

It was important to determine whether increased immunoregulation in the presence of the B6-derived *Idd3* allele was due to intrinsically better T_{REG} cells or due to T_{EFF} cells which are better inducers of T_{REG} cell suppressive function. Our

findings demonstrate that a "more suppressible" population of T_{EFF} cells is not responsible for *Idd3* allele-mediated diabetes protection. If this was the case, then CFSE-labelled BDC.Idd3 T_{EFF} cells would have proliferated less than BDC2.5 T_{EFF} cells when transferred in either NOD or NOD.B6 *Idd3* mice. Since the proliferation the CFSE-labelled T_{EFF} cells was dependent on the strain of recipient mice, rather than the origin of the donor cells, this excludes the possibility of more suppressible T_{EFF} cells. Instead, it appears as though peripheral tolerance mechanisms in the recipient mice are responsible for diabetes resistance. In order to determine whether increased T_{REG} suppressor function was responsible for increased peripheral tolerance in NOD.B6 *Idd3*, we utilized a highly synchronous antigen-specific model of T1D. The transfer of BDC2.5 T_{EFF} cells into immunodeficient mice induces diabetes with a mean onset of 8 days post transfer. Therefore by purifying $CD4^+$ total, T_{EFF} and T_{REG} cells from both BDC2.5 and BDC.Idd3 mice it was possible to perform various crisscross transfer experiments in order to determine if T_{REG} cells from mice containing the protective *Idd3* allele were intrinsically better suppressors of T1D. Although BDC2.5 mice show low onset of diabetes, transfer of transgenic $CD4^+$ T cells into immunodeficient hosts can induce diabetes quite efficiently. Since lymphopenia is a known catalyst of autoimmune disease (161), it is thought that the absence of lymphocytes in the immunodeficient recipients may be responsible for elevated diabetes onset in BDC2.5 $CD4^+$ T cell recipients. Whereas the transfer of BDC2.5 $CD4^+$ T cells from 6-8 week old mice caused diabetes in 71% of recipients, co-transfer of BDC2.5 T_{EFF} cells and BDC.Idd3 T_{REG} cells caused diabetes in only 31% of recipients. Since the T_{EFF} population is the same in these two groups of recipients, reduced diabetes onset can be directly attributed to increased suppressor function of the BDC.Idd3 T_{REG} cells. Similarly, 43% of BDC.Idd3 $CD4^+$ T cell recipients progressed to overt diabetes compared to 71% of BDC.Idd3 T_{EFF} cells + BDC2.5 T_{REG} cells recipients. Once again the T_{EFF} cell population is the same in both groups, and the BDC.Idd3 T_{REG} repeatedly display increased suppressor function. Additionally, since transfer of BDC2.5 $CD4^+$ T cells and co-transfer of BDC.Idd3 T_{EFF} cells + BDC2.5 T_{REG} cells into recipients caused similar diabetes onset, the

hypothesis of the protective *Idd3* allele inducing "more suppressible" T_{EFF} cells seems unlikely. Taken together, our findings demonstrate that the protective *Idd3* allele directly increases the intrinsic suppressor function of the T_{REG} population.

A recent study demonstrated that BDC2.5 T_{REG} cells suppressed late stages of diabetes, namely the progression from peri-insulitis to destructive insulitis (149). Interestingly, early stages of diabetes such as T_{EFF} cell activation and antigen driven proliferation of T_{EFF} cells were not affected by T_{REG} cell-mediated immunoregulation. In accordance with these findings, our data show the suppression of late stages of diabetes in NOD.B6 *Idd3* mice, suggestive of enhanced T_{REG} cell function. *In vivo*, the NOD.B6 *Idd3* mice contain $CD69^+$ and $CD25^+$ T_{EFF} cells in the pLN and immune cell infiltration of the pancreas. However, the increased suppressor function of T_{REG} cells appears to maintain steady-state insulitis whereas NOD mice progress to overt diabetes. The increased T_{REG}:T_{EFF} ratio exclusively in the pancreas of pre-diabetic NOD.B6 *Idd3* mice also supports previous findings demonstrating that BDC2.5 T_{REG} cells are functionally superior in the pancreatic lesion than in the pLN (148, 149). These studies imply that an inflamed target tissue may imprint T_{REG} cells with a distinct functional program. In accordance with these previous studies, the increased T_{REG} cell suppressor function in NOD.B6 *Idd3* mice induces a highly localized dominance of T_{REG} cells over T_{EFF} cells in the pancreatic islets. Taken together, our findings highlight the importance of the *Idd3* locus in the susceptibility to T1D since it reduces T_{REG} cell potency in NOD mice. This ultimately leads to the inability of NOD T_{REG} cells to adequately suppress the naturally arising self-reactive T_{EFF} cells in the immune system. Nevertheless, since NOD.B6 *Idd3* mice do not show complete protection form T1D, this reflects the complex polygenetic dysregulations underlying disease susceptibility.

Although it is it is reasonable to conclude that IL-2 is responsible for these reported differences in T_{REG} function, a direct demonstration is still necessary. Furthermore, since the *Il-21* gene is also present in the *Idd3* locus, it is possible

that differential expression of this cytokine may also be involved in diabetes protection (161). It is likely that the increased tolerance observed in NOD.B6 *Idd3* mice may reflect a synergistic effect of differential *Il-2* and *Il-21* gene expression compared to NOD mice. Although King *et al.* reported lymphopenia in 12 week old NOD but not NOD.B6 *Idd3* mice (161), we were unable to detect any differences in absolute numbers of $CD4^+$ T cells when comparing either NOD and NOD.B6 *Idd3* mice or BDC2.5 and BDC.Idd3 mice. This study also reported the reduced proliferation of CFSE-labelled islet-specific cells in the pLN of NOD.B6 *Idd3* mice compared to wild-type NOD mice. However, King *et al.* base the increased proliferation of cells in NOD mice to mild lymphopenia which fuels the expansion of the transferred cells. It should be noted that King *et al.* used 12 week old NOD recipients which are apparently lymphopenic, whereas we used younger non-lymphopenic 8 week old NOD recipients (161). Nevertheless, we cannot fully discount the possibility of varying lymphopenia in recipient mice as a cause for different proliferation of T_{EFF} cells in NOD and NOD.B6 *Idd3* recipients. Thus, dysregulation of IL-2 and IL-21 in NOD mice may independently contribute to the loss of self-tolerance.

To directly identify IL-2 as the cause of this differential tolerance, a NOD.IL-2^{B6} knock-in mouse would need to be generated, in which the entire NOD genome is intact except for the *Il-2* gene originating from the C57BL/6 mouse strain. If this mouse shows reduced diabetes onset and increased T_{REG} cell function, then IL-2 can be confirmed as a cause for increased tolerance. If this is the case, then it would be interesting to compare the effects of NOD- and C57BL/6-derived IL-2 on IL-2 receptor signalling in NOD T_{REG} cells. Monitoring the phophorylation of STAT-5 will reveal whether the C57BL/6-derived IL-2 is a more potent inducer of IL-2 receptor signalling. This will be important in determining whether the protective *Idd3* allele encodes a qualitatively superior IL-2 molecule or whether the *Il2* gene is more readily expressed. Alternatively, the routine administration of anti-IL-21 neutralizing antibody or anti-IL-21R (receptor) blocking antibody in both NOD and NOD.B6 *Idd3* mice, followed by a

comparative analysis of T_{REG}:T_{EFF} ratios in the pancreas may also help determine whether IL-2 is responsible for increased T_{REG} cell function.

The findings in this study demonstrate a clear role for T_{REG} cells in the protection conferred by the B6-derived *Idd3* allele. However, the direct impact of this genetic locus on T_{REG} cell function still remains unknown. It is possible that the B6-derived *Idd3* allele, which contains IL-2, induces either a better survival of T_{REG} cells or the enhancement of their suppressive function. To determine any differences in T_{REG} homeostasis, it would be interesting to inject NOD and NOD.B6 *Idd3* mice with BrdU, a pyrimidine analogue of thymidine, which is selectively incorporated into dividing cells. Using a BrdU-specific monoclonal antibody, it would be possible to monitor the percentage of proliferating T_{REG} cells within various organs. This would reveal whether NOD.B6 *Idd3* mice possess more actively proliferating T_{REG} cells than NOD mice. If this is the case, it would demonstrate the enhanced homeostasis and better "fitness" of the T_{REG} cells in the presence of the protective *Idd3* allele. Also, given the ability of IL-2 to upregulate genes involved in T_{REG} cell growth and metabolism (124), it would be interesting to use DNA microarray technology to compare genes expressed in freshly isolated T_{REG} cells from NOD and NOD.B6 *Idd3* mice. This type of comparative analysis may also reveal differential expression of various suppression-related genes such as *Il-10*, *Tgfb* or *Ctla4* (124, 148). This would help explain how the B6-derived *Idd3* alleles can render T_{REG} cells intrinsically better. Also, it will be important to eventually compare how the *Idd3* locus modulates T_{REG} cell-mediated suppression of the various steps leading to T1D. For this purpose, the adoptive transfer model will be an important tool since transferring CFSE-labelled islet-reactive cells will allow the tracking of T_{EFF} cell proliferation at early time points. By looking at early time points post-transfer, it will be possible to monitor how BDC2.5 and BDC.Idd3 TREG cells differ in their ability to suppress activation, proliferation and accumulation of T_{EFF} cells. This may also help answer whether high T_{REG}:T_{EFF} ratios in the pancreas of NOD.B6 *Idd3* mice

control the infiltration of additional T_{EFF} cells into the islets or the proliferation of T_{EFF} cells already present.

In conclusion, this study has determined that the *Idd3* allele can influence murine T_{REG} cell suppressor function but not frequency in vivo. Although the protective *Idd3* allele increases IL-2 production in activated T_{EFF} cells, the T_{REG} cells are intrinsically better suppressors of proliferation and diabetes onset. Moreover, protection from T1D in NOD.B6 *Idd3* mice correlates with increased T_{REG}:T_{EFF} ratios exclusively in the pancreas, at the site of tissue destruction. The data presented will undoubtedly help in understanding the impact of allelic variance on T_{REG} cell suppressor function and how its influence on progression of autoimmune diseases. If IL-2 is linked to our observed increase of T_{REG} cell function, these findings may help elucidate the role of the *CD25* gene in human susceptibility to T1D. If the IL-2 signaling pathway is an important determinant in both murine and human T1D, our findings may ultimately help develop novel therapies for patients afflicted with autoimmune diabetes.

Figures and Tables

FIGURE 1. Model for T1D onset in humans (modified from (4))

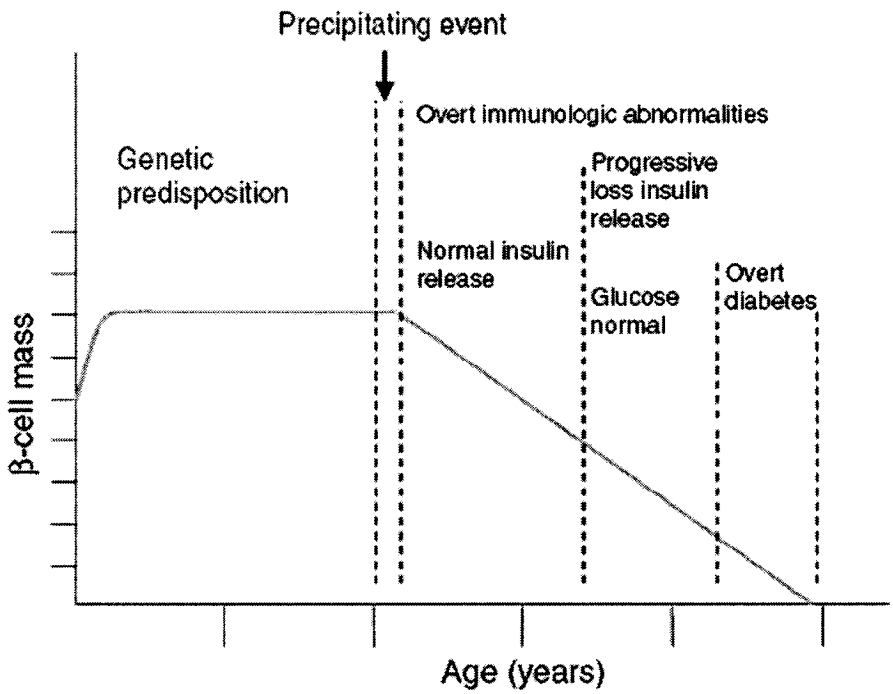
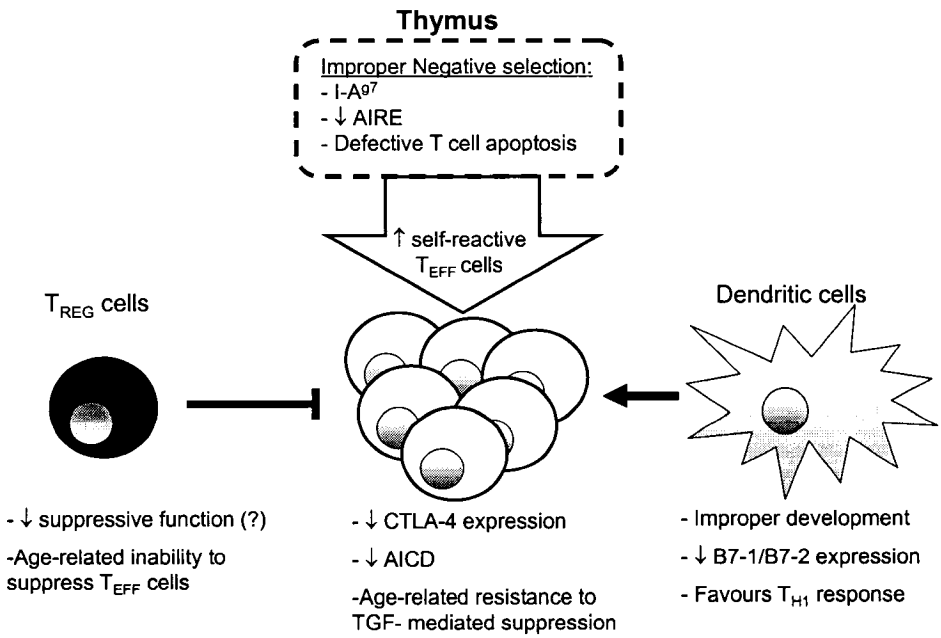
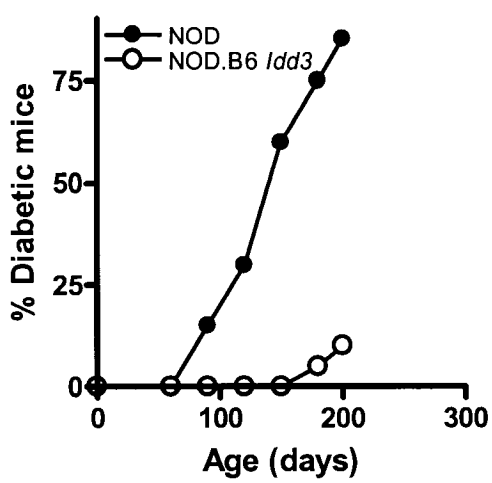


FIGURE 2. Factors responsible for pathogenic T_{EFF} cells in NOD mice



A

Diabetes Incidence



B

TH1 response

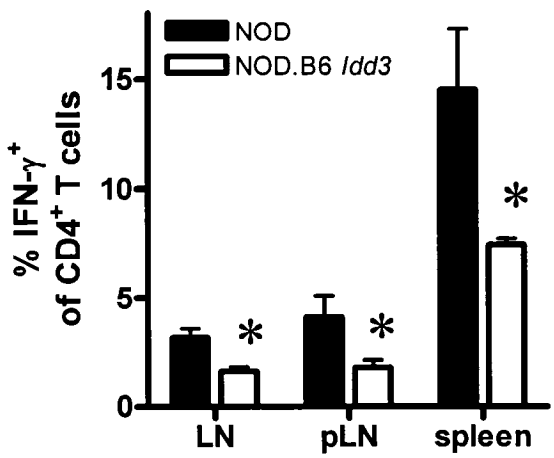


FIGURE 3. Adult NOD.B6 *Idd3* mice show reduced diabetes and halted disease progression compared to wild-type NOD mice.

A, Onset of diabetes in our colony of NOD and NOD.B6 *Idd3* mice (n=20). B, Frequency of $CD4^+$ T cells which were IFN-γ^+ in the various lymphoid organs of pre-diabetic 12 week old mice as determined by intracellular staining (average and standard deviation). Values represent pooled values from two independent experiments (n = 5). *$p < 0.01$

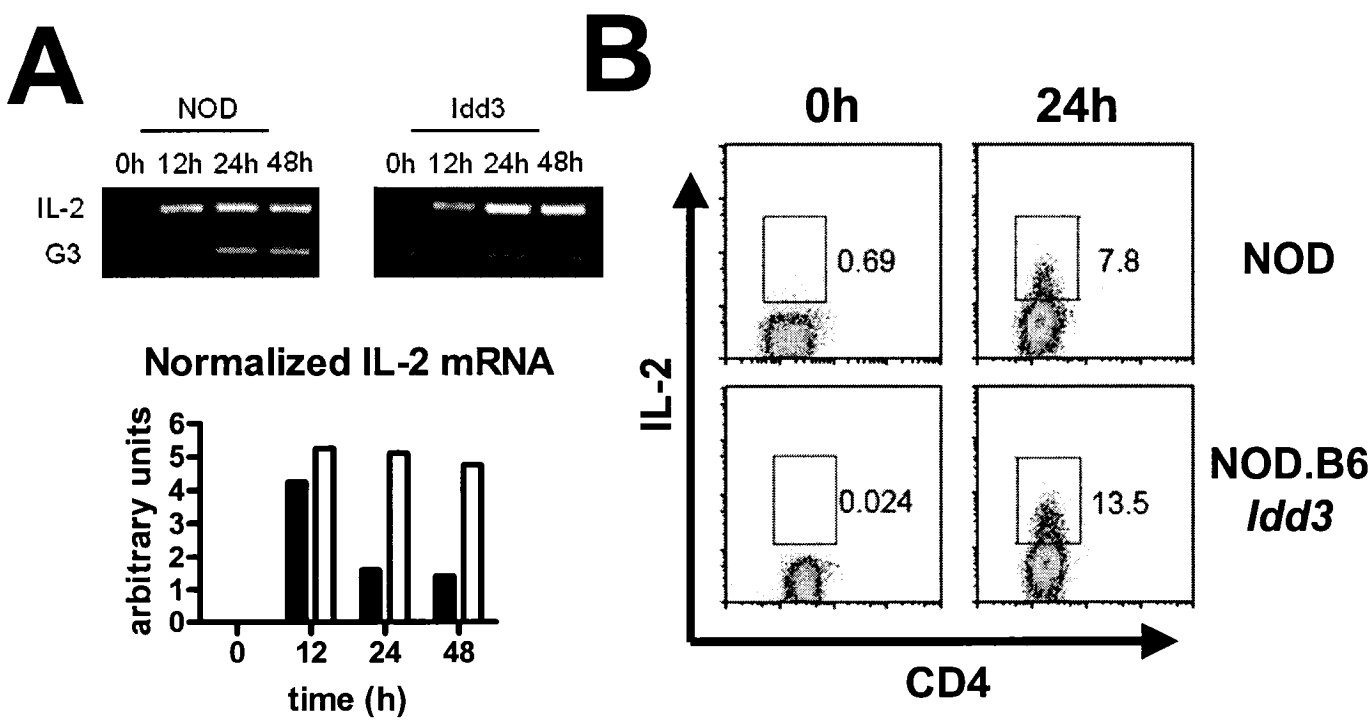

A
NOD
Idd3
0h 12h 24h 48h
0h 12h 24h 48h
IL-2
G3
Normalized IL-2 mRNA
arbitrary units
0
1
2
3
4
5
6
0
12
24
48
time (h)
B
0h
24h
IL-2
0.69
7.8
NOD
0.024
13.5
NOD.B6
Idd3
CD4

FIGURE 4. NOD.B6 *Idd3* $CD4^+CD25^-$ T_{EFF} cells show increased expression of IL-2.

A, $CD4^+CD25^-$ T cells isolated from 3-4 week old NOD (black bars) or NOD.B6 *Idd3* (white bars) mice were activated using plate-bound anti-CD3 and harvested at various time points. RNA was extracted from the cell samples and analyzed for IL-2 expression using semi-quantitative RT-PCR. Normalized values were obtained by densiometric analysis of bands present on the agarose gel and represent the IL-2 to G3 ratios for each lane. Data representative of five independent experiments comparing freshly sorted NOD and NOD.B6 *Idd3* T cells. B, Intracellular staining of freshly purified $CD4^+CD25^-$ T cells 24h after PMA/ ionomycin activation. Data representative of three separate experiments comparing freshly sorted T cells.

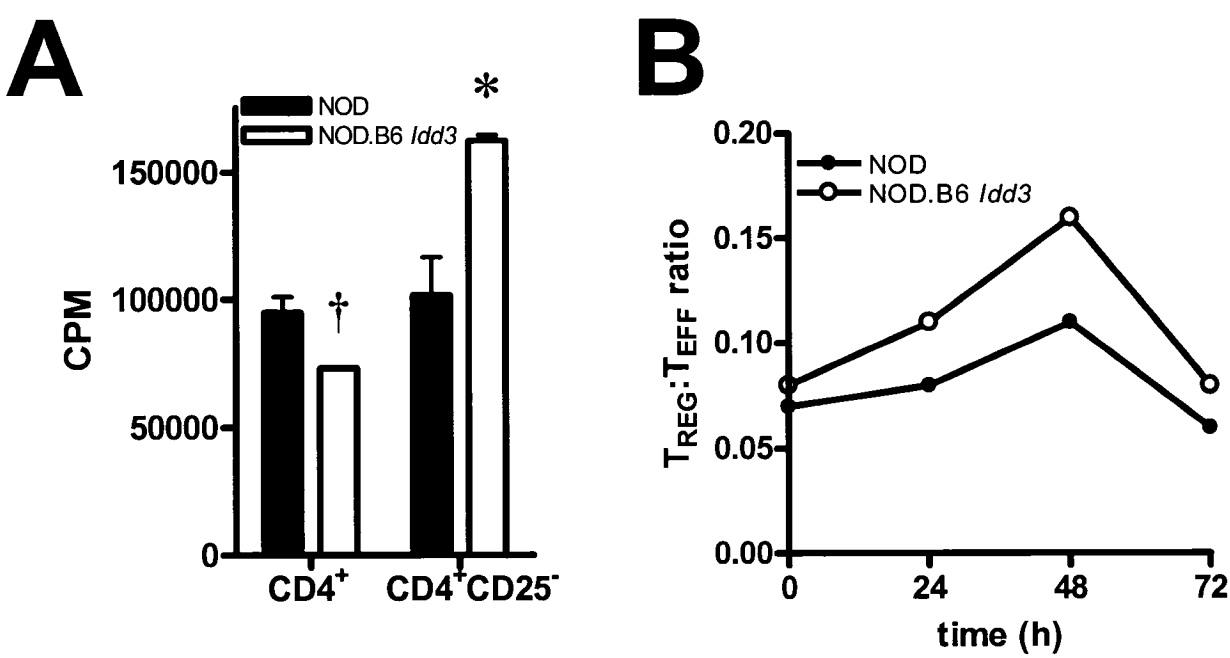

A
NOD
NOD.B6 Idd3
CPM
150000
100000
50000
0
*
†
CD4+
CD4+CD25-
B
NOD
NOD.B6 Idd3
TREG:TEFF ratio
0.20
0.15
0.10
0.05
0.00
0
24
48
72
time (h)

FIGURE 5. The *Idd3* locus affects the T_{REG}:T_{EFF} ratio subsequent to activation of $CD4^+$ T cells.

A, Proliferation of 5 x 10^4 T cells isolated from 3-4 week old NOD and NOD.B6 *Idd3* mice, activated using 1μg/ml of soluble anti-CD3 and 2 x 10^5 irradiated splenocytes, as assessed by thymidine incorporation at 72h. Data shown is the average of triplicate wells and representative of three independent experiments. B, Fold increase of $Foxp3^+$ cells within purified CFSE-labeled $CD4^+$ T cells (2.5 x 10^5) subsequent to activation using 1μg/ml of soluble anti-CD3 and irradiated splenocytes (1 x 10^6). Data representative of four independent experiments. * $p < 0.01$, † $p < 0.05$

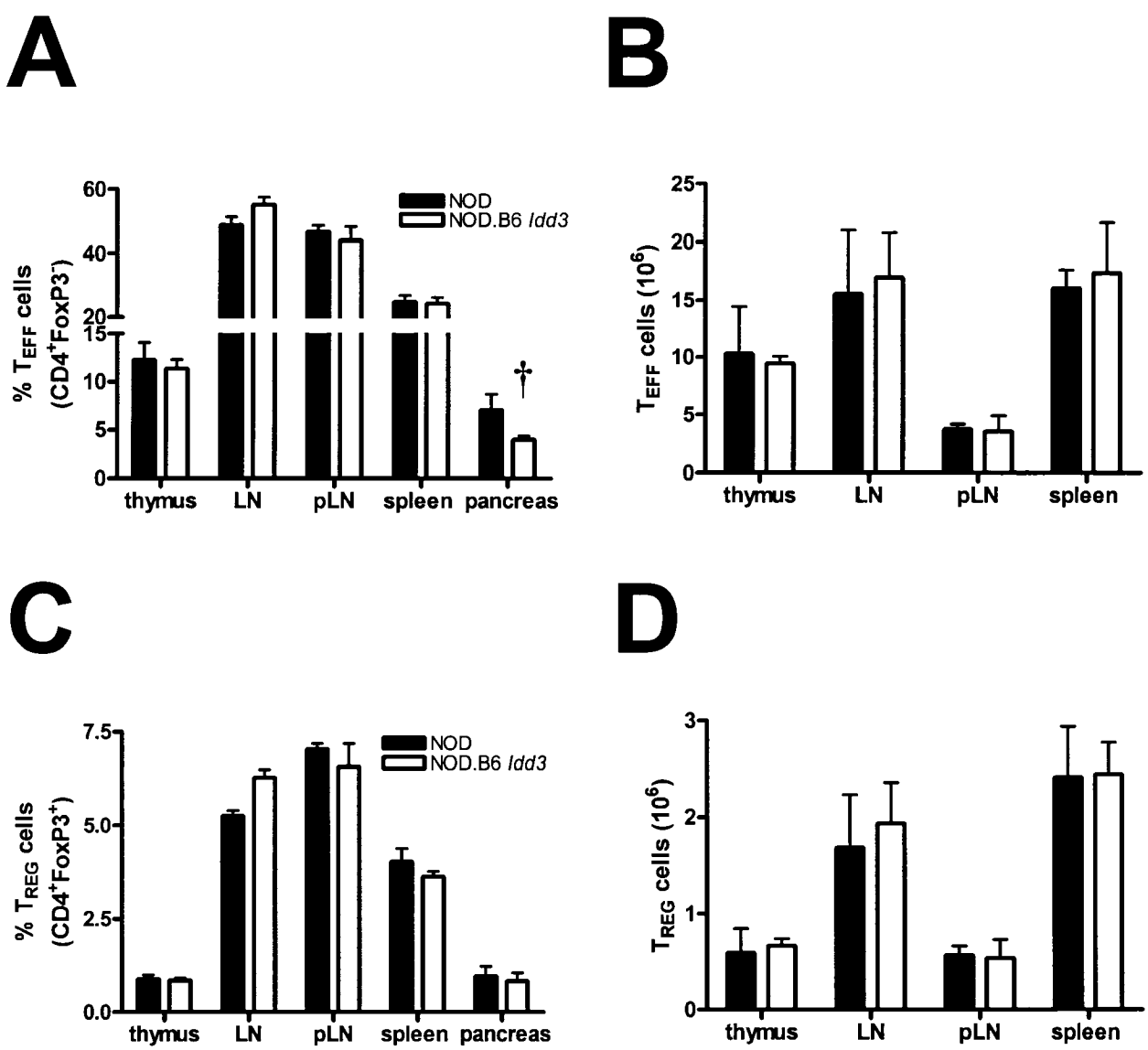

A
B
C
D
NOD
NOD.B6 Idd3
% TEFF cells (CD4+FoxP3-)
TEFF cells (10^6)
% TREG cells (CD4+FoxP3+)
TREG cells (10^6)
thymus
LN
pLN
spleen
pancreas

FIGURE 6. The protective *Idd3* locus does not increase the overall frequency or absolute number of T_{REG} cells.

Frequency of $CD4^+Foxp3^-$ T_{EFF} cells (A) and $CD4^+Foxp3^+$ T_{REG} cells (C) amongst total lymphocytes in various organs. Absolute number of T_{EFF} (B) and T_{REG} (D) cells as assessed by multiplying the total number of cells in each lymphoid organ with the frequency of the specific subset. Values represent the average of three mice and data representative of 3 independent experiments. †$p < 0.05$

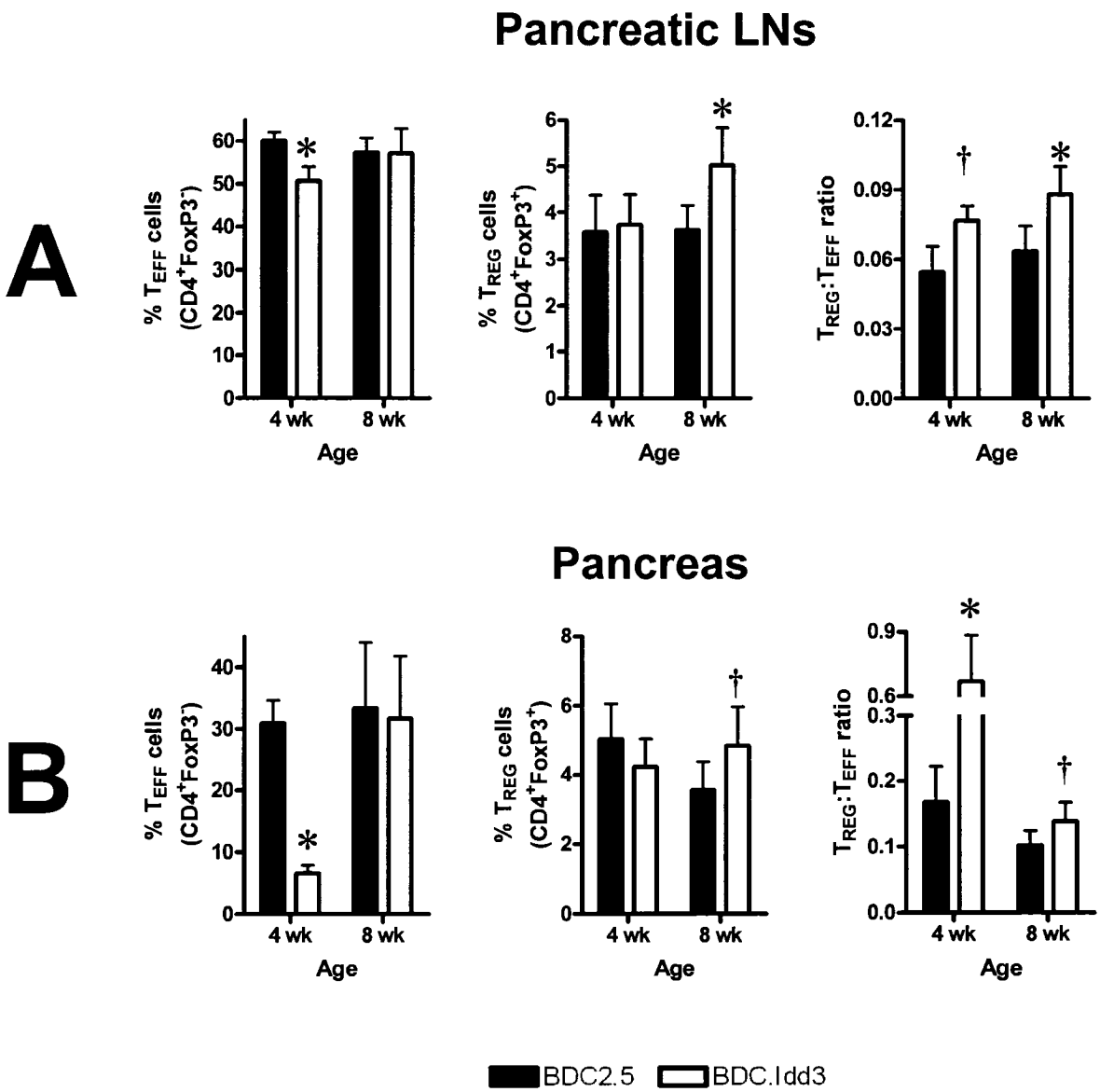
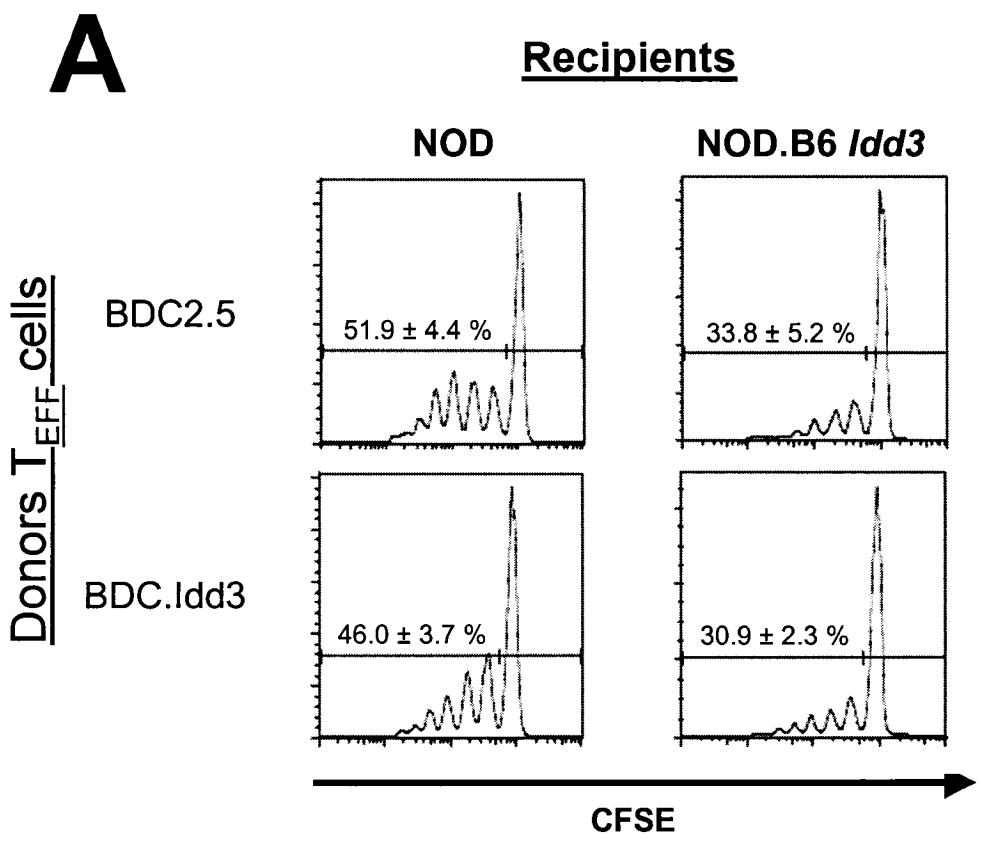

Pancreatic LNs
A
% TEFF cells (CD4+FoxP3-)
% TREG cells (CD4+FoxP3+)
TREG:TEFF ratio
4 wk
8 wk
Age
Pancreas
B
BDC2.5
BDC.Idd3

FIGURE 7. Young BDC.Idd3 transgenic mice show delayed accumulation and activation of T_{EFF} cells in the pLN and infiltration into the pancreas.

Frequency of T_{EFF} ($CD4^+Foxp3^-$) cells and T_{REG} ($CD4^+Foxp3^+$) cells amongst total lymphocytes in the pLN (A) and pancreas (B) of 4w old (n= 4 per strain) or 8w old (n=9 per strain) BDC2.5 and BDC.Idd3 mice. The corresponding T_{REG}:T_{EFF} ratios were calculated using the total frequency of each cell type within the organ. * $p < 0.01$, † $p < 0.05$


A
Recipients
NOD
NOD.B6 Idd3
Donors T_EFF cells
BDC2.5
BDC.Idd3
51.9 ± 4.4 %
33.8 ± 5.2 %
46.0 ± 3.7 %
30.9 ± 2.3 %
CFSE

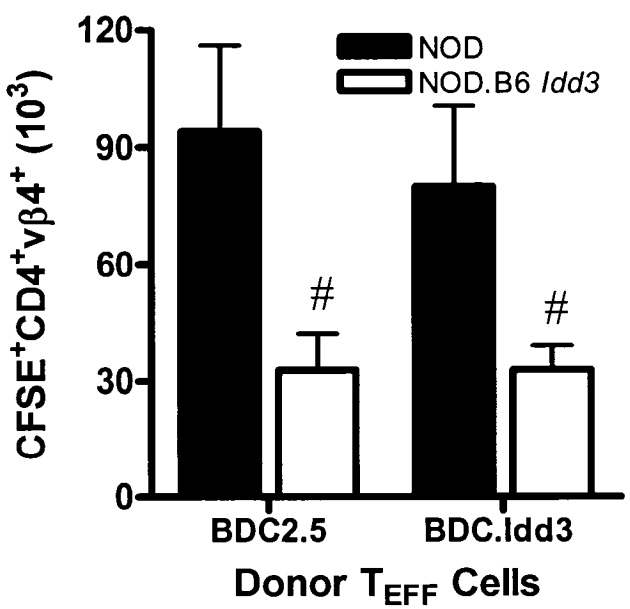

B
CFSE+CD4+vβ4+ (10^3)
120
90
60
30
0
NOD
NOD.B6 Idd3
#
#
BDC2.5
BDC.Idd3
Donor T_EFF Cells

FIGURE 8. The NOD.B6 *Idd3* mice are better suppressors of islet-reactive T_{EFF} cells in the pancreatic lymph nodes.

A, BDC2.5 or BDC.Idd3 lymphocytes were depleted of $CD25^+$ cells by magnetic bead cell-sorting, CFSE-labelled and 8 x 10^6 CD4 cells were adoptively transferred into NOD or NOD.Idd3 recipients. The pancreatic LNs were harvested four days later and proliferation of CFSE-labeled $CD4^+V\beta4^+$ T cells was determined. B, Absolute number of CFSE-labeled $CD4^+V\beta4^+$ T cells present in the pancreatic lymph node of recipient mice.(n=3-4 mice/group, representative of 3 separate experiments) # $p<0.02$

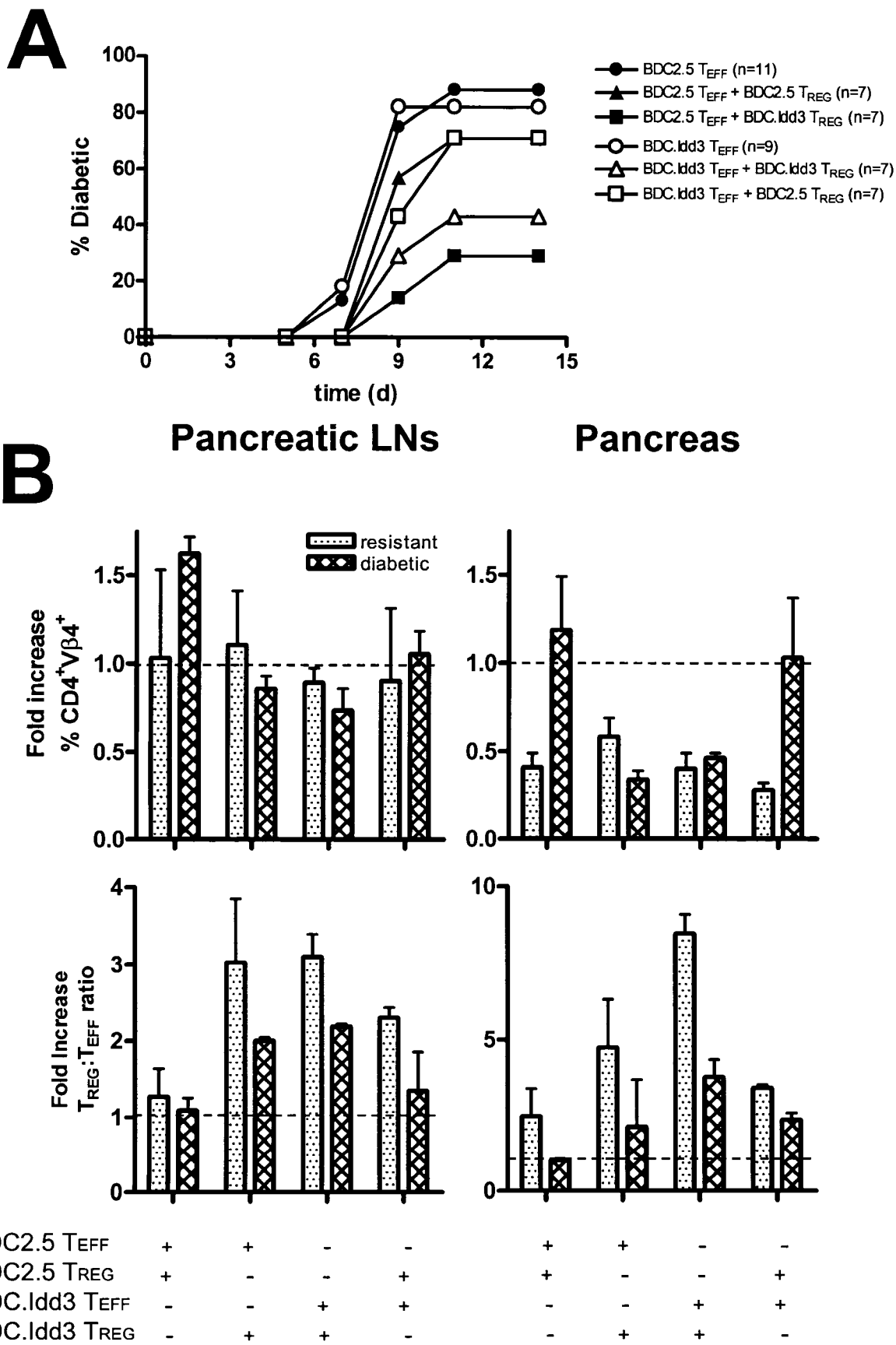

A
% Diabetic
time (d)
BDC2.5 T_EFF (n=11)
BDC2.5 T_EFF + BDC2.5 T_REG (n=7)
BDC2.5 T_EFF + BDC.Idd3 T_REG (n=7)
BDC.Idd3 T_EFF (n=9)
BDC.Idd3 T_EFF + BDC.Idd3 T_REG (n=7)
BDC.Idd3 T_EFF + BDC2.5 T_REG (n=7)
B
Pancreatic LNs
Pancreas
resistant
diabetic
Fold increase % CD4+Vβ4+
Fold Increase T_REG:T_EFF ratio
BDC2.5 TEFF
BDC2.5 TREG
BDC.Idd3 TEFF
BDC.Idd3 TREG

FIGURE 9. BDC.Idd3 $CD4^+$ T cells are less pathogenic than BDC2.5 $CD4^+$ T cells when transferred into immunodeficient mice and this is due to increased suppressor function of $CD4^+$ $CD25^+$ T_{REG} cells.

5 x 10^5 purified T cells from 6-8 wk old BDC2.5 and BDC.Idd3 mice were adoptively transferred into NOD.TCR$\alpha^{-/-}$ mice in various combinations and diabetes incidence was monitored every 2 days (A). At day 15, mice from each group were sacrificed and various organs were screened using flow cytometry to monitor T_{REG} and T_{EFF} cell frequency (B). Values shown reflect the fold modulation of cell frequencies relative to the pooled values of diabetic BDC2.5 and BDC.Idd3 T_{EFF} cell recipients.

TABLE 1. Congenic regions influencing type 1 diabetes susceptibility in humans (modified from (171))

Human type 1 diabetes loci with multiple replication studies.		
Human locus and chromosome location	Candidate causal variant	Functional phenotype of susceptibility allele
HLA class II, 6p21	HLA-DQ/DR	Major role of peptide-binding pockets P1, P4 and P9 in resistance/susceptibility
Insulin, 11p15.5	Probably insulin VNTR class I	VNTR class I associated with decreased thymic insulin expression
CTLA4, 2q33	CT60 (+6 230G>A, rs3087243)	Allele A associated with increased levels of soluble CTLA-4
PTPN22, 1p13	Arg620Trp (+1 858C>T, rs2476601)	Trp620 unable to bind to CSK

TABLE 2. Comparison of insulin-dependent diabetes in humans and NOD mice (modified from (10))

Characteristic	Humans	NOD mice
Genetic predisposition (MHC class II linkage)	+	+
Complex polygenic control	+	+
Environmental effects on gene penetrance	Probable	+
Disease transmissible via bone marrow	+	+
T-lymphocyte-driven insulitic lesions	+	+
Leucocytic infiltrates found in other organs	Sometimes	+
Defective peripheral immunoregulation	+	+
Humoral reactivity to beta cells	+	+
Endogenous retroviral genes expressed in beta cells	–	+
Diabetic ketoacidosis if untreated	+	Mild
Sex bias	–	+
Successful intervention therapies	In progress	+

TABLE 3. Impact of various cytokines on diabetes progression in NOD mice.

		Cytokine administration	Antibody Treatment	Knockout mice	Localized expression in the β islets cells
TH1 cytokines	IFN-γ		prevents diabetes	delays diabetes	accelerates diabetes
	TNF-α		prevention or delay of diabetes		prevents diabetes
	IL-12	Accelerates diabetes		no effect on diabetes	
TH2 cytokines	IL-4	Prevents diabetes		no effect on diabetes	prevents diabetes
	IL-10	Prevents diabetes		no effect on diabetes	accelerates diabetes

TABLE 4. Congenic regions affecting T1D susceptibility in NOD mice (modified from (171))

NOD locus	Chr.	Interval size[a]	Number of genes[b]	Candidate genes	Known gene function
Idd5.1	1	2.1 Mb	4	*Ctla4*	Negative T cell regulator
Idd5.2	1	1.52 Mb	45	*Nramp1/Slc11a1*	Endosomal/lysosomal acidification
Idd13	2	6 cM (H3a-Il1 interval)	NR	*B2m*	Required for antigen presentation by MHC class I molecules
Idd3	3	0.78 Mb	8	*Il2* *Il21*	T cell development and homeostasis
Idd17	3	1.1 cM	NR	NR	NR
Idd10	3	0.95 Mb	7	*Cd101*	Co-stimulatory receptor of T cells
Idd18	3	0.7 Mb	2	*Vav3*	Guanine nucleotide exchange factor involved in signaling of T and B cell receptors
Idd6	6	10 cM	NR	NK complex: *Lmp, Bcat1, Kras2*	Innate immune system; control cell proliferation
Idd9.1	4	39 cM	NR	*Lck*	T cell responsiveness
Idd11[d]	4	13 cM	NR	NR	NR
Idd9.2	4	3.7 Mb	13	NR	Candidate tumour suppressor gene
Idd9.3	4	1.2 Mb	13	*4-1bb*	Role in enhancing and regulating multiple immune cell types
Idd4	11	5.2 cM	NR	PAF-AHIb1 *Nos2* CC chemokines	Inflammatory responses
Idd14	13	>58 cM	NR	NR	NR
Idd23	17	~20 Mb	NR	NR	NR
Idd16	17	3.1 Mb	37	*Mapk13* *Mapk14* Class I K	Cell signalling, antigen presentation
Idd1	17	NR	NR	Class II A and E	Antigen presentation
Idd24	17	12.1 Mb	NR	NR	NR

Abbreviations: Chr, chromosome; NR, not reported.
[a] On average, each megabase (Mb) is approximately 0.5 centimorgan (cM).
[b] Where not reported, on average 1 Mb has 5–25 genes.
[c] HI Fraser *et al.*, unpublished.
[d] *Idd9.1* and *Idd11* are probably the same *Idd* locus.

		Number of cell divisions						
		0	1	2	3	4	5	6
Gate: CD4+	NOD	2.20%	5.50%	17.80%	15.60%	25.70%	**24.40%**	**7.30%**
	NOD.B6*Idd3*	2.10%	5.70%	**20%**	**17.50%**	**26.90%**	20.80%	5.60%

		Number of cell divisions						
		0	1	2	3	4	5	6
Gate: CD4+ Foxp3+	NOD	1.80%	4.30%	17.90%	28%	29.10%	12.20%	1.75%
	NOD.B6 I*dd3*	1.13%	4.65%	**20.90%**	28.10%	28.30%	11.70%	1.35%

		Number of cell divisions						
		0	1	2	3	4	5	6
Gate: CD4+ Foxp3-	NOD	2.20%	4.35%	17.80%	14.80%	25.50%	**25.10%**	**7.41%**
	NOD.B6 *Idd3*	1.72%	4.73%	**20%**	14.40%	26.40%	22.60%	5.92%

TABLE 5. CFSE dilution analysis of activated $CD4^+$ T cells from NOD and NOD.B6 *Idd3* mice 72h after activation.

The three tables represent the same population of activated $CD4^+$ T cells gated on either total $CD4^+$, $CD4^+Foxp3^+$ or $CD4^+Foxp3^-$ cells. Percentages shown reflect the distribution of cells at each division peak within the gated population. Values in bold highlight differences >1% between NOD and NOD.B6 *Idd3* cells for a given subset. Data representative of 3 independent experiments.

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APPENDIX