# Functional dynamics of $CD4^+Foxp3^+$ regulatory T cells throughout the progression of type 1 diabetes: lessons learned from the NOD mouse model.

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## DEDICATION

Αφιερωμενο στην μητερα μου, Μαρια Ψαρουδη,
και στον συζυγο μου, Δαμιανο Γιαννο. Σας λατρευω.

# ACKNOWLEDGMENTS

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Alexander Albanese, former M.Sc. student of the Piccirillo laboratory, participated in the design, performance, and analysis of data for the manuscript focusing on the impact of *Il2* allelic variants on $T_{reg}$ functions and resistance to type 1 diabetes (Chapter III). He also actively participated in the writing of the manuscript and gave insightful advice. It was a pleasure to collaborate with him on this project. Mara Kornete, a current M.Sc. student of the Piccirillo laboratory, played an important role in the performance of experiments for the manuscripts that focused on the impact of ICOS stimulation on $T_{reg}$ cells (Chapter IV) and the *Idd3*$^{B6}$-mediated effect of DC on $T_{reg}$ cell functions (Chapter V). Mara was always very generous with her time and displayed her devotion to the project openly. I would like to thank all other members, past and present, of the Piccirillo laboratory, namely Ekaterina Yurchenko, Eva d'Hennezel, Dr. Eva Bjur, Helen Mason, Dr. Moshe Ben Shoshan, Micheal Tritt, Jasmine Grenier, Maria da Sylva Martins for providing such a jovial working space in which to grow. I would like to recognize the unconditional support and advice that Ekaterina Yurchenko and Eva d'Hennezel have

given me for the duration of my PhD studies. They are great colleagues and even greater friends.

# ABSTRACT

Type 1 diabetes (T1D) results from the T cell-mediated destruction of the insulin-producing β-islets. $CD4^+CD25^+Foxp3^+$ regulatory T ($T_{reg}$) cells have emerged as a central control point in T1D progression. A correlation exists between T1D and T cell hyporesponsiveness at the time of insulitis, which results in a decline in IL-2. A growing body of evidence strongly demonstrates that IL-2 is an important signal for $T_{reg}$ cell competitive fitness. Congenic non-obese diabetic (NOD) mice introgressed with the *Idd3*$^{B6}$ genetic interval from non-autoimmune prone strain (C57/BL6) (*Idd3*$^{B6}$) exhibit a marked delay in disease incidence, onset and severity relative to wild-type (WT) NOD mice. The candidate gene within the *Idd3*$^{B6}$ locus is *Il2*. Considering the critical role of IL-2 in $T_{reg}$ cell functions, we hypothesized that the *Idd3*$^{B6}$ locus confers T1D protection by promoting the survival, function or recruitment of $T_{reg}$ cells. We show that NOD mice succumb to T1D due to a marked age-related decline in the compartment of cycling $T_{reg}$ cells due to IL-2 deficiency, a defect corrected by IL-2 therapy or the *Idd3*$^{B6}$ locus. Interestingly, inducible costimulator (ICOS), which is IL-2-dependent, is predominantly expressed by intra-islet resident $T_{reg}$ cells, favors their suppressive functions and maintains production of IL-10, as demonstrated by blockade and genetic ablation studies. Thus, ICOS imprints $T_{reg}$ cells with their suppressive signature phenotype. In contrast to T1D-protected animals, the expression of ICOS in the cycling $T_{reg}$ cell compartment also diminishes with age in WT NOD mice, suggesting that loss of ICOS signals correlates with waning of $T_{reg}$ cell functional potency. ICOS-ligand (ICOS-L) is expressed exclusively within the target organ by dendritic cells (DC) and its expression also declines over time, suggesting bidirectional ICOS/ICOS-L signalling and

conditioning between $T_{reg}$ cells and DC. Furthermore, DC from pancreatic sites of NOD mice exhibit differential costimulatory load and priming capabilities relative to T1D-resistant counterparts. *Idd3*$^{B6}$ DC, in contrast to WT NOD DC, transiently transcribe IL-2, which may be an initial non-T cell source of IL-2 for $T_{reg}$ cell functions. Thus, IL-2 shortage directly impinges the functional homeostasis of $T_{reg}$ cells by modifying the ICOS costimulation pathway and $T_{reg}$ cell/DC cross-talk, and in turn, contributes to T1D susceptibility.

# RESUME

Le maintien de la tolérance au soi implique des mécanismes centraux et périphériques assurés, notamment, par les cellules régulatrices T ($T_{reg}$), caractérisées par l'expression de CD25 et Foxp3. Des modifications numériques et/ou fonctionnelles de ces populations cellulaires pourraient être la cause de la rupture de tolérance au soi. Nous avons entrepris l'analyse numérique et surtout fonctionnelle de ces cellules $T_{reg}$ au cours du diabète de type 1 dans le modèle murin "non-obese diabetic" (NOD). Le diabète de type 1 est une maladie autoimmune aboutissant à une destruction totale des cellules bêta des îlots de Langerhans par les cellules T CD4 autoréactives. La prédisposition génétique et les facteurs de risque déclenchent le diabète insulino-dépendant. Les cellules $T_{reg}$ constituent le mécanisme prédominant de suppression contre les cellules T pathogènes. Les résultats tendent à montrer que les cellules $T_{reg}$ déploient leurs fonctions régulatrices, mais qu'elles déclinent avec l'âge. Le nombre de cellules $T_{reg}$ ainsi que leurs fonctions d'inhibition de la prolifération et sécrétion de cytokines inflammatoires des cellules T CD4 effectrices sont altérées chez la souris NOD. Par ailleurs, les défaults numérique et fonctionnel des cellules $T_{reg}$ que semblent présenter ces souris sont restitués pas des variantes alléliques d'IL-2, un facteur essentiel à l'homéostase des cellules $T_{reg}$, chez la souris congénique résistante au diabète NOD.B6 *Idd3*. En aval de la signalisation d'IL-2, les résultats dépeignent un rôle prépondérant pour la stimulation ICOS dans l'activation des fonctions régulatrices des $T_{reg}$. De plus, ICOS promeut l'expression de la cytokine immunomodulatrice IL-10, impliqué dans l'amortissement du diabète. La baisse d'ICOS par les cellules $T_{reg}$ coincide avec le diabète, démontrant une relation étroite entre la costimulation ICOS et

l'autoimmunité. La stimulation fournie par les cellules dendritiques (DC) est défectueuse chez la souris NOD, illustrée par l'expression moindre de molécules de stimulation, dont CD80, CD86, et surtout ICOS-ligand comparé à NOD.B6 *Idd3*. En revance, CD40 est surexprimé par les DC chez la souris NOD. En somme, une meilleure connaissance des altérations affectant ces cellules $T_{reg}$ devrait permettre d'entreprendre des stratégies thérapeutiques pour la prévention ou le renversement de la maladie en manipulant cette population cellulaire.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AICD | Activation-induced cell death |
| Aod2 | Autoimmune ovarian dysgenesis 2 |
| AML-1 | Acute myeloid leukemia-1 |
| AP-1 | Activator protein-1 |
| APC | Antigen presenting cell |
| ATP | Adenosine triphosphate |
| BB | Biobreeding |
| β2m | β2 microglobulin |
| B7.1 | CD80 |
| B7.2 | CD86 |
| Bcl-2 | B cell CLL/lymphoma 2 |
| BMDC | Bone marrow derived dendritic cell |
| cAMP | Cyclic adenosine monophosphate |
| CDR3 | Complementarity-determining region 3 |
| CD40L | CD40 ligand |
| Cetn4 | Centrin 4 |
| CIA | Collagen-induced arthritis |
| Crem | Cyclic adenosine monophosphate responsive element modulator |
| CTL | $CD8^+$ cytotoxic T lymphocyte |
| CTLA-4 | Cytotoxic T lymphocyte antigen 4 |
| CXCR3 | Chemokine C-X-C motif receptor 3 |

| | |
|---|---|
| DC | Dendritic cell |
| DEC205 | Decalectin 205 |
| DP | Double-positive (referring to thymocytes) |
| EAE | experimental autoimmune encephalomyelitis |
| Eae3 | Experimental allergic encephalomyelitis susceptibility |
| Erk | Extracellular signal-regulated kinase |
| Fgf2 | Fibroblast growth factor 2 |
| Foxp3 | Murine forkhead box p3 winged-helix transcription factor |
| $Foxp3^{gfp}$ | GFP expression under Foxp3 promoter control |
| FOXP3 | Human forkhead box p3 winged-helix transcription factor |
| Gab2 | Growth factor receptor bound protein 2-associated protein 2 |
| GAD | Glutamate dehydrogenase |
| GAS | Gamma-induced sequence |
| GATA-3 | (Th2-specific) transcription factor binds to GATA consensus sequence |
| GFP | Green fluorescent protein |
| GITR | Glucocorticoid inducible TNF receptor family-related protein |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| Grb2 | Growth factor receptor-bound protein 2 |
| HA | Hemagglutinin |
| HAT | Histone acetyl transferase |
| HLA | Human leukocyte antigen |
| HSC | Hematopoietic stem cells |
| Hsp60 | Heat shock protein 60 |
| IA2 | Insulinoma associated protein 2 |

| | |
|---|---|
| IAA | Insulin autoantibodies |
| IBD | Inflammatory bowel disease |
| ICOS | Inducible costimulatory molecule |
| ICOS-L | Inducible costimulatory molecule ligand |
| Idd | Insulin-dependent insulitis |
| IDO | Indoleamine oxygenase |
| IFIH1 | interferon induced with helicase C domain 1 |
| IFN | Interferon |
| IGRP | Glucose 6 phosphatase catalytic subunit related protein |
| IL | Interleukin |
| IL-2R | IL-2 receptor |
| IL-7Rα | IL-7 receptor α chain |
| IL-15Rα | IL-15 receptor α chain |
| IPEX | Immunodysregulation polyendocrinopathy enteropathy X-linked |
| IRF4 | Interferon regulatory factor 4 |
| iTreg | Induced $T_{reg}$ cells |
| Jak | Janus kinase |
| $K_d$ | Dissociation constant |
| KDP | Komeda diabetes-prone |
| LAP | Latency associated protein |
| LFA-1 | Leukocyte function-associated antigen-1 |
| LN | Lymph node |
| LYP | Lymphoid tyrosine phosphatase |

| | |
|---|---|
| mAb | Monoclonal antibody |
| MCC | Moth cytochrome c |
| MEK | Mitogen activated kinase kinase |
| MHC | Major histocompatibility complex |
| miRNA | Micro ribonucleic acid |
| MΦ | Macrophage |
| mTEC | Medullary thymic epithelial cells |
| NFAT | Nuclear factor of activated T cells |
| NF-κB | Nuclear factor kappa light chain enhancer of activated B cells |
| NK | Natural Killer cell |
| NK-T | Natural Killer T cell |
| NOD | Non-obese diabetic |
| $nT_{reg}$ cell | Naturally-ocurring thymic-derived regulatory T cell |
| Nu/nu | Athymic mice with nude phenotype |
| pancLN | Pancreatic LN |
| PI3K | phosphoinositide 3 kinase |
| PTPN22 | Protein tyrosine phosphatase, non-receptor type 22 |
| RAG | Recombination activating gene |
| RNA | Ribonucleic acid |
| RORγτ | Retinoic acid related orphan receptor γτ |
| Runx1 | Runt-related transcription factor 1 |
| SCID | Severe combined immunodeficiency |
| Shc | Src homologous and collagen |

| | |
|---|---|
| SLE | Systemic lupus erythrematosus |
| SNP | Single nucleotide polymorphism |
| SP | Single-positive (referring to thymocytes) |
| STAT | Signal transducer and activator of transcription |
| STZ | Streptozotocin |
| T1D | Type 1 diabetes |
| T-bet | (Th1 specific) T-cell-specific T-box transcription factor |
| TCR | T cell receptor |
| $T_{eff}$ cell | Conventional $CD4^+$ effector T cell |
| Tenr | Testis nuclear RNA-binding protein |
| TGF-β | Tumour growth factor β |
| Th | T helper cells |
| Th3 | Induced TGF-β-producing regulatory T cell |
| TNF | Tumour necrosis factor |
| Tr1 | Induced IL-10-producing regulatory T cell |
| $T_{reg}$ cell | $CD4^+CD25^+Foxp3^+$ regulatory T cell |
| VNTR | Variable number tandem repeats |
| WT | Wild-type |
| Zap70 | Zeta-chain (TCR) associated protein kinase |

# CONTRIBUTION OF AUTHORS

This thesis consists of a collection of four original papers, two of which are published and two will be submitted shortly. Each paper constitutes a separate chapter, which contains an introduction, material and methods, results and conclusion. A statement has been included in order to bridge each of the chapters. Unless otherwise stated, I, Evridiki Sgouroudis, under the close supervision of my supervisor, oversaw experimental design and execution, data analysis and manuscript preparation for the body of work presented here-in. I was directly responsible for the research in chapters III to V, with the exception of certain experiments, as stated below:

**Chapter II**

**Functional waning of naturally occurring CD4+ regulatory T-cells contributes to the onset of autoimmune diabetes.**

*Diabetes 2008 57(1):113-23*

Tritt M, Sgouroudis E, d'Hennezel E, Albanese A, Piccirillo CA.

Michael Tritt was responsible for figures 1, 2, 4A, B, D, 5, 6A-D. Alex Albanese generated the data for figures 3, 4E. I was responsible for figures 4C, 4F, 6D, 6E. Eva d'Hennezel was intimately involved in plotting the graphs. With support of my supervisor, I was particularly involved in the rationale development, experimental design, data analysis, writing of the paper, and publication revision process. My contributions to this manuscript served as a cornerstone for Chapter III.

## Chapter III

**Impact of protective IL-2 allelic variants on $CD4^+Foxp3^+$ regulatory T cell function *in situ* and resistance to autoimmune diabetes in NOD mice.**

*J. Immunology 2008 181(9):6283*

Sgouroudis E, Albanese A, Piccirillo CA.

I acknowledge that Alex Albanese, a former M.Sc. student of the Piccirillo laboratory and second author of the article, generated the data depicted in figures 1A, 1D, 2A, 2B, 3A, 5B. He also participated in data interpretation and manuscript preparation. With support of my supervisor, I was particularly involved in the rationale development, experimental design, data analysis, writing of the paper, and publication revision process. My contributions to this manuscript served as a cornerstone for Chapter IV.

## Chapter IV

**IL-2 dependent ICOS-mediated control of $CD4^+Foxp3^+$ regulatory T cell homeostasis and differentiation in pre-diabetic islets of NOD mice.**

Sgouroudis E, Kornete M, Piccirillo CA.

Mara Kornete, a current PhD student of the Piccirillo laboratory, generated the NOD $Foxp3^{gfp}$ and BDC2.5 $Foxp3^{gfp}$ mice by extensive backcrossing of C57BL/6 $Foxp3^{gfp}$ to NOD and BDC2.5 respectively. She also generated figures 3 and 5C, and actively participated in the interpretation of the data. With the support of my supervisor, I was particularly involved in the rationale development, experimental design, data analysis,

and writing of the paper. My contributions to this manuscript served as a cornerstone for Chapter V.

## Chapter V

### IL-2 production by dendritic cells augments Foxp3+ regulatory T cell function in autoimmune resistant NOD mice.

Sgouroudis E, Kornete M, Piccirillo CA.

Many of the bone marrow derived dendritic cell (BMDC) cultures were prepared by Mara Kornete. BDC2.5 $Foxp3^{gfp}$ mice generated by Mara were used to isolate $CD4^{+}Foxp3^{gfp-}$ $T_{eff}$ cells in order to assess *in vitro* Foxp3 conversion and IL-2 production (Fig.1C and Fig.4A). With support of my supervisor, I was particularly involved in the rationale development, experimental design, data analysis, and writing of the paper.

Under the guidance of my research supervisor, Dr. Piccirillo, I also wrote an extensive review on the impact of T cell intrinsic and extrinsic variables on the waning of $T_{reg}$ cell functions in the NOD mouse model:

*Sgouroudis E, Piccirillo CA. Control of type 1 diabetes by $CD4^{+}Foxp3^{+}$ regulatory T cells: lessons from mouse models and implications for human disease. (2009) Diabetes Metab Res Rev. (3):208-18.*

Furthermore, I participated in the rationale development, experimental design, data analysis and manuscript preparation in collaboration with the laboratory of Jeffrey Bluestone of the Diabetes Center in the University of California at San Francisco (UCSF):

*Tang Q, Adams JY, Penaranda C, Melli K, Piaggio E, Sgouroudis E, Piccirillo CA, Salomon BL, Bluestone JA. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. (2008) Immunity (5):687-97.*

I, Evridiki Sgouroudis, have read, understood and abided by all norms and regulations of academic integrity of McGill University.

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**CHAPTER IV *IL-2 dependent ICOS-mediated control of CD4$^{+}$Foxp3$^{+}$ regulatory T cell homeostasis and differentiation in pre-diabetic islets of NOD mice.***



***CHAPTER V IL-2 production by dendritic cells augments Foxp3+ regulatory T cell function in autoimmune resistant NOD mice.***

# CHAPTER I *GENERAL INTRODUCTION*

# *1.* Type 1 Diabetes: environmental and genetic factors underlie autoimmune disease.

## a. Introduction.

The immune system is the body's natural defence mechanism against invading foreign pathogens and must simultaneously mount effective immunity while maintaining tolerance to self-antigens [1]. The immune system has evolved to recognize the difference between self and non-self [1]. A breach in the mechanisms promoting tolerance may result in autoimmune diseases, such as type 1 diabetes (T1D) [1]. The precise etiology and initiating immunogens that launch autoimmunity remain unknown. Elucidation of the mechanisms of autoimmunity is fundamental in understanding how the immune system normally maintains tolerance [1].

T1D is a T cell-mediated autoimmune disease characterized by the destruction of the endocrine insulin-producing beta (β) cells of the pancreatic islets of Langerhans [1]. Clinical manifestations result when more than 90% of β cell mass is destroyed, although the new belief is that β cells are not destroyed, but rather lie dormant under inflammatory settings [2]. The destruction of the β cells by self-reactive T cells results is an absolute dependence of the patient on exogenous insulin to maintain glucose homeostasis [3]. Plasma glucose deregulation, persistent hyperglycemia and long-term complications (retinopathy, nephropathy, neuropathy) are a consequence of β-islet destruction [1]. T1D arises in genetically susceptible individuals and is preceded by a prodromal stage in which autoantibodies are detected in the blood, followed by subclinical β cell destruction and ultimately overt diabetes [3].

The discovery that autoimmune diabetes is mediated by autoreactive T cells occurred when an inflammatory lymphocytic infiltrate was initially detected in autopsy specimens of diabetic patients [4]. Pancreatic biopsies revealed that the infiltrate was mainly composed of $CD4^+$ and $CD8^+$ T cells, although B cells, dendritic cells (DC) and macrophages (MΦ) were also detected [5-7]. This was a preliminary indication that innate and adaptive immune systems act in concert to efficiently destroy the islets [5, 6]. These cells accumulate and are non-destructive until an unknown triggering event occurs, resulting in the destruction of the β cells [7]. MΦ and DC process and present antigen, which leads to the activation of β cell-specific cytotoxic $CD8^+$ T cells (CTL) and the generation of autoreactive $CD4^+$ effector T ($T_{eff}$) cells [3]. These cells release cytotoxic molecules (cytokines, granzyme B, perforin) and signal β cell death through the Fas pathway, sparing the α and δ cells [3, 7].

It is believed that insulin is the primary target autoantigen [6]. However, through epitope spreading, T cell clones and autoantibodies for various β cell-specific autoantigens are detected as disease progresses [6]. Indeed, $CD4^+$ and $CD8^+$ T cells specific for β islet antigens such as glutamate dehydrogenase (GAD), insulinoma-associated protein 2 (IA2) and glucose 6 phosphatase catalytic subunit related protein (IGRP) have been detected in the peripheral blood of diabetic patients [6]. Serological markers of anti-islet immunity such as IA2, insulin, and GAD are also present in diabetics [6]. In contrast to the inflammatory mediators secreted by $CD4^+$ $T_{eff}$ cells and CTL, autoantibodies are reported to not participate in disease pathogenesis in humans [6]. However, treatment of type 1 diabetics with rituximab, a B cell depleting monoclonal antibody (mAb), has yielded promising preliminary findings [8]. Autoantibodies are

incontestably of high predictive value, as they can appear up to a decade before diagnosis is confirmed [6]. Indeed, T1D-associated antibodies are detected in the blood of 70-80% of newly-diagnosed patients [6]. Also, the titer and number of different autoantibodies dictates the probability to develop diabetes [6]. They are detectable for some time after the onset of disease, but are subsequently lost in 50% of diabetic patients several years after diagnosis, suggesting that autoimmunity is driven by β islet antigens, which are destroyed over time [3].

### b. Epidemiology.

The World Health Organisation-sponsored study demonstrates that Canada has the fourth highest incidence of T1D world-wide with 23 cases/100 000/year (Figure 1) [9]. The disease manifests itself primarily in children, within the range of 0-14 years of age [9]. T1D is one of the most common chronic diseases in childhood, causing significant morbidity and mortality and enormous healthcare and economic costs [10]. In fact, the incidence in children under 5 years of age is predicted to double by 2020 [10]. An overall north-south geographical gradient is observed in Europe, North and South Americas, although exceptions do arise (Figure 1) [9]. The etiology of this north-south gradient is unknown, although climate differences and prevalent infections in the northern hemisphere relative to the southern hemisphere have been proposed [9]. Sun exposure is limited in the northern hemisphere and has been proposed as a factor in T1D susceptibility [11]. Vitamin D, which is provided by natural sunlight, is a well-known immunosuppressant [11]. Indeed, the non-obese diabetic (NOD) mouse, which spontaneously develops diabetes, is T1D-protected when treated with vitamin D

analogues [11]. Vitamin D has been shown to stimulate transforming growth factor β 1 (TGF-β1), which is an important factor in the conversion of $T_{eff}$ cells to induced regulatory T ($iT_{reg}$) cells, which are implicated as an important tolerance mechanism (discussed in section 5).

### c. Genetic predisposition.

Until the late 1950's, the incidence of T1D was relatively low and has since exploded world-wide [6]. An increase in the penetrance of major susceptibility genes or that of low-risk genes as a result of environmental changes may explain this rapid rise in incidence [6]. Strong clustering within families, such as a sibling recurrence rate of 6% and a concordance rate of 6-10% for dizygotic twins and 30-50% for monozygotic twins suggest that important genetic determinants are involved in T1D susceptibility [12, 13]. Association between several genetic variants and T1D susceptibility has been well documented. The major susceptibility gene is located within the human leukocyte antigen (HLA) region of chromosome 6. HLA-DR and DQ polymorphisms account for 50% of the total genetic contribution to disease [14]. Most T1D patients express HLA-DR3 or DR4 alleles, and approximately 30-40% of patients are DR3/DR4 heterozygous and carry the highest risk genotype [15]. Nevertheless, only a relatively small proportion (less than 10%) of these individuals progresses to clinical disease.

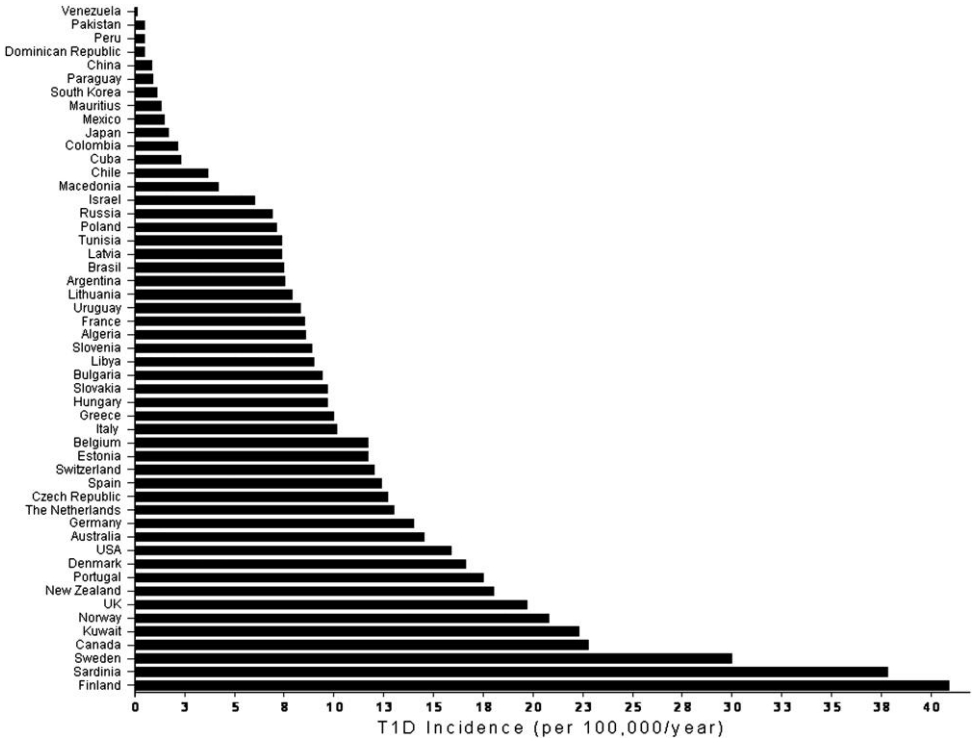



**FIGURE 1. The worldwide incidence of T1D in children under 14 years of age.** Adapted from Zipris *et al.,* 2009 [6].

This implies that additional factors are needed to trigger and drive β cell destruction in genetically predisposed subjects (discussed below). Mouse studies suggest that in genetically susceptible individuals, MHC class II molecules may poorly present self-peptides, thereby leading to inadequate negative selection of autoreactive T cell populations that could later become activated to elicit an islet-specific destructive autoimmune response [16, 17]. Poor peptide binding by MHC class II molecules may also lead to defective positive selection of $CD4^+CD25^+$ regulatory T ($T_{reg}$) cell populations that maintain tolerance [16, 17]. Many MHC class-I restricted epitopes have been identified in both mouse and man (Figure 2) [18], implicating a role for MHC class I

in the etiology of T1D [19]. However, the contribution of MHC class I variants is small relative to MHC II variants [19].

Other established non-HLA common variants include *Insulin, cytotoxic T-lymphocyte Antigen 4* (*Ctla4), Interferon induced with helicase C domain 1 (Ifih1), protein tyrosine phosphatase non-receptor type 22 lymphoid (Ptpn22)* and *Cd25* genes, whose overall effects are small relative to HLA genes [20]. Polymorphisms in most of the aforementioned gene products have an impact on fundamental immunological processes such as central and peripheral tolerance, as they alter the threshold of T cell activation [20]. The insulin gene on chromosome 11 is the most important non-HLA factor in disease susceptibility, accounting for 10% of the genetic contribution [21]. It is believed that genetically determined low thymic insulin levels predispose to T1D through less efficient negative selection of autoreactive insulin-specific T cells [22]. In third place for disease prediction is PTPN22 [23]. A polymorphism within *Ptpn22,* which encodes lymphoid tyrosine phosphatase (LYP), causes a less potent inhibition of T cell receptor (TCR)-mediated signalling [23]. This mutation alters the threshold of activation of thymocytes undergoing negative selection [23]. CTLA-4, an important moderator of co-stimulation, predisposes to T1D through an unknown mechanism [23]. As well, a non-synonymous polymorphism at IFIH1, a helicase that mediates interferon responses to viral ribonucleic acid (RNA), may alter immune responses to viral epitopes through molecular mimicry [23]. Lastly, polymorphisms within the *Cd25* gene strongly segregate with T1D [24-26]. The CD25 gene is ranked fourth in importance for genetic contribution to T1D [24-26]. Genetic variation within *Cd25,* which encodes the $\alpha$ chain of the interleukin (IL)-2 receptor, has also been reported to be associated with other

autoimmune diseases, such as multiple sclerosis [27] and juvenile idiopathic arthritis [28], underpinning the importance of IL-2 signals in tolerance. IL-2 signalling is crucial for the metabolic fitness of $T_{reg}$ cells, suggesting that slight variations in IL-2 expression may alter the potency of $T_{reg}$ cell suppressive function, tipping the balance to autoimmunity (discussed in section 6) [24-26]. Overall, the pathways that are directly conserved with human T1D genetic susceptibility include the HLA or MHC class II molecules, the IL-2 pathway, and T cell activation pathways, underpinning dysregulated immune responses as the culprit in loss of tolerance. Understanding the mechanisms involved in mediating defective immune responsiveness will shed light on potential therapeutic avenues.

### d. Environmental insults.

Despite the apparent genetic influence of disease susceptibility, T1D does not fit in the Mendelian pattern of inheritance and is considered a complex multifactorial disease. Indeed 85% of cases occur in individuals with no family history of diabetes [1]. Also, the development of T1D is increasing at a pace greater than what the genetic component can account for, implicating environmental insults as important factors in autoimmunity [29]. The most powerful evidence in humans that autoimmune diabetes is due to environmental factors comes from the study of identical twins. A considerable discordance in the incidence of T1D exists in monozygotic twins, as 60-70% of affected diabetic twins have an unaffected twin [30]. Furthermore, epidemiological migrant surveys have demonstrated that the incidence of T1D has increased in population groups who have moved from a low-incidence area to a high-incidence region, further

emphasizing the influence of environmental conditions [31]. Early exposure to cow's milk or gluten have also been linked to susceptibility to T1D [12].

T1D is a seasonal disease more prevalent in northern countries, with a high prevalence in the fall and winter months [6, 32]. T1D is most prominent in countries with large temperature differences between winter and summer months, such as Canada [6, 32]. This reinforces correlative studies that associate viral infections to T1D susceptibility [33]. In fact, a positive correlation has been posited between Coxsackie B virus (enterovirus), rubella, mumps, cytomegalovirus, Epstein-Barr virus, rotavirus infections and T1D [34-39]. The strongest correlation with T1D susceptibility is Coxsackie B virus infection. Antibodies against islet antigens often correlate with Coxsackie B virus expression [40]. This enterovirus can infect human pancreas and has been isolated from the pancreas of acute T1D patients, but not healthy human subjects [41]. In line with this evidence, enterovirus infections are more common in newly-diagnosed patients versus control subjects [42]. How viruses cause diabetes in humans is unclear, although molecular mimicry, bystander damage, epitope spreading and interaction with susceptibility genes have been suggested as potential mechanisms [43].

The hygiene hypothesis postulates that exposure to infectious agents in early life is necessary for the proper maturation of the immune system [44]. In the absence of antigenic exposure, the immune response is underdeveloped and, along with proper genetic susceptibility, autoimmunity ensues [44]. This is consistent with NOD mice, which are T1D-protected in "dirty" animal facilities [45]. Furthermore, when NOD mice are infected with certain pathogens that induce chronic infections, the spontaneous development of T1D is inhibited [29]. However, not all infections can provide long-

lasting protection from T1D [29]. For example, a glycoprotein nematode product, which harbours important immunoregulatory properties, can reverse autoimmune disorders such as collagen-induced arthritis (CIA), but not T1D in NOD mice [29]. Thus, although the hygiene hypothesis is a tempting explanation for autoimmune diseases, the supporting evidence is rather confusing and not clear-cut. Overall, genetic propensity along with environmental insults act in concert to establish clinical autoimmune disease [46].

### e. Immune defects in human T1D.

Immune responses towards islet antigens, compounded by genetic and environmental factors, largely contribute to pathogenesis of T1D. Abnormalities in the innate arm of the immune system have been documented, which may inadvertently prime autoreactive T cells and as such cause T1D [47]. Recent reports proposed aberrant function of monocytes and increased levels of MΦ-derived cytokines in at-risk and diabetic patients as a contributing factor to disease pathogenesis [47]. Reduced DC function [47] and numbers [48] have also been reported. Since DC process antigen and "instruct" the adaptive immune system, abnormalities in these cells could dictate development of T1D. Reduced production of IL-4, which reverses polarization of destructive T helper (Th)1 cells to protective Th2 cells, by natural killer (NK) and natural killer T (NK-T) cells in diabetics as compared with healthy controls has also been associated with T1D susceptibility [49, 50]. Apart from producing cytokines, NK cells might also contribute to the development of diabetes by directly interacting with MΦ, DC, T and B cells [47]. The last decade has seen an emergence of $CD4^+CD25^+$ $T_{reg}$

cells as a central control point in tolerance. A developmental or functional defect in this suppressive population has been proposed as an underlying mechanism in various autoimmune diseases, including T1D [51].

We must bear in mind that the nature and dynamics of the autoimmune response in human T1D patients remains poorly defined, in part due to data mainly generated from peripheral blood, far from the site of injury, justifying the importance of animal models of autoimmunity [3]. Luckily, several rodent models exist, which develop spontaneous T1D and have proven most useful as they are models of dysregulated immune responses to islets. The rodent models can be used to guide pathogenesis studies and immunotherapeutic trials.

## *2.* The Non-obese diabetic (NOD) mouse model.

### a. A brief introduction.

Three models have been developed for the study of spontaneous T1D: the BioBreeding (BB) rat, Komeda diabetes-prone (KDP) rat and the NOD mouse [52-54]. The KDP rat model is a two-gene model for the development of diabetes, as it was found that most of the genetic predisposition to diabetes is accounted for by 2 major susceptibility genes: *Mhc* and *Iddm/kdp1* [55], providing limited information for human T1D, which is a polygenic multifactorial disease. T1D development in the BB rat is similar to that in humans and NOD mice [56]. However, the BB rat is severely lymphopenic, with a reduced $CD4^+$ T cell pool and a nearly absent $CD8^+$ T cell compartment [56]. In that respect, the BB rat represents a drastically different immunological phenotype, which does not reconstitute that of human T1D [56]. The

NOD mouse model has proven to be far superior to rat models, since it shares the most features with human T1D, such as the involvement of autoreactive $CD4^+$ and $CD8^+$ T cells in the destruction of the islets, presence of autoantibodies directed against islet cells, defective MΦ and DC functions, the phenotype of the insulitic infiltrate, which will all be discussed in greater detail below [54]. Although these rodent models are very informative, it is important to note that none recapitulate all aspects of diabetogenesis in the genetically diverse human population. Lastly, diabetes can be chemically induced by streptozotocin (STZ) in non-autoimmune prone strains of mice, although one study using human islet grafts in mice demonstrated that human β islet cells, in contrast to their murine counterparts, are resistant to STZ, diminishing the relevance of this method [57].

The NOD mouse was created at the Shionogi Research Laboratories in Japan through vigorous brother-sister mating of a cataract-prone strain of mice (Jcl-ICR) [58]. The NOD mouse strain has a general propensity for autoimmune disorders, which extends beyond T1D [59, 60]. NOD mice are genetically prone to be globally immune dysregulated, and as such develop multi-organ autoimmunity including sialitis, thyroiditis, peripheral polyneuropathy, spontaneous thyroiditis, celiac disease and are susceptible to certain exeperimentally inducible autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) [59, 60].

Many $CD4^+$ and $CD8^+$ T cell epitopes have been identified in mouse and man. GAD65, proinsulin and IA-2 are the predominant $CD4^+$ T cell epitopes in both man and mouse [18]. The major $CD8^+$ T cell epitope in mouse is islet-specific IGRP in mouse and proinsulin/insulin in man [18]. The epitope distribution in mouse and man is represented

in Figure 2 [18]. The potential primary autoantigen in the NOD is thought to be insulin, more specifically peptide B9-23 [61]. Similar to human diabetes, low expression of insulin in the thymus of NOD mice has also been reported and may play a role in diabetes susceptibility, potentially skewing the T cell repertoire to β-islet reactivity [62]. In contrast to humans, NOD mice express two forms of insulin: Ins1 and Ins2. They are differentially expressed, with Ins2 expression exclusively in the thymus and Ins1 mainly in the pancreas, although trace amounts have been detected in the thymus [63]. Ins2 may be important for central tolerance induction, as $Ins2^{-/-}$ mice have a higher diabetes incidence relative to the wild-type (WT) NOD mice [63]. In stark contrast, $Ins1^{-/-}$ mice are completely protected relative to WT mice, suggesting that insulin is a primary autoantigen, although insulitis is still detected [64].

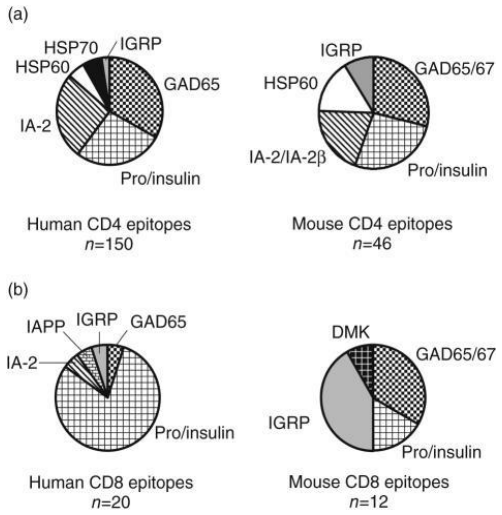



**FIGURE 2. Pie charts showing the antigenic distribution of (a) CD4+ and (b) CD8+ T cell epitopes in autoimmune diabetes in humans and mice.**

Adapted from Di Lorenzo *et al.*, 2007 [18].

The NOD mouse model has greatly facilitated the genetic dissection of T1D and serves as a prototypic model of human autoimmunity [54]. As in humans, MHC genes are the dominant genetic element predisposing toward disease in the NOD mouse model [65]. The NOD MHC class II haplotype that affords T1D susceptibility is H2-$A^{g7}$ [65]. Interestingly, the H2-$A^{g7}$ haplotype shares sequence and structural motifs with T1D-susceptible MHC gene products in humans [66]. The introduction of a different MHC class II haplotype onto the NOD background renders the strain resistant to T1D, but makes it susceptible to other autoimmune diseases, demonstrating the strong genetic propensity of this strain to autoimmunity [13]. For example, congenic NOD.2H4 mice, which express H2-$A^{k}$, spontaneously develop autoimmune thyroiditis [67]. H2-$A^{g7}$ thus appears to specifically engage in thymic generation and/or peripheral activation of diabetogenic T cell clones in NOD mice [65]. However, the NOD H2-$A^{g7}$ is not sufficient to confer diabetes when introduced into a non-autoimmune prone strain that lacks other NOD-related autoimmune susceptibility loci, making a strong case for the importance of non-MHC genes in diabetogenesis in the NOD [68].

Like their human counterparts, diabetes in NOD mice requires non-MHC susceptibility genes [69, 70]. Genome-wide scanning of loci involved in autoimmunity has led to the identification of multiple loci that overlap, giving rise to the concept of common autoimmunity genes [69]. T1D susceptibility is inherited through multiple genes, with a strong predisposition from genes affecting immune responses to β-islet cell antigens [69]. Over 20 diabetogenic or insulin-dependent diabetes (*Idd*) loci have been identified by genomic mapping of congenic NOD strains, which harbour defined genetic intervals from diabetes-resistant mouse strains (C57BL/6), although no single

gene is both necessary and sufficient [68, 69, 71]. *Idd* congenic mice represent an important tool in the elucidation of the impact of certain genes on T1D progression, and are invaluable for the identification of stages in the breakdown of self-tolerance in NOD mice [68, 69, 71].

The best-described *Idd* loci include *Idd1*, *Idd3*, *Idd5*. The *Idd1* locus has been mapped to the MHC class II region and confers the greatest susceptibility to T1D [72]. Among the non-MHC genes, the locus which confers the most susceptibility is the *Idd3* locus, which harbours *Il2* as the candidate. The *Idd5* locus is also note-worthy as it contains inducible costimulatory (*Icos*) and *Ctla4*, which are important mediators of activation and suppression of T cell priming [68, 73-75]. As in humans, the pathways that regulate the threshold of T cell activation are implicated in disease pathogenesis. Each individual *Idd* locus imparts partial resistance to T1D, and combinations of these *Idd* loci results in nearly complete protection against diabetes [68, 74-80]. The *Idd3* locus will be discussed in greater detail in section 12 as per the involvement of *Il2* allelic variants in T1D protection through the promotion of $Foxp3^+$ $T_{reg}$ cell functions.

### b. BDC2.5: a TCR transgenic NOD mouse model.

In order to provide a rapid synchronous system for the analysis of antigen-specific T cell responses *in vivo*, the BDC2.5 $CD4^+$ TCR transgenic mouse was created and carries the rearranged TCR $\alpha$ and $\beta$ ($V\alpha1V\beta4$) genes from a diabetogenic $CD4^+$ T cell clone isolated from a diabetic NOD mouse [81]. There is a near-complete allelic exclusion of the endogenous TCR $\beta$ locus and a partial allelic exclusion of the TCR $\alpha$

locus, enabling a TCR repertoire that is highly skewed for the transgene-encoded TCR specificity [81].

The BDC2.5 TCR is specific for an unknown β islet autoantigen [81]. BDC2.5 T cells are responsive to β islet cells and accumulated rapidly in the islets [81]. Since the T cells are transgenic and aggressively diabetogenic, accelerated diabetes in the BDC2.5 mouse was expected [81]. However, diabetes onset in BDC2.5 was delayed relative to NOD, which was surprising at the time [81]. It is now well established that the extensive lag time between the onset of disease and overt diabetes is due to, in part, $T_{reg}$ cells, which develop in virtue of endogenous TCR α chain recombination [82]. The insertion of a TCR with monospecificity into the NOD diabetes model has provided great insight into the diabetogenicity of a particular T cell clone. However, TCR transgenic mice do not allow for the evaluation of a particular TCR within a physiological polyclonal TCR repertoire.

### c. The natural course of T1D in the NOD and BDC2.5 mouse models.

In NOD mice, β cell autoimmunity progresses in well-defined stages or checkpoints. Prior to three weeks of age, no obvious islet pathology can be detected [54]. However, at three weeks of age, a mononuclear infiltrate of NK cells, B cells, DC, MΦ, $CD4^+$ and $CD8^+$ T cells, surrounds the islets of Langerhans, termed checkpoint 1 [54]. The insulitic phenotype at an early age is reminiscent of events in human diabetes, underpinning the strength of the NOD as a model for autoimmunity [54].

The activation of naïve β-islet specific T cells occurs in the draining pancreatic lymph node (pancLN), after which diabetogenic T cells migrate to the target organ

undergoing autoimmune attack [83]. Bystander T cells are not recruited to the islets, demonstrating that pancreatic infiltration and destruction is an antigen specific process [84]. Through epitope spreading, $CD4^+$ and $CD8^+$ T cells targeting a number of β-islet autoantigens are further recruited [18]. Despite advanced stages of insulitis, the majority of β cell mass remains intact. In fact, until overt diabetes, known as checkpoint 2, the autoreactive pool of T cells is held at bay [85]. The lag time between checkpoints 1 and 2 suggests that immunoregulatory mechanisms in the periphery act to prevent autoimmunity in these mice [85]. This also suggests that the diabetogenic process can be stopped at various stages during T1D progression [85]. Overall, a delicate balance of pathogenic and protective immune responses control T1D onset and progression. We will discover in a later section that one of the major immunoregulatory mechanisms that maintain the autoreactive T cell pool in check is $Foxp3^+$ $T_{reg}$ cells (Figure 3) [85].

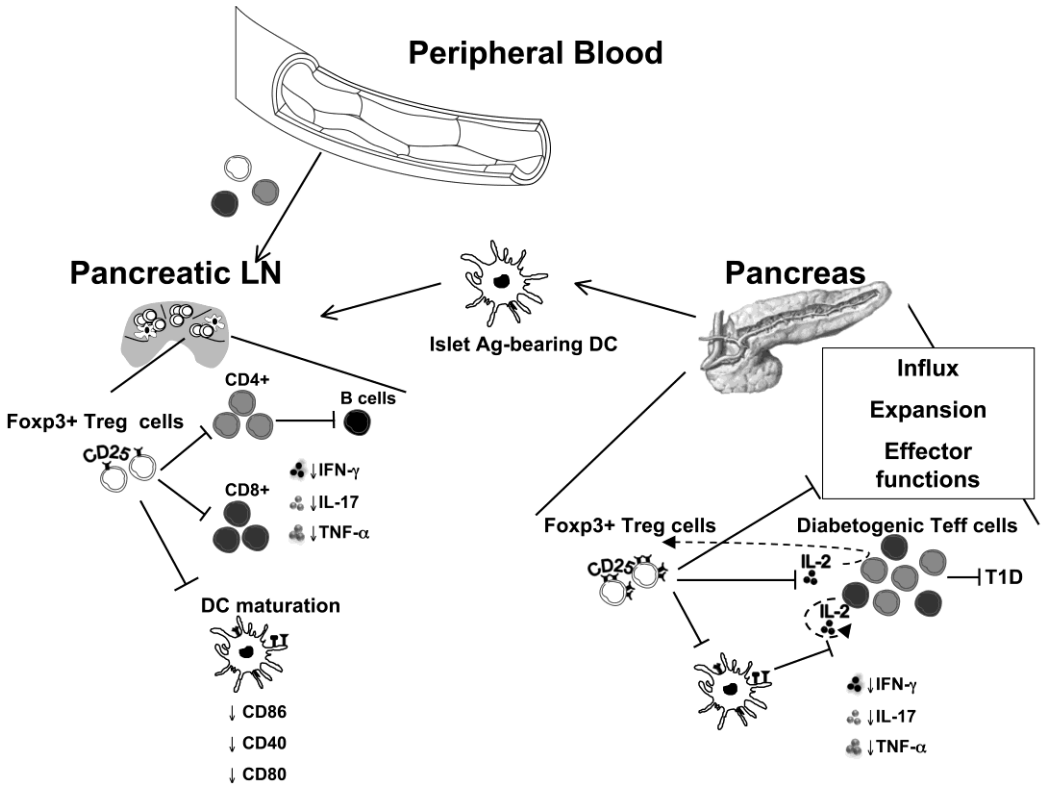



**FIGURE 3. Autoreactive T cell responses drive T1D in the NOD mouse model.** Tissue-derived DC uptake β-islet antigens, traffic to the draining pancLN where they activate islet-specific $T_{eff}$ and $T_{reg}$ cells and suppress various aspects of the diabetogenic response including the maturation of DC, the activation, expansion, differentiation and effector functions of $T_{eff}$ cells, and the activation of autoreactive B cells. $T_{eff}$ cell-derived IL-2 potentiates the inhibitory functions of $CD4^+Foxp3^+$ $T_{reg}$ cells. Quantitative or temporal differences in IL-2 production may precipitate diabetes by negatively affecting $T_{reg}$ cell functions, as IL-2 is essential for the development, maintenance and metabolic fitness of $T_{reg}$ cells. Quantitative and qualitative differences in $T_{reg}$ cells of NOD animals have been purported for the breakdown in self-tolerance mechanisms, which may be attributed to a decline in IL-2 at the time of insulitis. Adapted from Sgouroudis *et al.,* 2009 [86].

## *3.* Immunoregulatory dysfunction underlies loss of β cell tolerance.

### a. Defective central tolerance mechanisms in the NOD mouse.

Thymic selection shapes the peripheral T cell repertoire and represents one of the primary mechanisms of immune tolerance [87]. Most autoreactive T cells are eliminated by clonal deletion when the TCR is engaged with high affinity by MHC/self-peptide complexes encountered on thymic stromal epithelial cells [16, 17]. However, most healthy individuals have circulating autoreactive T cells, suggesting that self-reactive T cells can escape this process [87]. In the NOD mouse model, defective thymic selection may contribute to their propensity for autoimmunity by allowing more autoreactive T cells to leave the thymus [16, 17]. Experimentally, this theory has been supported by studies demonstrating abnormal deletion of thymocytes upon anti-CD3 mAb administration [88], diminished thymocyte signalling [89] and diminished thymocyte apoptosis *in vitro* [60]. Non-MHC genes may also act on autoreactive $CD4^+$ T cells to enable them to escape clonal deletion in the thymus and reach the pancreatic islets in elevated numbers [90]. This was later confirmed using the BDC2.5 model [59]. Alternatively, organ-specific antigens may be present in only trace amounts in the thymus or circulation, such that T cells escape thymic deletion due to inefficient MHC/self-peptide complex presentation [91]. The autoreactive T cells may also recognize epitopes that are only presented in the peripheral organ [92].

### b. Autoreactive T cell responses drive T1D in the NOD mouse.

The discovery that T1D is a T cell-dependent autoimmune disease was made using T cell-deficient $TCR\alpha^{-/-}$, neonatally thymectomized or athymic nude (*nu/nu)* mouse

models on the NOD background, which are T1D-protected [93-95]. In addition, $CD4^+$ T cell depletion through mAb treatment prevents diabetes onset [93-95]. The transfer of purified $CD4^+$ or $CD8^+$ T cells from diabetic mice into immunodeficient recipients induces T1D [96, 97], although the relative contribution of $CD4^+$ and $CD8^+$ T cells remains to be fully characterized. CTL are thought to play a role in effector function and to act early by causing sufficient β-islet destruction to promote robust $CD4^+$ T cell responses, although some CTL were sufficient to destroy β cells without the help of $CD4^+$ T cells [98, 99]. In NOD mice lacking $\beta_2$ microglobulin ($\beta_2m^{-/-}$), MHC class I expression was low and consequently very few $CD8^+$ T cells developed [100, 101]. No insulitis and overt diabetes was observed in $\beta_2m^{-/-}$ NOD mice, suggesting that $CD8^+$ T cells are essential to T1D pathogenesis [100, 101]. $CD8^+$ T cells are also believed to initiate the autoimmune response because histological studies demonstrate that they are the first lymphocytes to appear in the islets [102].

Th1 populations, associated with the lineage-specific T-box transcription factor (T-bet) and cytokines including interferon γ (IFN-γ), tumor necrosis factor α (TNF-α) and IL-2, are key mediators of β-cell autoreactivity [103]. The transfer of Th1 cells into neonatal mice caused diabetes and islet-reactive Th1 clones generated from $CD4^+$ TCR transgenic BDC2.5 mice drive aggressive diabetes [104]. In accordance with these findings, T-$bet^{-/-}$ mice are completely protected due to a defect in the activation of the $T_{eff}$ cell population [105]. In stark contrast, germline deletion of *Il12*, *Ifng* or *Ifng-receptor* did not affect the kinetics of T1D pathogenesis, suggesting a redundancy in classical Th1 cytokines [106-108].

Conversely, Th2 populations, associated with the transcription factor GATA-3 and cytokines IL-4 and IL-10, were thought to promote a dominant protective effect against autoimmunity [99, 109]. However, it seems to be more complex than a simple Th1/Th2 imbalance. For example, IL-$4^{-/-}$ and IL-$10^{-/-}$ NOD mice did not demonstrate an accelerated diabetes phenotype [99, 109]. Onset and incidence resembled that of their NOD counterparts, suggesting that T1D is not caused by a lack of protective Th2 cells and consequent Th1 polarization in the NOD mouse model [99, 109]. However, the cytokine production capability of Th2 cells, which infiltrate the pancreas of diabetic human patients, appears to be impaired as judged by the secretion of either IL-4 or IL-10 [110]. Overall, caution should be exercised when interpreting animal data for human therapeutic purposes.

### c. The contribution of T cell-extrinsic factors in T1D development in the NOD mouse.

Although the presence of T cells is essential for the destruction of the pancreatic islets, T cell-extrinsic factors contribute also to T1D [54]. In both human and rodent T1D, autoantibodies to β-islet antigens are detectable, but not pathogenic, as diabetes cannot be transferred by autoantibodies from new onset diabetic donors [54]. They are generated as a consequence of T cell-mediated attack of β-islets and they have remarkable predictive value [111]. However, as is the case with human diabetics, B cells are important in the development of autoreactivity, because B cell depletion by antibody treatment results in protection in NOD mice [8]. Furthermore, a germline mutation that prevents the maturation of B cells, also affords protection, indicating an as-yet undetermined role for B cells in T1D progression [54]. The most prevalent

autoantibodies, which are unequivocally linked to the progression of T1D, include insulin autoantibodies (IAAs), autoantibodies to GAD65, heat shock protein 60 (Hsp60) and the tyrosine phosphatase related IA-2 molecule [112]. Thus, although the contribution of B cells cannot be ignored, autoreactive T cell responses drive T1D pathogenesis in the NOD mouse.

It has been suggested that defects in antigen-presenting cell (APC) function contribute to the activation of autoreactive $CD4^+$ and $CD8^+$ T cells responsible for the destruction of pancreatic $\beta$ cells in T1D [18]. For example, hyper-inflammatory MΦ produce elevated amounts of cytokines such as IL-12p40, IL-1$\beta$, TNF-$\alpha$, and IFN-$\gamma$ [113, 114]. The soluble mediators produced by MΦ may also promote CTL killing [115, 116]. These islet-infiltrating hyper-inflammatory MΦ may also contribute to enhance $\beta$ cell damage by inducing the production of superoxide radicals in the islets, to which the $\beta$ cells are very sensitive, because they exhibit very low free radical scavenging activity [2].

Studies have also shown that overexpression of activating receptor NKG2D and its ligand RAE-1 on NK cells resulted in compromised NK cell functions, which contribute to T1D [117]. Defective NK-T cell populations have been suggested as a contributor to disease progression in the NOD, whose augmented activity promotes Th2 responses and results in the prevention of T1D [118]. Protective $DX5^+$ NK cells were also reported to play a protective role in T1D [119].

A significant body of evidence shows that phenotypic and functional abnormalities of DC underlie the risk of T1D in humans and in NOD mice [120-123]. In

the murine model, DC are the first leukocytes to infiltrate the islets during the insulitic phase, suggesting that they are required for the maintenance of insulitic lesions [2]. Consistently, DC depletion correlates with the reversal of lymphocytic infiltration of the islets and T1D protection [124]. In stark contrast, depletion of MΦ does not alter diabetes kinetics [125]. In addition to their role in the elimination of foreign invaders, DC have a well-established role in tolerance [126]. Phenotypic analysis of DC subsets revealed that NOD mice had significantly fewer CD8$\alpha^+$ DC relative to T1D-resistant strains [127]. Under steady-state non-inflammatory conditions, CD8$\alpha^+$ DC encountering apoptotic bodies due to normal cell turnover present self-peptides to induce tolerance [128]. Moreover, CD8$\alpha^+$ NOD DC exhibit impaired capacity to induce indoleamine 2, 3 dioxygenase (IDO)-mediated tryptophan catabolism. Reduced IDO activity limits the efficiency of DC to halt autoreactive T cell growth by tryptophan starvation [129]. Furthermore, recent reports have shown that activation of NK-T cells protects NOD mice through the NK-T cell-mediated induction of a protective DC population [118, 130]. Overall, NOD mice possess multiple, peripheral immune defects, which collectively contribute to spontaneous multi-organ autoimmunity.

Although the aforementioned T cell-extrinsic immunomodulatory factors contribute to disease pathogenesis, defects in $T_{reg}$ cell functions have emerged as one of the underlying mechanisms in the breakdown of self-tolerance to islet antigens. A brief introduction of $T_{reg}$ cells will be presented and then followed by a discussion on their incontestable role as mediators of tolerance in the NOD mouse model of autoimmune diabetes.

## *4.* $T_{reg}$ cells: Immunological switch in the outcome of immune responses.

### a. A historical perspective.

To prevent excessive or indiscriminate immune responses that might compromise the survival of the organism, several non-redundant central and peripheral regulatory mechanisms exist to maintain this delicate balance. Central tolerance mechanisms ensure the elimination of potentially aggressive T cells with specificity for autoantigens in the thymus by negative selection [87]. Extra-thymic peripheral mechanisms exist, such as immunological ignorance, anergy, immune deviation, apoptosis and cross-presentation, to maintain self-tolerance and avert autoimmunity [131]. However, in the last thirteen years, $CD4^+CD25^+$ regulatory T cells have emerged as the dominant mechanism against autoimmunity.

The development of destructive multi-organ specific autoimmunity in thymectomy of three-day old neonates lead to the hypothesis that a regulatory cell population generated in the thymus after three days of life existed, which held inappropriate immune responses at bay [132]. Initial studies demonstrated that $CD4^+$ T cells depleted of $CD5^{high}$, $CD45RC^{low}$ or $CD45RB^{low}$ in mice or RT6.1 in rats and transferred to an immunodeficient host could induce multi-organ specific autoimmunity [133-137]. In each aforementioned transfer system, reconstitution of these depleted populations, deemed regulatory, always reverted disease [133-137]. A subsequent seminal paper by Sakaguchi *et al*. demonstrated that the transfer of CD25-depleted $CD4^+$ T cells yielded a variety of autoimmune diseases including gastritis, insulitis, thyroiditis, reminiscent of the phenotype of day three thymectomized mice [138], and their reconstitution reverted

the phenotype [138-140]. The transfer of $CD4^+CD25^-$ T cells isolated from splenic cell suspensions into athymic nude mice produced autoimmune diseases at a higher incidence and in a wider spectrum of organs than the transfer of $CD5^{low}$ or $CD45RB^{high}$ T cells, suggesting that CD25 was a more restrictive marker than CD5 or CD45RB for the elusive regulatory population of T cells [133-140]. In fact, the $CD25^+$ T cells were confined within the $CD5^{high}$ and $CD45RB^{low}$ fraction of $CD4^+$ T cells [138]. This revealed CD25 as a definitive marker for the immunoregulatory population of T cells [138]. Consequently, the existence of naturally-occurring thymic-derived $CD4^+CD25^+$ ($nT_{reg}$) cells was formally acknowledged and recognized as a dominant mechanism of peripheral self-tolerance [138].

$CD4^+CD25^+$ $T_{reg}$ cells constitute 1-10% of $CD4^+$ T cells in mouse and man [141]. $CD4^+$ $T_{reg}$ cells constrict runaway responses to pathogens and allergens, maintain tolerance to obligate commensal flora, prevent graft-versus-host disease after bone marrow transplantation, and facilitate immune evasion of tumors, feto-maternal tolerance and allograft acceptance, demonstrating that $T_{reg}$ cells represent a critical immunological switch in the control of immune responses to self and non-self antigens [142-144]. Thus, $T_{reg}$ cells not only prevent autoimmunity, but also control a broad range of immune responses to non-self antigens *in vivo*.

### b. Requirements for ontogeny.

$nT_{reg}$ cells arise in the thymus and migrate to the periphery where they are poised for surveillance of self antigens, suggesting that their suppressive function is imprinted before antigenic exposure [145]. $CD4^+CD25^+$ thymic-derived $nT_{reg}$ cells ontogenically

become detectable in the periphery of normal mice a few days after birth, as neonatal thymectomy abrogates their thymic production [139]. Self-reactive T cells that have migrated to the periphery before neonatal thymectomy are thus free to activate, expand, and cause autoimmune disease [139].

The requirement of TCR signalling for suppressive activity of $nT_{reg}$ cells and the impact of $nT_{reg}$ cells on self-tolerance pointed to self-reactive specificity on their TCR [146]. Self-tissue recognition by $nT_{reg}$ cells was proposed to be required to maintain tolerance to target organs [146, 147]. Adoptive transfer of splenocytes from donor mice in which a target tissue has been removed by surgery or chemical treatment, prevented autoimmune attack of all tissues except the tissue missing from the animal used as a source of $nT_{reg}$ cells [146]. For instance, transferring splenic preparations from athyroid donors into thymectomized rats prevented diabetes development, but failed to protect against thyroiditis [146]. Furthermore, hemagglutinin (HA)-specific $CD4^+CD25^+$ $nT_{reg}$ cells proliferated only when transferred to HA-transgenic mice, not in wild-type recipients [148].

This strong evidence suggested a tight TCR repertoire skewed toward self-antigens [149]. Indeed, unlike conventional $T_{eff}$ cells, $nT_{reg}$ cells could be activated in response to self-peptides [149, 150]. Surprisingly, the TCRs of a large cohort of peripheral $nT_{reg}$ cells and $T_{eff}$ cells were sequenced and it was found that the repertoire was diverse, but distinct from that of conventional $T_{eff}$ cells, with only 15-20% overlap between the two groups [149]. Interestingly, the same TCR could be expressed by both self-reactive $T_{eff}$ cells and $T_{reg}$ cells [151]. However, the signals that lead to the development of protective T cells versus pathogenic T cells of the same antigen

specificity are unknown [151]. The TCR specificities of $nT_{reg}$ cells were similar between the thymus and spleen, but did not overlap with TCR specificities of $T_{eff}$ cells, suggesting that $nT_{reg}$ cells are thymic-derived and are not induced from $Foxp3^-$ precursors [149]. However, a more recent study claims that in fact $nT_{reg}$ cells express a polyclonal TCR repertoire as diverse as that of conventional T cells and do not preferentially recognize self-antigens [152]. This is supported by the observation that T cells expressing $nT_{reg}$ cell-derived TCRs (by retroviral transduction of cloned TCR derived from $nT_{reg}$ cells) caused wasting disease in lymphopenic mice [152].

### c. Costimulation in $T_{reg}$ cell development.

CD28-dependent costimulation represents a major contribution to the thymic generation of $nT_{reg}$ cells, as demonstrated by $CD28^{-/-}$ and $B7^{-/-}$ studies [153, 154]. CD28/B7 interactions may facilitate the development of $nT_{reg}$ cells by enhancing TCR signalling upon self-recognition through the enhancement of the avidity of the interactions between thymocytes and medullary thymic epithelial cells (mTECs) [155]. Another possibility resides in the necessity of CD28 signalling during the thymic selection process to produce IL-2 in sufficient quantities to promote proper $nT_{reg}$ cell development and homeostasis [155]. Tai *et al.* elegantly demonstrated through a series of bone marrow chimera reconstitutions that $CD28^{-/-}$ $nT_{reg}$ cells cannot develop in the presence of *in vivo* IL-2, underpinning the importance of CD28-mediated costimulation in $nT_{reg}$ cell development, independent of IL-2 [155].

This pointed to a crucial role for the molecular interactions that augment the overall avidity of a developing $nT_{reg}$ cell for mTECs. This evidence is substantiated by

$CD40^{-/-}$, CD40 ligand $(CD40L)^{-/-}$ and leukocyte function-associated antigen 1 $(LFA\text{-}1)^{-/-}$ studies [156-159]. The number of $CD4^+CD8^-CD25^+$ thymocytes is substantially reduced in CD40 and LFA-1 deficient mice and, to a lesser degree, in CD40L deficient mice [156-159]. How these interactions with thymic stromal cells contribute to the high avidity required by developing $nT_{reg}$ cells remains to be determined.

### d. Foxp3: The most stringent marker of $T_{reg}$ cells.

#### *i. The discovery of Foxp3.*

$T_{reg}$ cells can express a variety of cell surface markers such as CD25, CD62L, CD103, CTLA-4 and glucocorticoid TNF receptor family-related protein (GITR) [131]. Since these markers can be upregulated in conventional T cells, the use of these molecules as specific key markers in the detection of *bona fide* regulatory T cells was contested [131]. Therefore, an extensive search for a specific unambiguous surface marker was undertaken. In 2003, intranuclear forkhead box p3 (Foxp3), a transcription factor of the winged-helix family, was identified and is considered the 'master controller' of $T_{reg}$ cell development and homeostasis, at least in mouse [160-162]. Approximately 10-15% of $CD4^+$ T cells are $Foxp3^+$, and 50-80% of $CD4^+Foxp3^+$ T cells are $CD25^+$, in the periphery of normal naïve mice [163].

#### *ii. The role of Foxp3 in $nT_{reg}$ cell development.*

Foxp3 is believed to be the 'master regulator' of the $nT_{reg}$ cell lineage, as $Foxp3^{-/-}$ mice succumb to lymphoproliferative disorder and multi-organ specific autoimmunity at an early age reminiscent of the day three thymectomy phenotype, which can be

reversed by the infusion of $T_{reg}$ cells [160, 164, 165]. Approximately 5% of $CD4^+CD8^-$ single-positive (SP) thymocytes are $Foxp3^+$, and 70% of those are $CD25^+$ [163]. In bone marrow chimera reconstitution experiments with a mixture from wild-type and Foxp3-deficient bone marrow cells, $Foxp3^{-/-}$ cells failed to give rise to $CD4^+CD25^+$ $nT_{reg}$ cells, whereas Foxp3-intact bone marrow cells generated $nT_{reg}$ cells, which suppressed disease development [160]. In Foxp3 transgenic mice, which overexpress Foxp3, the number of $CD4^+CD25^+Foxp3^+$ $nT_{reg}$ cells increased dramatically [160].

Using reporter mice, which express green fluorescent protein (GFP) under the Foxp3 promoter ($Foxp3^{gfp}$) on MHC class $I^{-/-}$, MHC class $II^{-/-}$, and MHC class I/II double deficient backgrounds, it was shown that the expression of MHC class II is absolutely required for Foxp3 expression in SP $CD4^+CD8^-$ thymocytes, demonstrating that they undergo thymic selection through self-peptide/MHC complexes expressed on thymic stromal epithelial cells [166]. In double transgenic mice expressing a transgene-encoded ligand on stromal cells and T cells whose transgenic TCR has a high affinity for the specific ligand, almost all T cells differentiate to $CD4^+CD25^+$ $T_{reg}$ cells with suppressive properties [167]. In stark contrast, when the double transgenic mice expressed a TCR with a lower affinity for the ligand, $T_{reg}$ cells failed to develop [168]. This demonstrated that $T_{reg}$ cells require TCR-mediated selection on self-antigens of high affinity, implicating high affinity self-ligand as a requirement to launch signalling cascades to activate Foxp3 [167-169]. This was counterintuitive as conventional T cells with high affinity for self-antigens are eliminated by negative selection and only T cells whose TCR receives moderate signals are positively selected to populate the periphery [170]. This suggested that the threshold for positive selection of $T_{reg}$ cells differed from

conventional T cells and that the signal strength delivered to the TCR was between that required for positive and negative selection of conventional T cells [170]. Two possibilities could be envisioned: the $nT_{reg}$ cell lineage is driven by the positive selection of highly self-reactive T cells or $nT_{reg}$ cells are inherently resistant to negative selection [171]. Using an inducible promoter to quantitatively alter the degree of thymic expression of a moth cytochrome c (MCC)-derived antigen, one study showed that the number of $nT_{reg}$ cells did not change despite increasing degree of MCC expression. This was in contrast to conventional T cells, whose numbers drastically declined with increasing expression of MCC owing to deletion, suggesting that $nT_{reg}$ cells are inherently resistant to deletion [172]. A direct positive correlation was made between Foxp3 expression and TCR levels in $CD4^+CD8^-$ SP thymocytes, reinforcing the notion that Foxp3 induction is dependent on the strength of the TCR signals [173].

Intringuingly, recent findings have suggested that the role of Foxp3 in thymic development of $nT_{reg}$ cells is not as clear-cut as initially thought. For instance, thymocytes that are destined to become $nT_{reg}$ cells are not negatively selected in the absence of functional Foxp3 protein [174, 175]. These $nT_{reg}$ cells with a non-functional Foxp3 gene product egress from the thymus to populate the peripheral compartment [176]. In the absence of intact Foxp3, the $T_{reg}$ cells acquire aggressive autoreactive properties in the periphery, resulting in autoimmune disease [176]. Therefore, although a functionally intact Foxp3 protein is dispensable for proper thymic development of $nT_{reg}$ cells, it is indispensable for the stabilization of the $T_{reg}$ cell signature transcriptional program and suppressive phenotype in the peripheral compartment [175].

### *iii. The role of Foxp3 in $T_{reg}$ cell functions in the periphery.*

In order to determine the role of Foxp3 in $T_{reg}$ cell functions, many experiments were undertaken by independent groups. Murine T cells retrovirally transduced with Foxp3 acquired the $T_{reg}$ cell phenotype, as they were profoundly suppressive *in vitro* and *in vivo* [162]. This demonstrated that Foxp3 initiates and supports a gene expression profile that regulates the development and maintenance of $T_{reg}$ cells [162]. Conversely, mice generated with a T cell specific ablation of Foxp3 developed rampant autoimmunity [177]. Consistently, T cells transcribing Foxp3, but lacking functional Foxp3 protein due to the insertion of green fluorescent protein (GFP) in the coding region of Foxp3 ($GFP^{+}Foxp3^{null}$) or $T_{reg}$ cells whose Foxp3 has been conditionally knocked out, lose regulatory activity [174, 176, 178, 179]. Diminished or loss of Foxp3 expression leads to the acquisition of conventional $T_{eff}$ cell properties, including the production of inflammatory mediators such as IL-2, IL-17 and IFN-$\gamma$ [160, 162, 174, 176, 180, 181]. Foxp3 promotes its own transcription, as $GFP^{+}Foxp3^{null}$ $T_{reg}$ cells transferred into lymphophenic animals lose half of GFP expression [174, 176], although the possibility that these cells are converted to conventional $T_{eff}$ cells in the absence of functional Foxp3 protein cannot be excluded [182-184]. Human diphtheria toxin receptor under the control of the Foxp3 promoter leads to temporal loss-of-function of $T_{reg}$ cells when mice are treated with diphtheria toxin. Using this controlled lineage ablation approach, it was demonstrated that sustained Foxp3 expression in $T_{reg}$ cells is needed to maintain tolerance, as the elimination of 98% of $Foxp3^{+}$ $T_{reg}$ cells lead to rampant multi-organ specific autoimmunity [178, 185]. One group whose punctual loss-of-function approach could only eliminate 90% of $Foxp3^{+}$ $T_{reg}$ cells showed that

autoimmunity developed in neonates, but adults were spared by the immunosuppression exerted by the residual 10% $Foxp3^+$ $T_{reg}$ cells [179]. The aforementioned elegant studies demonstrated unequivocally that Foxp3 is essential to the suppressive function of $T_{reg}$ cells.

### *iv. The impact of FOXP3 in self-tolerance in humans.*

The human correlate of $Foxp3^{-/-}$ mice is is best represented in a rare immune condition known as immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX), characterized by multi-organ autoimmune disease. The frequency of $T_{reg}$ cells in IPEX patients is drastically reduced due to mutations in the *FOXP3* gene [186]. The IPEX syndrome is manifested by a signature collection of autoimmune diseases that typically present during early infancy in affected males, including insulin-dependent diabetes, eczema, food allergies [186]. An analogous X-chromosome-linked pathology was described for the scurfy mouse that carries a spontaneous mutation in the forkhead/winged-helix protein *Scurfin* gene [164]. Most FOXP3 mutations in IPEX patients are found within the forkhead DNA-binding domain [186]. In scurfy mice, a frameshift mutation reduces gene expression and encodes a truncated Foxp3 product lacking the forkhead domain [164]. Hemizygous human and scurfy females harbour both defective and intact $Foxp3^+$ $T_{reg}$ cells due to random inactivation of the X-chromosome, which exist as a genetic mosaicism [187]. Importantly, these females are healthy and do not exhibit intermediate disease phenotypes [187]. This indicates that residual $Foxp3^+$ $T_{reg}$ cells dominantly control self-reactive T cells in hemizygous females [187].

Although Foxp3 is the most stringent marker in murine $T_{reg}$ cells, the situation is less straight-forward in humans. Like their murine counterparts, human $CD4^+CD25^+$ $T_{reg}$ cells constitutively express high levels of FOXP3 [188-190]. Moreover, human $T_{reg}$ cells express an alternatively spliced isoform of FOXP3, which lacks exon 2 [191]. Although the precise function of these two isoforms remains to be fully defined, evidence indicates that their biological roles may not be equivalent [191]. In contrast to murine $T_{eff}$ cells, FOXP3 can be transiently upregulated in $CD4^+CD25^-$ human T cells [188-190]. This does not necessarily lead to suppressive activity, but rather to hyporesponsiveness [188-190]. Furthermore, induction of FOXP3 *ex vivo* in $CD4^+CD25^-$ human T cells does not necessarily confer a regulatory phenotype [192]. It appears that only the activated $CD4^+$ T cells that acquire and maintain high levels of FOXP3 exhibit suppressive activity, whereas those that fail to do so only transiently express FOXP3 [193]. Overall, caution should be exercised when extrapolating data from mouse models.

### *v. The molecular mechanism of Foxp3.*

As discussed above, Foxp3 expression is crucial for $T_{reg}$ cells to exert their suppressive activity and control immune responses to self and non-self antigens. In this section, we will discover that Foxp3 coordinates the establishment of a $T_{reg}$ cell "signature profile", which inhibits the expression of $T_{eff}$ cell-specific cytokines and promotes $T_{reg}$ cell-specific receptors and fitness factors [194]. In other words, Foxp3 acts as both a transcriptional activator and repressor, and mediates gene silencing and activation to enable efficient $T_{reg}$ cell suppression [194]. A thorough search for Foxp3 target genes revealed that Foxp3 indirectly controls approximately 700 hundred genes

and binds directly to 10% of them [194]. Foxp3-binding genes include those encoding signal transduction molecules (*zeta-chain TCR-associated protein kinase* (*Zap70*), *Ptpn22*), transcription factors (*cyclic adenosine monophosphate (cAMP) responsive element modulator* (*Crem*)), cytokines (*Il2*), cell-surface molecules (*Cd25*, *Ctla4*, *Fas ligand*) and enzymes involved in cell metabolism (*phosphodiesterase 3b*).

Upon $T_{eff}$ cell activation, nuclear factor of activated T cells (NFAT) translocates to the nucleus, where it associates with activator protein-1 (AP-1), nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and acute myeloid leukemia 1/runt-related transcription factor 1 (AML1/Runx1) to promote the expression of *Il2*, thus contributing to the activation and differentiation of conventional T cells [195]. Foxp3 binds to the minimal IL-2 promoter in association with NFAT to block IL-2 transcription [195]. Thus, $T_{reg}$ cells are incapable of producing their own IL-2, although they absolutely require IL-2 to exert their functions [195]. Foxp3 also activates $T_{reg}$ cell-specific *Cd25*, *Ctla4* and *Gitr* genes [174, 194]. Amino acid substitutions in the forkhead domain of Foxp3 that disrupt the Foxp3/NFAT interaction, impair the ability of Foxp3 to repress *Il2* and to activate $T_{reg}$ cell specific *Cd25*, *Ctla4* and *Gitr* genes, leading to the transition of $T_{reg}$ cell to a $T_{eff}$ cell phenotype [174, 194]. Downstream of the Foxp3/NFAT association, Foxp3 also interacts with AML1/Runx1, further contributing to the transcriptional repression of IL-2 in mouse and man [195, 196]. Therefore, if the Foxp3/AML1/Runx1 association is disrupted in $T_{reg}$ cells, AML1/Runx1 is free to associate with NFAT and promote IL-2 transcription [195, 196]. As a result, $T_{reg}$ cells lose their suppressive activity and gain $T_{eff}$ cell properties [195, 196]. Furthermore, genetic ablation of AML1/Runx1 or silencing of AML1/Runx1 in $T_{reg}$ cells negatively

affects Foxp3 function and results in autoimmune disease [171]. Therefore, Foxp3 maintains a positive feedback loop of its own expression through its interaction with AML1/Runx1 [197]. This set of data convincingly demonstrates that NFAT and AML1/Runx1 aid in the establishment of aspects of the regulatory program of $T_{reg}$ cells, such as the upregulation of *Cd25*, *Ctla4*, *Gitr* [195, 196]. Eos, a zinc finger transcription factor of the Ikaros family, is also expressed in $CD25^{+}Foxp3^{+}$ $T_{reg}$ cells and participates in the repression of IL-2 [198].

Transcriptional control exerted by Foxp3 also involves histone acetyl transferases (HATs). Upon TCR-mediated activation, histone deacetylation is induced by the association of Foxp3 to the *Il2* promoter, a process that inhibits chromatin remodeling and opposes gene transcription, resulting in the repression of *Il2* [199]. These data indicate that Foxp3 may regulate transcription through direct chromatin remodelling and show that signals from the TCR impact Foxp3 function.

$T_{reg}$ cells harbour a specific micro RNA (miRNA) profile under the control of Foxp3 [200]. Depletion of Dicer, an RNAse enzyme required for the processing of double-stranded RNA such as microRNAs, hampers development of $Foxp3^{+}$ $T_{reg}$ cells [200]. This elicits autoimmunity, implicating a role for miRNAs in thymic development of $T_{reg}$ cells in a Foxp3 dependent fashion [200]. Interestingly, Foxp3 also promotes the expression of Th2-specific transcription factor interferon regulatory factor 4 (IRF4) in order to suppress Th2 responses. Through its interaction with IRF4, Foxp3 blocks the expression of factors essential for Th2 differentiation [201]. Lastly, Foxp3 potentially interacts with retinoic acid orphan receptor $\gamma\tau$ (ROR$\gamma\tau$), the transcription factor intimately linked with the differentiation of Th17 cells, a subset of cells involved in the inflammatory

processes in various autoimmune disorders [202]. Thus, $T_{reg}$ cells, through their expression of Foxp3, block the differentiation of Th1, Th2 and Th17 cells, demonstrating their importance in the outcome of immune responses.

The maintenance of $T_{reg}$ cell stability rests on the epigenetic modification of the *Foxp3* locus. Two CpG islands within this locus that have been identified, located upstream of the promoter and first intron [203]. As opposed to conventional activated T cells, the CpG islands of $T_{reg}$ cells are unmethylated [203, 204]. Under homeostatic conditions, $T_{reg}$ cells receive positive signals and amplify their expression of Foxp3 [203]. In inflammatory settings, cytokines such as IL-6 reprogram Foxp3 expression, demonstrating that $T_{reg}$ cells exhibit plasticity, which is strongly dependent on the milieu [203]. Thus, Foxp3 expression is the sum of positive and negative signals imputed by $T_{reg}$ cells [203]. Taken together, strong evidence points to an irreplaceable role for Foxp3 in $T_{reg}$ cell functions, by interrupting the transcription machinery for conventional $T_{eff}$ cells, thereby converting them into $T_{reg}$ cells with distinct suppressive characteristics. However, how Foxp3 regulates the genes that imprint a $T_{reg}$ cell with a suppressive program remains unclear [203].

## *5.* Peripherally induced regulatory T ($iT_{reg}$) cells.

It was initially thought that murine $T_{reg}$ cells developed solely within the thymus as a terminally differentiated and functionally mature population. However, recent reports have demonstrated plasticity in the extra-thymic generation of $T_{reg}$ cells, which may have important implications for their role in immune homeostasis [205]. Under specific stimulatory conditions, conventional $CD4^+CD25^-$ T cells can be induced to become

$CD4^+CD25^+Foxp3^+$ $iT_{reg}$ cells outside of thymus, which exhibit characteristics similar to thymic-derived $nT_{reg}$ cells [205].

### a. Tr1 and Th3 $iT_{reg}$ cells.

T regulatory 1 (Tr1) and T helper (Th3) cells represent two unique subsets of $CD4^+$ $T_{reg}$ cells inducible from naïve T cells *in vitro* and *in vivo* in an IL-10-dependent process for the former and in virtue of oral tolerance for the latter. Antigen-specific Tr1 cells can be generated by activation in the presence of the immune-modulating cytokine IL-10 [206]. Persistent stimulation of conventional $CD4^+$ $T_{eff}$ cells *in vitro* by immature DC, cytokine-modified IL-10-producing DC or immunosuppressive drug-treated DC also results in the generation of Tr1 cells [207, 208]. Tr1 cells are also induced by bacterial products [209]. Tr1 cells secrete a pattern of cytokines distinct from that of Th1 and Th2 cells and are characterized by high levels of IL-10 and generally low levels of TGF-$\beta$ and IL-5 [206]. In addition, like $nT_{reg}$ cells, Tr1 cells are anergic, functionally suppressive *in vitro* and are able to prevent the development of experimentally-induced autoimmune diseases *in vivo* [206]. However, Tr1 cells do not express high levels of CD25 or Foxp3, suggesting that they represent a functionally distinct population of $T_{reg}$ cells [210, 211]. Tr1 exert their suppressive functions by secreting the same cytokines that induced their differentiation, i.e. IL-10 [206]. It was posited that Tr1 cells play a role in later stages of the immune response, since they are induced upon repeated antigen stimulation, as opposed to $CD4^+CD25^+$ $nT_{reg}$ cells, which are recruited and activated early during an immune response [206]. Thus, Tr1 cells may act later to dampen the immune response and maintain tolerance [206].

Th3 cells were first described in the context of administration of protein antigen via the oral route, suggesting that they play an importance role in tolerance to dietary antigens [212]. In contrast to Tr1 cells, Th3 cells express Foxp3 and require TGF-β for their induction and to exert their suppressive function [212].

### b. *De novo* induction of Foxp3$^+$ $T_{reg}$ cells: adaptable mode of tolerance.

Various elegant studies performed in recent years have convincingly demonstrated that $T_{reg}$ cells can be induced *de novo* from $CD4^+CD25^-Foxp3^-$ $T_{eff}$ cell precursors within the peripheral compartment. For example, continuous low-dose suboptimal administration of antigen via osmotic pump to antigen-specific recombination activating gene $(RAG)^{-/-}$ transgenic recipients induced the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ $T_{reg}$ cells [213]. In accordance with this finding, the transfer of antigen-specific naïve T cells into $RAG^{-/-}$ immunodeficient transgenic mice leads to the conversion of $CD4^+CD25^+Foxp3^+$ $T_{reg}$ cells [214]. Presentation of antigen that is targeted to immature DC via decalectin 205 (DEC205), can also result in the *de novo* differentiation of $CD4^+Foxp3^+$ $T_{reg}$ cells, suggesting a flexible and adaptable mode of establishing peripheral tolerance [215]. In fact, specific subsets of DC may be adapted to promote $T_{reg}$ cell conversion, as $CD8^+DEC205^+$ splenic DC produce TGF-β and can readily induce the conversion of naïve $T_{eff}$ cells into $Foxp3^+$ $T_{reg}$ cells [216]. In ovalbumin-specific TCR transgenic $RAG^{-/-}$ mice, which are devoid of $nT_{reg}$ cells, $Foxp3^+$ $iT_{reg}$ cells were generated in a TGF-β dependent fashion by the administration of ovalbumin in the drinking water, underpinning the importance of the conversion process in oral tolerance [217]. *In vivo* neutralization of TGF-β inhibited the conversion of $Foxp3^+$

$T_{reg}$ cells [217]. Therefore, TGF-β seems to be a key cytokine in the differentiation of $T_{reg}$ cells from conventional $T_{eff}$ cells.

Indeed, TGF-β dependent protocols have been developed to differentiate $Foxp3^{+}$ $T_{reg}$ cells *in vitro* from naïve conventional T cells in culture settings [202]. Not surprisingly, IL-2 has proven to be an indispensable factor in the *in vitro* conversion of $iT_{reg}$ cells [202]. In fact, TGF-β dependent conversion was negated in IL-2 neutralization studies or when $IL\text{-}2^{-/-}$ T cells were used, highlighting the importance of IL-2 in conversion, but also functions of $iT_{reg}$ cells [218]. $iT_{reg}$ cells present the typical characteristics of $nT_{reg}$ cells, such as expression of cell surface markers (CD25, CTLA-4, GITR, CD62L, $CD45RB^{low}$), anergy and suppression both *in vitro* and in several models of disease *in vivo* [205, 206].

### c. Reciprocity between the $iT_{reg}$ cell and Th17 cell lineages: a TGF-β dependent process.

Interestingly, the combination of TGF-β and IL-6 induces the differentiation of Th17 cells *in vitro* from naïve T cells, demonstrating that $iT_{reg}$ cells and Th17 cells antagonize the functions of one another due to their common requirement for TGF-β [202]. In humans, this conversion can be attained using TGF-β, IL-1 and IL-23 [219]. Upon TCR-mediated activation, TGF-β signalling leads to an intermediate stage early in the response characterized by the expression of both RORγτ and Foxp3, which physically interact with each other and the IL-17 promoter [220-222]. In the presence of IL-2, Foxp3 wins out by shutting down RORγτ [220]. Conversely, the presence of IL-6 induces RORγτ and effectively shutting down Foxp3 induction [220]. In fact, IL-6 can

induce the dedifferentiation of activated $nT_{reg}$ cells to Th17 cells [223]. This demonstrates the importance of this IL-6 in genetically reprogramming $Foxp3^{+}$ $nT_{reg}$ cells to Th17 cells [223], in a STAT3-dependent manner [224]. Thus, pro-inflammatory cytokines render murine $nT_{reg}$ cells unstable [223]. In contrast, human thymic $T_{reg}$ cells cannot be induced to convert to Th17 cells, suggesting that the ability to convert can only be acquired in the peripheral compartment [219]. However, the pretreatment of murine $iT_{reg}$ cells with IL-2 and TGF-β rendered them resistant to a Th17 cell shift, suggesting that $iT_{reg}$ cells are more resistant to the inflammatory milieu [225]. Collectively, these findings suggest that in the steady state TGF-β induces $iT_{reg}$ cells and maintains self-tolerance [226].

Taken together, high levels of IL-6 produced by innate immune cells in response to foreign pathogens block $T_{reg}$ cell induction and concomitantly induce Th17 cells to produce a strong pro-inflammatory response [226]. Therefore, IL-6 acts as the decisive factor in favor of the conversion of Th17 cells, but also overrides the functions of $T_{reg}$ cells, until clearance of infection [226]. Thus, understanding the factors that drive the differentiation of $T_{reg}$ cells versus Th17 cells may be useful in elaborating therapies that shift from destructive Th17 cell-mediated responses to $T_{reg}$ cell protective responses [227].

## *6.* Cytokine signals in $T_{reg}$ cell development, function and homeostasis.

### a. A historical overview.

The cytokines that promote $T_{reg}$ cell development, homeostasis and functions remained unclear, although TGF-β, IL-10, IL-4, IL7, IL-15 and IL-35 have been shown to participate in these processes, while IL-6 and IL-21 have proven to be inhibitory [228, 229]. A serendipitous finding demonstrated the pivotal role of IL-2 in $T_{reg}$ cell development and functions. It was believed that germline ablation of IL-2 or CD25 would result in defective activation and differentiation of conventional $T_{eff}$ cells [230, 231]. The phenotype that ensued from the deletion of IL-2 or CD25 was severe lymphoproliferative disorder, rather than immunodeficiency, suggesting that IL-2 functions in the regulation of peripheral tolerance [230, 231]. In humans, CD25 (IL-2 receptor α chain) deficiency is indistinguishable from the IPEX phenotype, reinforcing the contribution of IL-2 signalling in tolerance [232]. It was puzzling that mice and humans lacking IL-2 or the receptor for IL-2 did not exhibit serious defects in T cell differentiation and functions. Initial speculation suggested that this process was driven by the failure of IL-2 to sensitize T cells to undergo apoptosis [230, 231]. It was later demonstrated that the impairment of IL-2 signalling correlated with a drastic decrease of $T_{reg}$ cells, and infusion of this regulatory subset reverted lethal phenotype [233-235].

### b. The role of IL-2 in $T_{reg}$ cell development.

Studies initially claimed that IL-2 was essential for the development and homeostasis of $T_{reg}$ cells [236]. For example, restricted expression of IL-2 receptor (IL-

2R) β chain in the thymus of IL-2Rβ deficient animals resulted in protection from lethal autoimmunity, suggesting that IL-2 signalling is essential for $nT_{reg}$ cell development, but dispensable for $T_{reg}$ cell homeostasis [236]. This finding has since been challenged by two independent groups, who claim that IL-2 has a redundant function in $nT_{reg}$ cell development [237, 238]. They show that thymic generation is relatively normal in *Il2* or *Il2rα* deficient mice [237, 238]. Another group independently showed that $CD4^+CD8^-CD25^+$ SP thymocytes expressed almost normal amounts of Foxp3 mRNA from IL-2 deficient mice, indicating that IL-2 is dispensable for thymic development of $nT_{reg}$ cells [239]. In spite of these findings, IL-2 neutralization studies suggest that $nT_{reg}$ cells may require IL-2 at least for their survival in the medulla, where thymocytes reside for one to two weeks before thymic egress [240]. Interestingly, IL-2Rβ$^{-/-}$ mice exhibit a more profound defect in $nT_{reg}$ cell development relative to IL-2$^{-/-}$ mice due to defective signalling of both IL-2 and IL-15, which have a common requirement for IL-2Rβ for signalling [241]. Therefore, it was posited that IL-15 likely compensated for the absence of IL-2 in the thymus of IL-2 deficient mice [241]. In line with this evidence, IL-2 and IL-15 double knock-out mice harbor an $nT_{reg}$ cell developmental defect reminiscent of the IL-2Rβ deficiency [242]. Genetic ablation of the common γ chain ($\gamma_c$) results in multi-organ specific autoimmunity due to a marked decrease in $nT_{reg}$ cells, demonstrating that $\gamma_c$ cytokines (IL-2, IL-7, IL-15) are indinspensable for $nT_{reg}$ cell development [237]. Overall, IL-2 signalling is dispensable for $T_{reg}$ cell development but essential for their competitive fitness in the peripheral compartment.

### c. The impact of IL-2 in the peripheral fitness of $T_{reg}$ cells.

Strong evidence demonstrates that IL-2 is an important signal for the peripheral survival and suppressive function of $T_{reg}$ cells. IL-2 therapy or transfer of IL-2-treated IL-$2^{-/-}$ splenocytes averted lymphoproliferative disorder in IL-$2^{-/-}$ mice by enhancing $T_{reg}$ cell numbers [243]. The transfer of $CD25^{-/-}$ bone marrow precursor cells, which cannot respond to IL-2, in $RAG^{-/-}$ recipients resulted in death [234]. However, the infusion of $CD4^+CD25^+$ reverted the lethal phenotype [234]. Other studies showed that $CD4^+$ T cells from IL-$2^{-/-}$, which can respond to IL-2, prevented the development of EAE [244]. These studies demonstrated that insufficient $T_{reg}$ cell numbers can be reconstituted by the transfer of $T_{reg}$ cells or IL-2 treatment, in situations when IL-2 production or responsiveness is compromised [245]. Therefore, IL-2 contributes to the constriction of an immune response by promoting $T_{reg}$ cell functions [245]. Interestingly, the few $T_{reg}$ cells detectable in IL-$2^{-/-}$ mice exhibited elevated levels of IL-15 receptor $\alpha$ chain (IL-15R$\alpha$) and IL-7 receptor $\alpha$ chain (IL-7R$\alpha$), implicating compensatory mechanisms for the loss of IL-2 signalling [245].

Paradoxically, although $T_{reg}$ cells have a high dependence on IL-2 for their functions, they are incapable of producing their own IL-2 [237]. As a result, $T_{reg}$ cells must derive IL-2 from $T_{eff}$ cells in a paracrine manner in order to sustain their functions [237]. They are profoundly anergic in response to stimulation *in vitro* unless exogenous IL-2 is provided [237]. The hypo-proliferative state is intimately linked to their supression, as breaking anergy also results in a simultaneous loss in regulatory activity *in vitro* [237]. Since $T_{reg}$ cells readily proliferate *in vivo* under steady-state and lymphopenic conditions and are quite long-lived [246], this *in vitro* anergy reflects an

insufficiency of appropriate stimulatory signals. However, the possibility that Foxp3, a transcriptional repressor of IL-2, prevents chromatin remodelling of the IL-2 promoter cannot be excluded [195, 196].

IL-2 signalling activates the major pathway in T cells destined for their survival, in virtue of the activation of the Akt kinase, resulting in the upregulation of anti-apoptotic factors B cell CLL/lymphoma 2 (Bcl-2) and Bcl-$x_L$ [247]. It was first posited that the phenotype of the IL-2 deficient mice was due to defective survival of $T_{reg}$ cells in the absence of IL-2 [247]. A Bcl-2-overexpressing IL-$2^{-/-}$ transgenic mouse still exhibited reduced $T_{reg}$ cell numbers, disproving the belief that IL-2 was required predominantly for $T_{reg}$ cell survival [247]. Actually, the fundamental role of IL-2 signalling lies in its ability to upregulate Foxp3 expression [248]. $T_{reg}$ cells upregulate Foxp3 expression upon IL-2 stimulation through CD25 in both mouse and man [237]. Gene expression analysis demonstrates that IL-2 signalling, by upregulating Foxp3, is essential for the maintenance of expression of genes involved in regulation of cell growth and metabolism [237].

Thus, a tightly regulated feedback loop seems to be in place whereby $T_{reg}$ cells, requiring IL-2 produced by activated $T_{eff}$ cells for their maintenance and activation, limit the expansion of $T_{eff}$ cells by shutting down their production of IL-2 at the messenger RNA and protein level [249, 250]. $CD4^+CD25^+$ $T_{reg}$ cells shut down $T_{eff}$ cell-fuelled IL-2 transcription even in the presence of exogenous IL-2 [251]. When this negative loop is impaired, it may predispose to autoimmunity [252]. The emerging view on the role of IL-2 in T cell activation is that IL-2 is essential for T cell differentiation only in the instance when $T_{reg}$ cell suppression must be overcome [252]. Since IL-$2^{-/-}$ or $CD25^{-/-}$ mice lack

$T_{reg}$ cells, $T_{eff}$ cells are unleashed from $T_{reg}$ cell suppression and can therefore mount strong responses that lead to autoimmunity [252]. Overall, in the absence of $T_{reg}$ cells, control on T cell activation is reduced, so that the normal requirements for the differentiation of $T_{eff}$ cells are no longer critical [253].

### d. IL-2 signalling.

Activation through the TCR and co-stimulatory molecules such as CD28 results in the expression and production of IL-2 [254]. Upon TCR activation, CD28 costimulation plays a crucial role in IL-2 transcription and messenger RNA stabilization, which enhances IL-2 production such that T cell clonal expansion increases 1000-fold [254]. IL-2 is produced by activated $CD4^+$ T cells and, to a lesser extent, $CD8^+$ T cells and exerts its biological function by binding the IL-2 tripartite receptor (discussed below). IL-2 also has the ability to sensitize activated T cells to undergo activation-induced cell death (AICD) in FAS- or TNF-dependent pathways, a key mechanism of immune tolerance and the contraction of an immune response [255]. This demonstrates the central role that IL-2 plays in both the activation and suppression of immune responses [255].

The IL-2 receptor consists of three subunits:$\alpha$, $\beta$, and $\gamma_c$ chains [256]. The IL-2$\beta\gamma$ subunits compose the intermediate-affinity receptor and IL-2R$\alpha$ chain represents the low affinity receptor [256]. The $\alpha$ chain is characterized by a short cytoplasmic tail that does not transmit intracellular signals [256]. It acts to enhance the binding affinity of IL-2 to the IL-2 receptor complex, as the dissociation constant ($K_d$) is lower for the tripartite $\alpha\beta\gamma$ complex ($K_d=10^{-11}$) than that for the bipartite receptor complex ($K_d=10^{-9}$) [257].

Although neither the IL-2Rβ nor IL-2Rγ chains have intrinsic enzymatic function, they associate with Janus kinase (Jak)1 and Jak3 respectively upon activation [257]. Jak1 and Jak3 then phosphorylate multiple tyrosine residues found in the cytoplasmic tail of the IL-2Rβ and γ chains [257]. One of the tyrosine residues, Y-338, serves as a docking site for the adaptor protein src homologous and collagen (Shc), which leads to the recruitment of growth factor receptor-bound protein 2 (Grb2), resulting in the activation of the Ras/Raf/mitogen activated kinase kinase (Mek)/extracellular signal-regulated kinase (Erk) signalling cascade [258]. Shc also recruits growth factor receptor bound protein 2-associated protein 2 (Gab2), resulting in the activation of phosphoinositide 3 kinase (PI3K) [259]. The activation of the Ras/Raf/Mek/Erk and PI3K pathways results in cytokine transcription, survival, cell cycle entry and growth [259]. Importantly, phosphorylated tyrosine residues also act to recruit adaptor protein signal transducer and activator of transcription (STAT) 5 [260]. STAT5 dimerizes and translocates to the nucleus where it binds to DNA-binding site gamma-activated sequence (GAS) resulting in the transcription of mitogenic and survival genes [260]. IL-2Rα promoter is also a STAT5 target, suggesting a positive feedback loop where IL-2 signalling provides further IL-2R production [260].

STAT5 is activated downstream of IL-2 signalling and plays a pivotal role in the activation and differentiation of T cells [245]. STAT5 is also essential for the development and function of $T_{reg}$ cells, as constitutive activation of STAT5 in lymphocytes generates increased numbers of $T_{reg}$ cells and selective ablation of STAT5 results in a complete absence of $T_{reg}$ cells leading to autoimmunity [241, 261-263]. Transient over-expression of STAT5 rescues the reduction of $T_{reg}$ cell numbers in IL-$2^{-/-}$

mice, suggesting that STAT5 plays a key role in $T_{reg}$ cell homeostasis [247]. $T_{reg}$ cells exclusively activate STAT5, when provided with exogenous IL-2, as opposed to $T_{eff}$ cells, which induce PI3K, Ras and STAT5 upon activation [264]. STAT5-binding sites were identified in the Foxp3 promoter, although the biological significance remains to be determined [241, 261, 265]. A patient with a missense mutation in the *Stat5b* gene exhibited impaired expression of Foxp3 and hence $T_{reg}$ cell function leading to immune dysregulation, implicating STAT5 activation with human $T_{reg}$ cell maintenance [266]. Taken together, these findings make a strong case for the hypothesis that IL-2 signalling, in a STAT5-dependent manner, has a non-redundant role in $T_{reg}$ cell functions in both mouse and man.

## *7.* Mechanism of suppression of regulatory T cells.

### a. The characteristics of $T_{reg}$ cell suppressive activity *in vitro*.

In order to fully understand the impact of $T_{reg}$ cells on immune responses, great efforts have been made in order to determine the mechanism of suppression. As we will see in this chapter, $T_{reg}$ cells exert their suppressive activity employing various mechanisms. As a first step, an *in vitro* suppression assay was designed whereby the degree of suppression in a co-culture of $CD4^+CD25^+$ $T_{reg}$ cells and $CD4^+CD25^-$ $T_{eff}$ cells upon antigenic stimulation in the presence of APCs is evaluated as a read-out of proliferation of these cells [267]. $T_{reg}$ cells mediate suppression in an antigen-non-specific fashion [268]. In other words, once the $T_{reg}$ cells are appropriately stimulated, they can suppress T cells with different antigen specificities [268]. *In vitro,* $T_{reg}$ cells exert their suppressive activity in a contact-dependent fashion, as determined by

Transwell experiments [249]. The Transwell membrane permits soluble factor exchange, but inhibits direct physical contact [249]. $T_{reg}$ cells, when physically separated from the $T_{eff}$ cells, were incapable of exerting their suppression [249].

Initial reports claimed that TGF-β, expressed in its latent form on $CD4^+CD25^+$ $T_{reg}$ cells, was delivered to $T_{eff}$ cells and converted in its active form locally [269]. In stark contrast, blockade studies *in vitro* showed that $T_{reg}$ cells needn't produce TGF-β nor be responsive to it to exert their suppressive functions [270]. Upon activation, a high proportion of human and murine $Foxp3^+$ $T_{reg}$ cells express latency associated protein (LAP), which associates with the latent form of TGF-β [271, 272]. The upregulation of LAP implies the eventual conversion of latent TGF-β to its active form. Intriguingly, that is not the case, as the use of the most potent inhibitor of TGF-β did not alter the suppressive function of $T_{reg}$ cells [267]. One study suggested that latent TGF-β expressed on the surface of $T_{reg}$ cells participates in the conversion of $Foxp3^-$ T cells to $Foxp3^+$ $T_{reg}$ cells through a mechanism of infectious tolerance, although the possibility that TGF-β acts on DC to dampen their priming capabilities cannot be excluded [271]. A role for TGF-β in suppression has been attributed to human $CD4^+CD25^+$ $T_{reg}$ cells as well, although TGF-β blockade can never completely reverse suppression *in vitro* [273-275].

Parallel studies demonstrated that IL-4, IL-10, and TGF-β transcripts were more actively transcribed by $CD4^+CD25^+$ $T_{reg}$ cells in *in vitro* suppression assays, suggesting that these soluble mediators were involved in $T_{reg}$ cell-mediated suppressive activity [229]. However, neutralization of these immunosuppressive cytokines, alone or in

combination, did not abrogate $T_{reg}$ cell suppression *in vitro* [229]. Furthermore, the supernatant from the suppression co-culture failed to suppress the stimulation of $CD4^+CD25^-$ $T_{eff}$ cells [249, 250]. Lastly, $T_{reg}$ cells isolated from IL-$4^{-/-}$, IL-$10^{-/-}$ or TGF-$\beta^{-/-}$ mice were as effective as wild-type $T_{reg}$ cells in exerting suppressive functions, demonstrating unequivocally that soluble factors such as cytokines are not responsible for $T_{reg}$ cell suppression *in vitro* [249, 270].

Many other mechanisms have been proposed to operate, alone or in combination, for the control of immune responses *in vitro*. $Foxp3^+$ $T_{reg}$ cells have been shown to preferentially produce IL-35, a novel IL-12 related immunosuppressive cytokine, which can modulate intestinal inflammation [276]. Functional studies have shown that $T_{reg}$ cells may induce cytolysis or inactivate $T_{eff}$ cells or APCs through an ill-defined granzyme- or perforin-dependent mechanism [277, 278]. The delivery of a negative signal to responder T cells such as the upregulation of intracellular cAMP leads to suppression of T cell proliferation and IL-2 secretion [279]. More recently, cytokine competition by $T_{reg}$ cells has also been suggested to induce apoptosis in responding T cells on APC surfaces [280]. Furthermore, the expression of CD39 and CD73 by $Foxp3^+$ $T_{reg}$ cells results in the hydrolysis of available adenosine triphosphate (ATP) and yields adenosine, which is inhibitory to DC immunogenic functions [281]. This represents another potential mechanism of immune suppression [281].

### *b.* The role of soluble mediators in $T_{reg}$ cell suppressive functions *in vivo.*

The role of cytokines in $T_{reg}$ cell suppression *in vivo* appears to be context-dependent and many discrepancies exist depending on the experimental model [282,

283]. In a transfer model of inflammatory bowel disease (IBD), $CD4^+CD25^+$ $T_{reg}$ cells from IL-$10^{-/-}$ mice failed to prevent IBD [284]. In stark contrast, in a model of autoimmune gastritis, IL-10 was not required for $CD4^+CD25^+$ $T_{reg}$ cells to mediate suppression [283]. Selective disruption of IL-10 in $T_{reg}$ cells resulted in spontaneous colitis and inflammation only at environmental interfaces such as the lungs, skin and gut, but progression to autoimmune gastritis was not altered [282]. This pointed to differential mechanism of suppression by $T_{reg}$ cells, whereby IL-10 is essential to maintain homeostasis of $T_{reg}$ cells in the colon [282].

$CD4^+Foxp3^+$ $T_{reg}$ cells isolated from TGF-β deficient mice or from mice expressing a dominant-negative form of TGF-β receptor II were able to suppress intestinal inflammation [271, 285, 286]. Two independent groups demonstrated that mice with a T cell specific deletion of TGF-β developed IBD, suggesting that TGF-β is absolutely required for IBD protection and that it can be derived from a non-$T_{reg}$ cell source [287, 288]. These conflicting results demonstrate that discrepancies among various groups prevent a consensus on the role of TGF-β in $T_{reg}$ cell functions.

### c. A mode of $T_{reg}$ cell suppressive function through the down-modulation of DC functions *in vivo*.

Many mechanisms of $T_{reg}$ cell suppression involve the dampening of DC immunogenic functions, which is the only APC subset capable of promoting $T_{reg}$ cell functions [289]. Two-photon laser scanning microscopy enabled the examination of antigen-specific $T_{reg}$ cells and DC interactions directly in the lymph nodes [85, 290]. In the absence of $T_{reg}$ cells, $T_{eff}$ cells swarmed in the vicinity of the DC and arrested for a prolonged period of time. $T_{reg}/T_{eff}$ cell interactions were not observed [85, 290]. In the

same vein, DC exposed to *in vitro* TGF-β mediated $iT_{reg}$ cells had a diminished capacity to prime antigen-specific $T_{eff}$ cells in a model of gastritis [291]. Hence, $T_{reg}$ cells do not compete for antigen with $T_{eff}$ cells nor do they act directly on $T_{eff}$ cells to prevent their interaction with DC. They exert their suppressive actions on $T_{eff}$ cells indirectly by down-modulating the priming capabilities of DC. One study proposed that activated $T_{reg}$ cells may compete with antigen-specific $T_{eff}$ cells for DC interaction through an LFA-1-dependent mechanism [292]. LFA-1 plays an established role in T cell contact and activation and is expressed at high levels on murine $T_{reg}$ cells [292]. The net result of $T_{reg}$ cell/DC interaction via LFA-1 was the down-regulation of CD80 or CD86 by a CTLA-4-dependent mechanism [292].

### d. The role of CTLA-4/B7 interactions in $T_{reg}$ cell suppression.

Interestingly, $T_{reg}$ cells constitutively express CTLA-4, in contrast to naïve $T_{eff}$ cells, which upregulate CTLA-4 only upon activation [131]. Also, Foxp3 acts as a transcriptional enhancer of CTLA-4, strengthening the hypothesis that it is important in mediating $T_{reg}$ cell functions [174, 194]. The administration of CTLA-4 mAb abrogated the $CD4^+CD25^+$ $T_{reg}$ cell-mediated suppression of a mouse model of colitis [293]. Antigen-activated $T_{reg}$ cells may also engage B7 (CD80 and CD86) molecules expressed by DC via CTLA-4 in order to induce the activation of enzyme IDO, which catabolizes tryptophan to kynurenines, starving T cells and leading to their apoptosis [294]. In the same vein, $T_{reg}$ cells may limit DC-mediated priming of $T_{eff}$ cells by inhibiting the upregulation of CD80 and CD86 via CTLA-4, as CTLA-$4^{-/-}$ $T_{reg}$ cells were incapable of modulating costimulatory molecule expression on DC [295].

$T_{reg}$ cells employ various mechanisms in order to prevent *in vivo* pathologies such as inhibition of $T_{eff}$ cell differentiation, expansion and migration of autoreactive cells [85, 296, 297]. $T_{reg}$ cells suppress maturation and function of DC, inhibit cytotoxic functions of NK and NK-T cells, and block B cell proliferation and immunoglobulin production and isotype switching [267]. These mechanisms may also exhibit redundancy and depend on the degree of inflammation [267]. The dissection of the mechanism of suppression of antigen-specific $T_{reg}$ cells in the context of a specific inflammatory response may provide therapeutic tools required to retract the exaggerated immune response [267].

Overall, $T_{reg}$ cells have emerged as a critical immunological switch that determines the outcome of immune responses. $T_{reg}$ cells participate in maintaining immune homeostasis, as their depletion leads to defective immune responses in disparate diseases ranging from chronic parasite infection, autoimmunity and graft rejection [51]. $T_{reg}$ cells represent a dominant mechanism in the prevention of exaggerated autoimmune responses and a deficiency in $T_{reg}$ cell development and/or function may promote the activation, expansion, and recruitment of autoreactive T cells and the onset of autoimmunity [51]. We will now discuss the impact of defective $T_{reg}$ cell functions in diabetes pathogenesis.

## *8.* Defective regulatory T cells: major predisposing factor in the breakdown in self-tolerance in the NOD mouse model.

In both humans and mice, we have seen that $T_{reg}$ cells represent the underlying pivotal mechanism in the maintenance of self-tolerance, as IPEX patients and Scurfy or

Foxp3-deficient mice develop systemic autoimmunity due to a drastic deficiency in $T_{reg}$ cells [164, 177, 186]. Thus, the onset of any given autoimmune disorder is likely the result of environmental and genetic factors, compounded with a certain degree of $T_{reg}$ cell depletion [164, 177, 186]. In contrast to IPEX patients or Scurfy mice that harbour no $T_{reg}$ cells, NOD mice likely develop T1D due to subtle fluctuations in thymic development or fitness/stability of $T_{reg}$ cells in the peripheral compartment [298]. In NOD mice, which exhibit a strong genetic propensity for autoimmune disorders, $T_{reg}$ cells may be unstable and fitness factors such as IL-2 and DC-mediated costimulation may stabilize the $T_{reg}$ cell pool and prevent autoimmunity [299, 300].

### a. Thymic development of $T_{reg}$ cells is normal in NOD mice.

The possibility that the progression of T1D is a result of aberrant thymic development of $nT_{reg}$ cells was a rather obvious starting point in examining the underlying factor in the breakdown in self tolerance. It was postulated that skewing of thymic selection processes may be due to the unique structure and binding capabilities of MHC [301]. Indeed, the H2-$A^{g7}$ molecule contains a non-aspartic acid substitution at position 57 of the $\beta$ chain that substantially alters the repertoire of MHC binding peptides presented by this allele [65]. This may be a result of the MHC's promiscuous ability to bind an array of low affinity peptides [302]. This substitution is also seen in the HLA-DQ $\beta$ chain of human T1D patients [303]. Two studies report a failure to censure autoreactive T cells in the thymus by negative selection, suggesting that loss of self-tolerance may be attributed to a greater number of $T_{eff}$ cells exiting the thymus [60, 90]. However, thymic development of $nT_{reg}$ cells is normal in NOD mice, as one study

reported that the NOD background proved superior in generating $nT_{reg}$ cells in the thymus relative to a non-autoimmune prone strain, suggesting that central tolerance mechanisms are intact [302, 304]. Furthermore, the frequency and function of NOD $CD4^+Foxp3^+$ $nT_{reg}$ cells were comparable to diabetes-resistant mice (chapter II) [305], although one study suggests the contrary [306]. Discrepancies between these findings may be due to different experimental setups, use of different $T_{reg}$ cell-specific markers, as well as the age of the mice used in these studies. Despite the apparently normal thymic development and frequency of the NOD $nT_{reg}$ cell lineage, TCR diversity within the selected repertoire was markedly restricted relative to the C57BL/6 strain [307]. Hence, the restricted diversity of $nT_{reg}$ cells may place them at a disadvantage for the dampening of the diabetogenic $T_{eff}$ cells [307]. Overall, no consensus has been reached concerning the thymic development of $T_{reg}$ cells in the NOD mouse model and is due to different experimental procedures.

### b. $T_{reg}$ cells are functionally operative in NOD mice.

Compelling evidence points to an essential role for $CD4^+CD25^+Foxp3^+$ $T_{reg}$ cells in the maintenance of self-tolerance in NOD mice. Depletion of CD25-expressing T cells results in a marked acceleration of T1D [154]. Recently, Chen *et al.* reported that genetic ablation of Foxp3 in NOD mice resulted in an increased incidence and earlier onset of T1D compared to WT NOD mice [308]. These studies underscore the importance of $CD4^+CD25^+Foxp3^+$ $T_{reg}$ cells in the maintenance of self-tolerance in the NOD mouse. The $Foxp3^{-/-}$ study, although invaluable, did not address whether the infusion of $T_{reg}$ cells, which rescues from T1D, compensates for the primary deficit in

$T_{reg}$ cells believed to underlie T1D pathogenesis in these mice, or whether such injection was actually suppressing the global inflammation/pathology that likely arose as a secondary consequence of Foxp3 deficiency [308]. Interestingly, BDC2.5 mice are characterized by a lower incidence of diabetes relative to polyclonal NOD mice, while $RAG^{-/-}$ BDC2.5 animals, devoid of $T_{reg}$ cells, exhibit fulminant diabetes and no lag time between insulitis and overt diabetes [119]. Infusion of $CD4^+$ splenic T cells to $T_{reg}$ cell-deficient $RAG^{-/-}$ BDC2.5 mice protects against T1D by suppressing inflammation in the islets [119]. Collectively, this data supports the notion that $T_{reg}$ cells exist and exert suppression on diabetes progression in NOD mice.

Several lines of evidence show that $T_{reg}$ cells are functionally operative in prediabetic NOD mice. Many studies have reported an expansion of $CD4^+CD25^+$ T cells with apparent regulatory activity in inflamed tissues of adult prediabetic, and insulitic NOD mice. Also, several reports noted an increase in $T_{reg}$ cells or Foxp3 mRNA expression at the time of disease onset, coinciding with a proliferative burst of $T_{eff}$ cells, suggesting that inflammation drives $T_{reg}$ cell functions [309, 310]. It is unclear from these studies whether these $CD4^+CD25^+$ T cells are induced from $CD4^+Foxp3^-$ progenitors during pancreatic inflammation, or whether they emerged from the thymus-derived $nT_{reg}$ cell pool. One study addressed the issue of conversion by sequencing the highly-variable complementarity-determining region 3 (CDR3) of the Vα2 chain of thymic versus peripheral $T_{eff}$ and $T_{reg}$ cells [311]. This study reported that autoreactive $T_{eff}$ cells do not undergo conversion into $iT_{reg}$ cells in response to self-antigen in pancreatic sites, suggesting that $T_{reg}$ cells within pancreatic inflammatory sites are thymic-derived [311]. The role of conversion in the NOD mouse model is unclear and requires further

investigation using reporter Foxp3$^{gfp}$ (expression of GFP under the Foxp3 promoter) mice. Lastly, diabetes can be inhibited in transfer systems by *in vitro* cultured CD4$^{+}$CD25$^{+}$ $T_{reg}$ cells, with the highest regulatory activity attributed to the CD62L$^{high}$ fraction [312]. Transfer of such cells after long-term *in vitro* expansion has been shown to be protective, although it is unknown whether *in vitro* conditioning of $T_{reg}$ cells may adversely affect their physiological role [312].

### c. Mechanism of suppression of $T_{reg}$ cells in NOD mice.

$T_{reg}$ cells can control or limit the potency of activation of the $T_{eff}$ cell pool at various stages such as differentiation and/or proliferation during priming in the draining pancLN, IL-2 production, trafficking to the pancreas, or effector functions *in situ* (chapter II) [85, 119, 305, 308, 313, 314]. The presence of $T_{reg}$ cells did not block the entry of autoreactive T cells into the pancLN, but did block their migration to the T-B cell boundary within the pancLN, suggesting that $T_{reg}$ cells impinge the proper migration of autoreactive T cells [315]. Adoptive transfer systems have demonstrated an inverse correlation between the proliferation of $T_{eff}$ cells and the amount of $T_{reg}$ cells present in draining pancreatic sites [297, 316]. Indeed, in CD28$^{-/-}$ or B7$^{-/-}$ recipients devoid of $T_{reg}$ cells, robust proliferation of transferred antigen-specific BDC2.5 CD4$^{+}$CD25$^{-}$ $T_{eff}$ cells was observed [85, 308]. Reconstitution of $T_{reg}$ cells significantly blocked proliferation [313]. Other studies demonstrated that, although $T_{reg}$ cells do not block the proliferation of $T_{eff}$ cells, they reduce the production of IFN-$\gamma$ or the expression of chemokine C-X-C motif receptor 3 (CXCR3), required for the infiltration of the islets by the autoreactive pool [297]. Also, under the conditions where priming of $T_{eff}$ cells remained unaffected,

the differentiation of Th17 and TNF-$\alpha$ producing Th1 cells was markedly hindered by $T_{reg}$ cells (chapter II) [305]. Furthermore, the infiltration of $T_{eff}$ cells in the target organ was markedly enhanced in the absence of $T_{reg}$ cells (chapter II) [305]. These data suggest that $T_{reg}$ cells may maintain self-tolerance by preventing antigen-specific homing to pancreas and/or decrease the cycling and differentiation of autoreactive $T_{eff}$ cells [313]. A very recent study has shed more light on the targets of $T_{reg}$ cell mediated immunosuppression. Human diphtheria toxin receptor under the control of the Foxp3 promoter leads to temporal loss-of-function of $T_{reg}$ cells when mice are treated with diphtheria toxin [317]. Using this approach, the authors elegantly demonstrated that IFN-$\gamma$ responsive genes in $CD4^+$ T cells, but more importantly in NK cells, were upregulated in the absence of $T_{reg}$ cells [317]. More specifically, NK-mediated IFN-$\gamma$ was found to be prodiabetogenic [317]. Therefore, $T_{reg}$ cells also control innate signals to dampen the diabetogenic response.

One elegant study has eluded to the division of labour of $T_{reg}$ cell subsets by demonstrating that at least three distinct subsets of $T_{reg}$ cells exist, that control autoimmunity at different sites, as the depletion of each subset in immunodeficient recipients resulted in a distinct organ-specific autoimmune disease [318]. The $CD4^+CD45RB^{high}$ $T_{reg}$ cell subset was able to suppress colitis, with no effect on diabetes or gastritis [318]. Conversely $CD62L^{high}$ $T_{reg}$ cells were more potent than their $CD4^+CD62L^{low}$ counterparts at suppressing diabetes with no impact on colitis or gastritis [318]. Lastly, the $CD4^+CD25^+$ regulatory subset protected against gastritis and to a lesser extent diabetes with no effect on colitis [318]. Collectively, these data point to the diversity and organ selectivity of $T_{reg}$ cells controlling distinct autoimmune diseases.

### d. Evidence of $T_{reg}$ cell-mediated suppression of DC functions.

$T_{reg}$ cells preferentially interact with DC presenting β-islet antigens, directly addressed in NOD mice by means of 2-photon laser-scanning microscopy of pancreatic LN [85]. In this study, Tang *et al.* elegantly showed that *in vitro* expanded BDC2.5 $T_{reg}$ cells and DC form stable associations, which seemingly correlates with disruption of BDC2.5 $T_{eff}$ cell/DC cellular interactions in pancLN [85]. This suggests that $T_{reg}$ cell/APC interactions may in be part responsible for $T_{reg}$ cell-mediated protection [85]. However, a direct causal relationship between $T_{reg}$ cell/DC interactions and suppression of $T_{eff}$ cells has never been formally made [85]. Overall, these findings highlight the notion that DC are key players in the functions $T_{reg}$ cells *in vivo*, and strongly suggest that their activation state is a critical determinant of tolerance or autoimmunity [85]. An inability of DC to efficiently tolerize $T_{eff}$ cells and/or expand $T_{reg}$ cells may be one, among many, underlying causes of loss of β cell tolerance [85]. Thus, alterations in DC maturation or function may indirectly impact $T_{reg}$ cell homeostasis such that the balance is tipped towards autoimmunity [85]. Irrespective of the presence of $T_{reg}$ cells and their distinct underlying mechanisms of suppression, NOD mice do eventually develop diabetes, suggesting a $T_{reg}$ cell-dependent breakdown in self-tolerance.

## *9.* Functional waning of $CD4^+Foxp3^+$ $T_{reg}$ cells predisposes to T1D.

### a. Evidence in the NOD mouse model.

Although NOD mice harbour $T_{reg}$ cells capable of preventing disease progression early in life, pathogenic $T_{eff}$ cells proceed to immune-mediated destruction of the β-islet

cells [319, 320]. A central unanswered question is whether the primary predisposing factor in diabetes onset is due to a decline in $T_{reg}$ cell frequency and/or functions or from the overriding of such regulation by the uncontrolled activation and expansion of diabetogenic T cells [319, 320]. Although two studies has suggested a quantitative defect in the NOD contributes to diabetes progression [321, 322], many more hint to a progressive waning in $T_{reg}$ cell functions as a potential mechanism to elucidate diabetes pathogenesis, despite a stable cellular frequency of $T_{reg}$ cells (chapter II) [305, 309, 319, 321, 323]. Initially, it was found that $T_{reg}$ cells isolated from older diabetic NOD females are less suppressive relative to $T_{reg}$ cells isolated from younger NOD animals [309, 323]. Upon further investigation, transfer studies also demonstrated a resistance of diabetogenic T cells to $T_{reg}$ cell suppression [309]. In contrast, data from our lab demonstrates that $T_{reg}$ cells from older or overtly diabetic NOD animals, unlike prediabetic mice, were incapable of preventing diabetes transfer (chapter II) [305]. The possibility that they are overwhelmed by the $T_{eff}$ cell response or by the accumulation of $T_{reg}$ cell-resistant autoreactive T cells over time cannot be excluded. [305]

Accumulating evidence suggests that $T_{reg}$ cell functions wane with age in the NOD mouse model due to Foxp3 instability as a result of insufficient fitness factors (IL-2/costimulation). Using a dual reporter mouse model, an elegant study demonstrated that 10% of cells never stably express Foxp3 or lose Foxp3 expression after normal thymic development of $T_{reg}$ cells [324]. In line with this evidence, $iT_{reg}$ cells exhibit Foxp3 instability, which can lead to the generation of pathogenic T cells [300]. Therefore, attenuated expression of intra-islet Foxp3 would eventually result in autoimmunity, demonstrating that continuous and high levels of Foxp3 are required to prevent

autoimmune diabetes [325]. Taken together, these data suggest that $T_{reg}$ cells of NOD mice have a generalized fitness defect in inflammatory sites, which in turn predisposes to disease onset [306].

### b. Evidence in human diabetes.

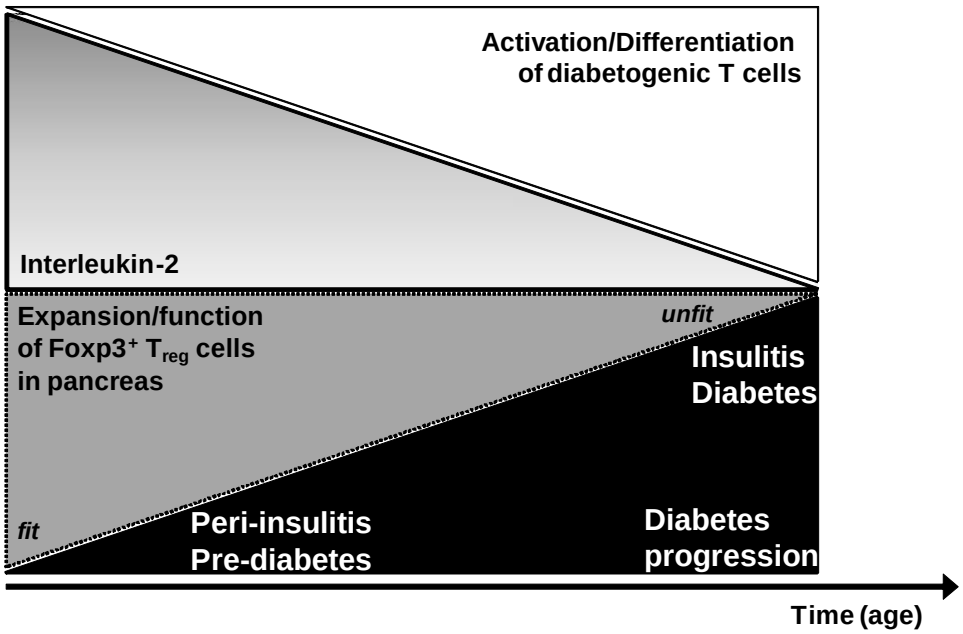
Conflicting data in human T1D patients demonstrate decreased $T_{reg}$ cell frequency in both recently diagnosed and long-standing patients [49], unaltered $T_{reg}$ cell frequency with marked decrease in suppressive activity *in vitro* [320, 326], and no differences at all compared to healthy controls [327]. There is evidence to support a loss of $T_{reg}$ cells suppressive activity and progressive resistance of $T_{eff}$ cells to $T_{reg}$ cell suppression [328-330], suggesting that these events are not mutually exclusive. Lower FOXP3 expression levels were detected in recently-diagnosed juvenile diabetes patients relative to healthy subjects, which points to an inherent defect in modulating early, as opposed to established, autoimmune responses [331, 332]. Interestingly, $T_{reg}$ cell defects in human autoimmune disorders are not limited to diabetes. $T_{reg}$ cells from patients suffering from rheumatoid arthritis, myasthenia gravis and multiple sclerosis exhibited decreased suppressive activity relative to $T_{reg}$ cells from healthy donors, reinforcing the notion that $T_{reg}$ cells govern self-tolerance by dampening the autoaggressive immune responses [333-335]. However, other reports highlight the lack of consensus on the role of $T_{reg}$ cells in autoimmune pathology in humans. For example, a greater proportion of $CD4^{+}Foxp3^{+}$ $T_{reg}$ cells was detected in patients suffering from ulcerative colitis as opposed to no differences in $T_{reg}$ cell frequencies in patients with autoimmune thyroid disease relative to healthy controls [336-338].

These differences can be explained by several reasons, including variations in the method of $T_{reg}$ cell isolation and purification, and the lack of functional tests on organ-specific $T_{reg}$ cells in blood. Thus, subtle functional differences in the $T_{reg}$ cell pool within sites of inflammation may not be adequately reflected in the peripheral blood compartment.

## *10.* T1D-protective and pro-fitness role of IL-2 in $T_{reg}$ cell functions.

Strong evidence suggests that inappropriate immunoregulation underlies the pathogenesis of T1D. Indeed, so far we have looked at the multiple defects in $T_{reg}$ cell functions in the NOD mouse model. The factors that contribute to the $T_{reg}$ cell-dependent breakdown in self tolerance must now be examined. Interestingly, NOD T cells respond normally to TCR activation until the onset of insulitis [339], at which point they become anergic, and sustain a drastic reduction in IL-2 production, which has recently been reported to occur in a model of murine systemic lupus erythrematosus [340]. Since IL-2 is essential for the fitness of $T_{reg}$ cells, a reduction in IL-2 in NOD mice may lead to an imbalance in the $T_{reg}/T_{eff}$ cell ratio, abrogating $T_{reg}$ cell function and subsequently enabling diabetogenic T cells to make the transition from insulitis to overt diabetes [341] (Figure 4). Interestingly, this decline in T cell activation and concomitant IL-2 production coincides with a time-dependent decrease in the cycling of $T_{reg}$ cells, suggesting that $T_{reg}$ cells are sensitive to IL-2 availability in prediabetic NOD mice [341]. Furthermore, IL-2 neutralization precipitates T1D by selectively depleting the $T_{reg}$ cell subset, reinforcing the importance of IL-2 in promoting $T_{reg}$ cell functions [342]. Consistently, low-dose administration of IL-2 in prediabetic female mice resulted in

enhanced frequency of Foxp3$^{+}$CD25$^{+}$ $T_{reg}$ cells, and this regimen resulted in T1D protection [341]. In stark contrast, the expression of IL-2 under the rat insulin promoter by pancreatic islets resulted in accelerated diabetes, suggesting that IL-2 levels dictate whether the balance is tipped toward self-tolerance or autoimmunity [343]. Interestingly, $T_{eff}$ cells isolated from early-onset T1D patients showed a marked reduction in IL-2 secretion [344]. This correlates with enhanced apoptosis of $T_{reg}$ cells relative to healthy controls, indicating that during the onset of disease, $T_{reg}$ cells in T1D may be exposed to a deficient cytokine milieu [344]. Taken together, variations in IL-2 may affect the balance between islet-specific autoreactive T cells and Foxp3$^{+}$ $T_{reg}$ cells, and consequently precipitate T1D [78, 344]. Based on recent findings, it is also possible that a functional deficiency in IL-2 signalling in the target organ may disturb the positive feedback loop that controls Foxp3 stability, such that $T_{reg}$ cells convert to $T_{eff}$ cells with high diabetic potential [300].




## FIGURE 4. The role of IL-2 in the progression of T1D.

The onset of diabetes coincides with a progression from peri-insulitis to destructive insulitis. At the onset of insulitis, the production of IL-2 declines, resulting in a waning of $T_{reg}$ cell functions. Consequently, diabetogenic T cells expand in the pancLN and traffic to the pancreas where they proceed to destroy the insulin-producing β-islets. These autoreactive T cells may also be refractory to $T_{reg}$ cell-mediated suppression and override their inhibition. These compounding factors may cooperate to precipitate the onset and progression of autoimmune diabetes. Adapted from Sgouroudis *et al.*, 2009 [86].

## *11.* Peripheral homeostasis of $T_{reg}$ cells in NOD mice.

### a. DC-mediated costimulation in $T_{reg}$ cell development and homeostasis.

Thus far, we have explored the importance of IL-2 signals in the competitive fitness of $T_{reg}$ cells. However, we must not neglect the pivotal role of costimulation in promoting $T_{reg}$ cell development and homeostasis by DC, the only APC capable of promoting $T_{reg}$ cell functions [153]. Interestingly, $T_{reg}$ cells have a greater requirement for costimulation relative to $T_{eff}$ cells, apparent from studies where the B7/CD28 pathway is disrupted, which leads to a marked decrease in $T_{reg}$ cell numbers [153, 154, 345]. Interestingly, germline ablation of CD40, a TNF family member, in NOD animals results in reduced numbers of $T_{reg}$ cells, which are not functional when tested *in vitro* [346]. Moreover, CD40L deficiency in NOD mice was shown to hinder the priming of anti-islet $CD8^+$ T cells and this defect was associated with the inhibition of DC maturation, resulting in T1D [157].

### b. The impact of dysregulated ICOS signalling on $T_{reg}$ cell homeostasis.

Clearly, costimulation plays a crucial role in self-tolerance, by influencing the development, function or homeostasis of $CD4^+Foxp3^+$ $T_{reg}$ cells. ICOS is the newest candidate involved in the regulation of autoimmune disorders, as it plays an important role in providing complementary signals to help fine-tune the effector function of $T_{reg}$ cells [347]. Individuals with ICOS deficiency, a rare disease in humans, exhibited reduced levels of $T_{reg}$ cells [348]. In accordance with this finding, ICOS and ICOS-L deficient mice also sustained drastic decrease in the proportion of $T_{reg}$ cells, suggesting that ICOS-mediated costimulation contributes to tolerance by promoting $T_{reg}$ cell

homeostasis [349, 350]. Also, $ICOS^+$ $T_{reg}$ cells display a strict propensity to undergo rapid apoptosis in culture unless signalled by ICOS-L [351]. Lastly, ICOS/ICOS-L interaction seems to be mandatory for anergy induction by tolerogenic DC in naïve human T cells [352]. Taken together, ICOS/ICOS-L interactions play a role in suppression of potentially harmful immune responses.

### *i. The role of ICOS signalling in T1D.*

Studies using ICOS-deficient mice have shown that ICOS is important for optimal IL-2 production, as $ICOS^{-/-}$ T cells exhibit defective activation and produce markedly reduced levels of IL-2 [347, 353]. However, these findings have been contested [354]. T cells activated in the presence of ICOS-L deficient APC [355] also exhibit reduced proliferation and IL-2 production, indicating that ICOS/ICOS-L interactions are essential for proper T cell functions [356]. Conversely, in addition to the TCR signal, IL-2 can further enhance ICOS expression [357], pointing to a positive feedback mechanism between ICOS and IL-2 production.

It has become increasingly clear that CD28 engagement and IL-2 expression are essential to maintain a functional $T_{reg}$ cell compartment in the NOD mouse, as demonstrated by the disruption of the B7/CD28 pathway or IL-2 neutralization studies [153, 154, 342]. Although CD28-dependent co-stimulation and IL-2 act in concert to promote $T_{reg}$ cell functions in the NOD, the impact of ICOS on the engagement of the $T_{reg}$ cell signature suppressive program could not be neglected. Given the importance of IL-2 in $T_{reg}$ cell functions and the positive feedback mechanism between IL-2 and ICOS

expression, it stands to reason that ICOS may participate in $T_{reg}$ cell homeostasis, survival or functions, in synergy with IL-2.

Curiously, genetic ablation or neutralization of ICOS in mice with a polyclonal repertoire (NOD) or monoclonal TCR repertoire (BDC2.5) yields drastically different clinical outcomes. For example, genetic deletion of ICOS in NOD mice resulted in T1D resistance, with no alterations in $CD25^{+}Foxp3^{+}$ $T_{reg}$ cell frequencies [358]. Consistently, ICOS blockade in NOD mice promoted disease reversal [359]. These studies confirm that the priming phase of autoreactive diabetogenic polyclonal T cells is dependent on ICOS costimulation. In stark contrast, ICOS signals are dispensable for the effector phase of autoreactive T cells, as ICOS blockade in overtly diabetic animals did not impact the kinetics of disease progression [359]. This indicates a role for ICOS in early activation events, suggesting that the timing of ICOS costimulation dictates the outcome of T1D pathogenesis. In line with this evidence, ICOS blockade was ineffective in preventing the destruction of islet allograft in the NOD mouse with established autoimmunity, due to sustained autoimmune responses [359]. In stark contrast to the disease phenotype seen in the NOD, targeting the ICOS pathway in the TCR transgenic BDC2.5 neonates resulted in accelerated diabetes phenotype, due to a decrease in the proportion of $T_{reg}$ cells directly within the lesion [360]. However, ICOS blockade at later time-points did not precipitate disease [360]. Taken together, these studies highlight the importance of understanding how ICOS regulates T cell responses during different phases of an immune response.

### *ii. IL-10 as a mechanism of ICOS-mediated self-tolerance in $T_{reg}$ cells.*

ICOS signalling has been linked to the induction of IL-10 secretion by $CD4^+$ T cells [361]. IL-10 was first identified as a product of Th2 cells that suppressed the differentiation and effector functions of Th1 cells [361]. IL-10 also exerts its immunosuppressive properties on DC and MΦ by down-modulating MHC class II and their B7 costimulatory markers, and in turn, limiting their priming capacity [361]. IL-10 blocks the initiation and continuation of the immune response by blocking both the innate and adaptive arms of immunity [361]. Thus, the main role of IL-10 is the containment inflammatory responses in order to minimize tissue damage in response to pathogens [361].

Over the years, various experimental systems have resulted in a great disparity on the effects of IL-10 in diabetogenesis in the NOD mouse. The administration of IL-10 mAb or exogenous IL-10 in NOD mice prevented insulitis [362, 363]. Systemic exposure of exogenous IL-10 during the effector phase of diabetes also inhibited disease, suggesting that IL-10 secretion diminishes as disease progresses [364, 365]. Consistently, blockade of the IL-10 receptor accelerated the onset of diabetes, suggesting that IL-10 signalling is a requirement for the containment of the diabetogenic response [363]. Surprisingly, the genetic ablation of IL-10 on the NOD background did not alter the kinetics of diabetogenesis [109]. Conversely, the overexpression of IL-10 in in the insulin-producing β cells in accelerated diabetes in NOD mice [366, 367], due to the induction of ICAM on pancreatic vascular endothelium, leading to the extravasation of autoreactive T cells to the inflamed sites [368].

The relevance of IL-10 in the progression of autoimmune disorders resurfaced when ICOS signalling was found to be linked to the induction of IL-10 secretion by $CD4^{+}Foxp3^{+}$ $T_{reg}$ cells in a model of allergen-induced airway hypersensitivity and T1D [360, 369]. Both groups suggested that ICOS-mediated IL-10 expression by $T_{reg}$ cells represented a mechanism of peripheral self-tolerance [360, 369]. IL-10 transcripts were highly expressed by $T_{reg}$ cells [360]. This suggested that $T_{reg}$ cells regulate autoimmune development in an ICOS-dependent manner directly in the prediabetic lesion through IL-10 production [360]. This does not preclude the possibility that IL-10 results in the induction of IL-10 producing Tr1 cells, which are often indistinguishable from thymic-derived $nT_{reg}$ cells. Also, whether ICOS expression induces IL-10 secretion in $T_{reg}$ cells or simply enhances the output of already differentiated IL-10 producing $T_{reg}$ cells remains to be elucidated [361]. Thus, many facets of IL-10 biology (kinetics of IL-10 secretion, the most important T cell and non-T cell IL-10 sources, IL-10 as a susceptibility gene) and its link to ICOS signalling in the NOD mouse model are largely unknown.

## *12.* Genetic determinants influencing T1D in the NOD mouse: the case for *Idd3*$^{B6}$ locus.

### a. The *Idd3*$^{B6}$ locus confers T1D protection in the NOD mouse.

To investigate the contribution of IL-2 in T1D susceptibility, the NOD.B6 *Idd3* congenic mouse model was established [76, 370]. The *Idd3*$^{B6}$ genetic interval, introgressed on the NOD background, harbors *Il2* allelic variants from a T1D-resistant mouse (C57BL/6) and exhibits 80% T1D-protection from diabetes [76, 370]. *Idd3*$^{B6}$ has been identified as a susceptibility locus for several autoimmune diseases, highlighting

the impact of IL-2 in self-tolerance [76, 370, 371]. Given the importance of IL-2 in $T_{reg}$ cell development, homeostasis and metabolic fitness of $T_{reg}$ cells discussed in the previous chapter, it stands to reason that one of the underlying mechanisms of T1D-resistance of the NOD.B6 *Idd3* mice is due to differential expression of IL-2 and the consequent impact on $T_{reg}$ cells. Multiple single nucleotide polymorphisms (SNPs) were identified within the *Il2* candidate gene, which segregate with susceptibility to diabetes [78, 372]. It was therefore hypothesized that polymorphisms that affect critical DNA regulatory elements and transcription of IL-2 may contribute to susceptibility and resistance to autoimmune disease by modifying the genesis, function and/or recruitment of $T_{reg}$ cells either at the level of the target organ or the draining lymph node [78].

### b. IL-2 SNPs account for susceptibility to T1D.

The *Idd3*$^{B6}$ locus was mapped to a 0.35-cM interval on proximal mouse chromosome 3 between the microsatellite markers D3Nds55 and D3Nds40b [372], further narrowed to 0.15cM 780kb interval [76] and 650kb [78]. The *Idd3*$^{B6}$ locus contains five known genes: *Il2*, *testis nuclear RNA-binding protein* (*Tenr)*, *Il21*, *Centrin 4* (*Cetn4)* and *Fibroblast growth factor 2* (*Fgf2)*, two predicted genes of unknown function (KIAA1109 and KIAA1371) and three pseudogenes [76].

Thirty-three disease-associated SNPs were identified in the 5' end of *Il2* [78, 372]. This 5' region of the *Il2* has locus control region-like activity that determines the competency of a cell to express *Il2* mRNA. Upon TCR-mediated activation, the 5' region of the *Il2* gene normally undergoes epigenetic changes during activation that regulate accessibility and recruitment of the transcription machinery. The SNPs identified within

the 5' region were suggested to alter chromatin accessibility negatively in NOD mice, as the IL-2 promoter is transcriptionally less active and correlates with decreased levels of IL-2 production in wild-type mice relative to T1D-resistant NOD.B6 *Idd3* [78]. Based on this elegant genetic study, two possible mechanisms were proposed to explain *Idd3*$^{B6}$-mediated T1D protection [78]. The first one involves faster transcription of *Il2* on a per cell basis owing to improved accessibility of transcription factors [78]. The other one rests on an increase in the fraction of cells that produce IL-2 owing to a reduction in the threshold of remodeling required for transcription [78]. In any case, the effect of *Idd3*$^{B6}$ is dose dependent rather than dominant or recessive, as heterozygote NOD$^{BL/6\ IL\text{-}2/NOD\ IL\text{-}2}$ mice present with an intermediate T1D phenotype [78].

Recently, 9 SNPs and 2 deletions were discovered upstream of the minimal IL-2 promoter that controls IL-2 expression and are candidates for the autoimmune disease susceptibility locus *autoimmune ovarian dysgenesis/Idd3/experimental allergic encephalomyelitis* (*Aod2/Idd3/Eae3*) [373]. Seven of the nine SNPs present in the proximal region of the minimal promoter were shown to regulate the enhanced transcriptional activity of the IL-2 promoter [373]. Differences in AP-1 binding proximal to and outside of the minimal promoter contribute to differences in IL-2 expression of $CD4^+$ T cells in resistant and autoimmune strains [373]. Overall, this suggests that the NOD haplotype of *Il2* SNPs predisposes to organ-specific autoimmunity by reducing IL-2 production by antigen-specific autoreactive T cells [373]. This correlates with the impairment of $T_{reg}$ cell functions, which are critically dependent on paracrine sources of IL-2.

One of the polymorphisms, the presence of a proline rather than serine at position 6 of the mature *Idd3*$^{B6}$ IL-2 protein, was associated with increased glycosylation, stabilizing the half-life of IL-2 [77]. However, the NOD.CZECH *Idd3*$^{B6}$ mouse, whose glycosylation pattern is identical to that of its wild-type counterpart, was T1D-resistant, suggesting that glycosylation differences do not account for the molecular basis of *Idd3*$^{B6}$ [78].

### c. The impact of the *Idd3*$^{B6}$ locus on the innate system.

The differential expression of IL-2 owing to the *Idd3*$^{B6}$ locus may also impact NK cells, because IL-2 augments their cytolytic activity by increased IFN-$\gamma$ production [374]. Indeed, one study claims that the protective effect of the *Idd3*$^{B6}$ genetic interval was almost solely attributed to CD11b$^{+}$CD11c$^{-}$ macrophage-mediated costimulation [375]. This study only focused on *in vitro* suppressive assays, and did not corroborate the findings *in vivo* [375]. Furthermore, this is the first study to claim that macrophages can activate $T_{reg}$ cell functions in the NOD mouse model and to lessen the importance of $T_{eff}$ cell-derived IL-2 on $T_{reg}$ cell fitness [375].

### d. IL-2 is an inhibitory factor for the differentiation of Th17 cells.

So far, the pleiotropic functions of IL-2 presented herein are bountiful and include immune contraction by the mechanism of AICD or through the maintenance of $T_{reg}$ cells, immune activation via clonal activation/expansion/survival of pathogen-specific or autoimmune CD4$^{+}$ or CD8$^{+}$ $T_{eff}$ cells [237, 247, 376]. Recently, IL-2 was also shown to inhibit the differentiation of Th17 cells, as disruption of IL-2 signalling by genetic ablation

or blockade of IL-2 or STAT5 results in elevated levels of Th17 cells [377]. Therefore, the action of IL-2 is two-fold in that it simultaneously blocks inflammation by preventing the generation and expansion of the highly inflammatory Th17 cells, while increasing the number of suppressive $T_{reg}$ cells [377]. Given the inhibitory effect of IL-2 on Th17 cell differentiation, it stands to reason that enhanced IL-2 levels in NOD.B6 *Idd3* animals may result in T1D-resistance due to, not only more potent $T_{reg}$ cell suppressors, but also to a significant decrease in the Th17 cell subset.

### e. The role of Th17 cells in diabetogenesis.

However, little information exists on the relative contribution of Th17 cells in T1D. Recent data showed increased IL-17 transcripts with age in NOD mice, suggesting that IL-17 correlates with established insulitis and diabetes [378]. Therapeutic reversal of T1D in the NOD mouse was associated with a decrease in Th17 cells, suggesting that Th17 cells play a pathogenic role in T1D [379]. Blockade of IL-17 or IL-25, an inhibitory factor of Th17 cells, during the effector phase of T1D pathogenesis resulted in protection, by decreasing intra-islet resident Th17 cells and enriching dominant $Foxp3^+$ $T_{reg}$ cells [380]. Monocytes in the blood of human diabetic patients spontaneously secrete IL-1$\beta$ and IL-6, cytokines important in Th17 cell induction [381]. Overall, Th17 cells seem to play a role in T1D pathogenesis, although they are not the predominant destructive T cell subset.

### f. SNPs within the *Il2RA* gene, not Il2, contribute to human T1D.

To date, no association between SNPs within the *Il2 gene* and human T1D susceptibility was made [382]. While IL-2 is not a susceptibility locus in human T1D, T1D susceptibility is associated with polymorphisms contained within the *Il2RA* (*Cd25*) gene, suggesting an important role for IL-2 signalling in human T1D pathogenesis [24]. It must be noted that this finding has been contested [382]. A recent study demonstrated that SNPs at the *Il2RA* locus were a major determinant of the age of diagnosis of Finnish T1D patients [383]. Surprisingly, the impact of the *Il2RA* SNPs had an effect at par with the HLA-DQ2/DQ8 genotype [383]. This is the first study to suggest that the *IL2RA* locus may control the susceptibility to disease and its time of occurrence [383]. Interestingly, polymorphisms within the *Il*2RA (*Cd25*) gene are associated with multiple autoimmune disorders such as MS, Grave's disease, autoimmune thyroid disease and juvenile idiopathic arthritis, reinforcing the pivotal role of IL-2 signalling in self-tolerance [28, 384-386].

Taken together, although the *Idd* congenic mice represent a prototypic model that allows for the dissection of the impact of individual genes on T1D pathogenesis, it is not all-encompassing. Overall, the *Idd3*$^{B6}$ locus is a strong genetic tool that links IL-2 as a pro-fitness factor to $T_{reg}$ cell functions.

## *13.* Immunotherapy.

### a. Exploiting $T_{reg}$ cells to cure T1D.

Although daily insulin therapy has extended the life expectancy and enabled the management of an otherwise life-threatening disease, diabetic complications still arise [387]. Furthermore, the incidence of T1D worldwide is rising at an alarming rate [387]. For example, in the UK and China, the increase in the incidence rate has reached over 70% per year in children under 14 years of age, highlighting the importance of developing therapeutic strategies [387]. The hope for such therapeutic avenues ideally is to reset immunoregulation in new-onset T1D subjects in order to halt the destruction of remaining β cells [387]. The β cells may then be given a chance to recover function and hopefully stop disease progression [387, 388].

This goal can be achieved by immune-based therapies such as potentiation of $T_{reg}$ cell function *in vivo* or by *in vitro* conditioning prior to infusion into affected individuals at risk or with overt disease [388]. Promising results in the NOD mouse model suggest that this is a feasible therapeutic approach. For example, naïve islet-specific $CD4^+Foxp3^-$ $T_{eff}$ cells can be induced into islet-protective $CD4^+Foxp3^+$ $T_{reg}$ cells, when expanded by DC in the presence of TGF-β [389]. A potential risk associated with reinfusion of *ex vivo* expanded or $iT_{reg}$ cells in T1D patients is the possibility of conversion of $T_{reg}$ cells to $T_{eff}$ cells [300]. A better approach is the antigen-specific $T_{reg}$ cell expansion *in vitro* by various experimental methods [390-393]. The reinfusion of these expanded $T_{reg}$ cells potently protected and even reverted T1D [390-393]. $T_{reg}$ cells from diabetic patients can be expanded and retain their functional capacities, suggesting that cell therapy is a possibility in the treatment of T1D [329].

Another method to promote $T_{reg}$ cell mediated T1D protection was revealed by the administration of CD3-specific antibodies in NOD mice [394]. Regeneration of the β cell mass was observed and correlated with increased $Foxp3^+$ $T_{reg.}$ cell numbers. Anti-CD3 mAb treatment alters the $T_{reg}/T_{eff}$ cell balance through activation-induced cell death of $T_{eff}$ cells and inactivation of Th1 cell subsets [395, 396], while sparing $T_{reg}$ cells and actually increasing their numbers in a TGF-β dependent manner [397]. Despite the unknown mechanism of β cell regeneration, this method reached clinical trials. In humans administration of hOKT3γ1 (a humanized Fc mutated anti-CD3 monoclonal antibody) halted disease progression for up to five years [398, 399].

### b. Exploiting DC to promote $T_{reg}$ cell immunoregulatory functions.

The capacity of DC to shape T cell responses makes them of great clinical interest, as immature DC expressing low levels of costimulation can induce anergy or tolerance [112]. DC provide an appealing target for manipulating β cell autoimmunity, as a growing body of evidence demonstrates that manipulating DC function may result in diabetes protection in the NOD mouse, either by skewing the destructive Th1 response to a protective Th2 response or by promoting $T_{reg}$ cell functions [86]. In the NOD, $CD4^+CD25^+CD62L^+$ $T_{reg}$ cells expanded by antigen-loaded DC were able to protect and even restore normoglycemia in overtly diabetic animals [391]. Thus DC transmit the proper signals to $T_{reg}$ cells to render them suppressive, even in a highly inflammatory environment. Beta-islet antigen pulsed DC, in the presence of TGF-β, can induce islet-protective $CD4^+Foxp3^+$ $T_{reg}$ cells from naïve islet-specific $CD4^+Foxp3^-$ $T_{eff}$ cells, suggesting that DC have an impact on the *de novo* generation of $T_{reg}$ cells in the

periphery [389]. Furthermore, these $iT_{reg}$ cells potently protected syngeneic islet grafts in overtly diabetic NOD mice, providing a potential therapeutic strategy for the remission of destructive autoimmunity [391, 392]. These treatments were most effective when administered at early stages of disease. The efficiency of the treatment is unknown long after the onset of clinical diabetes,

Another approach that has proven to be effective in T1D protection is the transfer of bone marrow-derived DC (BMDC) or *ex vivo* peptide-pulsed DC, which correlates with enhanced $T_{reg}$ cell frequencies [400]. Therefore, it stands to reason that using peptide-pulsed DC as an antigen-specific therapeutic approach to prevent or reverse autoimmunity by potentiating $T_{reg}$ cell functions and inducing tolerance is feasible [388]. The administration of conditioned “regulatory” DC to T1D patients has reached clinical trials, although no preliminary results are available [401].

However, there are many unresolved issues limiting widespread clinical application of $T_{reg}$ cell therapy for human T1D. First and foremost, diabetes develops even in the presence $T_{reg}$ cells [401]. Hence, a more rigorous examination of the inflammatory milieu is required to determine which factor(s) potentially inhibit $T_{reg}$ cell functions. Other issues include ill-defined mechanism(s) underlying $T_{reg}$ cell-mediated suppression, the divergent observations between *in vitro* versus *in vivo* findings performed in mice and humans, and the risk of unwanted suppression towards infectious pathogens and tumours. Furthermore, the unreliable nature of current markers of $T_{reg}$ cells in inflammatory contexts often confounds the analysis of many studies. The discovery and validation of more specific extracellular biomarkers for $T_{reg}$

cells, other than CD25, is imperative, as this will undeniably facilitate the ability to monitor $T_{reg}$ cellular frequency and function in the context of T1D [131]. This will enable the effectiveness of novel therapeutic strategies that modulate $T_{reg}$ function *in vivo* [131].

## Rationale, Hypothesis and Objectives

T1D is a T cell-dependent autoimmune disease characterized by the destruction of the insulin-producing β islet cells of Langerhans in the pancreas, leading to hyperglycemia and long-term complications such as nephropathy and neuropathy. NOD mice, which spontaneously develop T1D, exhibit a lag time between the establishment of insulitis and overt clinical T1D. The over-arching hypothesis of my thesis is that the intercellular dynamics and the inflammatory context within the β-islet microenvironment influence the global stability of $Foxp3^+$ $T_{reg}$ cell function, and subtle deficiencies in $T_{reg}$ cell function tips the balance towards T1D progression.

There are 4 general questions/objectives to this thesis:

***1. Is there a loss of $Foxp3^+$ $T_{reg}$ cell function in T1D?*** Many convincing studies demonstrate that immunoregulatory circuits, notably $CD4^+Foxp3^+$ $T_{reg}$ cells, represent a critical checkpoint in the pathogenesis of T1D. To date, it is unclear whether T1D progression is due to a decline in the frequency and/or suppressive functions of $T_{reg}$ cells or from the accumulation of $T_{reg}$ cell-resistant diabetogenic $T_{eff}$ cells. Maintenance of tolerance coincides with the capacity of $Foxp3^+$ $T_{reg}$ cells to home, accumulate and proliferate within the target organ. Convincing evidence from many groups, including our own, suggests that the underlying mechanism in the development of T1D can be imputed to an age-related loss of $T_{reg}$ cell expansion/survival in the β islets. As a result,

the $T_{reg}/T_{eff}$ cell balance is disrupted and unleashes the diabetogenic $T_{eff}$ cells, which proceed to destroy the β islets. *As a first step, we propose to examine the mechanisms that lead to the switch from tolerance to immunity by investigating potential variations in $T_{reg}$ cell development and function and attempt to correlate variations in $T_{reg}$ cell activity with T1D onset, incidence and severity.*

***2. What is the impact of Il2 allelic variation on $T_{reg}$ cell homeostasis and function?*** A growing body of evidence strongly demonstrates that IL-2 is an important signal for $T_{reg}$ cell fitness in the peripheral compartment, suggesting that alterations in IL-2 signalling may attenuate $T_{reg}$ cell function and provoke autoimmunity. At the time of insulitis, there is a well-documented dip in IL-2 production by the autoreactive $T_{eff}$ cell pool, which may negatively affect $T_{reg}$ cell fitness, and in turn lead to a break-down in self-tolerance. NOD.B6 *Idd3* or $CD4^+$ TCR transgenic BDC.*Idd3* mice, introgressed with the protective $Idd3^{B6}$ locus, show a marked resistance to T1D onset, incidence and severity. Interestingly, the *Il2* gene is the primary candidate for $Idd3^{B6}$-mediated T1D protection. We hypothesized that the $Idd3^{B6}$ locus mediates T1D protection by promoting $T_{reg}$ cell functions. *Therefore, the second objective of this project was to determine whether the $Idd3^{B6}$ genetic interval impacted $T_{reg}$ cell development and function, and whether this could be related to the disease resistance observed in NOD.B6 Idd3 mice.*

***3. Does ICOS maintain the functional stability of $Foxp3^+$ $T_{reg}$ in pre-diabetic β-islets?*** B7 or CD28 deficient NOD mice have reduced $T_{reg}$ cell numbers and manifest a more aggressive form of T1D than control littermates. This strongly suggests that $T_{reg}$ cells have a heightened requirement for costimulation relative to $T_{eff}$ cells. Although

CD28-dependent costimulation and IL-2 act in concert to promote $T_{reg}$ cell functions in NOD and $CD4^+$ TCR transgenic BDC2.5 mice, costimulatory molecules downstream of IL-2 signalling may engage the $T_{reg}$ cell signature suppressive program. Indeed, a positive feedback loop between IL-2 and ICOS, a recently identified member of the CD28 superfamily, has been documented and ICOS has been linked to various autoimmune disorders, including T1D. Indeed, ICOS blockade in BDC2.5 mice results in accelerated diabetes due to a decrease in $T_{reg}$ cell frequencies/functions. We hypothesize that ICOS signalling in $Foxp3^+$ $T_{reg}$ cells favors their IL-2-dependent proliferation and suppressive function by instructing their differentiation for IL-10 secretion within the target organ. *Our third objective was to evaluate the impact of ICOS on $T_{reg}$ cell functions, to determine the role of IL-2 in promoting the stability of $Foxp3^+$ $T_{reg}$ cell function in vivo, and to relate these pathways to T1D progression.*

***4. Are $Idd3^{B6}$ T1D-protective genes operative in $CD11c^+$ DC and do they contribute to tolerance in vivo?*** Interestingly, $T_{reg}$ cells can actively expand and mediate antigen-specific suppression after antigen presentation by DC both *in vitro* and *in vivo*, suggesting DC provide requisite signals for the potentiation of $T_{reg}$ cell functions. A growing body of evidence shows that NOD DC posess intrinsic DC defects, which may contribute to disease pathogenesis. Currently, the functional contribution of DC in $T_{reg}$ cell-mediated resistance to organ-specific autoimmunity is ill-defined. We hypothesize that the protective *Il2* allelic variants of the $Idd3^{B6}$ locus confer T1D protection by enhancing the tolerogenic properties of DC, which in turn promote $T_{reg}$ cell function and expansion. *Therefore, the last aim of this project was to characterize the capacity of $CD11c^+$ DC to prime and promote antigen-specific T cell responses in vitro*

*and in vivo.*

## CHAPTER II *Functional waning of naturally occurring CD4$^{+}$ regulatory T-cells contributes to the onset of autoimmune diabetes.*

## Bridging statement from Chapter I to II

Functional abrogation of $T_{reg}$ cells or targeted deletion of the *Foxp3* gene leads to accelerated diabetes, strongly implying a role for $Foxp3^+$ $nT_{reg}$ cells in the control of T1D pathogenesis. Although $T_{reg}$ cells were shown to represent a critical control point in T1D pathogenesis, many aspects of $T_{reg}$ cell functions remained unanswered. We wondered at which anatomical location (pancLN versus pancreas) and stage of islet-specific autoreactive $T_{eff}$ cell priming (activation/differentiation/homing to the target organ) $T_{reg}$ cells exerted their suppression. In this study, we also sought to examine whether quantitative or qualitative deficiencies in $CD4^+Foxp3^+$ $nT_{reg}$ cells leads to a failure to control the onset of T1D.

# Functional waning of naturally-occurring CD4$^+$ regulatory T cells contributes to the onset of autoimmune diabetes.

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**Running title:** Functional loss in CD4$^+$Foxp3$^+$ $T_{reg}$ cells drives T1D.

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## 1. Abstract.

**Objective:** In this study, we asked whether a possible quantitative or qualitative deficiency in naturally-occurring $Foxp3^+CD4^+$ regulatory T ($nT_{reg}$) cells, which display potent inhibitory effects on T cell functions *in vitro* and *in vivo*, may predispose to the development of T1D.

**Research Design and Methods:** We assessed the frequency and function of $Foxp3^+$ $nT_{reg}$ cells in primary and secondary lymphoid tissues in the NOD animal model of type 1 diabetes.

**Results:** We show that the cellular frequency of $Foxp3^+$ $nT_{reg}$ cells in primary and secondary lymphoid tissues is stable and does not decline relative to T1D-resistant mice. We show that thymic and peripheral $CD4^+CD25^+$ T cells are fully functional *in vivo*. We also examined the functional impact of $CD4^+Foxp3^+$ $nT_{reg}$ cells on the development of autoimmune diabetes, and demonstrate that $nT_{reg}$ cells do not affect the initial priming or expansion of antigen-specific diabetogenic T cells in pancreatic lymph nodes (pancLN), but regulate later events of diabetogenesis by preferentially localizing in the pancreatic environment where they suppress the accumulation and function of effector T ($T_{eff}$) cells. Finally, we show that $nT_{reg}$ cell potency appears to decline with age, as demonstrated by the absence of regulation during late events of diabetogenesis.

**Conclusion:** This study demonstrates that Foxp3-expressing $nT_{reg}$ cells in NOD mice regulate diabetogenesis, but temporal alterations in $nT_{reg}$ cell function promote immune dysregulation, and the onset of spontaneous autoimmunity.

## 2. Introduction.

NOD mice are characterized by spontaneous development of several autoimmune diseases including T1D, which occurs progressively through a T cell dependent infiltration and destruction of insulin-producing beta ($\beta$) islet cells of Langerhans, leading to insulin deficiency [1-4]. The extended time interval between establishment of insulitis and onset of disease suggests a progressive loss of peripheral regulatory mechanisms in prediabetic NOD mice prior to disease progression [1].

$CD4^+$ naturally-occurring $T_{reg}$ ($nT_{reg}$) cells have emerged as the predominant regulatory population mediating peripheral self-tolerance [5-7]. The majority of these cells constitutively express the IL-2R $\alpha$ chain (CD25), and represent 1-10% of thymic or peripheral $CD4^+$ T cells in mice and man [8-9]. Functional abrogation of $nT_{reg}$ cells increases immunity to tumors, allografts and pathogens, and results in the development of multi-organ-specific autoimmunity by an as-of-yet undefined mechanism [6]. Recent studies show that $nT_{reg}$ cells constitutively and specifically express the Foxp3 transcription factor marker, a critical molecular switch for $nT_{reg}$ cell development and function [10, 11]. Targeted deletions or natural mutations of the *Foxp3* gene leads to a deficiency of $nT_{reg}$ cells, and provokes the development of severe autoimmunity in Scurfy in mice and IPEX patients [10-13].

Several studies have implicated $nT_{reg}$ cells in prevention of T1D. Depletion of CD25-expressing T cells or disruption of the B7/CD28 pathway in NOD mice has been shown to decrease $CD4^+CD25^+$ $nT_{reg}$ cell frequency, and ultimately leads to an accelerated T1D onset [14, 15]. Other studies have correlated T1D resistance in aged

NOD mice with the expansion of CD25-expressing $CD4^+$ T cells with regulatory activity within inflamed pancLN [16]. Although $T_{reg}$ cell function has been examined in lymphoid tissues of pre-diabetic and insulitic NOD mice, it is unclear whether these $CD4^+CD25^+$ T cells are thymus-derived $nT_{reg}$ or inflammation-induced $T_{reg}$ cells [17]. Recently, Chen *et al.* reported that Foxp3-deficient NOD mice display a significantly increased incidence and earlier onset of T1D compared to normal NOD mice, strongly implying a role for $Foxp3^+$ $nT_{reg}$ cells in the control of T1D pathogenesis [18, 19]. Some central question remains: How and where do $nT_{reg}$ cells mediate tolerance to β-islet antigens and disease protection? Also, does the spontaneous onset of T1D in NOD mice reflect developmental or functional deficiencies in $nT_{reg}$ cells, consequently tipping the balance towards the activity of diabetogenic T cells and clinical T1D [18, 20, 21]?

In this study, we examined whether quantitative or qualitative deficiencies in $CD4^+Foxp3^+$ $nT_{reg}$ cells leads to a failure to control the onset of T1D. We show that the frequency of $nT_{reg}$ cells in primary and secondary lymphoid tissues is stable and does not decline relative to T1D-resistant mice. We demonstrate that thymic and peripheral $nT_{reg}$ cells from neonatal NOD mice are fully functional *in vivo*, and dramatically halt the onset of primary and established T1D. We show that $nT_{reg}$ cells affect neither the priming nor the expansion of antigen-specific diabetogenic T cells in pancLN, but regulate late events of the diabetogenic process by localizing in draining LN and pancreatic lesions, where they suppress the accumulation and function of diabetogenic $CD4^+$ T cells. Finally, we show that $nT_{reg}$ cell potency appears to decline with age. In summary, we show that qualitative, and not quantitative alterations in $Foxp3^+$ $nT_{reg}$ cells in NOD mice drive immune dysregulation, and the spontaneous onset of T1D.

## 3. Research design and methods.

***Mice.*** NOD, C57BL/6, NOD.TCR$\alpha^{-/-}$ and NOD.BDC2.5 mice were bred and maintained in pathogen-free conditions at the McGill University animal facility. NOD.TCR$\alpha^{-/-}$ and NOD.BDC2.5 TCR mice were initially a kind gift from Dr. Christophe Benoist (Harvard Univ., Boston, USA).

***Phenotypic analysis of CD4$^+$ T cells.*** T cells were stained with a variety of fluorochrome-conjugated or biotinylated mAbs, as previously described [22;23]: anti-CD4 (clone RM5), anti-CD25 (clone PC61.5) (eBioscience, San Diego, CA), anti-V$\beta$4 (clone CTVB4), anti-CD69 (clone H1.2F3), anti-CD44 (clone IM7), anti-CD62L (clone MEL14) (BD Bioscience, Mississauga, Ontario), and anti-Foxp3 (eBioscience, San Diego, CA). Stained cells were acquired on a FACSCalibur flow cytometer (BD, San Jose, CA). In adoptive transfer experiments, pancreata were digested with collagenase-V (Invitrogen, Burlington, Ont.), and T cells were separated from the digested tissue by centrifugation on a Lympholyte-M gradient (Cederlane), and then stained accordingly.

***Purification of CD4$^+$ T cell subsets*.** CD4$^+$ T cell subsets were isolated from spleen, thymus or lymph nodes on a FACSAria (BD, San Jose, CA) or AutoMACS (Miltenyi Inc, Auburn, CA) cell sorter as previously described [22;23]. The final purity was assessed on a FACSCalibur (BD, San Jose, CA), and was routinely over 98%.

***Intracellular cytokine production.*** Cells from the spleen and pancLN were stimulated 4-5 hr with PMA and ionomycin, and were treated with monensin for the last 2-3 hr of culture. After surface staining, cells were fixed and permeabilized, and then intracytoplasmic staining was performed using anti-mouse IL-2 mAb (JES6-5H4), anti-

mouse TNF-α mAb (MP6), or PE-labeled rat IgG1 isotype control (PharMingen, San Diego, CA). Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest and FlowJo software.

***Adoptive cell transfers.*** Purified $CD4^+$ T cell subsets were transferred intravenously, either alone or in combination, into recipient mice ($1–3x10^5$/mouse). In some experiments, donor T cells were labeled with CFSE to assess cell proliferation *in vivo*, as previously shown [24].

***Diagnosis of diabetes.*** Recipient mice were tested every 2-3 days for diabetes, as previously described [25]. Overt diabetes was defined as glycemia >300 mg/dl. Haemoglukotest kits were kindly provided by Roche Diagnostics (Laval, Canada).

***Histologic analysis.*** H&E stained histologic slides of pancreatic tissues were prepared and graded for insulitis as previously described [14, 25, 26]. Three randomly obtained levels of pancreas were analyzed in double-blind fashion and 12-15 islets per section were scored per time point. Statistical analysis was performed with the $x^2$ test.

## 4. Results.

### Normal thymic $CD4^+$ $nT_{reg}$ cell frequency and function in NOD mice.

Some reports suggested that the ability of $nT_{reg}$ cells to suppress T1D is either absent or poorly developed, thus promoting disease onset [14, 20, 21]. To determine if NOD mice developed $CD4^+Foxp3^+$ $nT_{reg}$ cells in their thymus, the frequency of thymic $CD4^+Foxp3^+$ $nT_{reg}$ cells was assessed in $CD4^{SP}$ T cells in prediabetic NOD and BDC2.5 mice relative to T1D-resistant C57BL/6 (B6) mice. We show that the proportion of $CD4^+Foxp3^+$ $nT_{reg}$ cells represents approximately 1% and 5% of $CD4^{SP}$ T cells in BDC2.5 and NOD mice, respectively, and does not decrease in adult mice (Fig. 1A). Total numbers of $CD4^+Foxp3^-$ or $CD4^+Foxp3^+$ T cells were stable between 10 and 25 days of age, and seemingly increased between 25 to 50 days of age in NOD mice only to reach levels not significantly different from C57BL/6 mice (data not shown). No differences were observed in $CD25^+$ or $CD25^-$ $Foxp3^+$ $T_{reg}$ cell from thymi of prediabetic mice compared to C57BL/6 mice (data not shown).

In order to assess NOD thymic $T_{reg}$ cell function, we transferred $5x10^5$ thymic $CD4^+CD25^-$ $T_{eff}$ cells in the presence or absence of $5x10^4$ thymic $CD4^+CD25^+$ $nT_{reg}$ cells from neonatal NOD mice into NOD.*scid* recipient mice. $CD4^+CD25^-$ $T_{eff}$ cells from pre-diabetic mice transferred diabetes at a 50% incidence 5-6 wks post-transfer, reaching a total incidence of 80-90% at 10 wks post-transfer (Fig. 1B). However, when thymic $CD4^+CD25^+$ $nT_{reg}$ cells were co-transferred, T1D onset and incidence were dramatically reduced (Fig. 1B), and $\beta$ islet cells were preserved (data not shown), suggesting that thymic $nT_{reg}$ cells, which are devoid of potential peripherally-activated $CD25^+$ $T_{eff}$ cells,

are indeed protective in NOD mice. To further assess thymic $nT_{reg}$ cell function in a synchronous, antigen-specific system, similar experiments were performed in the BDC2.5 TCR transgenic transfer model of T1D [26]. To this end, we transferred thymic $CD4^+CD25^-$ $T_{eff}$ cells from pre-diabetic, 3-4 wk old BDC2.5 mice, either alone or in the presence of thymic $CD4^+CD25^+$ $T_{reg}$ cells, into NOD.TCR$\alpha^{-/-}$ recipients. Thymic $CD4^+CD25^-$ $T_{eff}$ cells transferred diabetes as early as 11 days post transfer, with an incidence as high as 60-75% at 30 days post-transfer (Fig. 1B). In contrast, the presence of thymic $nT_{reg}$ cells in transferred $CD4^+$ T cells completely abrogated the onset of disease, and protected from T1D for at least 3-4 weeks post-T cell transfer (Fig. 1B). Thus, $nT_{reg}$ cells develop in the NOD thymus, and can control the onset of T1D.

**Peripheral $CD4^+$ $nT_{reg}$ cells maintain tolerance to β-islet cells in pre-diabetic NOD mice.**

We hypothesized that alterations in the numbers or function of peripheral $T_{reg}$ cells may precipitate T1D. We first enumerated $Foxp3^+$ $T_{reg}$ cells in LN and spleen of prediabetic NOD and BDC2.5 mice of various ages. In NOD mice, $Foxp3^+$ $T_{reg}$ cell represented an average of 16-20% of $CD4^+$ T cells in the spleen and 7-10% in the pancLN with variations between ages not significantly different from T1D-resistant B6 mice (Fig. 2A). Similarly, $nT_{reg}$ cells accounted for 9-11% and 4-7% of $CD4^+$ T cells from spleen and pancLN BDC2.5 mice, respectively (Fig. 2B). The total pool of $nT_{reg}$ cells in pancreatic sites gradually increased with age in both NOD and BDC2.5 mice but was comparable to age-matched B6 mice (data not shown), suggesting that a quantitative defect in peripheral $T_{reg}$ cells does not precede T1D onset.

We then assessed the function of $nT_{reg}$ cells from neonatal NOD mice (7-10d old). To this end, we transferred $5x10^5$ $CD4^+CD25^-$ $T_{eff}$ cells from prediabetic or diabetic mice into NOD.*scid* mice either alone or in the presence of $5x10^4$ $CD4^+CD25^+$ $nT_{reg}$ cells from neonatal, pre-diabetic NOD mice. $CD4^+CD25^-$ T cells transferred diabetes at a 50% incidence 5-6 wks post-transfer, reaching an incidence of 80-90% at 10 wks post-transfer (Fig. 2C). However, when $CD4^+CD25^+$ $nT_{reg}$ cells were co-transferred with either prediabetic (Fig.2C, left panel) or diabetic (Fig.2C, right panel) $CD4^+CD25^-$ $T_{eff}$ cells, T1D onset and incidence were dramatically reduced, and $\beta$ islet cells were preserved (data not shown). Therefore, neonatal $nT_{reg}$ cells are functional and have the potential of protecting NOD mice from primary and established T1D.

**Temporal decline in the function of peripheral $CD4^+Foxp3^+$ $nT_{reg}$ cells in BDC2.5 mice.**

We reasoned that functional changes in the periphery might nevertheless disrupt $nT_{reg}$ cell function and precipitate T1D over time. We evaluated the potential for an age-dependent variation in $nT_{reg}$ cell suppressor function in our BDC2.5 transfer model. $CD4^+CD25^+$ $T_{reg}$ cells were isolated from young or adult BDC2.5 mice, and co-transferred with $CD4^+CD25^-$ $T_{eff}$ cells from BDC2.5 mice into NOD.TCR$\alpha^{-/-}$ mice. We show that while $nT_{reg}$ cells from young BDC2.5 mice completely suppressed $T_{eff}$ cell-induced T1D, $nT_{reg}$ cells isolated from older BDC2.5 animals were completely ineffective at suppressing T1D (Fig. 3A). It was possible that the lack of regulation of T1D was due to the increased frequency of activated T cells among $CD4^+$ T cells. The expression of CD25, CD69 and CD44 activation markers on T cells was not significantly different between 3-4 wk and 6-8 wk old donors, and the cellular frequency of IL-2 or TNF-$\alpha$

secreting Foxp3$^-$ $T_{eff}$ cells was similar between both age groups suggesting that the waning $nT_{reg}$ function in older donors was not attributed to an increased contamination of activated $T_{eff}$ cells within $T_{reg}$ cell preparations (Fig. 3B and C). Furthermore, recipients of 6-8 week old donor $CD4^+CD25^-$ T cells did not demonstrate a higher diabetes incidence compared to 3-4 week old donor cells, excluding the possibility of reduced pathogenicity of younger $T_{eff}$ cells (Fig. 3A). Interestingly, regulation of T1D could be restored if an additional bolus of purified $CD4^+CD25^+$ $T_{reg}$ cells from 6-8 week old donor mice were infused in recipient mice (data not shown), suggesting that an increase in the circulating pool of $nT_{reg}$ cells could ultimately control T1D in older NOD mice. Our results do not exclude the possibility that alterations in the pathogenic potential of $T_{eff}$ cells from older mice may also contribute to T1D onset by heightening their resistance to regulation. Overall, these results suggest that while $nT_{reg}$ cells function normally in the periphery of neonatal NOD mice, the progression beyond insulitis is seemingly due to a time-dependent waning in the functional potency of $T_{reg}$ cells, allowing self-reactive $T_{eff}$ cells to escape regulation and initiate a destructive infiltration of the islets.

**$CD4^+$ $nT_{reg}$ cells do not affect antigen-induced priming of diabetogenic $CD4^+$ T cells in lymphopenic and non-lymphopenic hosts.**

The functional impact of $nT_{reg}$ cells on diabetogenic T cells during the prediabetic period is not thoroughly understood. $CD4^+$ $T_{reg}$ cells may potentially control diabetes progression by inhibiting the activation or clonal expansion of islet-specific $CD4^+$ T cells in the pancLN, altering $T_{eff}$ cell trafficking to the pancreas, or suppressing effector functions *in situ* [18, 19, 27]. To determine whether $nT_{reg}$ cells influence the priming of

diabetogenic T cells, NOD.TCR$\alpha^{-/-}$ recipient mice were transferred i.v. with $10^6$ CFSE-labelled $CD4^+CD25^-$ $T_{eff}$ cells in the presence or absence of $10^5$ $CD4^+CD25^+$ isolated by FACS from peripheral LN of 2-4 week old BDC2.5 mice. At 3 days post transfer, we examined the frequency of activated and proliferating islet-specific $CD4^+$ T cells in pancLN and non-draining LN by FACS, and in the presence or absence of $nT_{reg}$ cells. In this system, transferred $CD4^+$ $T_{eff}$ cells migrate to the pancLN (Fig. 4A), and the activation of $CD4^+V\beta4^+$ $T_{eff}$ cells (CD69 early marker expression) (Fig. 4B; 22-26% versus 3% in non-draining LN), as well as the frequency of proliferating T cells (CFSE dilution) (Fig. 4C; 32-35% versus 7% in non-draining LN), could only be detected in pancLN, confirming that T cell activation is antigen-specific. The frequency of $CD4^+V\beta4^+CD69^+$ T cells (21.6 versus 26%), as well as the frequency of dividing $CD4^+V\beta4^+$ T cells (32.4% versus 35.2%) in the pancLN remained unaffected in the presence of $nT_{reg}$ cells compared to recipient mice transferred with $T_{eff}$ cells alone, suggesting that antigen presentation and proximal TCR signals are not inhibited in $T_{reg}$ cell-protected mice (Fig 4A).

Previous studies have suggested that lymphopenic environments might impair the functional dissection of the mechanism of $nT_{reg}$ cells. To circumvent the possible confounding effect of homeostatic proliferation in our system, similar transfer experiments were also conducted in non-lymphopenic, wild-type NOD recipient mice. Our results show that the frequency of $CD4^+V\beta4^+$ T cells (0.41% versus 0.71%), as well as Ag-driven proliferation of $T_{eff}$ cells in the pancLN was unaffected by the presence of BDC2.5 $T_{reg}$ cells (53.1% versus 53.1%) (Fig. 4D-E), suggesting that $T_{reg}$ cells do not

control the diabetogenicity of $T_{eff}$ cells by altering their homing to draining pancLN, early activation or antigen-driven proliferation.

**Protection from T1D correlates with an increased expansion of $CD4^+Foxp3^+$ $nT_{reg}$ cells in pancreatic sites.**

Recent studies show that $nT_{reg}$ cells localize in sites of inflammation in order to mediate their protective effect [16, 18, 19, 27]. To determine whether $nT_{reg}$ cells home to, and expand within pancLN, NOD.TCR$\alpha^{-/-}$ or WT NOD recipient mice were transferred i.v. with $10^5$ CFSE-labelled $CD4^+CD25^+$ $nT_{reg}$ cells in the presence of $10^6$ $CD4^+CD25^-$ $T_{eff}$ cells isolated from 2-4 week old BDC2.5 mice, and the frequency of proliferating $T_{reg}$ cells in pancLN and non-draining LN was examined 3 days post T cell transfer. The frequency of BDC2.5 $Foxp3^+$ $nT_{reg}$ cells in recipients of $CD25^+$/$CD25^-$ cells was found to be similar in the pancLN and the non-draining LN of both recipients (Fig. 5A and C). The proportion of proliferating $Foxp3^+$ $nT_{reg}$ cells was significantly greater in pancLN than in non-draining LN in immunodeficient (43% versus 13%, respectively; Fig. 5B) and immunocompetent hosts (52.7% versus 20.5%, respectively; Fig. 5D), suggesting that $nT_{reg}$ cells actively expand in pancreatic sites.

As altered early T cell priming events cannot directly explain the protective effect of $nT_{reg}$ cells, we then hypothesized that $nT_{reg}$ cells accumulating in pancreatic sites may have a functional impact on $T_{eff}$ cells at later stages of the diabetogenic process. To this end, $CD4^+$ T cell subsets from thymus of BDC2.5 mice were adoptively transferred into NOD.TCR$\alpha^{-/-}$ mice as described above, and 30 days post-transfer the frequency of $CD4^+Foxp3^-$ $T_{eff}$ and $CD4^+Foxp3^+$ $nT_{reg}$ cells were examined in various LN

and pancreas. Although our results do not show an influence of $nT_{reg}$ cells on the initial rounds of proliferation of diabetogenic T cells within pancLN (Fig. 4 and 5), our results indicate that the absence of $CD4^+$ $nT_{reg}$ cells favors the infiltration/expansion of $T_{eff}$ cells in spleen (data not shown), pancLN and pancreas, with a 2-3 fold increase in the number of $CD4^+V\beta4^+$ T cells in these sites, compared to recipient mice co-transferred with $T_{eff}$ cells and $nT_{reg}$ cells (Fig. 6B). This increased accumulation of $T_{eff}$ cells correlated with the onset of diabetes, and may suggest a role for $nT_{reg}$ cells in the control of antigen-driven recruitment or proliferation of $T_{eff}$ cells in pancreatic sites. Our result show that protection from T1D correlates with a significantly increased proportion of $Foxp3^+$ $nT_{reg}$ cells in these sites, and mirrors the decline in $T_{eff}$ cell numbers suggesting that a crucial part of the protective role of $nT_{reg}$ cells resides in their ability to migrate to or expand in sites of autoimmune attack (Fig. 6B). While not entirely preventing the infiltration of $Foxp3^-$ $T_{eff}$ cells into the islets, $nT_{reg}$ cells nonetheless reduced the severity of insulitis, with a greater proportion of islets with preserved morphology (Fig. 6A). Interestingly, examination of the few recipient mice receiving only $CD4^+CD25^-$ $T_{eff}$ cells and which never developed T1D revealed that the proportion of $Foxp3^+$ $T_{reg}$ cells were similar to that observed in recipient mice co-injected with $nT_{reg}$ cells, suggesting that this protective $Foxp3^+$ $T_{reg}$ cell subset originated from the $CD25^-$ T cell donor fraction, and possibly expanded/differentiated within these sites, and ultimately ensure disease protection (Fig. 6B).

As young and adult $nT_{reg}$ cells differed in their protective effect, we then compared the differential ability of $Foxp3^+$ $nT_{reg}$ cells from 3-4 or 6-8 week old donors to accumulate in inflamed pancreatic sites. In recipients of 3-4 week old cells, the

frequency of $nT_{reg}$ cells in pancLN and pancreas was significantly greater than recipients receiving $T_{eff}$ cells alone and correlated with T1D protection (Fig. 6D and E). In stark contrast, $nT_{reg}$ cells from 6-8 week old donor mice accumulated less efficiently in the pancLN and pancreas, and correlated with the onset of T1D in the majority of recipient mice (Fig. 6D and E). In the few T1D-free mice receiving $nT_{reg}$ cells from 6-8 week old donor mice, we observed that the frequency of $nT_{reg}$ cells in the pancreas was comparable to recipient mice receiving 3-4 week old $nT_{reg}$ cells. Collectively, our results show that $nT_{reg}$ cells home to and expand within inflamed pancreatic sites where they constrict the size of the $T_{eff}$ cell pool and reduce the histopathological consequences of a destructive infiltration. Thus, a possible waning with age of $nT_{reg}$ homing or expansion within pancreatic sites could explain the onset of an uncontrolled $T_{eff}$ cell infiltration of the pancreas and T1D induction.

## 5. Discussion.

Foxp3$^+$ nT$_{reg}$ cells have been implicated as a central control point in T1D progression, and defects in their development or function may represent a major predisposing factor for spontaneous autoimmunity in NOD mice [2, 28, 29]. Here, we show that thymic and peripheral CD4$^+$CD25$^+$ nT$_{reg}$ cells can suppress disease in both normal NOD and BDC2.5 antigen-specific model of T1D. We also show that CD4$^+$CD25$^+$Foxp3$^+$ nT$_{reg}$ cells do not affect the priming of antigen-specific effector T cells in pancLN, but localize within insulitic lesions, where they suppress the infiltration of T$_{eff}$ cells. The cellular potency of nT$_{reg}$ cells, while fully operative in neonatal mice, declines with age despite a stable cellular frequency of Foxp3$^+$ nT$_{reg}$ cells in primary and secondary lymphoid tissues.

Recent studies stipulate that defective or reduced CD4$^+$CD25$^+$ T cell frequencies in autoimmune-prone hosts, including NOD mice, represent the primary predisposing factor to spontaneous autoimmunity [20, 21, 30, 31]. In most studies, the CD25 surface marker is frequently used for the monitoring of nT$_{reg}$ cell frequencies, albeit at a time point when pancreatic inflammation is well engaged. CD25 is an unreliable marker as activated CD4$^+$ T cells upregulate CD25, thus precluding its use as a tracking biomarker of nT$_{reg}$ cells in NOD mice. We observed that Foxp3$^+$CD4$^+$ nT$_{reg}$ cells, irrespective of CD25 expression, represent a stable pool in thymocytes, LN or spleen of neonatal and adult NOD mice, and is comparable to T1D-resistant BL6 mice, thus refuting the view that NOD mice have a developmental defect in nT$_{reg}$ cells. However, it is possible that a functional deficiency in nT$_{reg}$ cells may not be visible as a sudden decline in the

frequency of these cells in peripheral tissues, and may conceivably be a resultant to gaps in the TCR repertoire or gene polymorphisms modulating various effector functions [32]. Consistently, we show that $nT_{reg}$ cell function wanes with time as evidenced by their inability to prevent $T_{eff}$ cell infiltration in pancreatic sites, suggesting that the loss of Ag-driven homing or expansion of $nT_{reg}$ cells in pancreatic environments may represent an essential checkpoint in the progression to T1D. We cannot exclude the possibility that time-dependent changes in $T_{eff}$ cells may contribute to T1D onset [33].

An unresolved question relates to the location of $nT_{reg}$ cell-mediated tolerance induction *in vivo*. The ability of $nT_{reg}$ cells to localize within tissues to dampen the magnitude of $T_{eff}$ cell responses and prevent the histopathological consequences has been observed in models of infectious disease, IBD and tumors [34, 35]. Our results show that $nT_{reg}$ preferentially home to or expand within inflamed pancLN and islets of T1D-protected mice, and may indicate that this is the location where $nT_{reg}$ cells control the effector functions of the infiltrating diabetogenic $CD4^+$ T cells, albeit not completely preventing insulitis. Interestingly, a more significant reduction in the degree of insulitis was observed with thymic $nT_{reg}$ cells compared with peripheral $nT_{reg}$ cells (Fig. 1D vs 2F), suggesting that an increased functional potency may exist in the thymic $nT_{reg}$ cell compartment, as well as a potential waning of this functional potency in peripheral $nT_{reg}$ cells [21, 29]. It is conceivable that the thymic microenvironment provides the necessary developmental and homeostatic signals that may be lacking in the periphery of NOD mice. One study showed that pancreatic BDC2.5 $CD4^+CD25^+$ $T_{reg}$ cells abrogated disease induced by pancreatic $CD4^+CD25^-$ $T_{eff}$ cells in NOD.*scid* recipients, and the

majority of $T_{reg}$ cells were actively suppressing in the pancreas, rather than affecting the initial priming of the autoreactive T cells in the pancLN [19]. Similarly, *in vitro* expanded BDC2.5 $CD4^+CD25^+$ $T_{reg}$ cells suppressed T1D induced after transfer of diabetic NOD splenocytes into NOD.*scid* recipients, despite the fact that insulitis was nonetheless apparent in protected mice [36, 37]. Interestingly, the gene expression profile of islet-infiltrating $nT_{reg}$ cells differ from $nT_{reg}$ cells residing in the pancLN, suggesting that the target tissue engages unique transcriptional programs in $nT_{reg}$ cells, which might relate to their regulation in these sites [19]. It is unclear whether these distinct $nT_{reg}$ gene signatures occur as a result of their tissue localization or as a consequence of their own suppression. Target organs may confer unique regulatory pressures on infiltrating $T_{eff}$ cells, and may shape the type of regulation needed for disease resolution. Alternatively, different $Foxp3^+$ $nT_{reg}$ cell subsets may exist to operate in a tissue-specific fashion, or chemokine receptors like CCR5 or CCR6 may endow $nT_{reg}$ cells with a competitive advantage to enter more efficiently in pancLN [38-40].

$CD4^+$ $nT_{reg}$ cells may potentially suppress anti-islet T cell responses by affecting their activation and clonal expansion in pancLN. In our system, the initial activation of islet-specific $CD4^+$ T cells is unaffected in the presence of $nT_{reg}$ cells suggesting that antigen presentation, and TCR signals are not inhibited by $nT_{reg}$ cells in draining pancLN, a finding consistent with those from Chen *et al*. [19]. This is also in agreement with our observation that $nT_{reg}$ cells suppress T1D mediated by diabetic T cells, which likely traffic directly to islets, circumventing priming in the pancLN. In addition, we were unable to detect any changes in the frequency of proliferating diabetogenic $CD4^+$ T cells in the pancLN, either in the presence of absence of $nT_{reg}$ cells, suggesting that antigen-

induced priming of autoreactive T cells is not directly affected by $nT_{reg}$ cells. Paradoxically, the absolute number of $T_{eff}$ cells in pancreatic sites is dramatically increased in the absence of $nT_{reg}$ cells, suggesting that $nT_{reg}$ cells may restrain T cell clonal expansion, survival or homing at later events of diabetogenesis. Consistently, transfer of BDC2.5 T cells into thymectomized NOD.B7-$2^{-/-}$ recipients in conjunction with *in vivo* depletion of $CD25^+$ T cells, which resulted in an increased accumulation of $T_{eff}$ cells in the pancLN compared to control mice [41]. Alternatively, $T_{reg}$ cells were shown to control the pathogenicity of islet-specific, $CD8^+$ $T_{eff}$ cells by inhibiting DC maturation in the pancLN [42], and *in vitro* expanded BDC2.5 $T_{reg}$ cells have been shown to disrupt BDC2.5 T/DC cellular interactions in pancLN, suggesting that $nT_{reg}$ cell/APC interactions may be in part responsible for $nT_{reg}$ cell-mediated protection [27]. Lastly, we cannot formally exclude a more subtle effect on $T_{eff}$ cells in these sites such that $T_{eff}$ cells are now imprinted with a reduced pathogenic potential, which would reveal itself once they traffic to islets.

A recent study by Chen *et al.* used NOD mice harboring the scurfy mutation of the *Foxp3* gene ($Foxp3^{sf}$) to examine the functional role of $nT_{reg}$ cells in T1D [19]. As the mutation of Foxp3 impairs the development of $nT_{reg}$ cells, NOD.$Foxp3^{sf}$ displayed a significantly advanced onset of T1D compared to normal NOD mice, implying a role for $Foxp3^+$ $nT_{reg}$ cells in the control of T1D pathogenesis. However, this study did not address the possibility that the injection of wild-type, antigen-specific $T_{reg}$ cells, while rescuing from the early onset of T1D in Foxp3-deficient NOD mice, were compensating for the primary deficit in $nT_{reg}$ cells believed to exist in these mice, or whether such injection was actually suppressing the global inflammation that likely arose as a

secondary consequence of Foxp3 deficiency. Although the NOD.Foxp3$^{sf}$ model provides a system devoid of $nT_{reg}$ cells, the *Scurfy* mice also possess multiple immune defects, which likely have consequences on the physiopathology of T1D. Chang *et al.* has shown that a T cell extrinsic defect may contribute to the *Scurfy* and IPEX syndrome, since the Foxp3$^{sf}$ mutation in non-hematopoietic, thymic stromal cell leads to an ErbB2-dependent defective thymopoiesis [43]. Furthermore, it is unclear whether NOD.Foxp3$^{sf}$ mice possess aberrant antigen presentation or co-stimulation, which could combine to reduce the activation thresholds for Foxp3$^{sf}$ $T_{eff}$ cells and render them resistant to suppression [44, 45].

In conclusion, $nT_{reg}$ cells represent a master-switch regulating disease onset and progression in NOD mice, as abrogation of $nT_{reg}$ function can break T cell tolerance to β-islet antigens. Despite $nT_{reg}$ cells actively suppressing anti-islet T cell responses in the neonatal immune system, this suppression is ultimately insufficient to maintain tolerance to pancreatic antigens since autoimmunity ultimately ensues in these mice, as shown in recent studies [46, 47]. These studies may provide insights into the cellular basis of T1D susceptibility, may lead to the development of novel approaches to potentiate $nT_{reg}$ cell activity in autoimmune-prone hosts.

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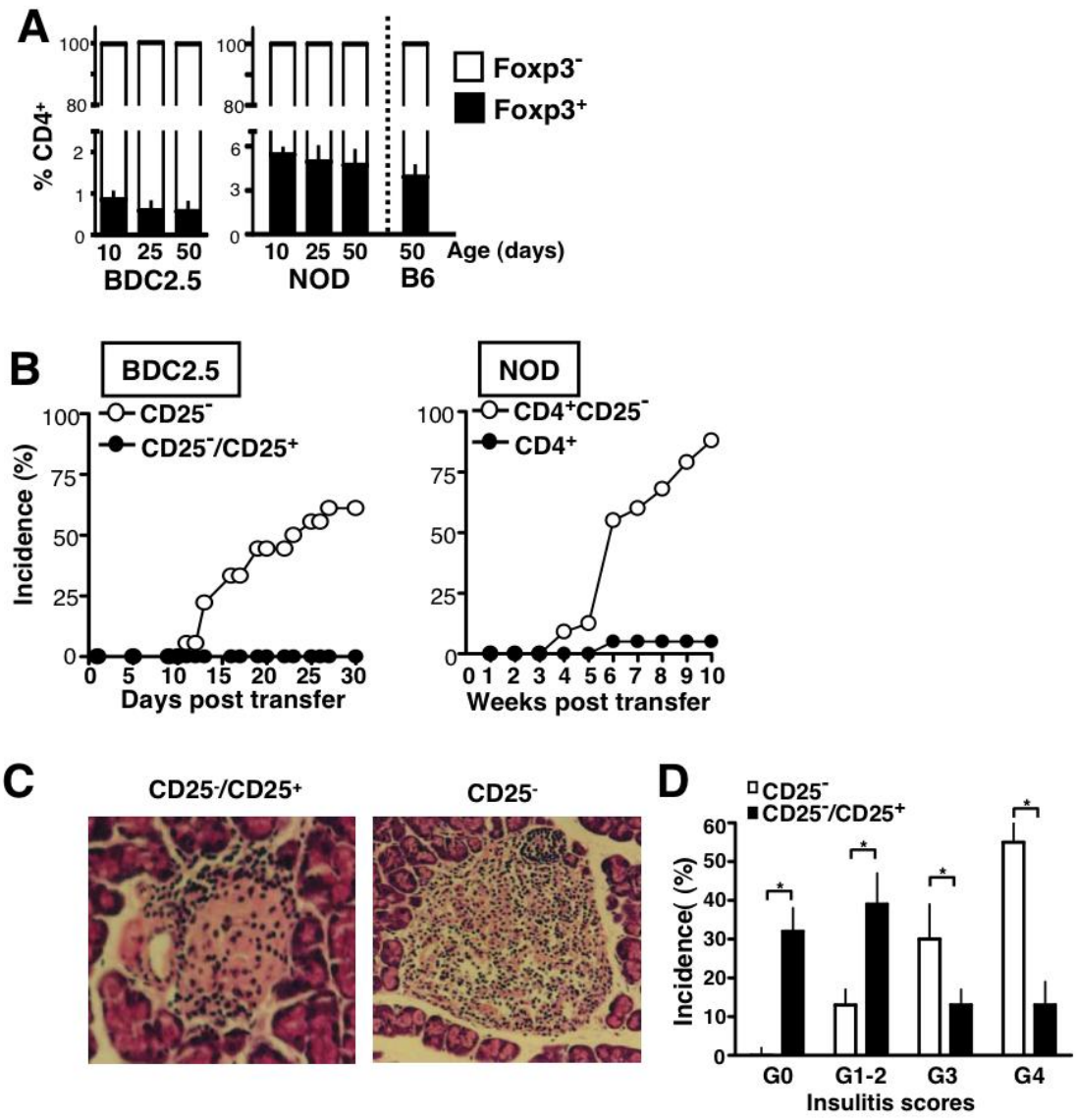
## 8. Legends.

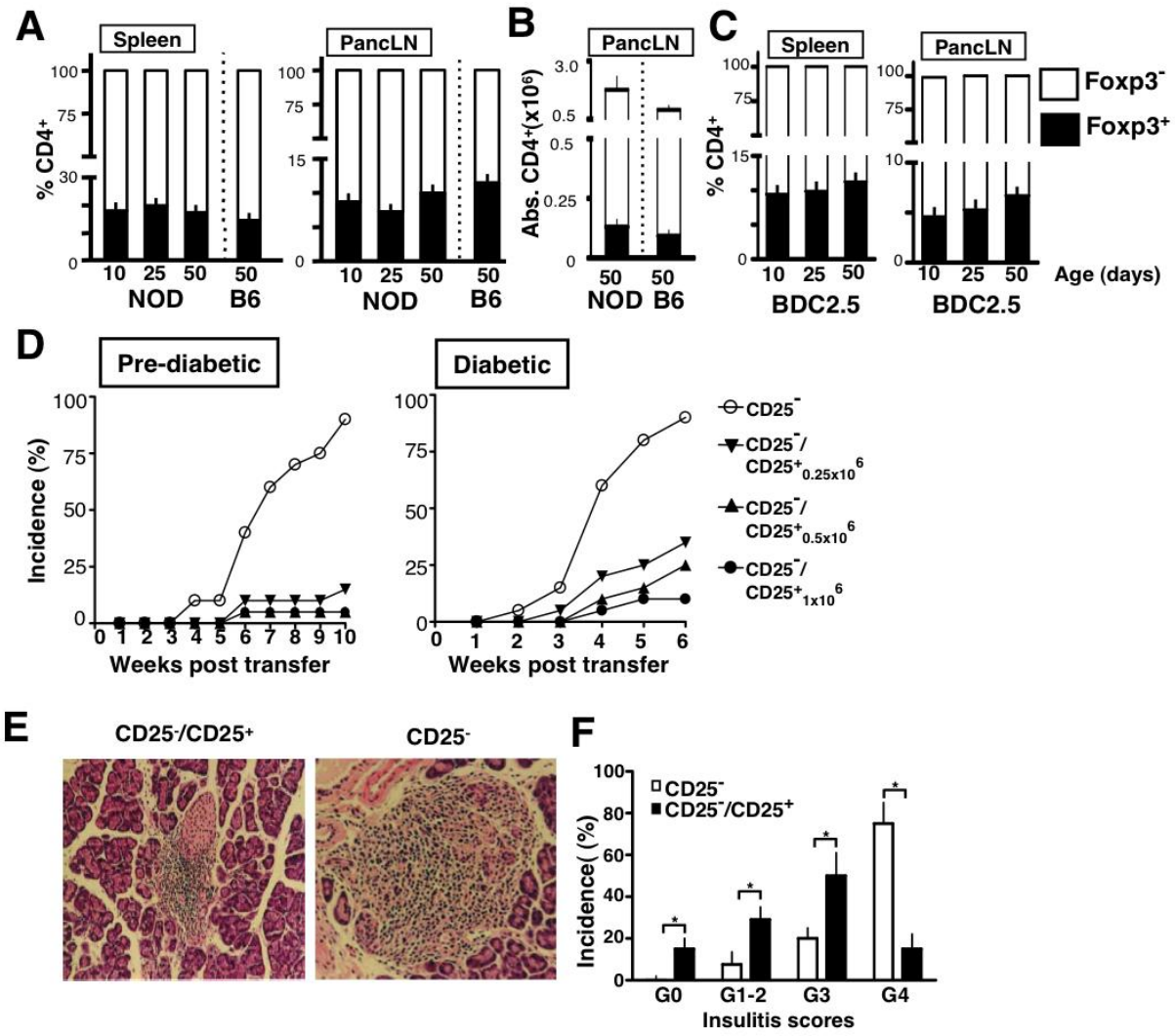
### FIGURE 1. Normal thymic $nT_{reg}$ cell frequency and function in pre-diabetic NOD and BDC2.5 mice.

(A). The frequency of $Foxp3^+$ and $Foxp3^-$ T cells within CD4 single positive ($CD4^{SP}$) thymocytes was determined in NOD and BDC2.5 mice at 10, 25 and 50 days of age, relative to diabetes-resistant 50 day-old C57BL/6 mice. Graphs represent pooled results of three separate experiments, with 3-5 mice analyzed per age group. Data are shown as a mean for both subsets and SD of the $Foxp3^+$ group are shown. (B). NOD.TCR$\alpha^{-/-}$ mice were transferred i.v. with 2.5 x $10^5$ $CD4^{SP}CD25^-$ (open circles) in the presence or absence of 2.5 x $10^4$ $CD4^{SP}CD25^+$ (closed circles) isolated from thymocytes of 2-4 week old BDC2.5 mice. Incidence of diabetes was assessed daily. Similarly, NOD.*scid* mice were transferred with $10^6$ $CD4^{SP}CD25^-$ either alone or with 0.5x$10^6$ $CD4^{SP}CD25^+$ $T_{reg}$ cells isolated from thymi of 10 day-old NOD mice. Incidence of diabetes was assessed weekly. Data is representative of three separate experiments.


A
% CD4+
Foxp3-
Foxp3+
10 25 50
BDC2.5
10 25 50
NOD
50
B6
Age (days)
B
BDC2.5
CD25-
CD25-/CD25+
Incidence (%)
Days post transfer
NOD
CD4+CD25-
CD4+
Weeks post transfer
C
CD25-/CD25+
CD25-
D
CD25-
CD25-/CD25+
Incidence( (%)
G0
G1-2
G3
G4
Insulitis scores

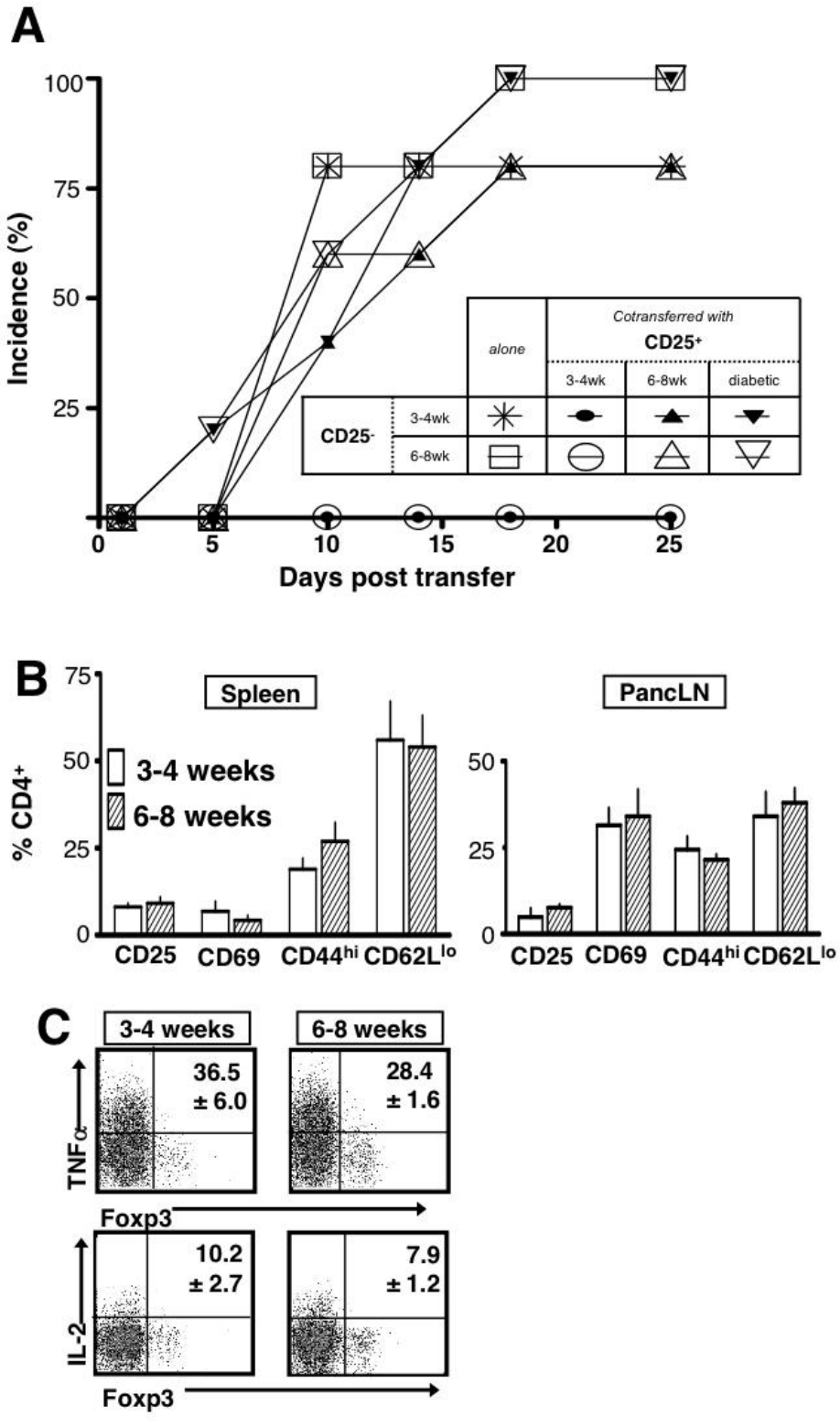
## FIGURE 2. Peripheral $CD4^+$ $nT_{reg}$ cells maintain tolerance to β-islet cells in pre-diabetic NOD and BDC2.5 mice.

The cellular frequency of $Foxp3^+$ and $Foxp3^-$ T cells within $CD4^+$ T cells in the spleen and pancLN was determined in NOD (A) and BDC2.5 (B) mice at 10, 25 and 50 days of age, relative to diabetes-resistant 50 day-old BL6 mice. Graph represents pooled results from 3-5 mice analyzed per age group, and data presented as the mean ± SD. (C). NOD.*scid* recipient mice were transferred with $10^6$ $CD4^+CD25^-$ from 10-day old prediabetic (left panel) or diabetic NOD mice (right panel) either alone or with 0.5-1x $10^6$ $CD4^+CD25^+$ $nT_{reg}$ cells isolated by FACS from peripheral LN of 10-day old prediabetic mice. The incidence of diabetes was monitored bi-weekly. Data represent pooled results of three separate experiments.


A
Spleen
PancLN
% CD4+
100
75
30
15
0
10 25 50 50
NOD B6
B
PancLN
Abs. CD4+(x10^6)
3.0
0.5
0.5
0.25
0
50 50
NOD B6
C
Spleen
PancLN
% CD4+
BDC2.5
Foxp3-
Foxp3+
Age (days)
D
Pre-diabetic
Diabetic
Incidence (%)
Weeks post transfer
CD25-
CD25-/ CD25+0.25x10^6
CD25-/ CD25+0.5x10^6
CD25-/ CD25+1x10^6
E
CD25-/CD25+
CD25-
F
CD25-
CD25-/CD25+
Incidence( (%)
G0
G1-2
G3
G4
Insulitis scores

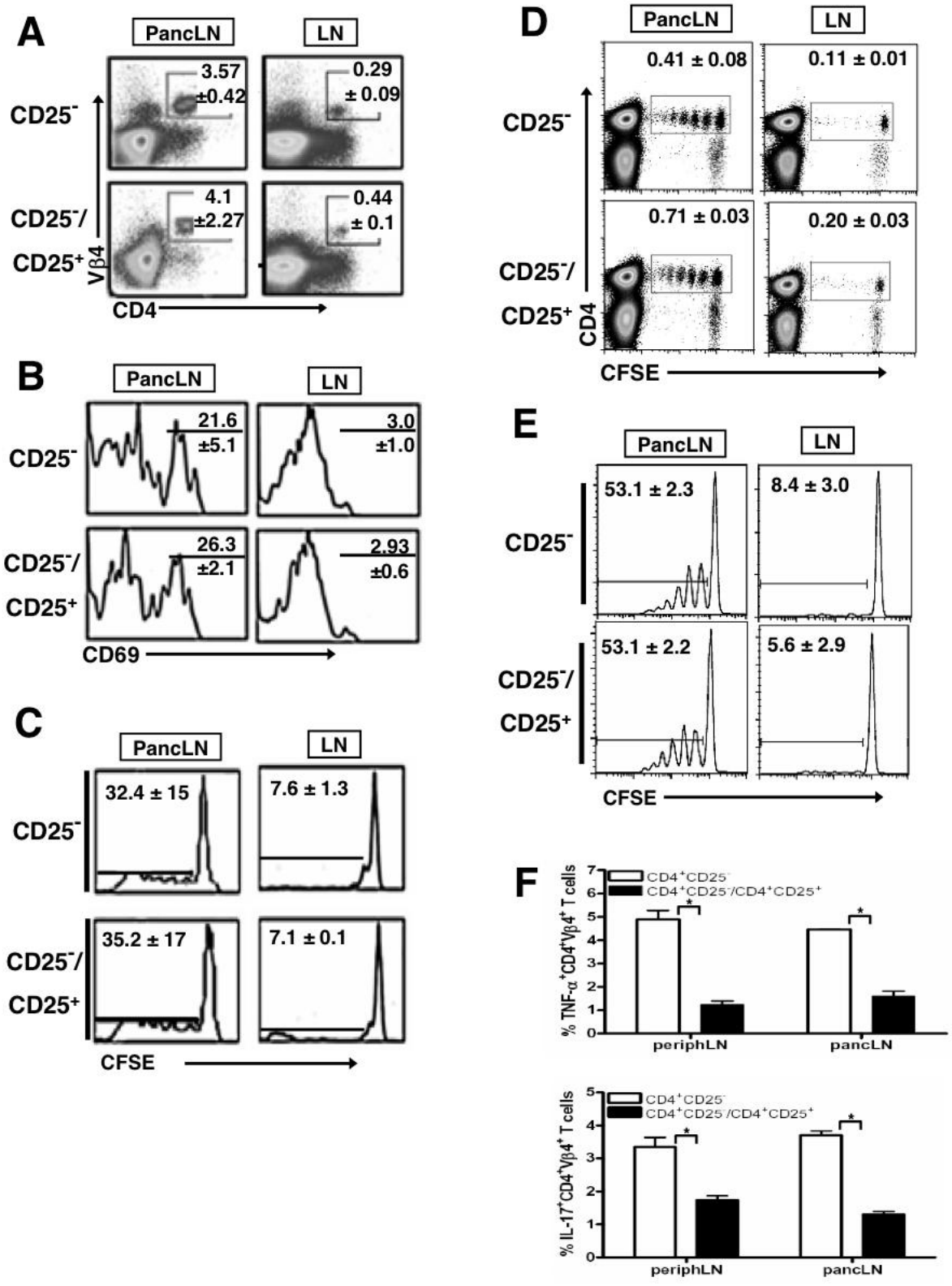
## FIGURE 3. Temporal decline in the function of $CD4^+Foxp3^+$ $nT_{reg}$ cells in the periphery of BDC2.5 mice.

(A). NOD.TCR$\alpha^{-/-}$ mice were transferred i.v. with $2.5x10^5$ $CD4^+CD25^-$ (open circles) in the presence or absence of $2.5x10^4$ $CD4^+CD25^+$ (closed circles) isolated from peripheral LN of either 3-4 week (left panel) or 6-8 week (right panel) old BDC2.5 mice. Incidence of diabetes was assessed every 24-48h. Data represent pooled results of three separate experiments. (B). Lymphocytes from spleen and pancLN of BDC2.5 mice at 3-4 (white bars) or 6-8 weeks of age (hatched bars) were stained for activation markers CD25, CD69, and CD44. (C). T cells were isolated from pancLN of 3-4 week-old (left panels) and 6-8 week old (right panels) BDC2.5 mice, stimulated for 4-5 hours with PMA/ionomycin, and co-stained for intranuclear Foxp3 and intracellular IL-2 and TNF-$\alpha$. Data represents mean percentage ± SD of $CD4^+V\beta4^+$ T cells for each marker. 5-8 mice were analyzed per age group.


A
100
75
50
25
0
5
10
15
20
25
Incidence (%)
Days post transfer
alone
Cotransferred with
CD25+
3-4wk
6-8wk
diabetic
CD25-
3-4wk
6-8wk
B
Spleen
PancLN
3-4 weeks
6-8 weeks
% CD4+
75
50
25
0
CD25
CD69
CD44hi
CD62Llo
C
3-4 weeks
6-8 weeks
36.5
± 6.0
28.4
± 1.6
TNFα
Foxp3
10.2
± 2.7
7.9
± 1.2
IL-2
Foxp3

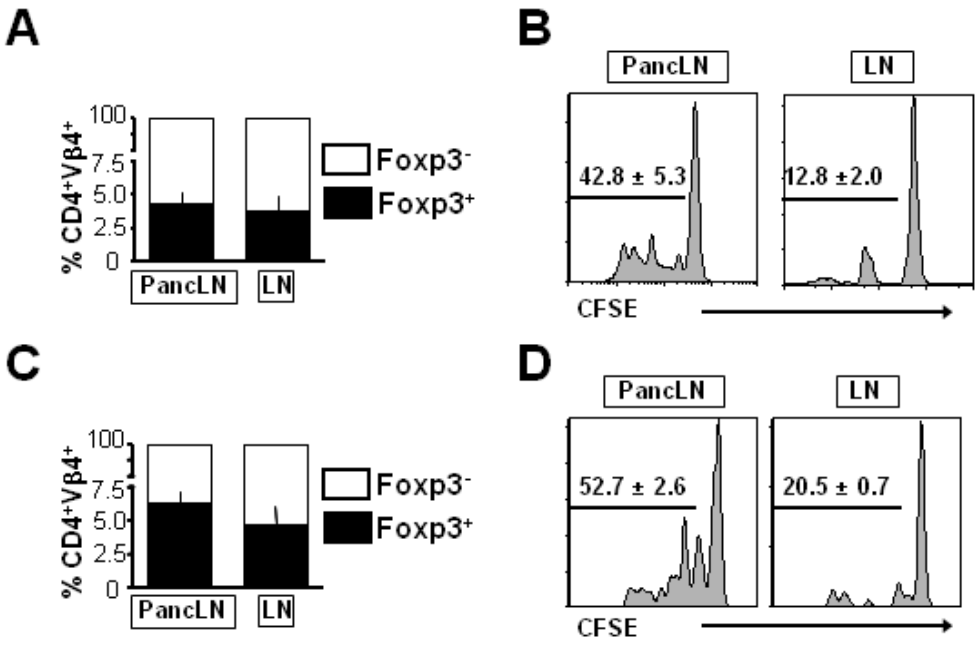
## FIGURE 4. $CD4^+$ $T_{reg}$ cells do not affect antigen-induced priming of diabetogenic $CD4^+$ T cells in lymphopenic and non-lymphopenic hosts.

NOD.TCR$\alpha^{-/-}$ (A-C) or prediabetic wild-type NOD recipient mice (D,E) were transferred i.v. with $10^6$ CFSE-labelled $CD4^{SP}CD25^-$ in the presence or absence of $2.5x10^4$ $CD4^{SP}CD25^+$ isolated by FACS from T cells derived from 2-4 week old BDC2.5 mice. Pancreatic and non-draining LN of recipient mice were harvested on day 3 post-T cell transfer, percentages of BDC2.5 $CD4^+V\beta4^+$ T cells, CD69 early activation marker expression on BDC2.5 $CD4^+V\beta4^+$ T cells (B) and the proliferative capacity (CFSE dilution profile) of $Foxp3^-$ BDC2.5 $CD4^+V\beta4^+$ T cells (C-E) were determined, in the presence or absence of BDC2.5 $nT_{reg}$ cells. Similar results were obtained in three independent experiments.


A
PancLN
LN
CD25-
CD25-/
CD25+
Vβ4
CD4
3.57 ±0.42
0.29 ± 0.09
4.1 ±2.27
0.44 ± 0.1
B
PancLN
LN
CD25-
CD25-/
CD25+
CD69
21.6 ±5.1
3.0 ±1.0
26.3 ±2.1
2.93 ±0.6
C
PancLN
LN
CD25-
CD25-/
CD25+
CFSE
32.4 ± 15
7.6 ± 1.3
35.2 ± 17
7.1 ± 0.1
D
PancLN
LN
CD25-
CD25-/
CD25+
CD4
CFSE
0.41 ± 0.08
0.11 ± 0.01
0.71 ± 0.03
0.20 ± 0.03
E
PancLN
LN
CD25-
CD25-/
CD25+
CFSE
53.1 ± 2.3
8.4 ± 3.0
53.1 ± 2.2
5.6 ± 2.9
F
CD4+CD25-
CD4+CD25-/CD4+CD25+
% TNF-α+CD4+Vβ4+ T cells
periphLN
pancLN
CD4+CD25-
CD4+CD25-/CD4+CD25+
% IL-17+CD4+Vβ4+ T cells
periphLN
pancLN

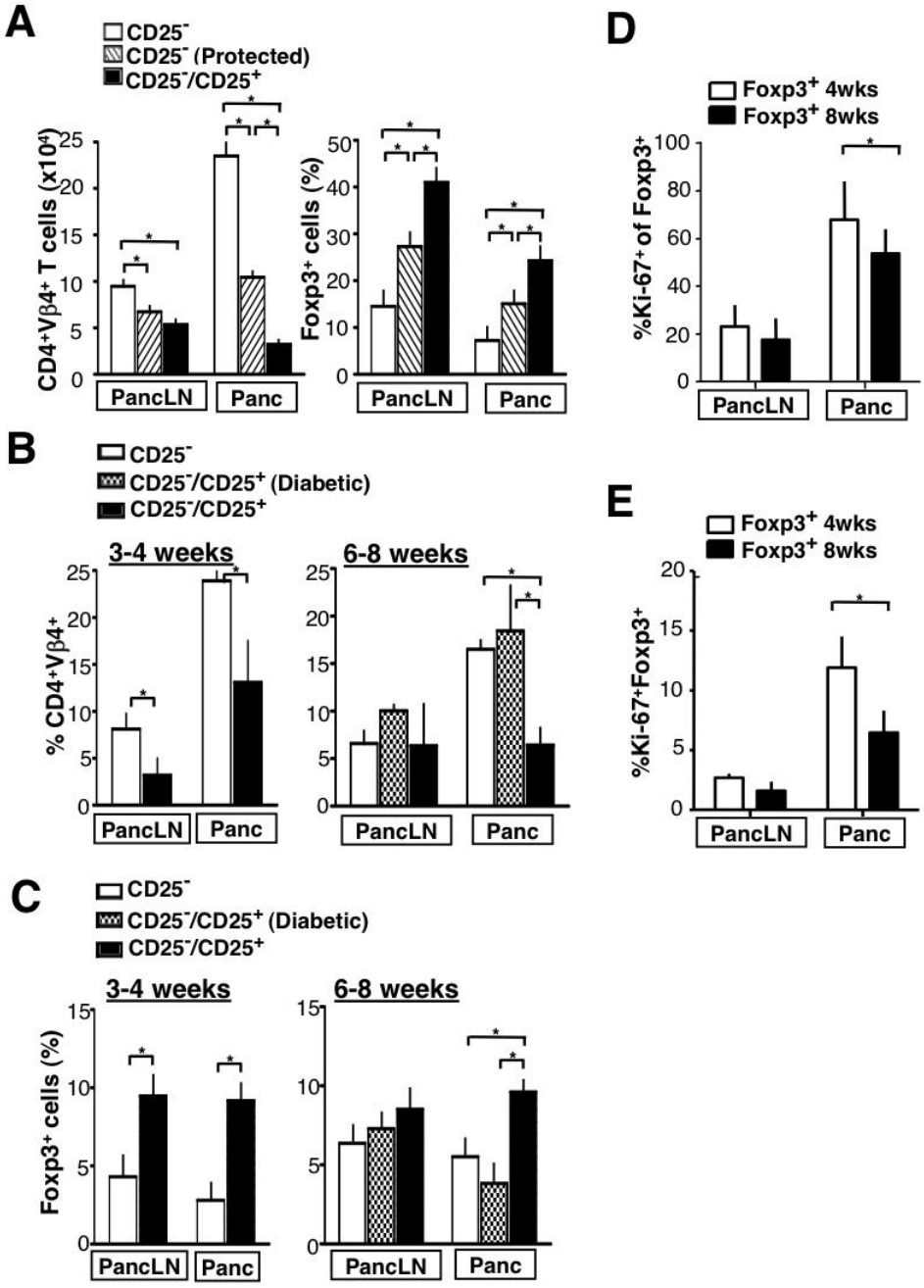
## FIGURE 5. $CD4^+$ $nT_{reg}$ cells expand in the pancreatic lymph nodes of lymphopenic and non-lymphopenic hosts.

NOD.TCR$\alpha^{-/-}$ (A, B) or prediabetic wild-type NOD recipient mice (C, D) were transferred i.v. with CFSE-labelled BDC2.5 T cells. Pancreatic and non-draining LN were harvested on day 3 post-T cell transfer, and the percentages of $Foxp3^+$ (A, C) and the frequency of proliferating $CD4^+V\beta4^+Foxp3^+$ T cells (B, D) was determined in pancreatic and non-draining LN are indicated. Similar results were obtained in three independent experiments.


A
100
7.5
5.0
2.5
0
% CD4+Vβ4+
Foxp3-
Foxp3+
PancLN
LN
B
PancLN
LN
42.8 ± 5.3
12.8 ±2.0
CFSE
C
100
7.5
5.0
2.5
0
% CD4+Vβ4+
Foxp3-
Foxp3+
PancLN
LN
D
PancLN
LN
52.7 ± 2.6
20.5 ± 0.7
CFSE

## FIGURE 6. Protection from T1D correlates with increased expansion of $CD4^+Foxp3^+$ $nT_{reg}$ cells in pancreatic sites.

(A). NOD.TCR$\alpha^{-/-}$ mice were adoptively transferred i.v. with 2.5 x $10^5$ $CD4^+CD25^-$ in the presence or absence of thymic 2.5x$10^4$ $CD4^+CD25^+$ isolated from 3-4 week old, prediabetic BDC2.5 mice. PancLN and pancreas were isolated 30 days post transfer from diabetic ($CD25^-$, white bars) and non-diabetic ($CD25^-$, hatched bars) mice in the absence of $nT_{reg}$ cells and in non-diabetic recipients in the presence of $nT_{reg}$ cells ($CD25^-/CD25^+$, black bars). Insulitis scores are shown (left panel). Data represents the mean of 3-5 mice analyzed, and 12 to 15 islets per pancreas were scored per time point. (B). The frequency of $CD4^+V\beta4^+$ T cells and $Foxp3^+$ $T_{reg}$ cells were assessed in these sites (middle and right panels). (C). NOD.TCR$\alpha^{-/-}$ mice were adoptively transferred i.v. with 2.5 x $10^5$ $CD4^+CD25^-$ in the presence or absence of peripheral 2.5 x $10^4$ $CD4^+CD25^+$ isolated from 3-4 week old or 6-8 week old prediabetic donors. Insulitis scores were assessed by as in (A). Percentages of $CD4^+V\beta4^+$ T cell (D) and the proportion of $Foxp3^+$ $T_{reg}$ cells (E) were determined in pancLN and pancreas of diabetic mice ($CD25^-$, white bars) in the absence of $T_{reg}$ cells, and non-diabetic ($CD25^-/CD25^+$, black bars) and diabetic ($CD25^-/CD25^+$, hatched bars) recipients in the presence of $T_{reg}$ cells. Error bars represent the mean ± SD. Similar results were obtained in three independent experiments.


A
CD25-
CD25- (Protected)
CD25-/CD25+
CD4+Vβ4+ T cells (x10⁴)
Foxp3+ cells (%)
PancLN
Panc
B
CD25-
CD25-/CD25+ (Diabetic)
CD25-/CD25+
3-4 weeks
6-8 weeks
% CD4+Vβ4+
PancLN
Panc
C
CD25-
CD25-/CD25+ (Diabetic)
CD25-/CD25+
3-4 weeks
6-8 weeks
Foxp3+ cells (%)
PancLN
Panc
D
Foxp3+ 4wks
Foxp3+ 8wks
%Ki-67+ of Foxp3+
PancLN
Panc
E
Foxp3+ 4wks
Foxp3+ 8wks
%Ki-67+Foxp3+
PancLN
Panc

## CHAPTER III *Impact of protective IL-2 allelic variants on $CD4^{+}Foxp3^{+}$ regulatory T cell function in situ and resistance to autoimmune diabetes in NOD mice.*

## Bridging statement from chapter II to III

In our first study, we demonstrated that qualitative defects in $T_{reg}$ cells predispose to diabetes. More specifically, we showed which aspects of T cell activation $T_{reg}$ cells modulate *in vivo*. Although $T_{reg}$ cells were incapable of hindering the activation and expansion of the diabetogenic T cells in draining pancreatic sites, they potently impaired their differentiation and accumulation in the target organ. The presence of $T_{reg}$ cells suppressed TNF-$\alpha$ and IL-17 effector cytokine production, thus strongly hindering the differentiation of disease-inducing T cells rather than their activation. Furthermore, the functional potency of intra-islet resident $T_{reg}$ cells, as per their proliferative capacity, decreased over time, strongly suggesting that $T_{reg}$ cell functions wane with age, enabling the transition from insulitis to overt diabetes. However, a reciprocal age-dependent increase in $T_{eff}$ cell resistance to $T_{reg}$ cell suppression could not be excluded. Although very informative, the article did not dissect the events that led to the waning of $T_{reg}$ cell functions and consequent breakdown in tolerance.

In collaboration with the laboratory of Dr. Bluestone (UCSF) we showed that intra-islet resident $T_{reg}$ cells displayed defective expansion and survival with time due to loss in Bcl-2 (anti-apoptotic factor) and CD25 (IL-2 receptor $\alpha$ chain) expression. The observed decline in $T_{reg}$ cells correlated with a concomitant increase in $T_{eff}$ cell infiltration resulting in a $T_{reg}/T_{eff}$ cell imbalance, leading destructive insulitis and T1D. The reduced CD25 levels on $T_{reg}$ cells suggested that local IL-2 deficiency could not maintain $T_{reg}$ cell fitness and resulted in their apoptosis, a phenotype reversed by prophylactic recombinant IL-2 therapy. These findings highlighted the crucial role that IL-2 plays in tolerance by maintaining a fit $T_{reg}$ cell pool.

In order to investigate the impact of IL-2 in T1D susceptibility, the NOD.B6 *Idd3* congenic mouse model, introgressed with the protective *Idd3*$^{B6}$ genetic interval, was employed. Genetic mapping studies have demonstrated that *Idd3*$^{B6}$ alleles confer delay in incidence, onset and severity of T1D. Incidently, the candidate gene within the *Idd3*$^{B6}$ genetic interval is *Il2*. Given the importance of IL-2 on $T_{reg}$ cell homeostasis and fitness, we hypothesized that the *Il2* allelic variants promoted the thymic development as well as the homing, expansion, and functions of $T_{reg}$ cells within pancreatic sites, which in turn conferred T1D protection.

# Impact of protective IL-2 allelic variants on CD4$^+$Foxp3$^+$ regulatory T cell function *in situ* and resistance to autoimmune diabetes in NOD mice.

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Keywords: CD4$^+$CD25$^+$ regulatory T cells, Foxp3, diabetes, tolerance, IL-2

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Email: Ciro.piccirillo@mcgill.ca

**Running title:** IL-2 allelic variants drive CD4$^+$ $T_{reg}$ cell-mediated T1D protection.

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## 1. Abstract.

T1D susceptibility is inherited through multiple insulin-dependent diabetes (*Idd*) genes. NOD.B6 *Idd3* congenic mice, introgressed with an *Idd3* allele from T1D-resistant C57BL/6 mice (*Idd3*$^{B6}$), show a marked resistance to T1D compared to control NOD mice. The protective function of the *Idd3*$^{B6}$ locus is confined to the *Il2* gene, whose expression is critical for naturally-occurring CD4$^{+}$Foxp3$^{+}$ regulatory T (nT$_{reg}$) cell development and function. In this study, we asked whether *Idd3*$^{B6}$ protective alleles in the NOD mouse model confer T1D resistance by promoting the cellular frequency, function or homeostasis of nT$_{reg}$ cells *in vivo*. We show that resistance to T1D in NOD.B6 *Idd3* congenic mice correlates with increased levels of IL-2 mRNA and protein production in antigen-activated diabetogenic CD4$^{+}$ T cells. We also observe that protective *Il2* allelic variants (*Idd3*$^{B6}$ resistance allele) also favor the expansion and suppressive functions of CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cells *in vitro*, as well as restrain the proliferation, IL-17 production and pathogenicity of diabetogenic CD4$^{+}$ T cells *in vivo* more efficiently than control nT$_{reg}$ cells. Lastly, the resistance to T1D in *Idd3*$^{B6}$ congenic mice does not correlate with an augmented systemic frequency of CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cells but more so with the ability of protective *Il2* allelic variants to promote the expansion of CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cells directly in the target organ undergoing autoimmune attack. Thus, protective, *Il2* allelic variants impinge the development of organ-specific autoimmunity by bolstering the IL-2 producing capacity of self-reactive CD4$^{+}$ T cells, and in turn, favor the function and homeostasis of CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cells *in vivo*.

## 2. Introduction.

Type 1 diabetes (T1D) is a T cell-dependent autoimmune disease resulting in the destruction of the insulin-producing beta (β) islet cells of Langerhans in the pancreas, leading to insulin deficiency [1, 2]. Studies in non-obese diabetic (NOD) mice show that the lag time between the establishment of insulitis and overt clinical T1D onset may result from a progressive loss of immunoregulatory mechanisms, which include naturally occurring $CD4^+$ regulatory T ($nT_{reg}$) cells [3-6]. $CD4^+$ $nT_{reg}$ cells, constitutively expressing CD25 and the Foxp3 transcription factor [7-9], represent a major mechanism of peripheral self-tolerance, as their functional abrogation increases immunity to tumors, grafts and pathogens, and induces multi-organ-specific autoimmunity[10]. Defects in $CD4^+Foxp3^+$ $nT_{reg}$ cell development or function promote T1D susceptibility [11, 12] and have been implicated as a central control point in T1D progression.

T1D susceptibility is inherited through multiple genes, with a strong predisposition for those affecting T cell responses to β islet cells [1-3]. Genomic mapping studies of congenic NOD strains, which harbor defined genetic intervals from T1D-resistant mice, have identified at least 20 insulin-dependent diabetes (*Idd*) regions that collectively contribute to disease susceptibility [13, 14], although no single gene is both necessary and sufficient for complete disease protection. The $Idd3^{B6}$ locus, which maps to a 650 kb interval in the proximal region of chromosome 3, represents a major genetic determinant conferring T1D susceptibility [15-17]. NOD.B6 *Idd3* mice, introgressed with the protective T1D-resistant $Idd3^{B6}$ locus, show a marked resistance to reduced T1D onset [15, 18, 19], while also affecting susceptibility to other organ-

specific autoimmune diseases like EAE and ovarian disease provoked by day 3 thymectomy [20, 21]. Interestingly, fine mapping and positional cloning studies of the *Idd3*$^{B6}$ locus have demonstrated that the *Il2* gene is the primary candidate for *Idd3*-mediated protection in a model of CD8$^+$ T cell-induced T1D and a likely contributor to increased CD4$^+$ nT$_{reg}$ activity in these mice [19, 22]. Although modest increases in their cellular frequency and *in vitro* function were observed, a detailed assessment of the impact of protective *Il2* allelic variants (*Idd3*$^{B6}$ resistance allele) on the *in vivo* function of CD4$^+$Foxp3$^+$ nT$_{reg}$ cells was not described [19, 22].

A growing body of evidence strongly demonstrates that IL-2 is an important signal for CD4$^+$Foxp3$^+$ nT$_{reg}$ cells development, function, homeostasis and competitive fitness of nT$_{reg}$ cells *in vivo*. It is suggested that alterations in IL-2 signaling may attenuate nT$_{reg}$ cell function and provoke autoimmunity [23]. Furthermore, B7.1/B7.2 or CD28 deficient NOD mice have reduced CD4$^+$Foxp3$^+$ nT$_{reg}$ cell numbers and manifest a more aggressive form of T1D than control littermates [24, 25], while systemic IL-2 neutralization provokes autoimmune neuropathy and accelerated T1D in NOD mice [26]. Moreover, T cells from prediabetic NOD mice have reduced T cell proliferative and IL-2 production capabilities, hallmark features, which coincide with a skewing towards pathogenic, β-cell–specific Th1 cell effector function [27]. Recently, we have shown that Foxp3$^+$ nT$_{reg}$ cell function declines with age in NOD mice, despite stable cellular frequencies of Foxp3$^+$ nT$_{reg}$ cells, and that a possible loss in nT$_{reg}$ cell expansion in inflammatory sites may perturb the equilibrium between effector and nT$_{reg}$ cells within pancreatic sites, and amplifies local immune diabetogenic responses [12]. Interestingly, Tang *et al.* clearly demonstrated that administration of low-dose IL-2 promoted nT$_{reg}$ cell

survival and protected NOD mice from developing T1D [28].

Currently, there is limited understanding in the regulation of T1D progression. Defining the mechanisms underlying the protective effects of T1D susceptibility gene variants, such as *Idd3*$^{B6}$, is critical to understand how genetic variation may impinge natural checkpoints in T1D progression. Here, we hypothesized that the protective *Il2* allelic variants (*Idd3*$^{B6}$ locus) confers T1D protection by supporting $CD4^+$ $nT_{reg}$ cell function and expansion *in vivo*. We show that *Idd3*$^{B6}$ controls IL-2 secretion by islet-reactive $CD4^+$ effector T ($T_{eff}$) cells, favouring the cycling and function of $CD4^+Foxp3^+$ $nT_{reg}$ cells locally in the pancreas, which in turn affords resistance to T1D. Thus, T1D genes may directly impinge the functional homeostasis of $CD4^+Foxp3^+$ $nT_{reg}$ cells, and in turn, contribute to T1D susceptibility.

## 3. Materials and Methods.

***Mice.*** Mice strains were maintained in SPF conditions at McGill University. NOD.TCR$\alpha^{-/-}$ and BDC2.5 CD4$^+$ transgenic (Tg) mice were a gift from Christophe Benoist (Harvard Univ., Boston, USA). BDC2.5 TCR transgenic NOD mice contain a monoclonal, β-islet-specific CD4$^+$ T cell repertoire (Vα1/Vβ4), thus providing a rapid, synchronous system for the analysis of antigen-specific T cell responses *in vivo.* NOD.B6 *Idd3* congenic mice (line#1098) were obtained from Taconic Farms, and BDC.*Idd3* mice were generated by in-house breeding.

***Cell purification*.** CD4$^+$, CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cell subsets were purified from lymph node (LN) or spleens using the autoMACS cell sorter (Miltenyi Biotech, San Diego, CA) or FACSAria flow cytometer (BD Biosciences, Mississauga, Ontario), as described previously [29].

***Flow cytometry****.* Stainings were done with the following fluorochrome-conjugated or biotinylated mAbs: anti-CD4 (clone RM5), anti-CD25 (clone PC61), anti-Vβ4 (clone CTVB4) (eBioscience, San Diego, CA). Anti-Foxp3 (clone FJK-16s) and anti-Bcl-2 (clone 10C4) (eBioscience) intracellular staining was performed according to the manufacturer's protocol (eBioscience). Stained cells were acquired on a FACSCalibur (BD Biosciences) and analyzed with Flowjo software.

***Ki-67 proliferation analysis.*** Pancreata were digested with collagenase type IV (Invitrogen) at 37°C, extensively washed in HBSS, followed by a 10-minute incubation at 37°C in non-enzymatic dissociation solution (Invitrogen, Burlington, Canada). Cells

were stained with anti-Ki-67 (clone B56) (BD Biosciences), anti-CD4, Foxp3 and CD25 (eBioscience).

***Adoptive transfers***. FACS-purified $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were transferred intravenously, either alone or in combination, into NOD.TCR$\alpha^{-/-}$, NOD or NOD.B6 *Idd3* recipient mice (1:10 $nT_{reg}/T_{eff}$ ratio; 2-3x$10^6$/mouse), as previously described (12). In some cases, T cells were CFSE labeled (Invitrogen), and expansion of donor T cells was evaluated, as previously described [30].

***In vitro proliferation assays***. Proliferation assays were performed by culturing $CD4^+$ T cells from NOD or BDC2.5 mice (5x$10^4$) in 96-well flat-bottom microtiter plates with irradiated, spleen cells (1-2x$10^5$) and soluble anti-CD3 (1μg/ml) or BDC2.5 mimetope (RVRPLWVRME) for 72h at 37$^o$C in 5% $CO_2$. Cell cultures were pulsed with 1μCi $^3$H-TdR for the last 6-12h and analyzed as previously shown [29]. All experiments were repeated at least 3 times. Suppression assays were performed by culturing cell-sorted $CD4^+CD25^-$ BDC.*Idd3* T cells with titrated numbers of highly-purified BDC2.5 or BDC.*Idd3* $CD4^+CD25^+$ $nT_{reg}$ cells, irradiated APCs and BDC2.5 mimetope (10ng/mL) for 72h. Cell cultures were pulsed with 1μCi $^3$H-TdR for the last 6-12h.

***Intracellular cytokine production***. Purified T cell subsets were stimulated 4-5h with PMA and ionomycin. In some instances, T cells were isolated from the pancreatic or distal LN of recipient mice following adoptive transfer, and were activated *ex vivo* overnight with bone marrow-derived DC (BMDC) and BDC2.5 mimetope, and treated with Golgi-Stop (BD Biosciences) for the last 2-3h of culture. Intracellular cytokine staining (ICS) was performed using fluorochrome-conjugated anti-mouse mAb IL-2

(clone JES6-5H4), IFN-γ (clone XMG1.2), IL-17 (clone eBio1787) (eBioscience), or appropriate isotype controls (BD Biosciences), as previously shown [12].

***Diagnosis of diabetes.*** Blood glycemia levels were determined every 2-3 days with Haemoglukotest kits (Roche Diagnostics, Laval, Canada), and T1D was diagnosed at values >300 mg/dl.

***RT-PCR.*** Analysis of IL-2 gene expression in resting and activated $CD4^+$ T cell subsets was achieved by normalizing the IL-2 densitometric value with the intensity of the G3PDH amplicon for each sample, and reported as arbitrary IL-2/G3PDH ratios, as previously described [29].

***Statistical analysis****.* Results are expressed as mean ± SD. Analyses were performed with a Student's *t* test, except for diabetes incidences where the Kaplan-Meier survival test was used. Values of $p<0.05$ were considered significant.

## 4. Results.

### Resistance to the progression of T1D in NOD.B6 *Idd3* congenic mice correlates with increased production of IL-2 by autoreactive $CD4^+$ T cells.

We confirm that NOD.B6 *Idd3* mice have a delayed onset and incidence of T1D compared to WT NOD mice [15]. Female NOD mice start to develop T1D by 14 wks of age and incidence reaches 85% by 28 wks. In contrast, only 10% of NOD.B6 *Idd3* female mice were diabetic by 28 wks of age, with the earliest onset occurring at 25 wks of age (Fig.1A), a finding consistent with the T1D protection seen in BDC.*Idd3* mice (data not shown). We then assessed whether differences in the production of TNF-$\alpha$ and IFN-$\gamma$ by $CD4^+$ Th1 cells, important inflammatory mediators in this model, correlated with T1D protection in NOD.B6 *Idd3* mice. We show a significant, albeit modest, decrease in the percentage of $CD4^+V\beta4^+$IFN-$\gamma^+$ T cells in peripheral LN (7.57±0.30% versus 5.83±0.26%, $p$=0.001), pancLN (8.52±0.46% versus 7.14±0.20%, $p$=0.03) and in spleen (data not shown) of pre-diabetic BDC.*Idd3* mice compared to BDC2.5 control mice following antigen-specific stimulation (Fig.1B). Moreover, we also observe a substantial decrease in the frequency of $CD4^+V\beta4^+$TNF-$\alpha^+$ T cells in peripheral LN (7.85±0.97% versus 5.09±0.17%, $p$=0.008), pancLN (7.51±0.43% versus 3.36±0.6%, $p$=0.002) and in spleen (data not shown) of BDC.*Idd3* mice compared to BDC2.5 controls under similar stimulatory conditions (Fig.1B). The T1D resistance in NOD.B6 *Idd3* mice also correlated with a significant reduction in $CD4^+$ T cell infiltration in the pancreas compared to WT NOD mice (1.04±0.3% versus 7.38±1.9%; $p\leq0.004$) (Fig.1C), an observation also made in BDC.*Idd3* (data not shown). Thus, the T1D-resistance in

*Idd3*$^{B6}$ congenic mice correlates with a significant reduction in the accumulation of $T_{eff}$ cells, particularly Th1 cells in lymphoid tissues or pancreas.

As the *Il2* gene is the strongest and primary candidate for T1D protection in the *Idd3*$^{B6}$ locus and it has been shown that the *Il2* promoter from *Idd3*$^{B6}$ alleles possesses some sequence variation compared to the susceptible NOD allele [31, 32], we wondered whether the *Idd3*$^{B6}$ interval increased IL-2 gene transcription and protein production in activated $CD4^+$ T cells of NOD.B6 *Idd3* mice. To this end, $CD4^+$ $T_{eff}$ cells from prediabetic NOD and NOD.B6 *Idd3* mice were activated with plate-bound anti-CD3, and IL-2 expression was assessed by RT-PCR in resting or TCR-stimulated conditions (Fig.1D). In contrast to unstimulated conditions, *Il2* expression levels in NOD.B6 *Idd3* $CD4^+$ $T_{eff}$ cells, albeit modestly greater than NOD T cells by 12h of TCR engagement, were approximately 2-3-fold greater than their NOD $CD4^+CD25^-$ T cell counterparts by 24-48h post-TCR stimulation. This increased IL-2 expression in NOD.B6 *Idd3* $CD4^+$ T cells also correlated with their augmented proliferation compared to NOD controls at 72h post-stimulation (Fig.1D and Fig.2A). To determine whether increased IL-2 transcription correlated with increased IL-2 production in *Idd3*$^{B6}$ congenic mice, $CD4^+$ $T_{eff}$ cells from prediabetic NOD and NOD.B6 *Idd3* or BDC2.5 and BDC.*Idd3* mice were activated with PMA/ionomycin or BMDC in the presence of BDC2.5 mimetope, respectively, and IL-2 protein production was determined by FACS 24h post-stimulation. Our results show that activated T cells revealed an at least 2-fold increase in the fraction of cells producing IL-2 in *Idd3*$^{B6}$ T cells compared to NOD control T cells without affecting the IL-2 mean fluorescence intensity in $CD4^+$ T cells of either genotype, suggesting that *Idd3*$^{B6}$ does not control the overall amount of IL-2 on a per

cell basis (Fig.1E). Thus, the protective *Idd3*$^{B6}$ allele causes an increase in the frequency of activated CD4$^+$ T cells producing IL-2, consistent with a recent study documenting similar increases in diabetogenic CD8$^+$ T cells from NOD.B6 *Idd3* mice [22].

**Protective *Idd3*$^{B6}$ alleles augment CD4$^+$Foxp3$^+$ nT$_{reg}$ function *in vitro.***

Given the critical role of IL-2 in CD4$^+$Foxp3$^+$ nT$_{reg}$ cell functions, we then hypothesized that the increased production of IL-2 by activated NOD.B6 *Idd3* T cells may potentiate nT$_{reg}$ cell suppressive function and restrain the proliferative capacity of responding CD4$^+$ T cells. We first assessed the proliferation of CD4$^+$ T cells from NOD and NOD.B6 *Idd3* wild-type mice or BDC2.5 and BDC.*Idd3* CD4$^+$ TCR transgenic mice, whose monoclonal TCR repertoire is specific for an as-of-yet unknown pancreatic β islet antigen [33], activated with irradiated APC and soluble anti-CD3, or with the BDC2.5 mimetope respectively. NOD or BDC2.5 CD4$^+$ T cells exhibited greater proliferation relative to total NOD.B6 *Idd3* and BDC.*Idd3* CD4$^+$ T cells under similar stimulatory conditions (Fig.2A). Consistently, depletion of NOD.B6 *Idd3* or BDC.*Idd3* CD4$^+$CD25$^+$ T cells resulted in a more significant increase in proliferation than similar treatments in T cells from NOD or BDC2.5 mice (data not shown).

To address whether the reduced proliferation observed in *Idd3*$^{B6}$ CD4$^+$ T cells was a consequence of an increased CD4$^+$Foxp3$^+$ nT$_{reg}$ cell pool within activated CD4$^+$ T cells, we determined the frequency of dividing CD4$^+$Foxp3$^+$ nT$_{reg}$ cells within the total CD4$^+$ T cell pool subsequent to anti-CD3 or mimetope stimulation by CFSE dilution analysis (Fig.2B). Our results show no difference in the percentage of CD4$^+$Foxp3$^+$ nT$_{reg}$

between unactivated $CD4^+$ T cells from NOD and NOD.B6 *Idd3* or BDC2.5 and BDC.*Idd3* (Fig.2B). However, activated $CD4^+$ T cells from NOD.B6 *Idd3* and BDC.*Idd3* revealed a significantly increased proportion of $CD4^+Foxp3^+$ $nT_{reg}$ cells at 72h post-activation when compared to NOD and BDC2.5 T cells, consistent with the reduced proliferation of $CD4^+$ $T_{eff}$ cells (Fig.2A,B). Notably, the higher percentage of $CD4^+Foxp3^+$ $nT_{reg}$ cells within the activated $CD4^+$ T cell pool from $Idd3^{B6}$ congenic mice correlated with increased proliferation (11.4±1.2 versus 7.7±0.6; $p \leq 0.009$) (Fig.2C), and increased numbers (data not shown) of $CD4^+Foxp3^+$ $nT_{reg}$ cells suggesting that $Idd3^{B6}$ $CD4^+Foxp3^+$ $nT_{reg}$ cells are intrinsically more potent in their function than WT NOD controls.

We then performed *in vitro* suppression assays to directly assess whether enhanced $CD4^+CD25^+$ $nT_{reg}$ cell-mediated suppression is affected in BDC.*Idd3* congenic mice. Our results show that $CD4^+CD25^+$ $nT_{reg}$ cells from BDC.*Idd3* mice were more efficient than BDC2.5 controls in suppressing anti-CD3-induced T cell proliferation at all $nT_{reg}/T_{eff}$ cell ratios examined, a finding consistent with $CD4^+CD25^+$ $nT_{reg}$ cells from NOD.*Idd3* mice (Fig.2D and data not shown). Overall, these findings demonstrate the ability of the protective $Idd3^{B6}$ allele to favor the function of $CD4^+Foxp3^+$ $nT_{reg}$ cells, which in turn dampen the proliferation of activated $CD4^+$ $T_{eff}$ cells *in vitro* more efficiently than in their BDC2.5 counterparts.

**Expansion of islet-reactive $CD4^+$ T cells is dampened in NOD.B6 *Idd3* mice.**

We sought to assess the impact of the protective $Idd3^{B6}$ locus on diabetogenic $CD4^+$ $T_{eff}$ cell activation and expansion *in vivo*. To this end, $CD4^+CD25^-$ $T_{eff}$ cells from

BDC2.5 or BDC.*Idd3* mice were CFSE-labeled, adoptively transferred into NOD or NOD.B6 *Idd3* recipients, and proliferation monitored by CFSE dilution. BDC2.5 and BDC.*Idd3* $T_{eff}$ cells proliferated and accumulated abundantly in the pancLN of NOD and NOD.B6 *Idd3* recipient mice (Fig.3A,B), but not in other peripheral LN (data not shown), confirming that T cell priming was β-islet antigen specific. Our results also reveal a more significant reduction in the antigen-driven proliferation and total accumulation of both BDC2.5 and BDC.*Idd3* $T_{eff}$ cells in the pancLN of NOD.B6 *Idd3* mice compared to NOD controls (Fig.3A,B). Interestingly, BDC.*Idd3* $T_{eff}$ cell antigen-induced proliferation was as efficient as that of BDC2.5 $T_{eff}$ cells in NOD recipient mice. This suggests that priming of diabetogenic T cells was not affected by the action of the *Idd3*$^{B6}$ allele in donor $T_{eff}$ cells. Instead, suppression of islet-reactive $CD4^+$ $T_{eff}$ cell priming is seemingly dependent on the presence of the protective *Idd3*$^{B6}$ allele in recipient mice (Fig.3A,B). Thus, the protective *Idd3*$^{B6}$ allele promotes a more suppressive environment, which impedes the activation and accumulation of antigen-specific, diabetogenic $CD4^+$ $T_{eff}$ cells *in vivo*.

In order to exclude the possibility that DC from *Idd3*$^{B6}$ congenic mice might be more tolerogenic in nature, and may be, on their own, responsible for the suppressive environment in these mice, we activated BDC2.5 $CD4^+$ T cells *in vitro* with $CD11c^{high}$MHC class $II^+$ DC purified from draining pancLN or spleen of BDC2.5 or BDC.*Idd3* mice, and in the presence of BDC2.5 mimetope. Our results show that splenic or pancLN DC from *Idd3*$^{B6}$ congenic mice were as potent in inducing antigen-specific T cell proliferation as DC from NOD controls (Fig.3C), suggesting that the suppressive environment conferred by the protective *Idd3*$^{B6}$ locus is independent of more tolerogenic DC in *Idd3*$^{B6}$ congenic mice.

**The differentiation of diabetogenic, IL-17-producing CD4$^+$ T cells in pancreatic lymph nodes is suppressed in NOD.B6 *Idd3* congenic mice.**

Since the proliferative capacity of autoreactive CD4$^+$ $T_{eff}$ cells was suppressed more efficiently in NOD.B6 *Idd3* congenic mice than control NOD mice, we wondered whether the differentiation of diabetogenic CD4$^+$ T cell pool would also be affected by protective *Idd3*$^{B6}$ alleles. While inflammatory cytokines produced by Th1 cells and DC, like IFN-$\gamma$, IL-12 and TNF-$\alpha$, are important mediators of $\beta$-islet destruction and play a pivotal role in the development of the insulitic lesions [34], recent studies show that IL-17-producing CD4$^+$ T (Th17) cells are important mediators of pathogenesis in various autoimmune disorders [35, 36]. Moreover, IL-17 mRNA transcripts have been shown to increase with the onset and severity of T1D [35, 36]. We hypothesized that these important inflammatory mediators were suppressed by the presence of the protective *Idd3*$^{B6}$ allele. To this end, BDC2.5 or BDC.*Idd3* CD4$^+$ T cells were injected into NOD or NOD.B6 *Idd3* recipients, and 4 days post-transfer, distal and pancLN cells were activated with mimetope-pulsed DC *ex vivo* and the production of IL-17 in T cells assessed by intracellular cytokine staining (ICS). Our results show that when BDC2.5 CD4$^+$ T cells were transferred into WT NOD recipients, a 6.5-fold enhancement in the frequency IL-17 producing CD4$^+$ T cells was observed (10.67%±4.2 versus 1.61±0.5; $p \leq 0.02$) compared to BDC2.5 or BDC.*Idd3* CD4$^+$ T cells transferred into NOD.B6 *Idd3* recipient mice. This suggested that the presence of the *Idd3*$^{B6}$ locus in either donor T cells or recipient mice was sufficient to severely hamper the differentiation of IL-17 producing CD4$^+$ T cells (Fig.4). Interestingly, IL-17 production was markedly hindered when the protective *Idd3*$^{B6}$ allele was present either exogenously within the donor cells

or endogenously within the recipient animals (Fig.4). These findings strongly suggest that the *Idd3*$^{B6}$ locus impacts the diabetogenic T cell pool by hindering its differentiation.

**The *Idd3*$^{B6}$ locus drives CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cell suppressive function and T1D protection.**

We then sought to directly determine whether CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cells from *Idd3*$^{B6}$ congenic mice were intrinsically better inhibitors of diabetogenic T cells and T1D *in vivo*. To this end, we transferred CD4$^{+}$CD25$^{-}$ T$_{eff}$ cells from BDC2.5 or BDC.*Idd3* mice into NOD.TCR$\alpha^{-/-}$ recipients either alone or in combination with BDC2.5 or BDC.*Idd3* CD4$^{+}$CD25$^{+}$ nT$_{reg}$ cells at physiological1T$_{reg}$/10T$_{eff}$ cell ratios, and the onset of diabetes was monitored. Recipient mice transferred with BDC2.5 or BDC.*Idd3* T$_{eff}$ cells alone developed T1D simultaneously between day 11-14 and with similar incidence, suggesting that the *Idd3*$^{B6}$ allele did not affect the diabetogenic potential of T$_{eff}$ cells *in vivo* (data not shown). Similarly, BDC2.5 and BDC.*Idd3* T$_{eff}$ cells accumulated with similar frequencies in the pancreas of recipient mice, further confirming that the *Idd3*$^{B6}$ allele does not directly impede the influx of islet-reactive CD4$^{+}$ T$_{eff}$ cells *in vivo* (data not shown). Interestingly, 80% of the recipients receiving BDC2.5 T$_{eff}$ and nT$_{reg}$ cells, developed diabetes by day 20 post-transfer, demonstrating that WT BDC2.5 nT$_{reg}$ cells are unable to maintain long-term self-tolerance (Fig.5A). Intriguingly, BDC2.5 CD4$^{+}$CD25$^{+}$ nT$_{reg}$ cells were capable of significantly reducing T1D incidence (40%) when co-transferred with BDC.*Idd3* T$_{eff}$ cells, suggesting that the presence of the *Idd3*$^{B6}$ allele in islet-reactive CD4$^{+}$ T$_{eff}$ cells is capable of potentiating the function of BDC2.5 CD4$^{+}$ nT$_{reg}$ cells *in vivo*. In stark contrast, NOD.TCR$\alpha^{-/-}$ recipient mice receiving BDC.*Idd3* CD4$^{+}$CD25$^{+}$ nT$_{reg}$ cell populations, irrespective of the genotype of the T$_{eff}$ cell

group, remained completely T1D-free for up to 20 days post-transfer, suggesting that BDC.*Idd3* $CD4^+CD25^+$ $nT_{reg}$ cells are more efficient at controlling the onset of T1D (Fig.5A). Thus, these results show that $Idd3^{B6}$ allelic variants drive the development of intrinsically more potent $CD4^+Foxp3^+$ $nT_{reg}$ cells, which in turn, impact disease progression and resistance to T1D.

Studies in various mouse models of disease indicate that $nT_{reg}$ cells can localize in sites of inflammation in order to mediate their protective effect [30, 37-39]. Given the increased suppression of T1D in mice receiving BDC.*Idd3* $nT_{reg}$ cells, we then asked whether this protection was due to increased accumulation of $CD4^+Foxp3^+$ $nT_{reg}$ cells in the pancreas. To this end, non-diabetic NOD.TCR$\alpha^{-/-}$ mice receiving $CD4^+$ $T_{eff}$ and $nT_{reg}$ cells from BDC2.5 or BDC.*Idd3* donors were sacrificed on day 15 post transfer, and the cellular frequency of $nT_{reg}$ and $T_{eff}$ cells was analyzed in the pancreas and various lymphoid organs. Our results show that no significant differences are detected in the overall frequencies of $CD4^+V\beta4^+$ T cells in the pancLN and pancreas of recipient mice regardless of $T_{eff}$ and $nT_{reg}$ cell origin, although frequencies of $CD4^+Foxp3^-$ $T_{eff}$ cells in the pancLN and pancreas of recipient mice were significantly reduced in the presence of BDC.*Idd3* $nT_{reg}$ cells (data not shown). Strikingly, mice receiving BDC.*Idd3* $CD4^+CD25^+$ $nT_{reg}$ cells showed increased frequencies of $CD4^+Foxp3^+$ $nT_{reg}$ cells in the pancLN and pancreas compared to mice receiving BDC2.5 $CD4^+Foxp3^+$ $nT_{reg}$ cells (Fig.5B). Interestingly, a significantly greater frequency of intra-pancreatic $CD4^+Foxp3^+$ $nT_{reg}$ cells was observed in co-transfers with BDC.*Idd3* $T_{eff}$ cells compared to BDC2.5 $T_{eff}$ cells, indicating that $Idd3^{B6}$ allelic variants in diabetogenic T cells on their own may not be sufficient to promote $CD4^+Foxp3^+$ $nT_{reg}$ cell activity and may act in a $nT_{reg}$ cell-

intrinsic fashion (Fig.5B). Thus, the decreased accumulation of $CD4^+Foxp3^+$ $nT_{reg}$ cells in the pancreas of BDC2.5 mice may suggest that they may be impaired in their ability to delay disease progression due to reduced functional capacities *in situ*.

We next determined whether this preferential accumulation of BDC.*Idd3* $CD4^+Foxp3^+$ $nT_{reg}$ cells in the pancreas also occurred in non-lymphopenic BDC2.5 and BDC.*Idd3* mice. While percentage of $CD4^+Foxp3^+$ $nT_{reg}$ cells in non-draining lymphoid sites in BDC2.5 and BDC.*Idd3* mice did not differ, BDC.*Idd3* mice show significantly higher frequencies of $nT_{reg}$ cells in the pancreas compared to BDC2.5 (28.8%±11.1% versus 11.4±3.9%; $p<0.0002$) (Fig.5C, right panel) and this correlated with a significantly reduced percentage of islet-specific $CD4^+$ $T_{eff}$ cells in the pancreas (19.0±9.9% versus 32.3±7.9%; $p<0.005$), and albeit to a lesser extent, in the pancLN, (52.7±7.8% versus. 67.8±8.2%; $p<0.005$) (Fig.5C, left panel). Overall, our results show that protective $Idd3^{B6}$ alleles favor high frequencies of $nT_{reg}$ cells in the target organ, which suppress the accumulation of islet-specific $CD4^+$ $T_{eff}$ cells and prevent the induction of T1D.

**The $Idd3^{B6}$ allele does not enhance resistance to apoptosis in $nT_{reg}$ cells.**

We show that T1D protection in BDC.*Idd3* mice correlates directly with an increased proportion of $CD4^+Foxp3^+$ $nT_{reg}$ cells in the target organ of prediabetic mice. Recently, we showed that T1D is not attributed to quantitative fluctuations in $CD4^+Foxp3^+$ $T_{reg}$ cells but more so to a temporal loss in the capacity of $CD4^+Foxp3^+$ $nT_{reg}$ cells to expand in pancreatic sites which in turn unleashes the diabetogenic potential of effector T cells [12]. We hypothesized that $Idd3^{B6}$ resistance alleles augment

the homeostasis of $nT_{reg}$ cells locally in the pancreas, correcting for the homeostatic "defect" in the BDC2.5, and consequently suppressing the function of diabetogenic T cells *in situ*. The *Idd3*$^{B6}$-mediated quantitative change in $nT_{reg}$ cells may result from a heightened resistance to apoptosis, consequently leading to their accumulation in pancreatic sites. To this end, we examined by FACS various lymphoid organs and the pancreas of pre-diabetic NOD and NOD.B6 *Idd3* or BDC2.5 and BDC.*Idd3* mice for the expression of Bcl-2, a critical mediator in the anti-apoptotic pathway. Our results show that neither the frequency of Bcl-2-positive $CD4^+Foxp3^+$ $nT_{reg}$ cells nor the amount of Bcl-2 produced in $CD4^+Foxp3^+$ $nT_{reg}$ cells varied between NOD and NOD.B6 *Idd3* (Fig.6, left panel) or BDC2.5 and BDC.*Idd3* mice (Fig.6, right panel). Hence, the protective *Idd3*$^{B6}$ alleles do not modulate $nT_{reg}$ cell functions by mediating their resistance to apoptosis.

**The *Idd3*$^{B6}$ locus potentiates $nT_{reg}$ cell proliferation *in vivo*.**

Since the increased proportion of $nT_{reg}$ cells in the pancreas of protected BDC.*Idd3* mice could not be attributed to prolonged survival, we hypothesized that an enhanced local expansion of $nT_{reg}$ cells could explain the protective phenotype observed in animals containing the *Idd3*$^{B6}$ interval. In order to evaluate the impact of the *Idd3*$^{B6}$ locus on $nT_{reg}$ cell expansion in draining pancLN, CFSE-labeled BDC2.5 or BDC.*Idd3* $CD4^+$ T cells were transferred into NOD or NOD.B6 *Idd3* recipients and proliferation was monitored. The greatest $nT_{reg}$ proliferation was observed when both the donor cells and recipient animals originated from *Idd3*$^{B6}$ congenic mice (41.62±10.1%), suggesting that both $nT_{reg}$ cell-intrinsic and extrinsic factors cooperated to yield enhanced proliferative capacity (Fig.7A). Interestingly, BDC.*Idd3*

$CD4^+V\beta4^+Foxp3^+$ $nT_{reg}$ cells accumulated in greater numbers in draining pancreatic sites, irrespective of the genotype of the recipient animals, confirming that the *Idd3*$^{B6}$ locus drives $nT_{reg}$ cell expansion and promotes their accumulation in pancLN (Fig.7B). The presence of donor $nT_{reg}$ cells in this system did not impede the activation of the diabetogenic T cell pool, as $T_{eff}$ cells from both genotypes exhibited similar proliferative profiles, irrespective of the origin of the recipient animal (data not shown). Interestingly, the accumulation of $CD4^+V\beta4^+Foxp3^-$ $T_{eff}$ cells was drastically reduced in NOD.B6 *Idd3* recipients irrespective of the genotype of the donor T cells (Fig.7C), demonstrating that the NOD.B6 *Idd3* environment is tolerogenic and impedes the expansion of diabetogenic $T_{eff}$ cells. Thus, this data highlights the importance of the *Idd3*$^{B6}$ locus in promoting $nT_{reg}$ cell proliferation and restraining the expansion of diabetogenic $T_{eff}$ cells.

**_Il2_ allelic variants promote the cycling of $CD4^+Foxp3^+$ $nT_{reg}$ cells directly in the pancreas.**

Since a greater proportion of $nT_{reg}$ cells accumulated and proliferated in draining pancreatic sites of NOD.B6 *Idd3* animals, we wondered whether the observed increased frequency of *Idd3*$^{B6}$ $nT_{reg}$ cells within the target organ (Fig.5C, right panel) was attributed to more efficient expansion *in situ*. To examine this possibility, we determined the cellular frequency of cycling $CD4^+Foxp3^+$ $nT_{reg}$ cells, as determined by the Ki-67 proliferation marker, in the spleen, non-draining mesenteric LN, pancLN and pancreas of BDC2.5 and BDC.*Idd3* mice. Our results show a marked decrease in the proportion of cycling $CD4^+Foxp3^-$ $T_{eff}$ cells within the pancreas of BDC.*Idd3* relative to WT BDC2.5 mice (17.4±4.0% versus 28.0±3.8%; $p$ <0.00001) (Fig.8A), and similar cycling differences could not be detected in spleen and distal LN suggesting that T1D

protection in BDC.*Idd3* mice correlates directly with the increased proportion of $CD4^+Foxp3^+$ $nT_{reg}$ cells in the target organ. In addition, although no significant differences were observed in non-draining and draining lymphoid sites between genotypes, the proportion of cycling $nT_{reg}$ cells was markedly enhanced within the pancreas of BDC.*Idd3* mice relative to WT BDC2.5 mice, and correlated with the frequency of CD25-expressing cycling $nT_{reg}$ cells (13.4±3.1% versus 6.5±2.5%; $p<0.0001$) (Fig.8B). This suggests that an increase in IL-2 in the inflammatory milieu, generated by the diabetogenic $T_{eff}$ cell pool, drives the upregulation of CD25, potentiating $nT_{reg}$ cell suppressive functions, a finding consistent with the observed local expansion of $CD4^+Foxp3^+$ $nT_{reg}$ cells within inflammatory sites seen in other mouse models [10, 37, 38, 41, 42]. More importantly, the decline in cycling $CD4^+Foxp3^-$ $T_{eff}$ cells in BDC.*Idd3* mice correlates directly with an increased proportion of cycling $CD4^+Foxp3^+$ $nT_{reg}$ cells (12.0±0.8% versus 4.4±1.9%; $p=1.1x10^{-8}$), suggesting that the proliferative potential of $nT_{reg}$ cells correlates directly with their functional potency, and is strongly indicative that $nT_{reg}$ cells are actively suppressing autoreactive $CD4^+Foxp3^-$ $T_{eff}$ cells within the target organ (Fig.8). Collectively, our data strongly suggests that a regulatory feedback loop initiated by IL-2-producing self-reactive $Idd3^{B6}$ $CD4^+$ T cells favors the preferential expansion and function of $CD4^+Foxp3^+$ $nT_{reg}$ cells within the target organ, in turn increasing the $T_{reg}/T_{eff}$ cell ratio and tipping the balance to self-tolerance.

## 5. Discussion.

$CD4^+Foxp3^+$ $nT_{reg}$ cells have been implicated as a central control point in the pathogenesis of T1D in NOD mice [30, 43]. T1D resistance also correlates with the expansion of $CD4^+CD25^+$ T cells within pancLN and insulitic lesions, and with a consequential decrease in the priming, expansion or differentiation of $T_{eff}$ cells in these sites [30, 41, 44, 45]. Developmental or functional defects of $CD4^+Foxp3^+$ $nT_{reg}$ cells in autoimmune-prone hosts may represent a major predisposition factor for spontaneous T1D [46, 48].

Fine mapping studies have established *Il2* as the primary genetic determinant of disease protection operative in the *Idd3*$^{B6}$ locus [22, 31]. Considering the critical role of IL-2 in $nT_{reg}$ cell functions, we hypothesized that *Idd3*$^{B6}$ protective alleles impart potent resistance to T1D by potentiating $nT_{reg}$ cell-mediated regulation of diabetogenic T cells. We found that the protective *Idd3*$^{B6}$ allele, relative to the NOD allele, augments the amount of IL-2 mRNA and protein produced by diabetogenic $CD4^+$ $T_{eff}$ cells, and affords resistance to spontaneous and $CD4^+$ T cell-induced T1D. While the frequency of $CD4^+Foxp3^+$ $nT_{reg}$ cells is not affected, and their functional potency is increased in *Idd3*$^{B6}$ congenic mice, we make the novel finding that that *Idd3*$^{B6}$ protective alleles primarily favor T1D disease resistance by heightening the cycling and function of $CD4^+Foxp3^+$ $nT_{reg}$ cells locally within the inflammatory environment of the pancreas. Collectively, we show that the T1D-protective *Idd3*$^{B6}$ allele variants dictate the amount of IL-2 production by diabetogenic $CD4^+$ $T_{eff}$ cells, which initiates a regulatory feedback loop driving the functional homeostasis of $CD4^+Foxp3^+$ $nT_{reg}$ cells in the target organ.

IL-2 is now viewed as an important signal for the development, function, and competitive fitness of $nT_{reg}$ cells *in vivo* [23, 26]. As $CD4^+Foxp3^+$ $nT_{reg}$ cells fail to make IL-2, their primary source of IL-2 *in vivo* is likely from $CD4^+$ $T_{eff}$ cells [29, 50, 51]. Indeed, mice deficient for B7/CD28, CD40/CD40L, IL-2, IL-2R$\alpha/\beta$, or STAT5A/B have drastically reduced $nT_{reg}$ cell numbers and suffer from severe autoimmunity [24, 25, 52-57]. Consistently, *in vivo* neutralization of IL-2 in NOD mice actually precipitates the onset and incidence of T1D [26]. Interestingly, NOD T cells respond normally to TCR activation until 4 wks of age, at which point they become anergic, and sustain a drastic reduction in IL-2 production, coinciding with the onset of insulitis [3]. This reduced IL-2 expression and activity in NOD mice may abrogate $nT_{reg}$ cell function and subsequently enable diabetogenic T cells to transition from insulitis (checkpoint 1) to overt T1D (checkpoint 2). Thus, temporal alterations in IL-2 expression in the prediabetic phase of NOD mice may influence $nT_{reg}$ cell development, and ultimately affect T1D onset [12, 23, 26].

Our results show that the protective effect of $Idd3^{B6}$ requires the presence of $CD4^+Foxp3^+$ $nT_{reg}$ cells, since depletion of $CD4^+CD25^+$ $nT_{reg}$ cells (90% of $Foxp3^+$ $nT_{reg}$ cells) from $CD4^+$ T cells unleashes the diabetogenic potential of BDC.*Idd3* $T_{eff}$ cells *in vivo*. Although our data shows that the $Idd3^{B6}$ environment conditions $nT_{reg}$ cells to be more suppressive, the protection conferred by the $Idd3^{B6}$ allele is neither dominant nor recessive, but more so dose dependent in nature [15, 22]. In this instance, the $Idd3^{B6}$ allele would likely result in higher IL-2 levels, particularly in the local environment of inflammation, which in turn would affect $nT_{reg}$ cell homeostasis and function in order to assure disease protection. Interestingly, recent evidence has demonstrated that IL-2 is

a potent inhibitor of Th17 cell differentiation *in vitro* and *in vivo* [58]. Our data would also suggest that the increased production of IL-2 by T cells from *Idd3*$^{B6}$ mice might promote protection to T1D by hindering Th17 cells while simultaneously promoting CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cells. Thus, our model would suggest that the presence of protective *Idd3*$^{B6}$ alleles permit diabetogenic CD4$^{+}$ T cells to produce sufficient IL-2 to optimally promote the expansion and function of CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cells in the pancLN and pancreas, and in turn blocking the diabetogenic process in the target organ. Consistently, administration of low-dose IL-2 promoted nT$_{reg}$ cell survival and protected NOD mice from developing T1D [28].

Studies have shown that the “susceptible” and “resistant” IL-2 differ in their N-terminal sequence, correlating with putative, differential glycosylation states, suggesting that IL-2 variants may be functionally distinct, potentially affecting the synthetic rate, protein folding/half-life, binding affinity or signalling of IL-2 [32]. Yamanouchi *et al*. recently demonstrated that several SNPs within the 5’ region of the NOD haplotype of *Il2* promoter collectively influence the competency of activated CD8$^{+}$ T cells to initiate IL-2 transcription or secrete IL-2 protein in activated self-reactive T cells [22]. This increased IL-2 expression by the *Idd3*$^{B6}$ allele in activated T cells may be related to improved assembly/activation of the transcription machinery during T cell activation [59, 60], in turn enabling more efficient IL-2 transcription or secretion in T cells with the downstream effect of increasing the cellular frequency of IL-2 secreting CD4$^{+}$ T cells in a given immune response. More importantly, the sole presence of *Idd3*$^{B6}$ protective alleles in diabetogenic CD4$^{+}$ T$_{eff}$ cells is able to partially correct the “defective fitness” of CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cells in NOD hosts (Fig.5A), indicating that *Idd3*$^{B6}$ allelic variants in

diabetogenic T cells are important contributors in self-tolerance mechanisms. These results do not exclude a possible $CD4^+$ $nT_{reg}$ cell-intrinsic role for protective *Idd3*$^{B6}$ alleles.

The capacity of $nT_{reg}$ cells to localize directly within inflamed tissues to dampen immune responses has been shown in various models of infectious disease, inflammatory bowel disease and tumors [37, 39, 40]. Similarly, $CD4^+Foxp3^+$ $nT_{reg}$ cells block the diabetogenic process, in part, by localizing within insulitic lesions, where they suppress the function of $T_{eff}$ cells [12, 30, 45, 61]. We show that $nT_{reg}$ cells from BDC.*Idd3* mice preferentially expand within the pancreas of T1D-protected mice, where they control the effector functions of infiltrating diabetogenic $CD4^+Foxp3^-$ T cells. This would suggest that the loss of Ag-driven homing, activation or expansion of $nT_{reg}$ cells in pancreatic sites may represent an essential checkpoint in the T1D progression, and that the protective *Idd3*$^{B6}$ alleles correct for this defect in NOD.B6 *Idd3* mice. It is unknown whether *Idd3*$^{B6}$ engages unique transcriptomes in infiltrating $nT_{reg}$ cells, particularly with regards to genes affecting metabolism, cell cycle, homing, and survival of $nT_{reg}$ cells.

In conclusion, we link the T1D-protective effect of *Idd3*$^{B6}$ with a more potent $CD4^+Foxp3^+$ $nT_{reg}$ cell compartment, particularly with regards to its ability to promote regulatory function in the local inflammatory environment of the pancreas. Our results are potentially relevant to human T1D considering that some studies have suggested a reduction of $nT_{reg}$ cell number and/or function in individuals with T1D [62]. In human T1D, the genes that encode for CTLA-4, insulin, and PTPN22 map to T1D susceptibility, and although an association with *Il2* in human T1D has not been made, a recent study by Qu *et al.* identified 2 SNPs in *Il2ra/CD25*, which correlate with T1D susceptibility in

humans [63-66]. Thus, the control of organ-specific autoimmunity is critically dependent on the dominant regulation of self-reactive T cells, and that in genetically susceptible subjects with a defective $CD4^{+}Foxp3^{+}$ $nT_{reg}$ cell compartment, an increase in IL-2R signalling may diminish T1D risk. This study illustrates that some T1D susceptibility genes may alter the balance between pathogenic and $nT_{reg}$ cell populations and ultimately contribute to T1D pathogenesis.

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## 9. Figure legends.

### FIGURE 1. Resistance to the progression of T1D in NOD.B6 *Idd3* congenic mice correlates with increased production of IL-2 by autoreactive CD4+ T cells.

(A) NOD.B6 *Idd3* mice exhibit drastically reduced incidence and onset of diabetes relative to the WT NOD mice (n=20). (B) Decreased frequency of IFN-$\gamma$ or TNF-$\alpha$ secreting $CD4^+$ T cells in BDC.*Idd3* congenic mice. Lymph node cells were isolated from pre-diabetic, BDC2.5 or BDC.*Idd3* mice (3-4 wk old), were co-cultured with BMDC (4:1 ratio) and BDC2.5 mimetope (100ng/mL), and the frequency of IFN-$\gamma$ and TNF-$\alpha$ producing $CD4^+V\beta4^+$ T cells was determined by ICS (n=5). Numbers represent the percentage of $CD4^+V\beta4^+$IFN-$\gamma^+$ or $CD4^+V\beta4^+$TNF-$\alpha^+$ cells. Results represent the mean ± SD. * $p \leq 0.008$, † $p<0.05$ difference from control BDC2.5 IFN-$\gamma$ or TNF-$\alpha$ producing $CD4^+$ T cells. (C) The $Idd3^{B6}$ locus drastically reduces the $CD4^+$ T cell infiltration in the pancreas of *Idd3* congenic mice. Pancreata from 3-4 week-old NOD or NOD.B6 *Idd3* mice were processed as described in Materials and Methods. The profiles (1.04±0.3% vs. 7.38±1.9%; $p \leq 0.004$) are representative of 3 (n=3) separate independent experiments. (D) The $Idd3^{B6}$ locus leads to increased IL-2 gene transcription in $CD4^+$ T cells. $CD4^+CD25^-$ T cells were isolated from pooled LN of 3-4 wk old NOD or NOD.B6 *Idd3* mice, and were activated under plate-bound anti-CD3 (5$\mu$g/ml) conditions. At various time points, total RNA was extracted and RT-PCR analysis of IL-2 relative to G3PDH gene expression was performed as described in Materials and Methods. (E) Increased IL-2 protein secretion in activated $CD4^+$ T cells from NOD.B6 *Idd3* or BDC.*Idd3* mice. $CD4^+CD25^-$ T cells from pooled LN of 3-4 wk old NOD and NOD.B6 *Idd3* or BDC2.5 and BDC.*Idd3* mice were stimulated with PMA/ionomycin or BMDC (4:1 ratio) and BDC2.5 mimetope (100ng/mL) respectively and ICS for IL-2 was performed. Data are representative of at least 3 separate experiments. Results represent the mean ± SD. $p<0.01$, difference with NOD IL-2 producing $CD4^+CD25^-$ T cells after 24h activation.

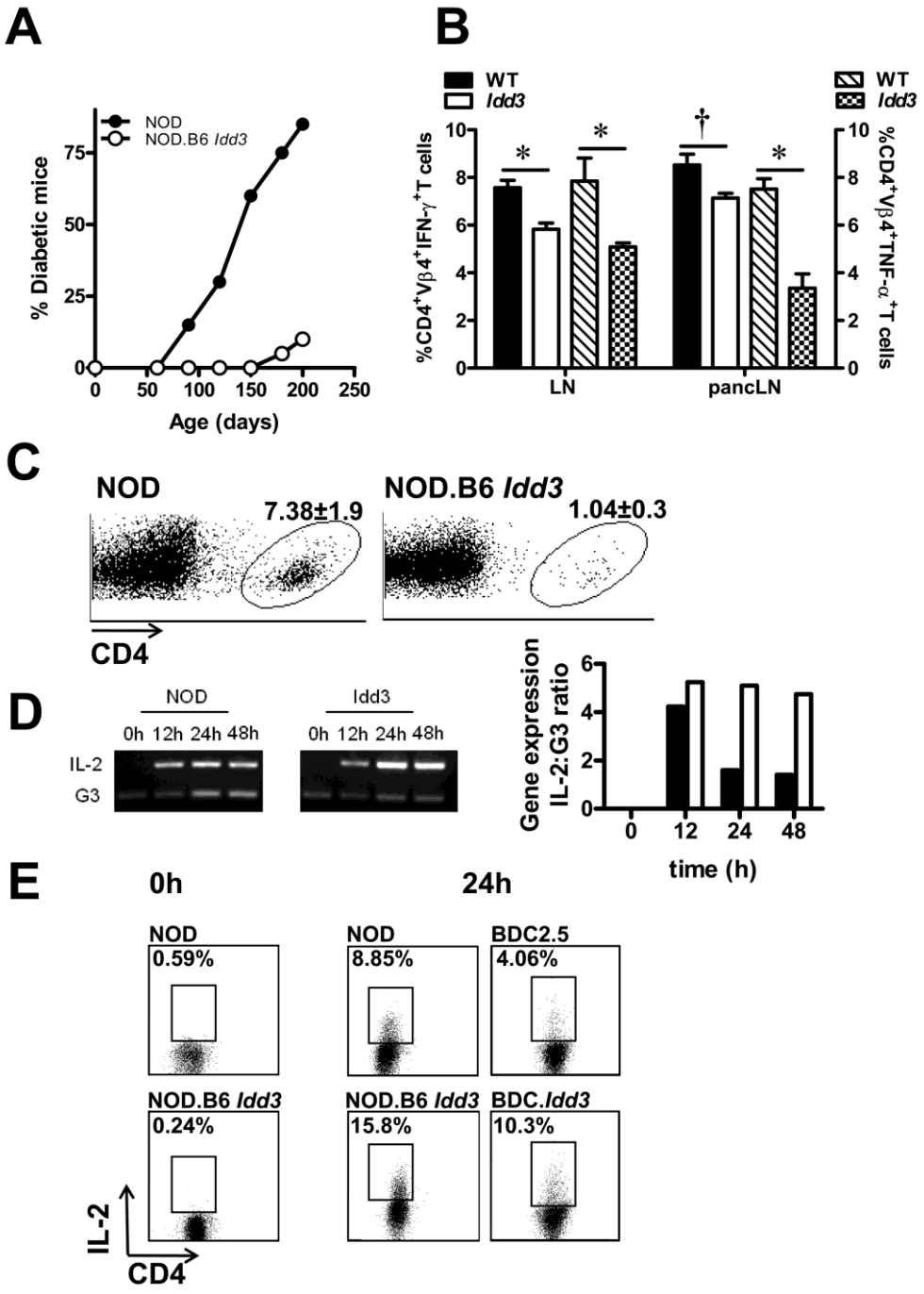
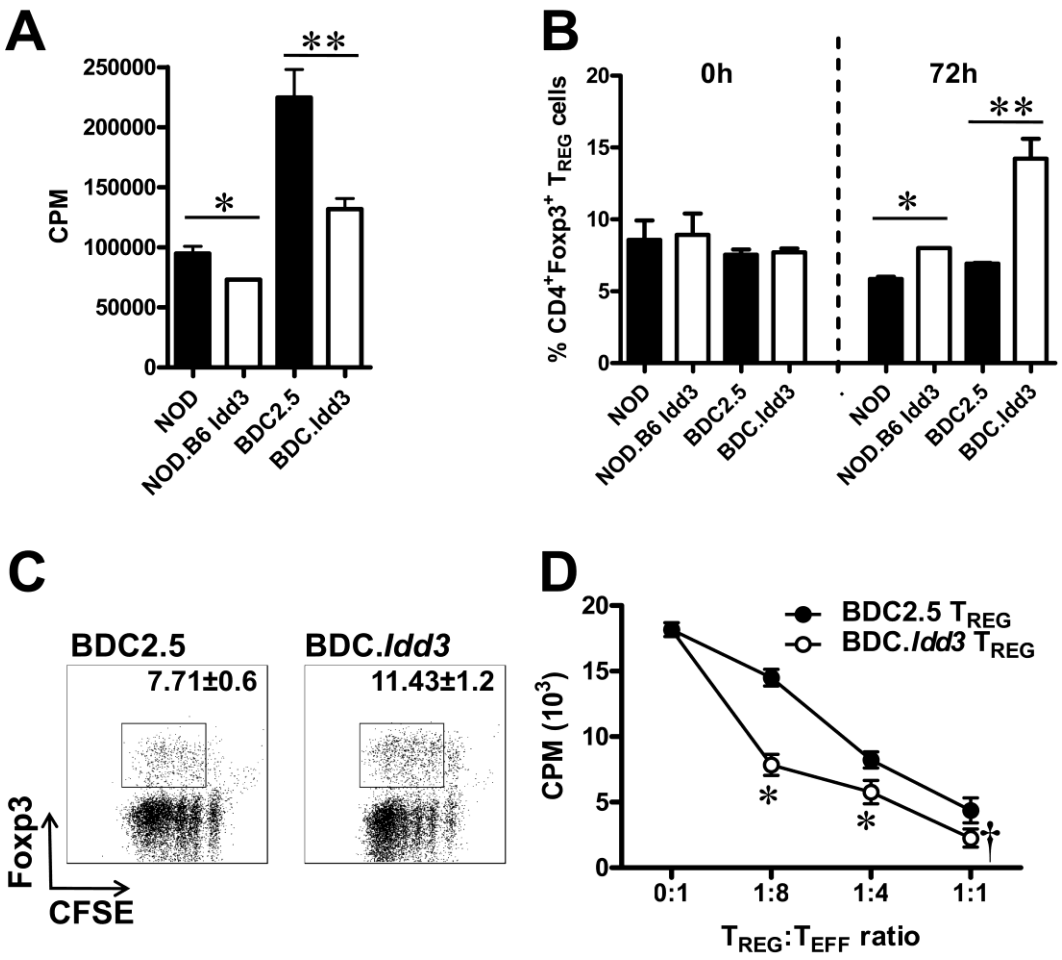

A
NOD
NOD.B6 Idd3
% Diabetic mice
Age (days)
B
WT
Idd3
%CD4+Vβ4+IFN-γ+T cells
%CD4+Vβ4+TNF-α+T cells
LN
pancLN
C
NOD
7.38±1.9
NOD.B6 Idd3
1.04±0.3
CD4
D
NOD
Idd3
0h 12h 24h 48h
IL-2
G3
Gene expression
IL-2:G3 ratio
time (h)
E
0h
24h
NOD
0.59%
NOD
8.85%
BDC2.5
4.06%
NOD.B6 Idd3
0.24%
NOD.B6 Idd3
15.8%
BDC.Idd3
10.3%
IL-2
CD4

**FIGURE 2. Protective *Idd3*$^{B6}$ alleles augment CD4$^+$Foxp3$^+$ nT$_{reg}$ cell function in activated CD4$^+$ T cells *in vitro*.**

(A) CD4$^+$ T cells from *Idd3*$^{B6}$ congenic mice are hypoproliferative *in vitro* compared to NOD controls. CD4$^+$ T cells (5x10$^4$) were isolated from LN of 3-4 wk old WT or NOD.B6 *Idd3* congenic mice, and activated with irradiated splenocytes (2x10$^5$) and anti-CD3 (1μg/ml). CD4$^+$ T cells from BDC2.5 and BDC.*Idd3* mice (1x10$^5$) were activated in the presence of LPS-matured BMDC (2.5 x 10$^4$) pulsed with the BDC2.5 mimetope (300ng/ml). In all instances, proliferation was assessed by thymidine incorporation at 72h post-activation. Results represent the mean ± SD, * $p$<0.01 difference with NOD or BDC2.5 T$_{eff}$ cell proliferation. (B) Decreased proliferation of T cells in NOD.B6 *Idd3* and BDC.*Idd3* congenic mice relative to WT NOD and BDC2.5 respectively correlates with an augmented proportion of CD4$^+$Foxp3$^+$ nT$_{reg}$ cells in activated CD4$^+$ T cells. CD4$^+$ T cells (1x10$^5$) from LN of NOD and NOD.B6 *Idd3* or BDC2.5 and BDC.*Idd3* mice were activated with irradiated splenocytes (4 x 10$^5$) and anti-CD3 (1μg/ml) or BDC2.5 mimetope-pulsed (300ng/ml) BMDC, respectively. Results represent the mean ± SD. * $p$<0.01 difference with control NOD or BDC2.5 Foxp3 frequencies 72h post-activation. (C) CFSE-labeled BDC2.5 and BDC.*Idd3* CD4$^+$CD25$^{+/-}$ T cells were activated for 72h with mimetope-pulsed (100ng/mL) BMDC, and the frequency of dividing CD4$^+$Vβ4$^+$Foxp3$^+$ T cells was assessed by FACS. $p$<0.009 difference with BDC2.5 dividing T$_{reg}$ cells (D) CD4$^+$CD25$^+$ nT$_{reg}$ cells from BDC.*Idd3* are more suppressive *in vitro* than their BDC2.5 control littermates. CD4$^+$CD25$^-$ responder T cells from BDC.*Idd3* congenic mice (5x10$^4$) were stimulated with anti-CD3 (1μg/ml) and irradiated spleen cells (2x10$^5$) in the presence or absence of titrated numbers of CD4$^+$CD25$^+$ nT$_{reg}$ cells from BDC2.5 or BDC.*Idd3* mice. Data are representative of three separate experiments. Results represent the mean ± SD. * $p$<0.02 and **†** $p$<0.04 difference with WT control BDC2.5 T$_{eff}$ cell proliferation.


A
CPM
250000
200000
150000
100000
50000
0
*
**
NOD
NOD.B6 Idd3
BDC2.5
BDC.Idd3
B
% CD4+Foxp3+ TREG cells
20
15
10
5
0
0h
72h
*
**
NOD
NOD.B6 Idd3
BDC2.5
BDC.Idd3
NOD
NOD.B6 Idd3
BDC2.5
BDC.Idd3
C
BDC2.5
BDC.Idd3
7.71±0.6
11.43±1.2
Foxp3
CFSE
D
BDC2.5 TREG
BDC.Idd3 TREG
CPM (10^3)
20
15
10
5
0
*
*
†
0:1
1:8
1:4
1:1
TREG:TEFF ratio

## FIGURE 3. The expansion and accumulation of islet-reactive CD4+ T cells is dampened in NOD.B6 *Idd3* mice.

CFSE-labeled $CD4^+CD25^-$ T cells ($3x10^6$) from BDC2.5 or BDC.*Idd3* mice were adoptively transferred into NOD or NOD.B6 *Idd3* recipients. The pancLN of recipient mice were harvested on day 4 post-transfer, and the proliferative capacity (A) and absolute numbers (B) of donor $CD4^+V\beta4^+$ T cells were determined in recipient mice (n=3-4 mice/group). Similar results were obtained in three independent experiments. Results represent the mean ± SD. * $p<0.02$ difference between recipients receiving BDC2.5 $T_{eff}$ cells or recipients receiving BDC.*Idd3* $T_{eff}$ cells. (C) DC from draining pancreatic sites do not drive the $Idd3^{B6}$-mediated effect on $T_{eff}$ cell expansion. In order to examine the impact of DC on the proliferative capacity of $CD4^+$ T cells, $CD11c^{high}$MHC $II^+$ DC were purified from draining pancLN and spleen of 3-4w old BDC2.5 or BDC.*Idd3* mice and plated at a 1:4 ratio with highly purified BDC2.5 $CD4^+$ T cells in the presence of BDC2.5 mimetope (100ng/mL) for 72h. Proliferation was assessed by thymidine incporporation. No significance (n.s.) was observed between groups.

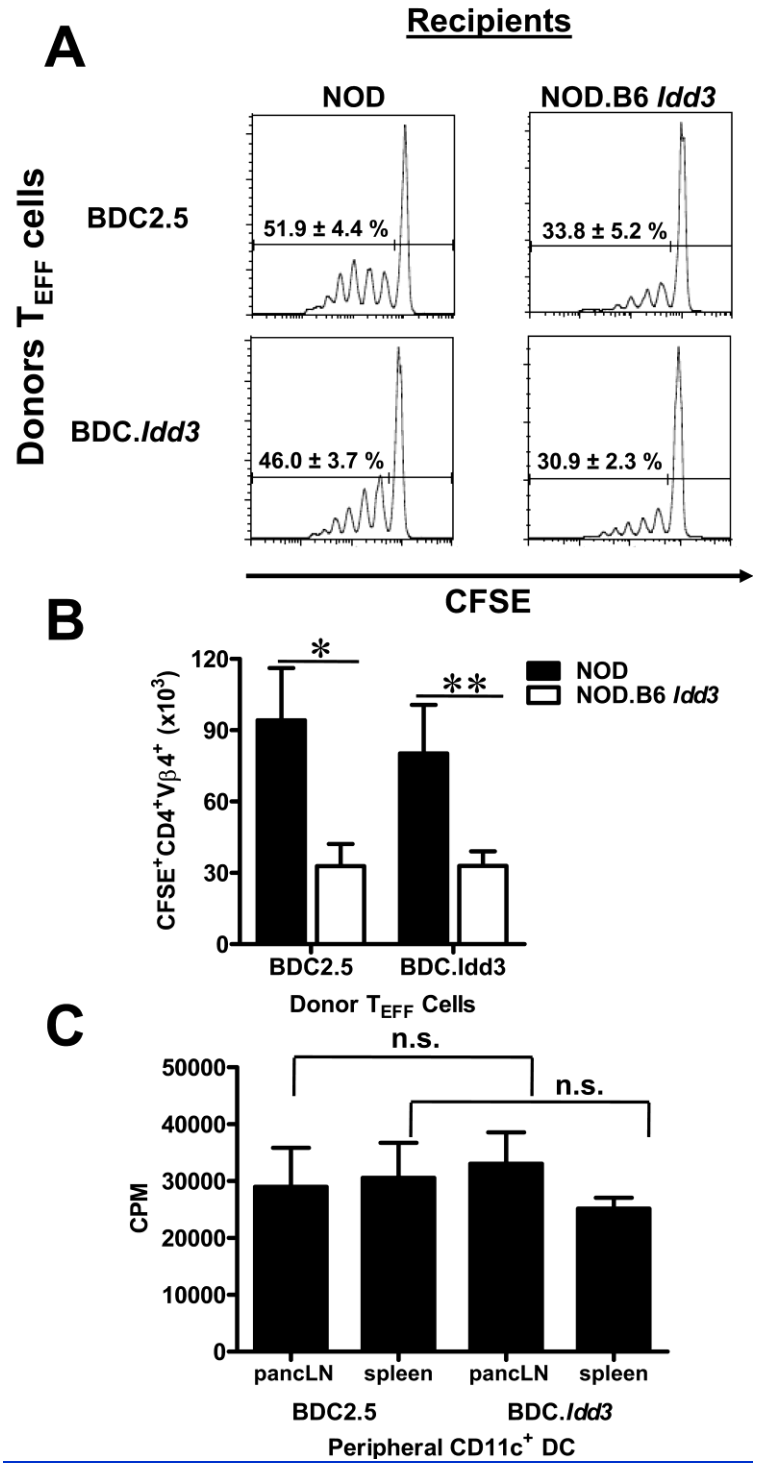
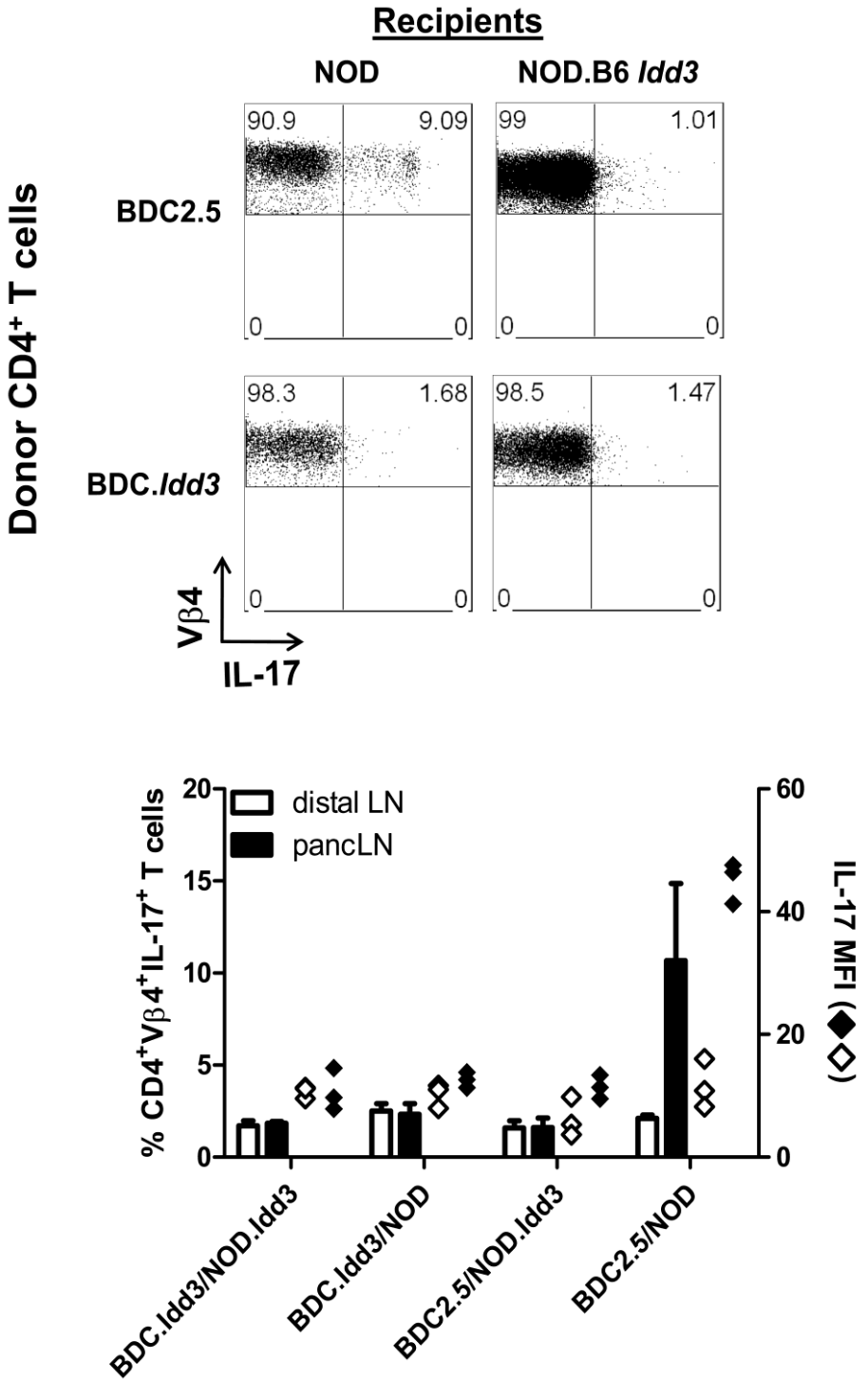

A
Recipients
NOD
NOD.B6 Idd3
Donors T_EFF cells
BDC2.5
BDC.Idd3
51.9 ± 4.4 %
33.8 ± 5.2 %
46.0 ± 3.7 %
30.9 ± 2.3 %
CFSE
B
CFSE+CD4+Vβ4+ (x10^3)
120
90
60
30
0
*
**
NOD
NOD.B6 Idd3
BDC2.5
BDC.Idd3
Donor T_EFF Cells
C
n.s.
n.s.
CPM
50000
40000
30000
20000
10000
0
pancLN
spleen
pancLN
spleen
BDC2.5
BDC.Idd3
Peripheral CD11c+ DC

**FIGURE 4. The differentiation of diabetogenic, IL-17-producing CD4$^+$ T cells in pancreatic lymph nodes is suppresses in NOD.B6 *Idd3* congenic mice.**

CFSE-labeled CD4$^+$ T cells from BDC2.5 or BDC.*Idd3* 6-8w old donor mice were adoptively transferred to NOD or NOD.B6 *Idd3* recipients. Four days post-transfer, pancLN and distal mesenteric LN of recipient animals were collected and plated (1x10$^6$) in the presence of LPS-matured, BDC mimetope-pulsed (100ng/mL) BMDC (5x10$^5$) for 24h. Cells were subsequently collected and stained for Vβ4, CD4 and ICS was performed for IL-17 production. Depicted are representative profiles of cells gated on CD4$^+$CFSE$^+$ T cells (top panel). Results represent the mean ± SD and MFI (open and closed diamonds) (bottom panel). On the x-axis, data are reported as genotype of cells transferred/genotype of recipient.


Recipients
NOD
NOD.B6 Idd3
Donor CD4+ T cells
BDC2.5
BDC.Idd3
90.9
9.09
99
1.01
98.3
1.68
98.5
1.47
0
Vβ4
IL-17
distal LN
pancLN
% CD4+Vβ4+IL-17+ T cells
IL-17 MFI (◆◇)
20
15
10
5
60
40
20
BDC.Idd3/NOD.Idd3
BDC.Idd3/NOD
BDC2.5/NOD.Idd3
BDC2.5/NOD

## FIGURE 5. The *Idd3*$^{B6}$ locus potentiates CD4$^+$Foxp3$^+$ nT$_{reg}$ cell suppressive function and T1D protection *in vivo*.

(A) NOD.TCR$\alpha^{-/-}$ recipient mice were adoptively transferred with FACS-sorted BDC2.5 or BDC.*Idd3* CD4$^+$CD25$^-$ T$_{eff}$ cells (5x10$^5$) in the presence or absence (data not shown) of BDC2.5 or BDC.*Idd3* CD4$^+$CD25$^+$ (5x10$^4$) nT$_{reg}$ cells from peripheral LN of 6-8 wk old donor mice. Blood glucose levels in recipient mice (n=7 recipients/group) were monitored for diabetes incidence every 48 hrs post-transfer. Results represent the mean ± SD. $p$<0.0001 difference between control NOD.TCR$\alpha^{-/-}$ recipient mice receiving BDC2.5 T$_{eff}$/BDC2.5 nT$_{reg}$ and those receiving BDC.*Idd3* T$_{eff}$/BDC.*Idd3* nT$_{reg}$. $p$<0.05 difference in diabetes onset between control NOD.TCR$\alpha^{-/-}$ recipient mice receiving BDC2.5 T$_{eff}$/BDC2.5 nT$_{reg}$ and those receiving BDC.*Idd3* T$_{eff}$/BDC2.5 nT$_{reg}$ cells. (B) Increased accumulation of CD4$^+$Foxp3$^+$ nT$_{reg}$ cells in pancreatic sites of mice receiving BDC2.5 or BDC.*Idd3* nT$_{reg}$ cells. NOD.TCR$\alpha^{-/-}$ recipient mice (n=2-4 mice/group) were adoptively transferred as in (A), and on day 16 post-transfer, mice from each group were sacrificed, pancLN and pancreas were harvested, and the cellular frequency of islet-specific Foxp3$^+$ nT$_{reg}$ cells within the CD4$^+$V$\beta$4$^+$ T cell compartment was determined by FACS. * $p$<0.01, † $p$<0.05 difference with control NOD.TCR$\alpha^{-/-}$ mice receiving BDC2.5 or BDC.*Idd3* T$_{reg}$ cells along with BDC2.5 T$_{eff}$ or those receiving BDC2.5 or BDC.*Idd3* T$_{reg}$ cells along with BDC.*Idd3* T$_{eff}$ cells. (C) Total frequency of islet-specific CD4$^+$V$\beta$4$^+$ T cells (left panel) and CD4$^+$V$\beta$4$^+$Foxp3$^+$ nT$_{reg}$ cells (right panel) was determined in the pancLN and pancreas of 3-4 wk old BDC2.5 and BDC.*Idd3* mice (n=10). Data are representative of the compilation of several independent experiments. Results represent the mean ± SD. * $p$=0.0005 difference with control BDC2.5 mice.

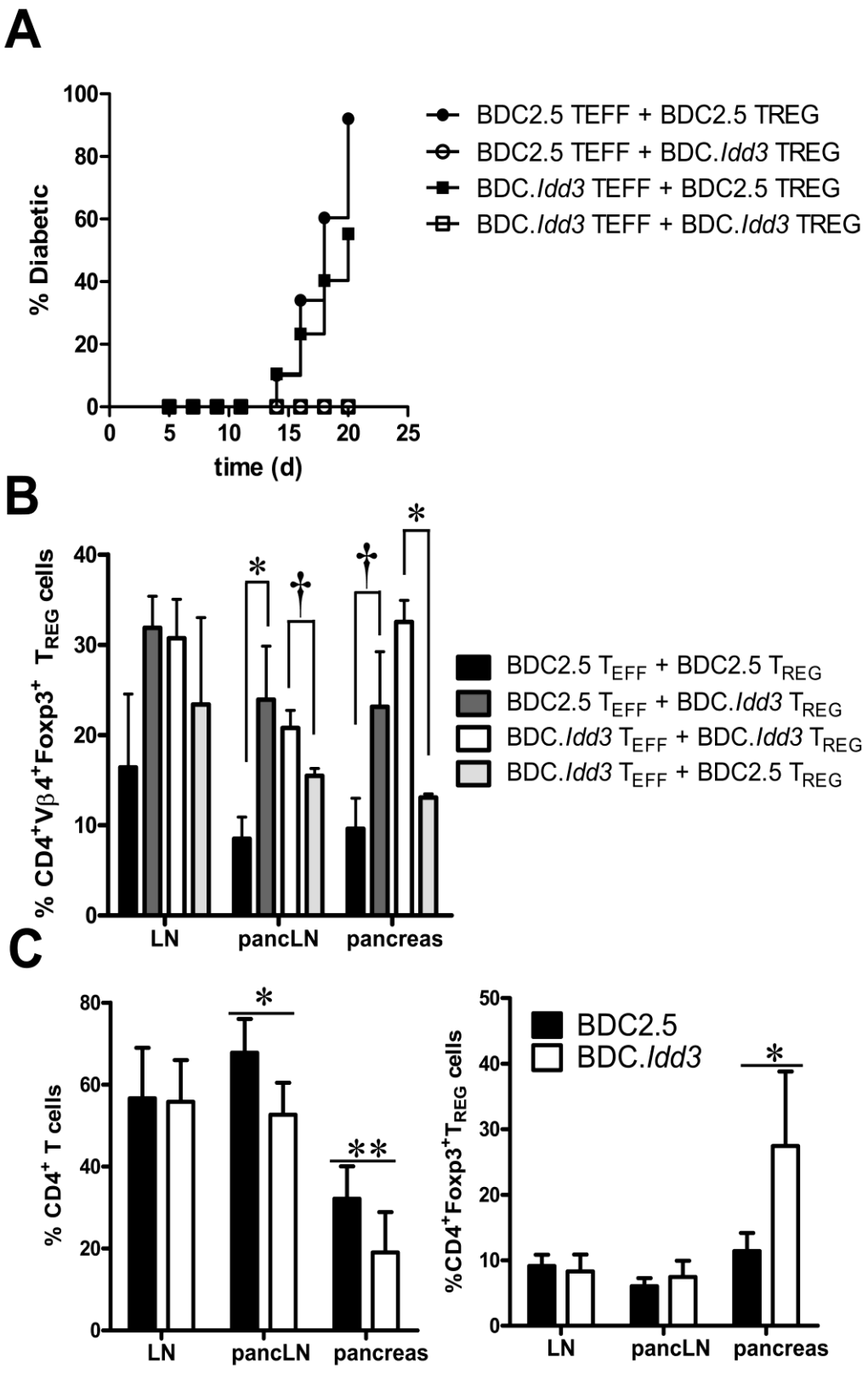

A
% Diabetic
time (d)
BDC2.5 TEFF + BDC2.5 TREG
BDC2.5 TEFF + BDC.Idd3 TREG
BDC.Idd3 TEFF + BDC2.5 TREG
BDC.Idd3 TEFF + BDC.Idd3 TREG
B
% CD4+Vβ4+Foxp3+ TREG cells
LN
pancLN
pancreas
BDC2.5 TEFF + BDC2.5 TREG
BDC2.5 TEFF + BDC.Idd3 TREG
BDC.Idd3 TEFF + BDC.Idd3 TREG
BDC.Idd3 TEFF + BDC2.5 TREG
C
% CD4+ T cells
%CD4+Foxp3+TREG cells
BDC2.5
BDC.Idd3
LN
pancLN
pancreas

**FIGURE 6. *Idd3*$^{B6}$ alleles do not increase Bcl-2-dependent resistance to apoptosis in CD4$^{+}$Foxp3$^{+}$ nT$_{reg}$ cells.**

Cell suspensions of distal mesenteric LN, spleen, pancLN and pancreata of 3 wk-old NOD and NOD.B6 *Idd3* (left panel) or BDC2.5 and BDC.*Idd3* (right panel) mice (n=3-5) were stained with CD4, Foxp3 and Bcl-2 and analyzed by FACS. Data is reported as the MFI values of individual animals.

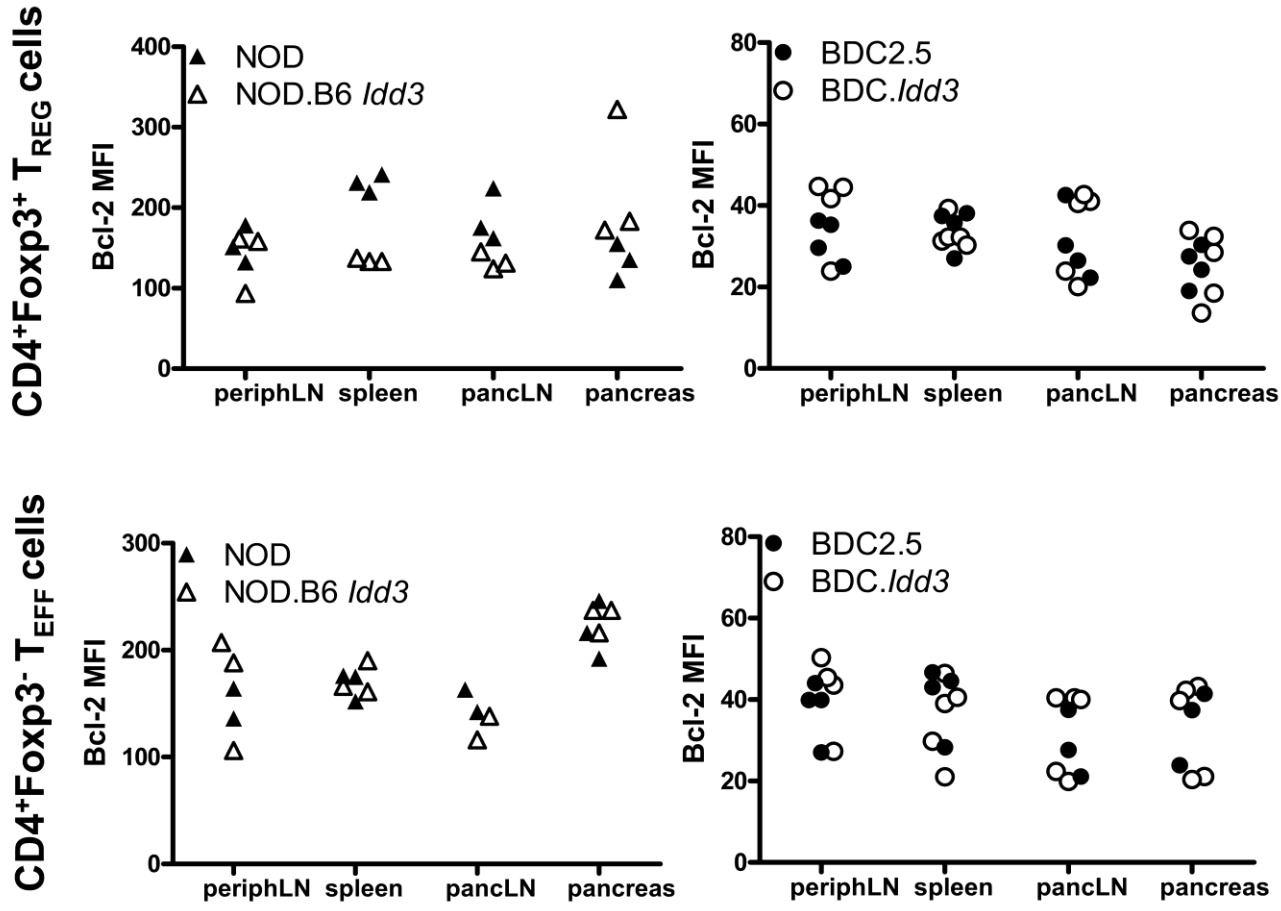

CD4+Foxp3+ TREG cells
Bcl-2 MFI
NOD
NOD.B6 Idd3
BDC2.5
BDC.Idd3
periphLN
spleen
pancLN
pancreas
CD4+Foxp3- TEFF cells

**FIGURE 7. The *Idd3*$^{B6}$ environment preferentially promotes the proliferation of CD4$^+$Foxp3$^+$ nT$_{reg}$ cells in draining pancreatic sites.**

(A) In order to evaluate the impact of the *Idd3*$^{B6}$ environment on $T_{reg}$ cell proliferation, CD4$^+$ T cells from LN cell suspensions from BDC2.5 and BDC.*Idd3* animals were purified, CFSE-labeled and injected into 6-8w old NOD or NOD.B6 *Idd3* recipients. Four days post-transfer, the pancLN were collected and stained for Vβ4, Foxp3 and CD4. Depicted are the representative CFSE profiles (A), absolute numbers and frequency (B) of 3 independent experiments (n=3) of cells gated on CD4$^+$Vβ4$^+$Foxp3$^+$ nT$_{reg}$ cells. (C) Absolute numbers of cells gated on CD4$^+$ Vβ4$^+$Foxp3$^-$ T$_{eff}$ cells.

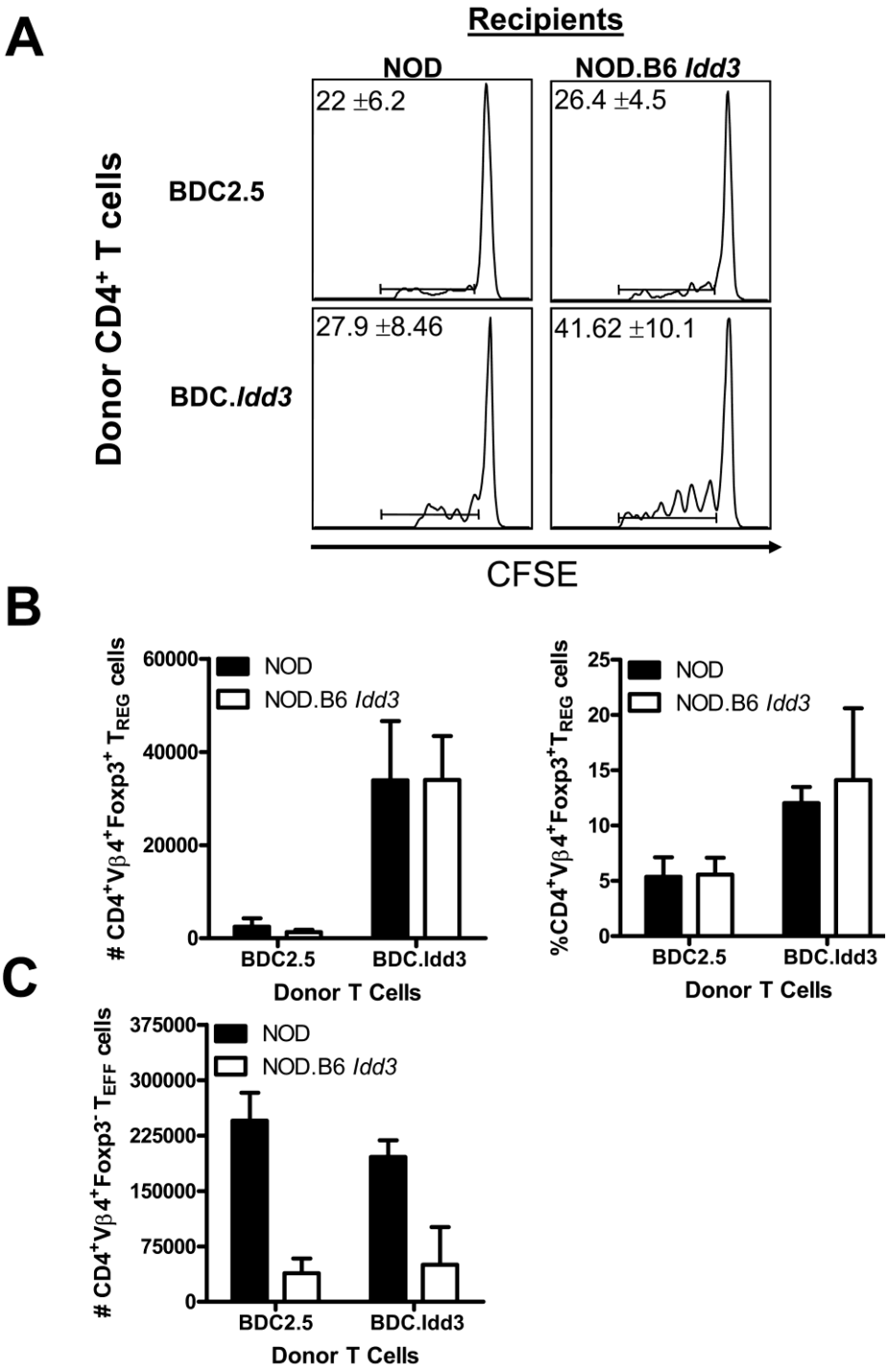
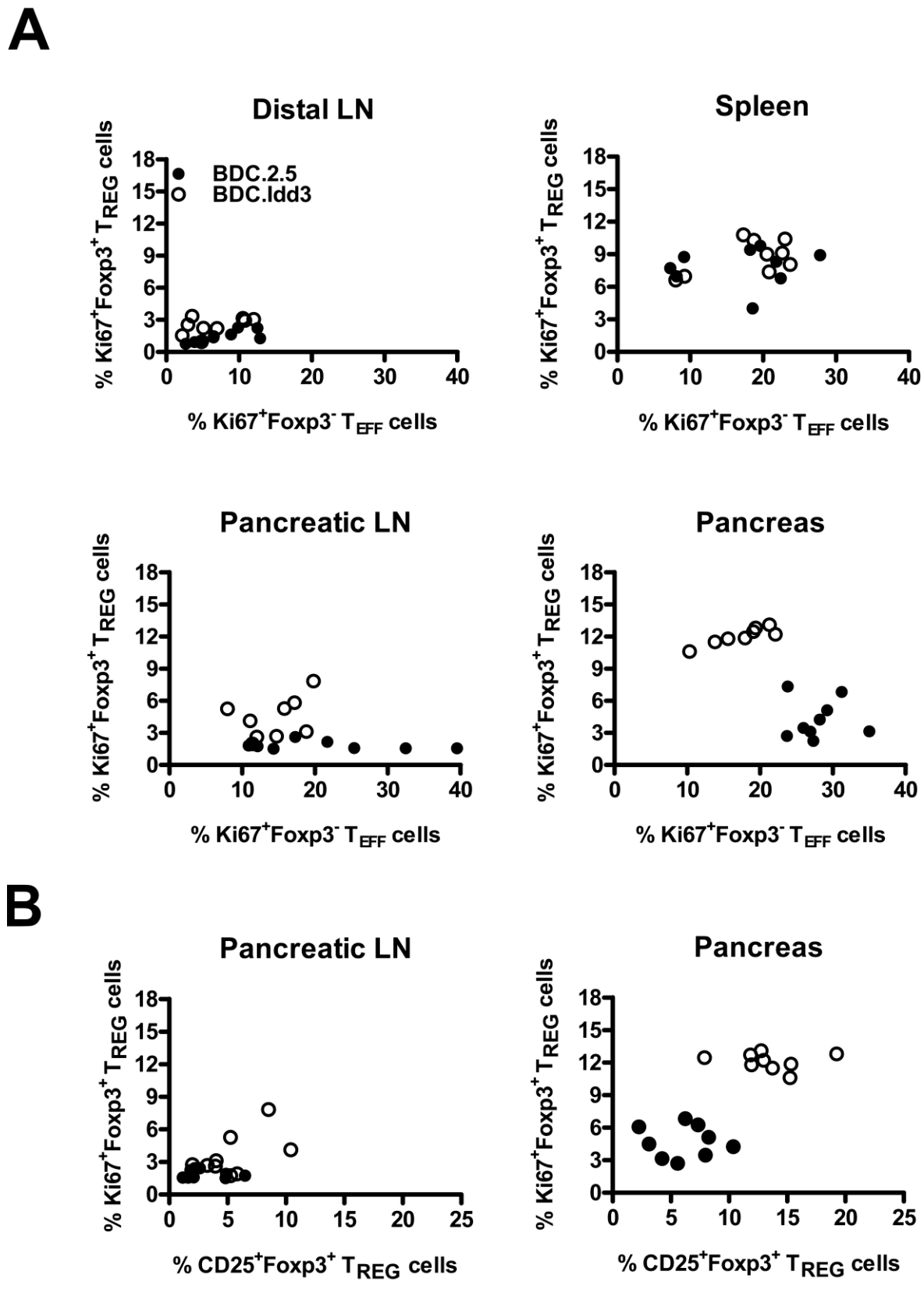

A
Recipients
NOD
NOD.B6 Idd3
22 ±6.2
26.4 ±4.5
27.9 ±8.46
41.62 ±10.1
BDC2.5
BDC.Idd3
Donor CD4+ T cells
CFSE
B
# CD4+Vβ4+Foxp3+ TREG cells
60000
40000
20000
0
NOD
NOD.B6 Idd3
BDC2.5
BDC.Idd3
Donor T Cells
%CD4+Vβ4+Foxp3+TREG cells
25
20
15
10
5
0
C
# CD4+Vβ4+Foxp3- TEFF cells
375000
300000
225000
150000
75000
0

**FIGURE 8. *Il2* allelic variants promote the cycling of CD4$^+$Foxp3$^+$ nT$_{reg}$ cells directly in the pancreas.**

Cell suspensions of distal mesenteric LN, spleen, draining pancLN and pancreas of 3 wk-old BDC2.5 and BDC.*Idd3* animals were stained with CD4, Foxp3, CD25 and Ki-67 and analyzed by FACS. Data is reported as cycling Foxp3$^-$ T$_{eff}$ cells relative to cycling Foxp3$^+$ nT$_{reg}$ cells in (A) and Foxp3$^+$CD25$^+$ T$_{reg}$ cells relative to cycling nT$_{reg}$ cells in (B). Each data point is representative of pooled lymphoid organs of two individual mice. Results represent the mean ± SD. *$p$ <0.00006 difference with control BDC2.5 mice.


A
Distal LN
BDC.2.5
BDC.Idd3
% Ki67+Foxp3+ TREG cells
% Ki67+Foxp3- TEFF cells
Spleen
Pancreatic LN
Pancreas
B
Pancreatic LN
Pancreas
% CD25+Foxp3+ TREG cells

# CHAPTER IV *IL-2 dependent ICOS-mediated control of $CD4^+Foxp3^+$ regulatory T cell homeostasis and differentiation in pre-diabetic islets of NOD mice.*

## Bridging statement from chapter III to IV

IL-2 exerts contradictory functions, since it acts to promote the expansion and differentiation of $T_{eff}$ cells while mediating $T_{reg}$ cell functions. Chapter III demonstrated that $T_{eff}$ cell-derived IL-2 is essential for the expansion and immunosuppressive capacities of $T_{reg}$ cells. $T_{reg}$ cells in turn constrict the diabetogenic response by dampening the proliferation and effector function of the $T_{eff}$ cells. The age-related decline in $T_{reg}$ cell functions in the NOD or $CD4^+$ T cell transgenic BDC2.5 mouse, which correlates with T1D progression, is due to IL-2 shortage, restored by the protective *Il2* allelic variants of the $Idd3^{B6}$ locus. Therefore tight regulation of IL-2 is imperative to maintain tolerance.

Although the IL-2 mediated effect on $T_{reg}$ cell functions was very informative, the molecular mechanism involved in the decline in $T_{reg}$ cell suppressive activity remained elusive. We hypothesized that $T_{reg}$ cell functions declined due to events downstream of inappropriate IL-2 signalling. It is well known that IL-2 and CD28-mediated costimulation act in concert to promote $T_{reg}$ cell functions. Indeed, disruption of the CD28/B7 pathway precipitates T1D due to a drastic decline in $T_{reg}$ cells, highlighting the importance of costimulation in $T_{reg}$ cell development and homeostasis. These studies do not exclude the participation of CD28-dependent non-redundant costimulation molecules such as ICOS, which is a strong inducer of the immunomodulatory cytokine IL-10.

ICOS regulates autoimmune diseases in several experimentally-induced models of autoimmunity, making a strong case for a role for ICOS in T1D. A study in 2004 by Herman *et al.* demonstrated that ICOS mRNA is preferentially expressed by $CD4^+CD25^+CD69^-$ $T_{reg}$ cells within inflamed, pancreatic sites and that ICOS blockade precipitates T1D, which correlates with an altered $T_{reg}/T_{eff}$ balance in pancreatic sites. However, it was unclear from this study how ICOS surface protein expression differed in $Foxp3^+$ and $Foxp3^-$ T cell subsets. Thus, ICOS may dampen the diabetogenic response and thus be a key player in tolerance to β-islet antigens.

Since a positive regulatory feedback loop exists between IL-2 and ICOS, we wondered whether an IL-2 shortage in the pancreas, which is a contributing underlying cause of T1D, would influence ICOS expression in $T_{reg}$ cells, and consequential suppressive activity, in the inflammatory pancreatic milieu. More specifically, we sought to characterize ICOS expression and its impact on $T_{reg}$ cell homestasis and function throughout T1D progression. We also assessed the functional impact of *Il2* allelic variation (congenic T1D-protected NOD.B6 *Idd3* mice) on ICOS expression and functional stability of $T_{reg}$ cells with T1D progression.

To answer these questions, we made use of two novel mouse models developed in our laboratory: NOD.$Foxp3^{gfp}$ and BDC2.5 $Foxp3^{gfp}$. Prior to the development of these mouse models, $T_{reg}$ cells were sorted based on CD25 expression. However, conventional $T_{eff}$ cells also upregulate CD25 upon activation, suggesting that highly-purified fractionated $CD4^+CD25^+$ $T_{reg}$ cells always comprised traces of contaminating

activated $T_{eff}$ cells. The NOD.Foxp3$^{gfp}$ and BDC2.5 Foxp3$^{gfp}$ mouse models enabled the cell separation of *bona fide* Foxp3$^{+}$ $T_{reg}$ cells based on GFP expression.

# IL-2 dependent ICOS-mediated control of $CD4^+Foxp3^+$ regulatory T cell homeostasis and differentiation in pre-diabetic islets of NOD mice.

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## 1. Abstract.

T1D susceptibility in NOD mice is associated with an age-related waning of naturally-occurring $CD4^+Foxp3^+$ regulatory T ($T_{reg}$) cell functions. A deficiency in IL-2, critical for the metabolic fitness of $T_{reg}$ cells, contributes to intra-islet $T_{reg}$ cell dysfunction and progressive loss of self-tolerance to islet antigens. ICOS blockade in neonatal BDC2.5 transgenic mice disrupts the $T_{reg}/T_{eff}$ cell balance, and exacerbates T1D, suggesting that ICOS may be important in controlling $T_{reg}$ cell function in pre-diabetes. We hypothesized that ICOS represents a critical factor in the stabilization of $Foxp3^+$ $T_{reg}$ cell functions within islets, and that IL-2 may sustain optimal ICOS-mediated costimulation necessary for $T_{reg}$ cell functions. Here, we show that $Foxp3^+$ $T_{reg}$ cells in prediabetic islets preferentially express ICOS, and that $ICOS^+Foxp3^+$ $T_{reg}$ cells dominate in prediabetic islets in contrast to $Foxp3^-$ T cells. $ICOS^+Foxp3^+$ $T_{reg}$ cells are endowed with greater suppressive functions than $ICOS^-Foxp3^+$ $T_{reg}$ cells, are high expressers of IL-2R$\alpha$, and cycle vigorously in pre-diabetic islets, in contrast to $ICOS^-Foxp3^+$ $T_{reg}$ cells. Interestingly, ICOS also drives the differentiation of Ag-specific IL-10 producing $Foxp3^+$ $T_{reg}$ cells in islets in contrast to $ICOS^-Foxp3^+$ $T_{reg}$ cells. Moreover, the progression from pre-diabetes to T1D is associated with a concomitant temporal loss in ICOS expression on intra-islet $Foxp3^+$ $T_{reg}$ cells and consequential loss in IL-10 secretion by these cells. Moreover, IL-2 therapy or protective *Il2* gene variation, factors promoting T1D protection, bolsters the pool of $ICOS^+Foxp3^+$ $T_{reg}$ cells in islets. Thus, ICOS-ICOS-L interactions, in cooperation with IL-2, stabilize $Foxp3^+$ $T_{reg}$ cell homeostasis and differentiation islets.

## 2. Introduction.

Type 1 diabetes (T1D) is a chronic autoimmune disease resulting from a T cell-dependent destruction of the insulin-producing β-islets of Langerhans [1, 2]. The non-obese diabetic (NOD) mouse model develops spontaneous T1D and shares many features with human T1D, such as development of autoantibodies and hyperglycemia [1]. The NOD mouse exhibits profound dysregulated immune responses and a progressive loss in immunoregulatory mechanisms are thought to underlie the pathogenesis of T1D [3]. Regulatory T ($T_{reg}$) cells, which constitutively express Foxp3 and CD25 [4-6], represent a major mechanism of peripheral tolerance and have been implicated as a central point in T1D progression, as depletion of CD25-expressing cells or genetic ablation of Foxp3 results in accelerated T1D [7, 8].

Studies point to a progressive waning in $T_{reg}$ cell functions, despite a stable cellular frequency of $T_{reg}$ cells, as a trigger of T1D onset and progression [9-12]. Recently, we showed that T1D progression is associated with a temporal loss in the specific capacity of $CD4^+Foxp3^+$ $T_{reg}$ cells to expand/survive in β-islets, which in turn perturbs the $T_{reg}/T_{eff}$ cell balance and unleashes the anti-islet immune responses [12, 13]. Moreover, a deficiency in IL-2, a cytokine essential for the function and fitness of $T_{reg}$ cells within islets, was shown to trigger this defective function of $T_{reg}$ cells, in turn provoking a $T_{reg}/T_{eff}$ cell imbalance in islets [13]. Consistently, low dose IL-2 treatment of NOD mice restored CD25 expression and survival in intra-islet $T_{reg}$ cells, and led to T1D prevention [14]. These results are also reminiscent of earlier studies showing that T cells from prediabetic NOD mice become hypoproliferative and poor IL-2 producers at

the onset of insulitis, a time-point coinciding with the waning of $T_{reg}$ cell functions [15].

Foxp3$^{+}$ $T_{reg}$ cell function in NOD mice is costimulation-dependent as disruption of CD28-B7 pathway abrogates $T_{reg}$ cell development and homeostasis and leads to acceleration of T1D in NOD mice [16, 17]. The contribution of other costimulatory pathways is currently not well understood. Of particular interest is inducible costimulator (ICOS), a CD28-superfamily related molecule, which plays an important role in T cell activation/survival [18]. ICOS favors IL-4 and IL-10 secretion in Th2 cell responses [18, 19], and blockade of the ICOS-ICOS-ligand (ICOS-L) pathway during the induction of Th1-driven experimental autoimmune encephalomyelitis (EAE) exacerbated disease by enhancing IFN-$\gamma$ production [20]. Interestingly, ICOS mRNA is abundantly expressed in pancreatic lymph nodes (pancLN) of T1D-protected BDC2.5 transgenic mice, whose TCR is specific for an unknown $\beta$ islet antigen [21]. Moreover, ICOS blockade in BDC2.5 neonates exacerbated T1D by disrupting $T_{reg}/T_{eff}$ cell balance in pancreatic lesions suggesting that ICOS may be important in controlling $T_{reg}$ function in pre-diabetic islets [21]. However, ICOS blockade or deficiency in neonatal NOD mice leads to T1D resistance, despite normal frequencies of CD25$^{+}$Foxp3$^{+}$ $T_{reg}$ cells, suggesting a role for ICOS in the activation of effector T ($T_{eff}$) cells in polyclonal repertoires [22]. Consistently, ICOS$^{-/-}$ $T_{eff}$ cells activate poorly and produce markedly reduced levels of IL-2 [23].

IL-2 is known to enhance ICOS expression on activated T cells, suggesting that a positive feedback loop exists between IL-2 and ICOS signalling pathways [24]. In this study, we hypothesized that ICOS represents a critical factor in the stabilization of Foxp3$^{+}$ $T_{reg}$ functions within islets, and that IL-2 may sustain optimal ICOS-mediated costimulation necessary for $T_{reg}$ cell functions. We show that ICOS is preferentially

expressed by $T_{reg}$ cells in prediabetic islets, in contrast to Foxp3$^-$ or Foxp3$^+$ T cells in pancLN or Foxp3$^-$ in islets. Islet-derived ICOS$^+$Foxp3$^+$ $T_{reg}$ cells dominate in prediabetic islets, and are endowed with greater suppressive functions than their ICOS$^-$Foxp3$^+$ $T_{reg}$ counterparts *in vitro*. In addition, ICOS$^+$Foxp3$^+$ $T_{reg}$ cells are highly enriched for CD25 expression and cycle vigorously in prediabetic islets in contrast to ICOS$^-$Foxp3$^+$ $T_{reg}$ cells. We also show that ICOS drives the differentiation of Ag-specific IL-10 producing Foxp3$^+$ $T_{reg}$ cells in islets in contrast to ICOS$^-$Foxp3$^+$ $T_{reg}$ cells, which secrete little IL-10 in response to polyclonal or Ag-specific stimulation. Moreover, the progression to T1D is associated with a temporal loss in ICOS and IL-10 expression on intra-islet Foxp3$^+$ $T_{reg}$ cells, perturbs their functional stability in pre-diabetic islets and unleashes autoimmunity. Interestingly, the marked decline in the frequency of intra-islet ICOS$^+$Foxp3$^+$ $T_{reg}$ cells over time is readily corrected by IL-2 therapy or protective *Il2* gene variation, suggesting that low IL-2 bio-availability in pancreatic sites may be a cause for the sub-optimal ICOS-mediated signals in $T_{reg}$ cells with age. ICOS-L is exclusively expressed by pancreatic CD11c$^+$ DC, and a progressive loss of ICOS-L$^+$CD11c$^+$ DC occurs within islets with age. Thus, ICOS favors IL-2 dependent proliferation/survival of Foxp3$^+$ $T_{reg}$ cells, stabilizes the Foxp3$^+$ $T_{reg}$ cell pool, and instructs their differentiation for IL-10 secretion in islets. Overall, events downstream of IL-2 signalling, such as ICOS co-stimulation, are important in maintaining $T_{reg}$ cell functions and self-tolerance.

## 3. Materials and Methods.

***Mice.*** Mice strains were maintained in SPF conditions at McGill University. BDC2.5 $CD4^+$ transgenic (Tg) mice were a generous gift from Christophe Benoist (Harvard Univ., Boston, USA). BDC2.5 $Foxp3^{gfp}$ reporter mice were generated by backcrossing BDC2.5 mice at least 10 generations to the C57BL/6 $Foxp3^{gfp}$ knock-in reporter mice [27]. NOD.B6 *Idd3* congenic mice (line#1098) were obtained from Taconic Farms, and BDC.*Idd3* mice were generated by in-house breeding. NOD.$ICOS^{-/-}$ mice (line#008376) were obtained from Jackson laboratories.

***Cell purification*.** $CD4^+CD25^-$/$CD4^+CD25^+$ or $CD4^+Foxp3^{gfp+}$/$CD4^+Foxp3^{gfp-}$ T cell subsets were purified from LN or spleens using the autoMACS cell sorter (Miltenyi Biotech, San Diego, CA) or FACSAria cell sorter (BD Bioscience), as described previously [28].

***Adoptive transfers***. BDC2.5 $CD4^+$ T cells ($5x10^5$) were transferred *i.v.* into NOD.TCR$\alpha$ knock-out immunodeficient recipients. Blood glycemia levels were determined every 2–3 days with Hemoglukotest kits (Roche Diagnostics), and T1D was diagnosed at values >300mg/dl. At defined time-points, cell suspensions of pancLN and pancreata from diabetic and diabetes-free mice were stained for anti-CD4, anti-V$\beta$4, anti-ICOS, anti-CD25, anti-Ki-67 and anti-Foxp3, as previously shown [13].

***Flow cytometry****.* Stainings were done with the following fluorochrome-conjugated or biotinylated monoclonal antibodies (mAbs): anti-CD4 (clone RM5), anti-V$\beta$4 (CTVB4), anti-CD25 (clone PC61), anti-ICOS (7E.17G9), anti-CD11c (p150/90), anti-CD86 (GL-

1), anti-ICOS-L (HK5.3) (eBioscience, San Diego, CA). Anti-Foxp3 (FJK-16s), anti-TNF-$\alpha$ (MP6-XT22), anti-IL-10 (JES5-16E3) (eBioscience, San Diego, CA), anti-IL-2 (JES6-5H4), and anti-Ki-67 (B56) (BD Bioscience, Mississauga, Ontario) intranuclear staining was performed according to the manufacturers' respective protocols. Stained cells were acquired on a FACSCalibur (BD Bioscience, Mississauga, Ontario) and analyzed with Flowjo software.

***In vitro functional assays.*** Suppression assays were performed by culturing $CD4^+CD25^-$ $T_{eff}$ cells ($1x10^5$) with titrated numbers of $CD4^+CD25^+$ $T_{reg}$ cells from WT NOD or NOD.$ICOS^{-/-}$ mice in 96-well round-bottom microtiter plates with irradiated APCs ($4x10^5$) and $\alpha$-CD3 (1$\mu$g/mL) for 72h. Cell cultures were pulsed with 1$\mu$Ci $^3$H-TdR for the last 12h-16h of culture. All experiments were repeated at least 3 times. In some cases, $ICOS^-$ or $ICOS^+$ $CD4^+CD25^+$ $T_{reg}$ cells were isolated from BDC2.5 mice, and stimulated with irradiated APCs and BDC2.5 mimetope (RVRPLWVRME) (2ng/mL) in the presence or absence of neutralizing anti-ICOS mAb (7E.17G9) (10$\mu$g/mL) (BD Bioscience, Mississauga, Ontario) for 72h at 37$^o$C in 5% $CO_2$. The aforementioned functional assays were repeated using BDC2.5 $Foxp3^{gfp}$ reporter mice by FACS sorting $T_{reg}$ cells based on GFP expression.

***Intracellular cytokine production.*** Cell-sorted CFSE-labeled BDC2.5 $CD4^+CD25^-$ $ICOS^-$ T cells were stimulated with NOD BMDC at a ratio of 4:1 and mimetope (100ng/mL) in the presence or absence of neutralizing anti-ICOS or anti-IL-2 mAb (10$\mu$g/mL) for 48h or 72h, and treated with Golgi-Stop (BD Bioscience, Mississauga, Ontario) for the last 2-3h of culture. Intracytoplasmic staining was performed using anti-

IL-2, anti-TNF-$\alpha$ or appropriate isotype controls. Expansion was evaluated 4 days post-activation by CFSE dilution as previously shown [12]. In order to determine the impact of ICOS on IL-10 secretion, two approaches were undertaken. Cell suspensions of pancLN and pancreas were activated *in vitro* for 4h using PMA/Iono and Golgi plug, as described in [22]. Highly-purified BDC2.5 Foxp3$^{gfp+}$ $T_{reg}$ cells fractionated based on ICOS expression were activated in the presence of irradiated APCs and BDC2.5 mimetope (10ng/mL) for 72h and Golgi plug was added in the last 2-3 hours of culture. Intracytoplasmic staining was performed using anti-IL-10 mAb (JES5-16E3) (BD Bioscience, Mississauga, Ontario) following manufacturer's instructions.

***In vivo treatment with IL-2.*** Three-week-old female BDC2.5 mice were injected with PBS or 5µg of human recombinant IL-2 (rhIL-2) for 5 consecutive days in the peritoneal cavity as previously described [14]. On the seventh day of treatment, pancLN and pancreata were harvested, treated and stained for anti-CD4, anti-CD25, anti-ICOS, anti-Foxp3, anti-Ki-67 for T cell analysis, as previously described [14].

***Statistical analysis****.* Analyses were performed with a Student's *t* test. Values of $p<0.05$ were considered significant.

## 4. Results.

### Preferential accumulation of ICOS$^+$Foxp3$^+$ $T_{reg}$ cells in pre-diabetic islets.

ICOS blockade in BDC2.5 mice leads to a disruption of the $T_{reg}/T_{eff}$ cell balance in pancreatic sites and results in T1D exacerbation, suggesting that ICOS may potentially play a role in $T_{reg}$ cell-mediated self-tolerance [23]. While ICOS mRNA is abundantly expressed in pancLN of T1D-protected BDC2.5 mice, expression of ICOS protein in T cell subsets was never directly demonstrated [23]. We first sought to characterize ICOS expression within Foxp3$^+$ and Foxp3$^-$ T cell subsets derived from pancreas, the tissue undergoing autoimmune attack, draining pancLN, and distal LN at the time of insulitis in 3-week-old female BDC2.5 mice. We observed a substantial increase in the frequency of Foxp3$^+$ICOS$^+$ $T_{reg}$ cells within the CD4$^+$ T cell compartment of the pancreas relative to draining pancreatic sites (8.8%±1.8 vs 1.46%±0.7; $p \leq 0.01$) (Fig.1A, left panel), and corresponding to 57%±10.2 and 18.9%±4.5 respectively within the total Foxp3$^+$ $T_{reg}$ cell pool (Fig.1A, middle panel) ($p \leq 0.002$). The striking difference in ICOS expression on Foxp3$^+$ $T_{reg}$ cells within the pancreas relative to the pancLN suggested that $T_{reg}$ cells within the pancreatic lesion acquire distinctive functional and phenotypical properties, in stark contrast to Foxp3$^-$ or Foxp3$^+$ T cells in pancLN or Foxp3$^-$ in islets (Fig. 1A). In accordance with this finding, the mean fluorescence intensity (MFI) of ICOS on $T_{reg}$ cells exhibited a significant upregulation in the pancreas relative to the draining pancLN sites (144.3±11.9 vs 38.5±15.9; $p \leq 0.001$) (Fig.1A, right panel), suggesting that ICOS is induced on Foxp3$^+$ $T_{reg}$ cells in pancreatic lesions, and may potentially play an active role in $T_{reg}$ cell functions directly within the organ undergoing autoimmune attack.

Only a small proportion of ICOS$^+$ $T_{eff}$ cells in draining pancLN and pancreas was

detected within the $CD4^+$ T cell pool (0.96%± 0.4 vs 5.3%±1.5) (Fig.1A, left panel) and more specifically within the $Foxp3^-$ T cell compartment (1%±0.4 vs 6%±1.6) (Fig.1A, middle panel), suggesting that ICOS-dependent costimulation may not be required for the priming and function of autoreactive T cells in these sites. Consistently, the MFI of ICOS within the $Foxp3^-$ $T_{eff}$ cell compartment did not differ significantly between draining pancLN and pancreatic sites (18.8±2.2 vs 24.2±3.8) (Fig.1A, right panel). In stark contrast, the MFI of ICOS, although comparable between $T_{eff}$ and $T_{reg}$ cells in pancLN, exhibited a plunging drop in $T_{eff}$ cells relative to $T_{reg}$ cells of the pancreas (24.2±3.8 vs 144.3±11.9; $p \leq 0.002$) (Fig. 1A, right panel), suggesting that there may be a $T_{reg}$ cell-specific role for ICOS, which could condition their function in inflamed pancreatic sites.

**$ICOS^+Foxp3^+$ $T_{reg}$ cells in pre-diabetic islets are $CD25^{high}$ and cycle vigorously *in vivo.***

IL-2 signaling is critical for Foxp3 expression, expansion and function in $T_{reg}$ cells, and failure to respond to IL-2 may negatively affect $T_{reg}$ cell activity *in vivo* [29-31]. Numerous studies show that $CD4^+CD25^+$ $T_{reg}$ cells are readily detected in the pancLN and pancreas of T1D-protected NOD mice [32]. Thus, we then examined CD25 expression on $ICOS^+$ or $ICOS^-Foxp3^+$ $T_{reg}$ cell subsets in pancLN and pancreas of pre-diabetic BDC2.5 mice. Only a negligible proportion of $Foxp3^+$ $T_{reg}$ cells were $CD25^+ICOS^+$ in pancLN (7.5%±0.8) (Fig.1B, left panel), while a significant fraction of $Foxp3^+$ $T_{reg}$ cells strikingly co-express CD25 and ICOS in the pancreas (53.8%±5.2) (Fig.1B), demonstrating that the $Foxp3^+$ $T_{reg}$ cell infiltrates in pancreas and pancLN tissues are phenotypically different. Moreover, the level of CD25 expression on $ICOS^+Foxp3^+$ $T_{reg}$ cells was also greater than their $ICOS^-$ counterparts suggesting that they may be more sensitive to IL-2 signals (Fig. 1B right panel).

$T_{reg}$ cells have been shown to expand rapidly in pancLN and pancreas as a result of antigenic challenge or ensuing inflammation [7, 23]. We therefore sought to examine whether ICOS expression was associated with a higher degree of $Foxp3^+$ $T_{reg}$ cell expansion within the pancreatic lesion as determined by expression of the Ki-67 mitotic marker. Our results show that a sizeable proportion of cycling $T_{reg}$ cells expressed ICOS in the pancreatic lesion of pre-diabetic, BDC2.5 mice relative to draining pancLN (24.4%±4.5 vs 9.2%±3.3; $p \leq 0.001$) (Fig.1C). Interestingly, the $ICOS^+$ fraction of $Foxp3^+$ $T_{reg}$ cells exhibited a much greater proliferative potential relative to the $ICOS^-$ compartment of $Foxp3^+$ $T_{reg}$ cells (53.1%±5.67 vs 16.9%±4.2; $p \leq 0.001$) (Fig.1C, right panel). Furthermore, ICOS expression was found exclusively within the $CD62L^{low}CD44^{high}$ effector memory compartment of $Foxp3^+$ $T_{reg}$ cells, irrespective of their proliferative potential (data not shown). Overall, these results show that ICOS is preferentially expressed in pancreas-resident $Foxp3^+$ $T_{reg}$ cells, possibly conferring onto these cells a greater capacity to proliferate in inflamed sites.

**ICOS-expressing $Foxp3^+$ $T_{reg}$ cells display an augmented suppressive function *in vitro.***

These results begged the question on the role of ICOS in the functional potency of $T_{reg}$ cells. In order to address this question, we sought to characterize the functionality of $T_{reg}$ cells based on their ICOS expression by performing *in vitro* suppression assays. $CD4^+CD25^+ICOS^+$ $T_{reg}$ cells were more potent than $CD4^+CD25^+ICOS^-$ $T_{reg}$ cells at suppressing $CD4^+CD25^-ICOS^-$ $T_{eff}$ cells at all ratios examined (Fig.2A, left panel). In order to ensure that the $CD25^+ICOS^+$ $T_{reg}$ cell fraction was not contaminated with activated $CD25^+ICOS^+$ $T_{eff}$ cells, *bona fide* $Foxp3^+$ $T_{reg}$ cells from BDC2.5 $Foxp3^{gfp}$ reporter mice were FACS sorted based on ICOS and GFP expression, and were

assessed for their ability to suppress the proliferation of Foxp3$^{gfp-}$ $T_{eff}$ cells *in vitro* in an antigen-specific manner. Foxp3$^{gfp+}$ICOS$^{+}$ $T_{reg}$ cells exhibited greater suppressive capacity relative to their ICOS$^{-}$ counterparts (Fig.2A, right panel). We then examined the effector role of ICOS in $T_{reg}$ cell suppressive activity *in vitro* by performing the above-described suppression assay in the presence or absence of an anti-ICOS blocking antibody. Interestingly, ICOS blockade did not reverse suppression mediated by Foxp3$^{gfp+}$ICOS$^{+}$ $T_{reg}$ cells. However, a modest, albeit significant, abrogation of suppression was observed when Foxp3$^{gfp+}$ICOS$^{-}$ $T_{reg}$ cells were used (Fig.2B).

In order to further dissect the role of ICOS in $T_{reg}$ cell-mediated suppression, $T_{eff}$ and $T_{reg}$ cells from WT NOD or NOD.ICOS deficient (-/-) mice were cultured in a criss-cross fashion in standard suppression assays, as described above. Our results show that WT NOD CD25$^{+}$ICOS$^{+}$ $T_{reg}$ cells were more potent suppressors of WT $T_{eff}$ cells compared to NOD.ICOS$^{-/-}$ $T_{reg}$ cells. In stark contrast, ICOS$^{-/-}$ $T_{reg}$ cells were as potent as WT $T_{reg}$ cells, irrespective of ICOS expression, in suppressing ICOS$^{-/-}$ $T_{eff}$ cells, suggesting that ICOS$^{-/-}$ $T_{eff}$ cells are more amenable to suppression that WT $T_{eff}$ cells (Fig.2C, right panel). The differences in susceptibility to $T_{reg}$ cell-mediated suppression between ICOS$^{-/-}$ $T_{eff}$ cells and WT $T_{eff}$ cells could not be attributed to defective priming, as ICOS$^{-/-}$ $T_{eff}$ cells activated as robustly as WT $T_{eff}$ cells (data not shown). Overall, these results show that while ICOS is not required in the effector phase of T cell suppression *in vitro*, it may be required to imprint the suppressive activity of $T_{reg}$ cells.

**ICOS controls the differentiation of IL-10-producing Foxp3$^+$ $T_{reg}$ cells in pre-diabetic islets.**

ICOS expression on activated murine and human T cells enables them to preferentially produce IL-10 [33, 34]. In support of this finding, ICOS-deficient patients show a severe reduction in IL-10 production, reinforcing the impact of ICOS in IL-10 secretion [35]. IL-10 is an important immunomodulatory cytokine, which has been shown to be critical for the control of T1D in the BDC2.5 model [36]. The intimate link between ICOS and IL-10 led to the hypothesis that the ICOS$^+$Foxp3$^+$ $T_{reg}$ cell subset wards off autoimmunity through the production of IL-10. In order to elucidate the mechanisms involved in preventing T1D progression, we examined whether ICOS expression favored IL-10 production in Foxp3$^+$ $T_{reg}$ cells of pre-diabetic mice. To this end, T cells from pancLN and pancreas of Foxp3$^{gfp}$ NOD mice were activated *in vitro* and ICOS expression correlated with IL-10 production in recently activated T cells. The substantial fraction of Foxp3$^{gfp+}$ $T_{reg}$ cells expressed ICOS post-activation (83%±6.8) (Fig.3A), a pattern of ICOS expression similar to that seen in pancreatic Foxp3$^+$ $T_{reg}$ cells examined directly *ex vivo*. More importantly, a substantial proportion of ICOS-expressing CD4$^+$ T cells produced IL-10 (14.8%±1.8) and strikingly, 47.2%±6.4 of Foxp3$^{gfp+}$ $T_{reg}$ cells secreted IL-10, upon *in vitro* polyclonal stimulation (Fig.3B, right panel). Moreover, the frequency of IL-10-secreting Foxp3$^+$ $T_{reg}$ cells is significantly greater in the pancreas than in their pancLN counterparts, which produce little IL-10 (Fig.3B).

In order to confirm this finding, BDC2.5 Foxp3$^{gfp+}$ $T_{reg}$ cells were fractionated based on ICOS expression and activated by WT bone-marrow derived dendritic cells (BMDC) in an antigen-specific manner. Our results show that only ICOS$^+$ $T_{reg}$ cells were able to

secrete IL-10 following antigen-specific stimulation (19%), in contrast to ICOS$^-$ $T_{reg}$ cells (Fig.3C) or Foxp3$^-$ $T_{eff}$ (data not shown), which failed to secrete this cytokine. Thus, ICOS expression controls the differentiation of IL-10-producing Foxp3$^+$ $T_{reg}$ cells in pre-diabetic islets.

**ICOS-dependent IL-2 production in effector T cells.**

Several publications reported a reduction or even complete lack of IL-2 production by ICOS$^{-/-}$ T cells stimulated by WT DC or WT T cells stimulated by ICOS-L$^{-/-}$ DC *in vitro* [25, 37]. Since IL-2 is a key survival factor for T cells and more specifically promotes the metabolic fitness of $T_{reg}$ cells, this finding prompted us to measure and compare IL-2 levels in the presence or absence of ICOS stimulation. ICOS blockade of BDC2.5 CD4$^+$Foxp3$^{gfp-}$ $T_{eff}$ cells activated in an antigen-specific manner did not impact activation levels, based on CD25 expression, demonstrating that ICOS is dispensable for initial priming events of the diabetogenic T cell pool (Fig.4A). However, ICOS blockade drastically reduced IL-2 levels in activated $T_{eff}$ cells (36.7%±1.2 vs 20.1%±3.1) (Fig.4A). The reduction in cytokine production was specific to IL-2, as TNF-$\alpha$ levels remained unaffected in the presence of ICOS blockade (18.4%±0.8 vs 16.9%±2), suggesting that ICOS preferentially promotes IL-2 production in activated $T_{eff}$ cells (Fig.4A). Interestingly, although IL-2 production was reduced, proliferation of CD4$^+$Foxp3$^{gfp-}$ $T_{eff}$ cells was not compromised by ICOS blockade, suggesting that the residual IL-2 produced by these cells is sufficient for the priming of the effector pool in these cultures (Fig.4B). Conversely, IL-2 is also important for T cell activation via ICOS upregulation [402], prompting us to determine whether IL-2 blockade impacted ICOS expression. Indeed, IL-2 blockade, although drastically reducing activation levels based on CD25

expression (62.1%±1.1 vs 41.9%±0.7), resulted in an attenuation in ICOS expression levels (Fig.4C), demonstrating a positive feedback loop between IL-2 and ICOS signalling pathways in $T_{eff}$ cells. Thus, ICOS-dependant costimulation in activated $T_{eff}$ cells may amplify IL-2 production and in turn, impact $T_{reg}$ cell activity *in vivo*. Taken together, our results indicate that ICOS may have dual functions in $CD4^+$ T cell subsets: promote IL-10 production in $Foxp3^+$ $T_{reg}$ cells, while promoting IL-2 production in $T_{eff}$ cells.

**Temporal loss in ICOS expression and IL-10 production in $Foxp3^+$ $T_{reg}$ cells coincides with T1D progression.**

Since BDC2.5 mice eventually develop diabetes due to functional changes in $T_{reg}$ cells with age, we hypothesized that ICOS represents a critical factor in the stabilization of $Foxp3^+$ $T_{reg}$ functions within islets. We explored the possibility that breakdown in self-tolerance may be attributed to, among other factors, an age-related decline in ICOS expression of $T_{reg}$ cells. We focused on the checkpoints known to demarcate the initiation (3-4wks) and progression of disease (12wks) in the BDC2.5 model. We observed a decline in the proportion of ICOS-expressing $Foxp3^+$ $T_{reg}$ cells within the $CD4^+$ T cell infiltrate of the pancreas (8.2%±2.3 vs 3.4%±0.4; $p \leq 0.03$) (Fig.5A, right panel). Furthermore, in stark contrast to the pancLN, the intensity of the ICOS signal also exhibits a remarkable decline with age (144.3±11.9 vs 50.7±17.6; $p \leq 0.003$). As with pre-diabetic 3wk-old mice, only a small proportion of $T_{eff}$ cells expressed ICOS in 12-week old BDC2.5 mice, suggesting that ICOS expression does not correlate with the diabetogenicity of the $T_{eff}$ cell subset (data not shown). Taken together, T1D progression correlates with a drastic decrease in the frequency of $Foxp3^+ICOS^+$ $T_{reg}$

cells within the pancreatic lesion.

In order to evaluate whether the decline in the frequency of $ICOS^+Foxp3^+$ $T_{reg}$ cells with age is attributable to a corresponding drop in their proliferative capacity, we assessed $T_{reg}$ cell expansion in the pancreatic sites of pre-diabetic 12wk-old mice. We observed that the expansion, as determined by the proliferation marker Ki-67, of ICOS-expressing $Foxp3^+$ $T_{reg}$ cells is dampened directly within the tissue (Fig.5B) (17.6%±3.5 vs 26.4%±4.5; $p \leq 0.03$) in pre-diabetic 12wk-old BDC2.5 mice relative to 4wk-old counterparts. This trend is not observed in draining pancLN (5.4%±0.5 vs 9.2%±3.3) (Fig. 5B). Overall, in contrast to the draining pancreatic sites where priming events are believed to occur [32], a substantial proportion of ICOS-expressing $T_{reg}$ cells is actively proliferating in response to the inflammation within the pancreatic lesion of young prediabetic mice. However, as T1D progresses, $T_{reg}$ cells exhibit distinct phenotypic and functional differences and as such are unable to constrict the diabetogenic pool and reduce the consequences of destructive infiltration.

We have previously shown that BDC2.5 $T_{reg}$ cells cannot maintain long-term tolerance, as 60% of immunodeficients recipients receiving BDC2.5 $T_{eff}$ and $T_{reg}$ cells at physiological ratios developed diabetes by 20 days post-transfer [13]. We wondered whether this attenuated regulatory activity could be attributed to $T_{reg}$ cell-specific loss of ICOS expression. Therefore, we sought to investigate the impact of ICOS on the functional stability of $T_{reg}$ cells. To this end, we transferred $CD4^+CD25^-$ $T_{eff}$ cells along with $CD4^+CD25^+$ $T_{reg}$ cells from BDC2.5 mice into NOD.TCR$\alpha^{-/-}$ immunodeficient recipients and monitored disease onset. By 18 days post-transfer, 60% of recipients became diabetic (data not shown). Diabetic and non-diabetic NOD.TCR$\alpha^{-/-}$ recipients

were sacrificed and the cellular frequency of ICOS-expressing Foxp3$^+$ $T_{reg}$ cells in the pancreas was analyzed in both groups. Interestingly, the frequency and mean fluorescence intensity (MFI) of ICOS expressed by Foxp3$^+$ $T_{reg}$ cells sustained a drastic loss in diabetic relative to non-diabetic mice (Fig.6C), suggesting that the progression to T1D correlates with a drastic drop in ICOS expression in Foxp3$^+$ $T_{reg}$ cells, particularly in the pancreas, and that ICOS expression drives $T_{reg}$ cell suppression. Furthermore, self-reactive $T_{eff}$ cells of both diseased and protected mice expressed similar low levels of ICOS (Fig.6C). ICOS MFI was also attenuated in $T_{eff}$ cells relative to $T_{reg}$ cells, irrespective of the disease status of the recipient mice.

Herman *et al.* demonstrated that IL-10 and ICOS transcripts were highly expressed by pancreatic CD25$^+$CD69$^-$ $T_{reg}$ cells relative to their draining LN counterparts [23]. Our results show that ICOS expression drives IL-10 production by pancreatic Foxp3$^+$ $T_{reg}$ cells. We wondered whether the loss in ICOS expression with T1D progression dampened the capacity of $T_{reg}$ cells to produce IL-10 in the BDC2.5 model [32]. To this end, T cells from pancreas of diabetic and non-diabetic mice were reactivated *ex vivo,* and the secretion of IL-10 was assessed by FACS. Interestingly, the marked drop in the frequency and expression of ICOS by Foxp3$^+$ $T_{reg}$ cells within the pancreas of diabetic mice correlated with a 3-fold decline in their capacity to produce IL-10 (Fig.6C, right panel). This suggests that ICOS drives the differentiation of IL-10 producing $T_{reg}$ cells, which in turn enables them to exert their suppressive functions and thwart T1D development.

**Protective *Il2* allelic variants promote the emergence of ICOS$^+$Foxp3$^+$ $T_{reg}$ cells in the pancreas.**

We and others have demonstrated that IL-2 deficiency is one of the underlying mechanisms in the age-related waning of $T_{reg}$ cell functions and loss of self-tolerance in NOD mice [13, 14]. Since IL-2 drives ICOS expression, we sought to determine whether *Il2* allelic variants of T1D-protected congenic BDC.*Idd3* mice could restore ICOS expression on $T_{reg}$ cells. Within the pancreatic infiltrate at the time of insulitis, we observed more than a two-fold enhancement in the proportion of ICOS$^+$Foxp3$^+$ $T_{reg}$ cells (21.3%±4.4 vs 8.2%±2.3; $p \leq 0.001$) and ICOS MFI (282.3±54 vs 144.3±11.9; $p \leq 0.04$) in 4wk-old BDC.*Idd3* mice relative to their WT counterparts (Fig.6A, right panel). This suggests that increased $T_{eff}$ cell-derived IL-2 production potentiates ICOS expression within the $T_{reg}$ cell subset, which may drive the dampening of the autoimmune response. Furthermore, as was the case with WT BDC2.5 mice, only a small proportion of ICOS-expressing $T_{reg}$ cells was observed in non-draining LN (data not shown) and draining pancLN (0.52%±0.2), suggesting that tissue-specific signals modify the phenotype of $T_{reg}$ cells. Although we observed a substantial drop in the frequency of ICOS-expressing Foxp3$^+$ $T_{reg}$ cells in BDC.*Idd3* pre-diabetic 12wk-old relative to 4wk-old animals, the frequency remained elevated relative to 12wk-old WT counterparts (11.1%±2.3 vs 3.4%±0.4; $p \leq 0.005$) (Fig.6A, right panel).

We sought to determine whether the enhanced proportion of ICOS-expressing Foxp3$^+$ $T_{reg}$ cells coincided with more robust T cell proliferation. Interestingly, only a

small proportion of ICOS-expressing $T_{reg}$ cells was actively proliferating in the draining pancLN counterparts, both in 4wk-old and 12wk-old BDC.*Idd3* animals (data not shown). However, within the target organ, a two-fold enhancement in the fraction of actively proliferating ICOS-expressing $Foxp3^+$ $T_{reg}$ cells was noted in 4wk-old BDC.*Idd3* relative to the WT BDC2.5 counterparts (39.1%±5.7 vs 24.4%±4.5; $p<0.01$) (Fig.6B). This suggests that $Idd3^{B6}$-mediated $T_{eff}$ cell-derived IL-2 drives the effector functions of $T_{reg}$ cells directly within the target organ through the upregulation of ICOS. This significant difference is maintained at later time points with disease progression (22.3%±2.8 vs 14.7%±3.4; $p<0.04$) (Fig.6B). Overall, *Il2* allelic variation potentiates IL-2 production in $T_{eff}$ cells and ICOS expression on $Foxp3^+$ $T_{reg}$ cells, which in turn, promotes $T_{reg}$ cell functions and T1D protection.

**Protective *Il2* allelic variants promote the upregulation of ICOS-L on $CD11c^+$ DC within the pancreatic lesion.**

$T_{reg}$ cell/DC cross-talk represents an important mechanism for the control of autoimmunity *in vivo* [38]. We therefore wondered whether the exclusive expression of ICOS on the $T_{reg}$ cell subset within the pancreas paralleled the upregulation of ICOS-L on pancreatic $CD11c^+$ dendritic cells (DC) over time, as they are the primary APC capable of efficiently promoting $T_{reg}$ cell functions [39]. The frequency of ICOS-L expressing $CD11c^+$ DC was negligible in all secondary lymphoid organs examined (data not shown), including draining pancLN. Strikingly, within the target organ undergoing autoimmune attack, more than half of $CD11c^+$ DC expressed ICOS-L (63.7%±3.4) (Fig.6C). Furthermore, the upregulation of ICOS-L was restricted to $CD11c^+$ DC, as only an inconsequential proportion of other APCs such as macrophages and B cells

expressed ICOS-L (less than 1%) (data not shown), implying an important cross-talk between resident intra-islet $T_{reg}$ cells and DC. Consistent with the decline in ICOS-expressing Foxp3$^{+}$ $T_{reg}$ cells over time, we observed a modest, albeit significant, decreased proportion of ICOS-L$^{+}$ DC in the pancreatic lesion with age (63.7%±3.4 vs 46.3%±4.1; $p \leq 0.05$) (Fig.6C).

Interestingly, the *Idd3*$^{B6}$ locus modestly restores the frequency (81.3%±2.2 vs 63.7%±3.4, $p \leq 0.05$) and expression (86.8±14.7 vs 57.2±4.7; $p \leq 0.05$) of ICOS-L on CD11c$^{+}$ DC (Fig.6C). This trend is maintained over time in frequency of ICOS-L$^{+}$ CD11c$^{+}$ (70.6%±0.7 vs 46.3%±4.1; $p \leq 0.05$) and ICOS-L MFI (108.2±33.6 vs 61.6±7.4; $p \leq 0.05$) by the T1D-protective *Idd3*$^{B6}$ alleles. Taken together, the tissue-specific expression of ICOS-L on DC suggests that ICOS/ICOS-L interactions are actively playing a role in restraining diabetogenic events. These interactions subside with age and result in autoimmune destruction of the pancreas, although it is unclear whether the loss of ICOS-L expression on CD11c$^{+}$ DC is causal or consequential to the disease process.

**Low-dose IL-2 therapy restores ICOS expression in Foxp3$^{+}$ $T_{reg}$ cells within the pancreatic lesion.**

ICOS is an important regulator of T cell activation, differentiation and function [40]. Indeed, ICOS$^{-/-}$ T cells fail to produce IL-2, reinforcing the notion that ICOS-mediated IL-2 production may impact $T_{reg}$ cells *in vivo* [25]. In order to confirm whether IL-2 deficiency is the underlying cause of the temporal loss of ICOS expression observed in the $T_{reg}$ cell subset within the pancreas, a prophylactic IL-2 therapeutic regime known to

be T1D-protective was initiated in pre-diabetic BDC2. 5 mice. To this end, a low dose of rhIL-2 was administered to WT 3-4wk-old mice, and the frequency of $Foxp3^+$ $T_{reg}$ cells, relative to ICOS expression, was monitored by FACS. In stark contrast to draining pancLN, we observe a significant increase in the proportion of $Foxp3^+$ $T_{reg}$ cells within the pancreatic infiltrate of mice administered IL-2 relative to PBS controls (13.3%±3.1 vs 9.4%±1.9; $p \leq 0.03$) (Fig. 7A). The proportion of $Foxp3^+ICOS^+$ $T_{reg}$ cells within the $CD4^+$ T cell infiltrate of the pancreas was also enhanced in IL-2-treated BDC2.5 animals relative to PBS-treated controls (5.85%±2 vs 2.37%±0.7; $p \leq 0.003$), and comparable to levels observed in unmanipulated BDC.*Idd3* mice (5.2%±2.92 vs 5.85%±2) (Fig.7B, left panel). Furthermore, the proportion of $CD25^+ICOS^+$ within the $Foxp3^+$ compartment was enhanced in IL-2 treated mice vs PBS controls (37.3%±7.7 vs 20.4%±4; $p \leq 0.002$) (Fig.7B, right panel), again to levels observed in BDC.*Idd3* mice (37.3%±7.7 vs 32.9%±6.6). Overall, this data strongly suggests that IL-2 drives ICOS expression on $T_{reg}$ cells, whose enhanced effector functions afford T1D protection by restraining the diabetogenic $T_{eff}$ cell pool.

## 5. Discussion.

Foxp3$^{+}$ $T_{reg}$ cells are implicated as a major mechanism of peripheral tolerance in NOD mice. Indeed, studies show that $T_{reg}$ cells dampen the progression of autoimmune T1D by limiting the priming, differentiation, migration or effector functions of autoreactive $T_{eff}$ cells either pancLN or directly in target organ [7, 12, 28, 36-38]. However, inappropriate immunoregulation in prediabetes, possibly due to developmental defects or age-related waning of $T_{reg}$ cell functions, is thought to underlie the predisposition to T1D [9, 11, 12, 39]. Numerous studies indicate that an age-related waning in $T_{reg}$ cell functions, despite a stable cellular frequency of $T_{reg}$ cells, is a trigger of T1D progression [9, 11, 12, 39]. Consistently, we and others have shown that a progressive loss in the balance between diabetogenic $T_{eff}$ and $T_{reg}$ cells in pancreatic sites unleashes the expansion, differentiation and effector function of islet-reactive $T_{eff}$ cell, consequently leading to overt diabetes [10, 13]

Foxp3$^{+}$ $T_{reg}$ cell development and function are heavily dependent on costimulation, as demonstrated by the exacerbated diabetes onset in CD28$^{-/-}$ or B7$^{-/-}$ NOD mice [16, 17]. ICOS, a member of the CD28 superfamily, has emerged as a critical costimulatory molecule in autoimmunity. ICOS has been implicated in the clinical course of various autoimmune disorders including EAE and collagen-induced arthritis (CIA) [41, 42]. Surprisingly, genetic ablation or blockade of ICOS at various time-points during disease progression yields drastically different clinical outcomes, demonstrating that the mode of action of ICOS differs in the context of various chronic inflammatory diseases [20, 40-42]. The ICOS/ICOS-L costimulatory pathway also appears to play an important role in the control of autoimmune responses in T1D. ICOS mRNA is highly expressed in

pancLN of T1D-protected BDC2.5 mice, and ICOS blockade in neonatal, prediabetic BDC2.5 mice precipitates T1D by favoring an imbalance between $T_{reg}/T_{eff}$ cells in pancreatic sites [21]. In contrast, ICOS blockade in WT NOD neonates, which harbor a polyclonal T cell repertoire, protects against diabetes [43]. Similarly, genetic ablation of ICOS in NOD mice results in a markedly reduced onset and incidence of disease, demonstrating that ICOS may also be implicated in the induction of disease [22]. In contrast, ICOS blockade in new-onset diabetic NOD mice did not reverse diabetes [43]. Taken together, ICOS regulates the onset and progression of insulitis and diabetes, and it remains unknown how ICOS differentially regulates $T_{eff}$ and $T_{reg}$ cell responses during different phases of an anti-islet immune response [21, 22, 43].

These findings prompted us to evaluate the phenotypic changes of islet-resident $T_{reg}$ cells during the progression of T1D. In this study, we show that ICOS is predominantly expressed in $Foxp3^+$ $T_{reg}$ cells that infiltrate the pancreas, in contrast to $Foxp3^+$ or $Foxp3^-$ T cells in various lymphoid tissues including pancLN. We also show that intra-islet $ICOS^+$ $T_{reg}$ cells of nenonatal BDC2.5 mice present a drastically different phenotype relative to their draining pancLN counterparts, as indicated by their high levels of CD25 expression and active proliferative capacity *in situ*. This is consistent with a study by Herman *et al.*, which demonstrated that the gene expression profile of islet-infiltrating $T_{reg}$ cells was drastically different from pancLN resident $T_{reg}$ cells, suggesting that the inflammatory environment of the target tissue directs unique transcriptional programs in $T_{reg}$ cells that may be related to their mechanism of action *in situ* [21]. Moreover, ICOS blockade in this system altered the gene signature of $T_{reg}$ cells suggesting that ICOS activity is necessary for the establishment of tolerance in

pre-diabetes [21]. Therefore, $ICOS^{+}Foxp3^{+}$ $T_{reg}$ cells dominate the prediabetic islet and restrain the destructive autoimmune response of $T_{eff}$ cells directly within the pancreatic lesion.

We and others have shown that T1D progression correlates with a temporal loss in the capacity of $CD4^{+}Foxp3^{+}$ $T_{reg}$ cells to expand/survive in β-islets, which in turn perturbs the overall $T_{reg}/T_{eff}$ cell balance and unleashes the anti-islet immune responses [10, 12, 13]. Moreover, a deficiency in IL-2, essential for the function of $Foxp3^{+}$ $T_{reg}$ cells within islets, was shown to render $T_{reg}$ cells unfit and functionally defective, in turn provoking a $T_{reg}/T_{eff}$ cell imbalance in islets [13, 44]. Interestingly, earlier studies showed that T cells from prediabetic NOD mice become hypoproliferative and poor IL-2 producers at the onset of insulitis [15, 45]. Moreover, we show that this defective $T_{reg}$ function within the pancreatic milieu of NOD mice could be readily restored by the protective *Il2* allelic variants within the $Idd3^{B6}$ locus [13] or low dose IL-2 treatment of NOD mice [14].

As IL-2 is known to enhance ICOS expression on activated T cells, we reasoned that a positive feedback loop exists between IL-2 and ICOS signaling pathways, and a disruption of this regulatory loop could destabilize $T_{reg}$ cell function in inflamed pancreatic milieu [24]. In line with this evidence, we show that the more potent $T_{reg}$ cells from T1D-resistant BDC.*Idd3* mice expressed more elevated levels of ICOS than their WT counterparts, underscoring the link between $T_{reg}$ cell suppressive function and the ICOS pathway. Moreover, a low dose IL-2 regimen in BDC2.5 mice at the time of insulitis restored ICOS levels on intra-islet $Foxp3^{+}$ $T_{reg}$ cells, comparable to those of BDC.*Idd3* mice. Thus, it is imperative for $T_{reg}$ cells to receive the proper fitness signals,

like IL-2, in order to maintain suppressive activity *in situ*. The limited availability of IL-2 may result in "defective" $ICOS^{low}$ expressing $Foxp3^+$ $T_{reg}$ cells with limited capacity to contain the diabetogenic response. Taken together, variations in IL-2 production/activity in inflamed pancreatic sites may restore the balance between islet-specific autoreactive T cells and $Foxp3^+$ $T_{reg}$ cells by modulating ICOS expression.

Unlike CD28, ICOS is seemingly not involved in thymic development of $T_{reg}$ cells, as NOD.$ICOS^{-/-}$ mice exhibit the same proportion of thymic $Foxp3^+$ $T_{reg}$ cells as ICOS-sufficient mice (unpublished results) [16, 46, 47]. In contrast, ICOS is essential for the control of the pool size of $T_{reg}$ cells within the periphery, and ICOS engages a genetic program within $T_{reg}$ cells which instructs its suppressive phenotype [46]. However, the possibility that ICOS is essential for the survival of $T_{reg}$ cells cannot be excluded. This is in accordance with observations reporting reduced proportion of $T_{reg}$ cells under homeostatic steady-state conditions, in the absence of ICOS [46]. Indeed, $ICOS^-$ $T_{reg}$ cells may be more sensitive to inflammatory mediators released within the lesion and thus to undergo apoptosis. Indeed, $T_{reg}$ cells display a strict propensity to undergo rapid apoptosis in culture, unless signaled by ICOS-L [48].

A striking correlation between ICOS expression and the capacity of T cells to produce IL-10 has been documented [18]. In a mouse model of asthma [21, 27, 32], the development of antigen-induced IL-10 producing $T_{reg}$ cell and consequential suppression of allergen-induced airway hyperreactivity was dependent on an intact ICOS-ICOS-L pathway [27]. Moreover, IL-10 has been shown to be immunoprotective in T1D [36], however, the potential for $T_{reg}$ cells to produce IL-10 has not been extensively examined. Here, we report that the production of IL-10 in $Foxp3^+$ $T_{reg}$ cells is restricted

within the ICOS$^+$Foxp3$^+$ $T_{reg}$ cell subset in contrast to ICOS$^-$Foxp3$^+$ $T_{reg}$ cells or Foxp3- Teff cells. The greater propensity for ICOS$^+$Foxp3$^+$ $T_{reg}$ cells to cycle in inflamed pancreatic sites, in conjunction with their dependence on IL-2, may promote the differentiation of the IL-10-secreting phenotype in islet-reactive Foxp3$^+$ $T_{reg}$ cells from recently activated Foxp3$^+$ $T_{reg}$ cells in pancreatic sites. In contrast, we also make the novel observation that ICOS functions differentially in Foxp3$^-$ $T_{eff}$ cells, primarily by potentiating IL-2 production, which may in turn bolster ICOS-mediated signals in Foxp3$^+$ $T_{reg}$ cells. Moreover, the loss of ICOS expression and consequential IL-10 production by antigen-specific Foxp3$^+$ $T_{reg}$ cell with T1D progression indicates that ICOS directly promotes IL-10 production by Foxp3$^+$ $T_{reg}$ cells. While this result strongly suggests that the temporal loss in IL-10$^+$Foxp3$^+$ $T_{reg}$ cells in islets contributes to T1D progression, it does not exclude the more remote possibility that the loss in ICOS/IL-10 expression by Foxp3$^+$ $T_{reg}$ cells is a consequence of the ongoing autoimmune response in these sites. Experiments are currently in progress in our laboratory to assess the function of ICOS$^{-/-}$ Foxp3$^+$ $T_{reg}$ cells throughout T1D progression.

The exclusive expression of ICOS-L on intra-pancreatic CD11c$^+$ DC coincides with the enhanced expression of ICOS on Foxp3$^+$ $T_{reg}$ cells directly within the pancreatic lesion, suggesting a dynamic interplay between DC and $T_{reg}$ cells, specifically under inflammatory settings. We observe that the age-related decline in ICOS expression on $T_{reg}$ cells parallels the loss of ICOS-L expression on DC, coincident with the break-down in self-tolerance. The role of ICOS-L in licensing DC to become tolerogenic is unknown and requires functional studies in order to elucidate its role in the control of immune responses within inflamed target organs. Taken together, $T_{eff}$ cell-mediated

inflammatory events may drive regulation by $T_{reg}$ cells through the modulation of ICOS, which in turn may license DC to be more tolerogenic via ICOS/ICOS-L interactions.

Diminished expression of ICOS in $T_{reg}$ cells of recently diagnosed human T1D patients supports the link between ICOS costimulation and the potency of the $Foxp3^{+}$ $T_{reg}$ cell compartment [49]. Our findings extend our current understanding of the role of ICOS in T1D pathogenesis and suggest that IL-2 insufficiency results in declining levels of ICOS expression, which correlates with waning of $T_{reg}$ cells and coincides with break-down in self-tolerance. The elucidation of mechanisms involved in maintaining self-tolerance will shed light on our current understanding of autoimmune disorders, and provides possible therapeutic tools to combat diseases such as T1D.

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## 8. Figure legends

### FIGURE 1. Preferential accumulation of ICOS$^+$Foxp3$^+$ $T_{reg}$ cells in pre-diabetic islets.

Cell suspensions of draining pancLN and pancreas of three-week-old BDC2.5 mice were prepared and the frequencies of (A) CD4$^+$Foxp3$^+$ICOS$^+$, (B) CD4$^+$Foxp3$^+$ICOS$^+$CD25$^+$ and (C) cycling (Ki67) CD4$^+$Foxp3$^+$ICOS$^+$ T cells were analyzed by FACS. Results represent the mean ± SD. Data are representative of at least three separate experiments. $^{**}p \leq 0.02$, $^{***}p \leq 0.002$.

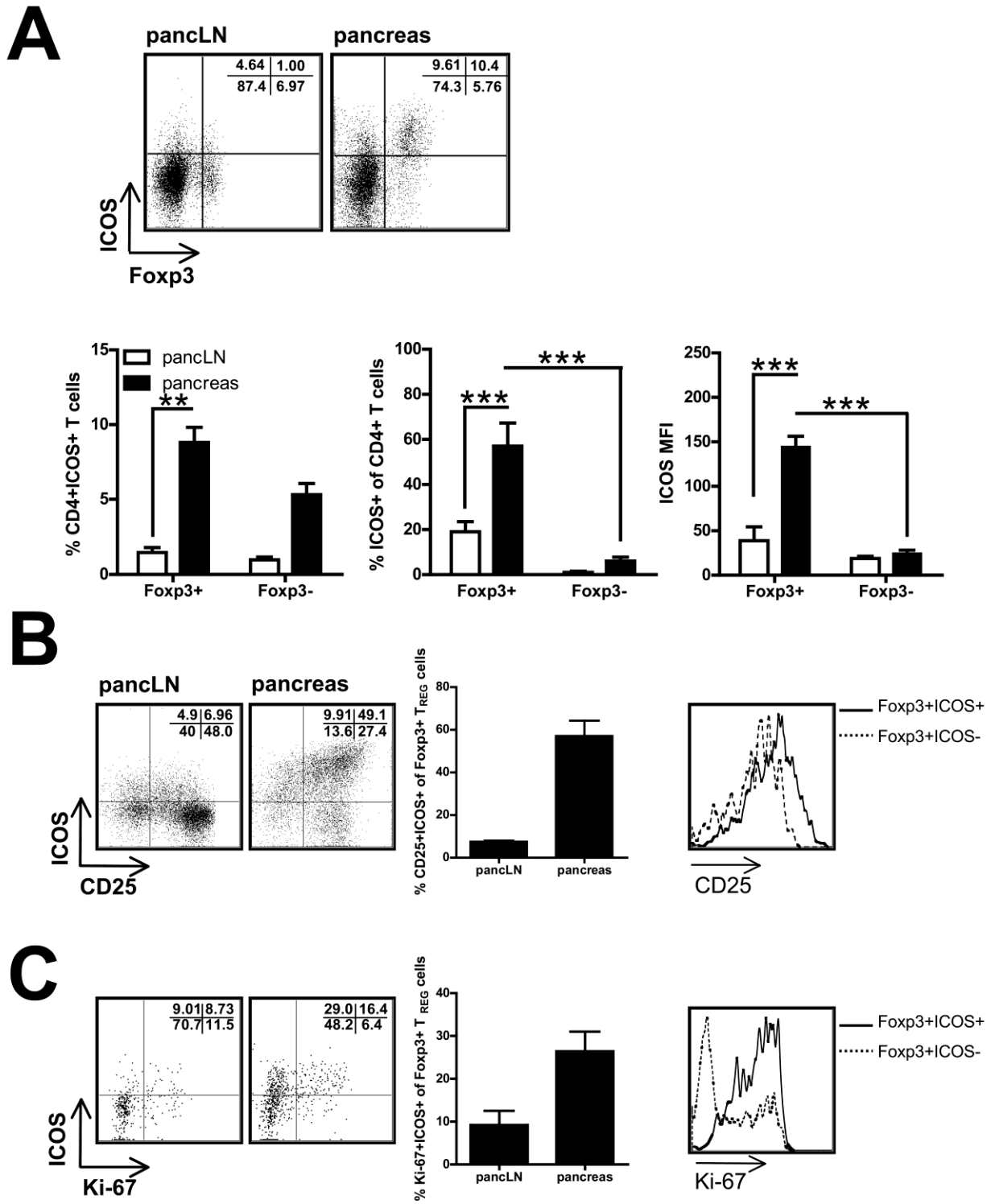
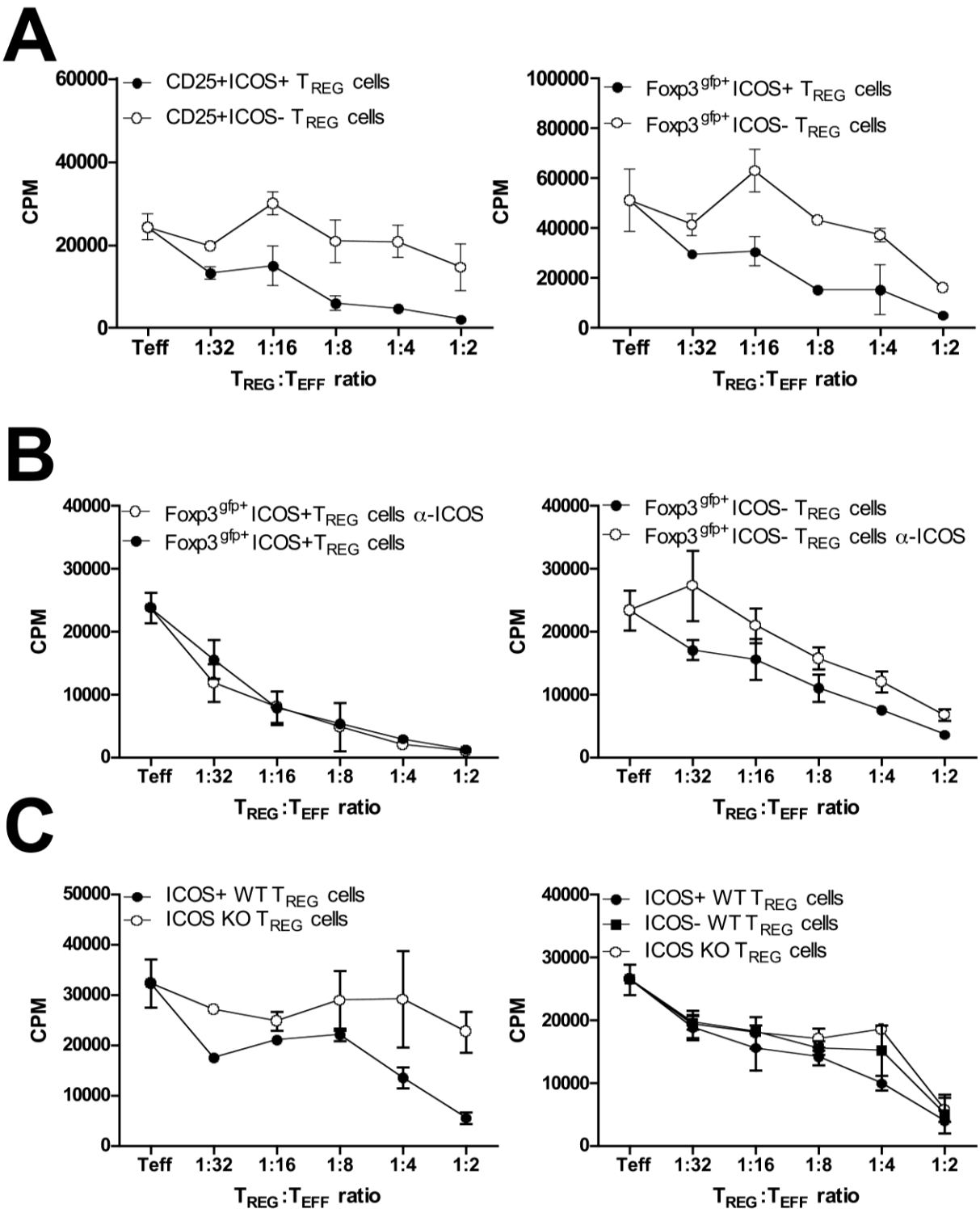

A
pancLN
pancreas
4.64
1.00
87.4
6.97
9.61
10.4
74.3
5.76
ICOS
Foxp3
pancLN
pancreas
% CD4+ICOS+ T cells
**
Foxp3+
Foxp3-
% ICOS+ of CD4+ T cells
***
***
ICOS MFI
***
***
B
pancLN
pancreas
4.9
6.96
40
48.0
9.91
49.1
13.6
27.4
ICOS
CD25
% CD25+ICOS+ of Foxp3+ TREG cells
pancLN
pancreas
Foxp3+ICOS+
Foxp3+ICOS-
CD25
C
9.01
8.73
70.7
11.5
29.0
16.4
48.2
6.4
ICOS
Ki-67
% Ki-67+ICOS+ of Foxp3+ TREG cells
pancLN
pancreas
Foxp3+ICOS+
Foxp3+ICOS-
Ki-67

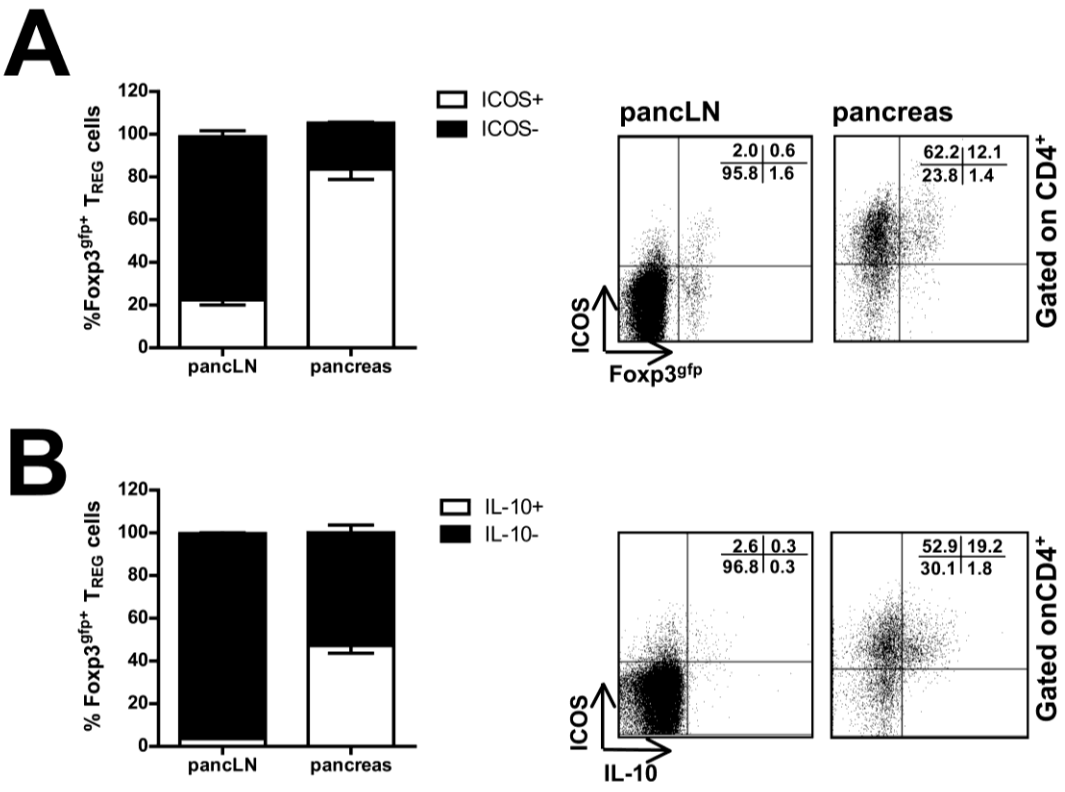
**FIGURE 2. ICOS-expressing Foxp3$^+$ $T_{reg}$ cells display an augmented suppressive function *in vitro* relative to their ICOS$^-$ counterparts.**

BDC2.5 CD4$^+$CD25$^-$ICOS$^-$ responder T cells (5x10$^4$) were stimulated with BDC2.5 mimetope (2ng/ml) and irradiated T cell-depleted spleen cells (2x10$^5$) in the presence or absence of titrated numbers of ICOS$^+$ or ICOS$^-$ CD4$^+$CD25$^+$ $T_{reg}$ cells (A, left panel). In order to exclude contaminating CD25$^+$ICOS$^+$ $T_{eff}$ cells from the $T_{reg}$ cell population, the suppression assay was repeated as in (A) using FACS-purified Foxp3$^{gfp-}$ICOS$^-$ responder T cells and Foxp3$^{gfp+}$ICOS$^+$ or Foxp3$^{gfp+}$ICOS$^-$ $T_{reg}$ cells (A, right panel). (B) In order to confirm that ICOS impacts the suppressive capacity of $T_{reg}$ cells, the suppression assay described in (A) was performed in the presence or absence of blocking anti-ICOS mAb (left panel: ICOS$^+$Foxp3$^{gfp+}$ $T_{reg}$ cells; right panel: ICOS$^-$Foxp3$^{gfp+}$ $T_{reg}$ cells). (C) CD4$^+$CD25$^-$ICOS$^-$ responder T cells (5x10$^4$) from WT (left panel) or ICOS$^{-/-}$ (right panel) NOD mice were stimulated with $\alpha$-CD3 (1$\mu$g/ml) and irradiated spleen cells (2x10$^5$) in the presence or absence of titrated numbers of ICOS$^+$, WT ICOS$^-$ CD4$^+$CD25$^+$ $T_{reg}$ cells or ICOS$^{-/-}$ CD4$^+$CD25$^+$ $T_{reg}$ cells. $^3$H-dTR was added in the last 8-12 hours of culture and suppression was assessed based on thymidine incorporation. Data are representative of three separate experiments. Results represent the means ± SD.


A
CD25+ICOS+ T$_{REG}$ cells
CD25+ICOS- T$_{REG}$ cells
CPM
60000
40000
20000
0
Teff
1:32
1:16
1:8
1:4
1:2
T$_{REG}$:T$_{EFF}$ ratio
Foxp3$^{gfp+}$ICOS+ T$_{REG}$ cells
Foxp3$^{gfp+}$ICOS- T$_{REG}$ cells
100000
80000
60000
40000
20000
0
B
Foxp3$^{gfp+}$ICOS+T$_{REG}$ cells α-ICOS
Foxp3$^{gfp+}$ICOS+T$_{REG}$ cells
40000
30000
20000
10000
0
Foxp3$^{gfp+}$ICOS- T$_{REG}$ cells
Foxp3$^{gfp+}$ICOS- T$_{REG}$ cells α-ICOS
C
ICOS+ WT T$_{REG}$ cells
ICOS KO T$_{REG}$ cells
50000
40000
30000
20000
10000
0
ICOS+ WT T$_{REG}$ cells
ICOS- WT T$_{REG}$ cells
ICOS KO T$_{REG}$ cells

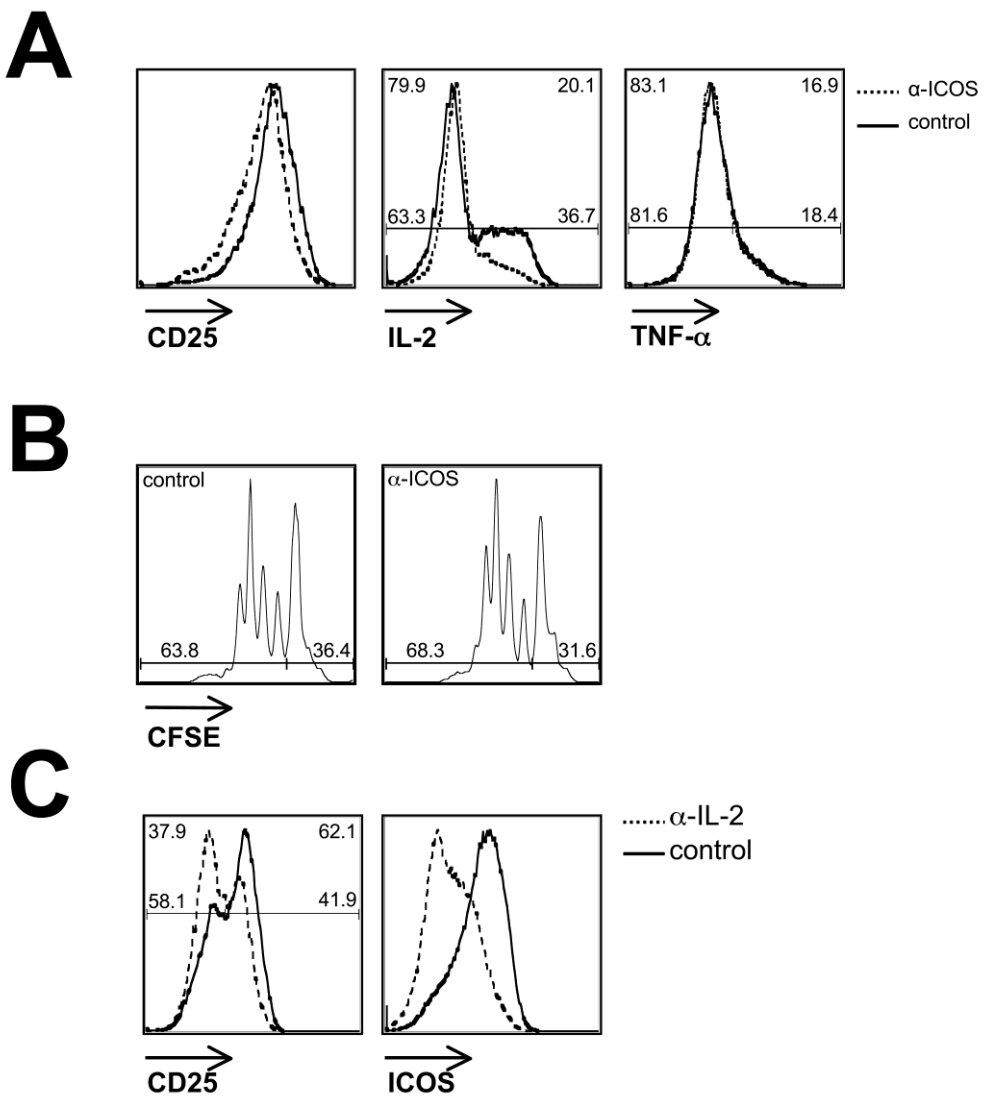
## FIGURE 3. ICOS controls the differentiation of IL-10-producing Foxp3$^{+}$ $T_{reg}$ cells in pre-diabetic islets.

PancLN (A) and pancreas (B) cell suspensions from 4w old NOD Foxp3$^{gfp}$ reporter mice were activated *in vitro* for 4h with PMA/Iono, and the frequency of IL-10 producing $T_{reg}$ cells, relative to ICOS expression, was determined by FACS. (C) FACS-purified ICOS$^{+}$ or ICOS$^{-}$ Foxp3$^{gfp+}$ $T_{reg}$ cells were activated in the presence of WT BMDC at a 4:1 ratio in the presence of BDC2.5 mimetope (10ng/mL) for 72h. Golgi plug was added in the three last hours of culture and the frequency of IL-10-producing $T_{reg}$ cells, relative to ICOS expression, was determined by FACS.


A
%Foxp3gfp+ TREG cells
120
100
80
60
40
20
0
pancLN
pancreas
ICOS+
ICOS-
pancLN
pancreas
2.0 0.6
95.8 1.6
62.2 12.1
23.8 1.4
ICOS
Foxp3gfp
Gated on CD4+
B
% Foxp3gfp+ TREG cells
120
100
80
60
40
20
0
pancLN
pancreas
IL-10+
IL-10-
2.6 0.3
96.8 0.3
52.9 19.2
30.1 1.8
ICOS
IL-10
Gated onCD4+
C
Foxp3gfp+ ICOS+
Foxp3gfp+ ICOS-
IL-10
Foxp3gfp+ ICOS+
Foxp3gfp+ ICOS-
81.0 19.0
1.15 0.06
98.1 1.91
1.48 0.03
Foxp3gfp
IL-10

## FIGURE 4. ICOS-dependent IL-2 production in effector T cells.

FACS-purified, CFSE-labeled Foxp3$^{gfp-}$ICOS$^{-}$ $T_{eff}$ cells (1x10$^{6}$) were activated by NOD WT BMDC at a ratio of 4:1 and BDC2.5 peptide (30ng/mL), in the presence or absence of blocking anti-ICOS mAb for 48h or 72h. (A) The frequency of IL-2 or TNF-$\alpha$ producing T cells was determined by FACS. (B) Proliferation of activated T cells in (A) was assessed by CFSE dilution analysis. (C) Cells were activated as in (A) in the presence of blocking anti-IL-2 antibodies and the level of ICOS expression determined by flow cytometry. Data are representative of both time-points and have been repeated at least 3 times.


A
79.9
20.1
63.3
36.7
83.1
16.9
81.6
18.4
α-ICOS
control
CD25
IL-2
TNF-α
B
control
α-ICOS
63.8
36.4
68.3
31.6
CFSE
C
37.9
62.1
58.1
41.9
α-IL-2
control
CD25
ICOS

## FIGURE 5. Temporal loss in ICOS expression and IL-10 production in Foxp3$^+$ $T_{reg}$ cells coincides with T1D progression.

(A-B) Cell suspensions of pancLN or pancreas of neonatal and adult BDC2.5 mice were stained with anti-CD4, anti-Foxp3, anti-ICOS, and anti-Ki-67 and analyzed by flow cytometry. Results represent the mean ± SD. Data are representative of at least three separate experiments. (C) BDC2.5 $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were adoptively transferred into immunodeficient NOD.TCR$\alpha^{-/-}$ recipients (n=10), and the onset of diabetes was monitored daily. 18 days post-transfer, cell suspensions of pancLN and pancreas of T1D-protected and diabetic mice were reactivated *in vitro* by PMA/Iono. The cells were collected post-activation and stained for anti-CD4, anti-V$\beta$4, anti-ICOS, anti-IL-10 and anti-Foxp3 for analysis on FlowJo software.

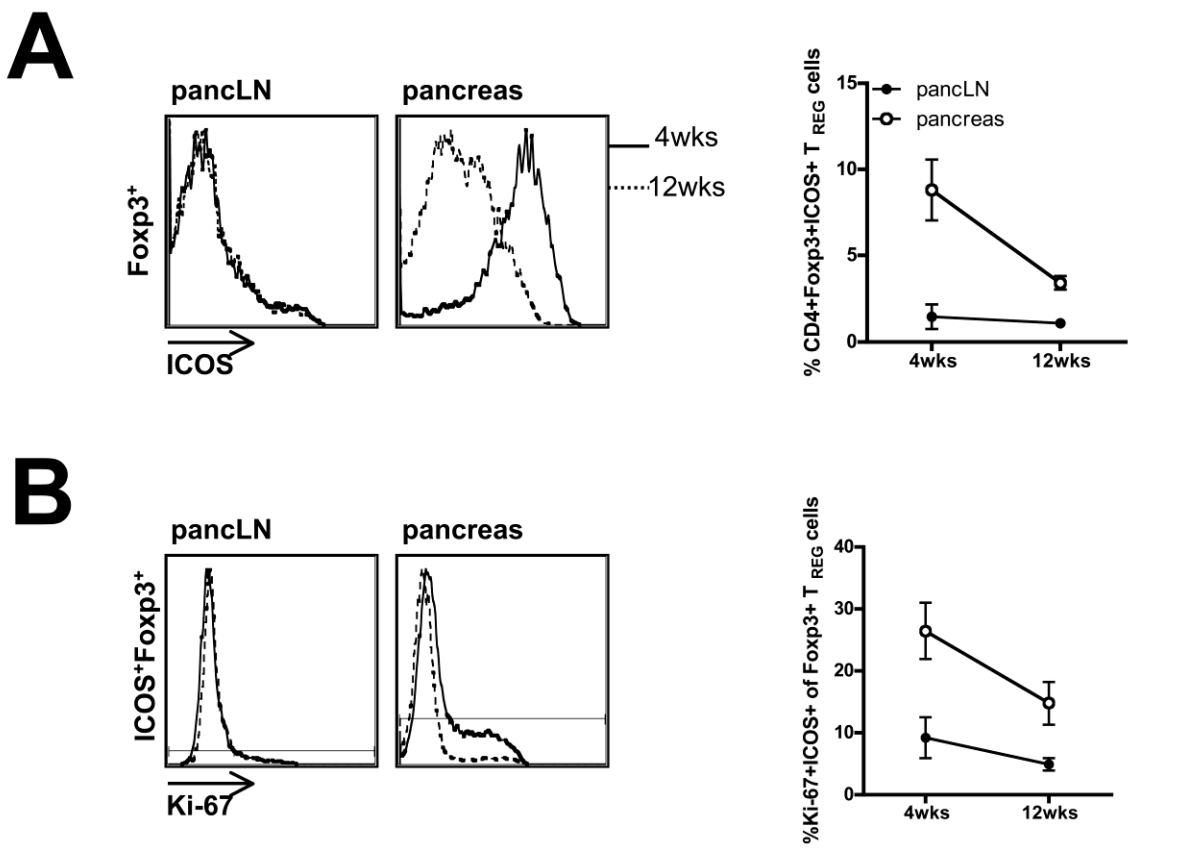
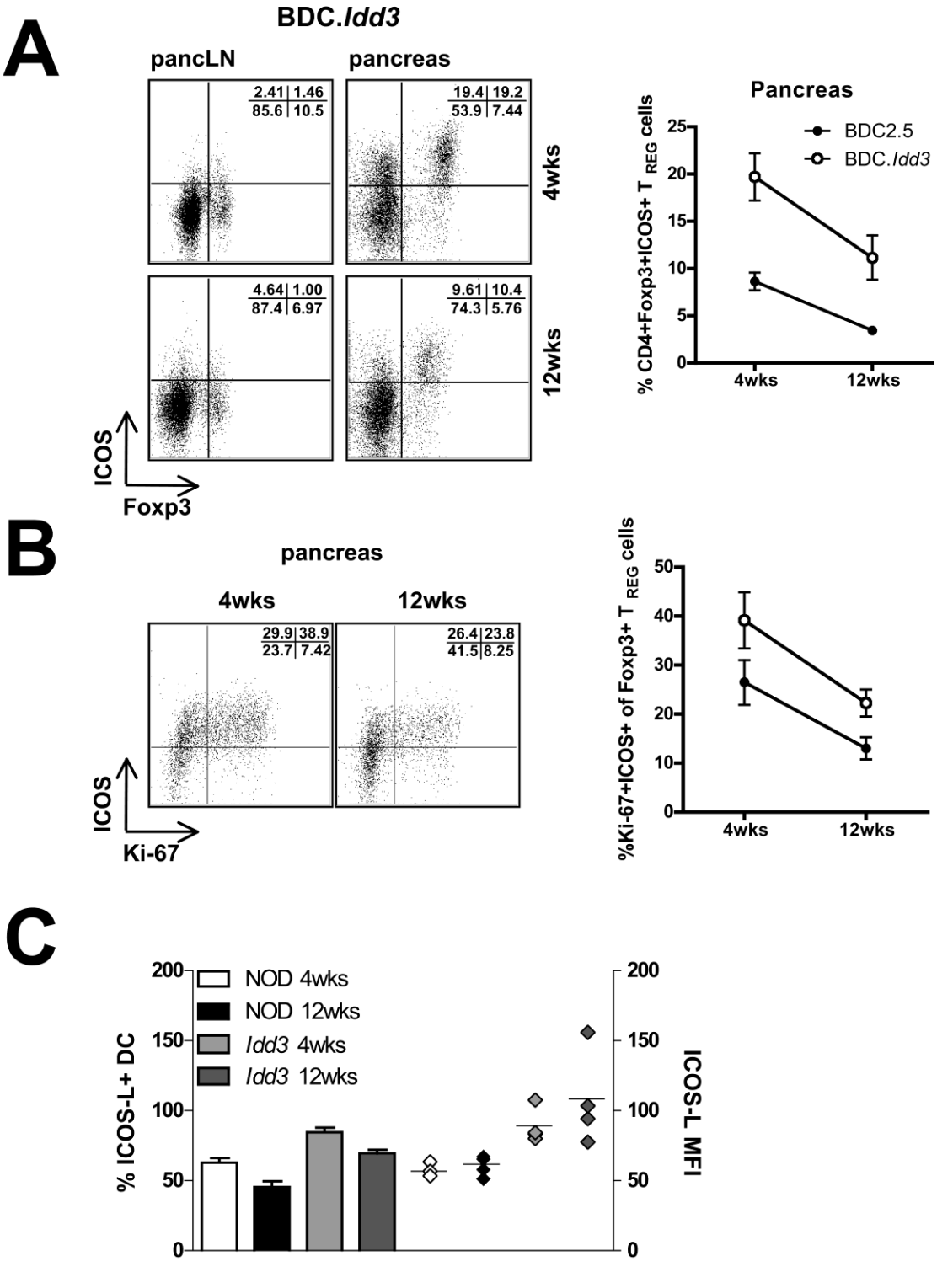

A
pancLN
pancreas
Foxp3+
ICOS
4wks
12wks
% CD4+Foxp3+ICOS+ T REG cells
pancLN
pancreas
4wks
12wks
B
pancLN
pancreas
ICOS+Foxp3+
Ki-67
%Ki-67+ICOS+ of Foxp3+ T REG cells
4wks
12wks
C
Diabetic
Non-Diabetic
ICOS
% of IL-10
ICOS MFI
Diabetic
Non-Diabetic
% of IL-10+ cells
ICOS MFI
CD4+ Foxp3+
CD4+ Foxp3-

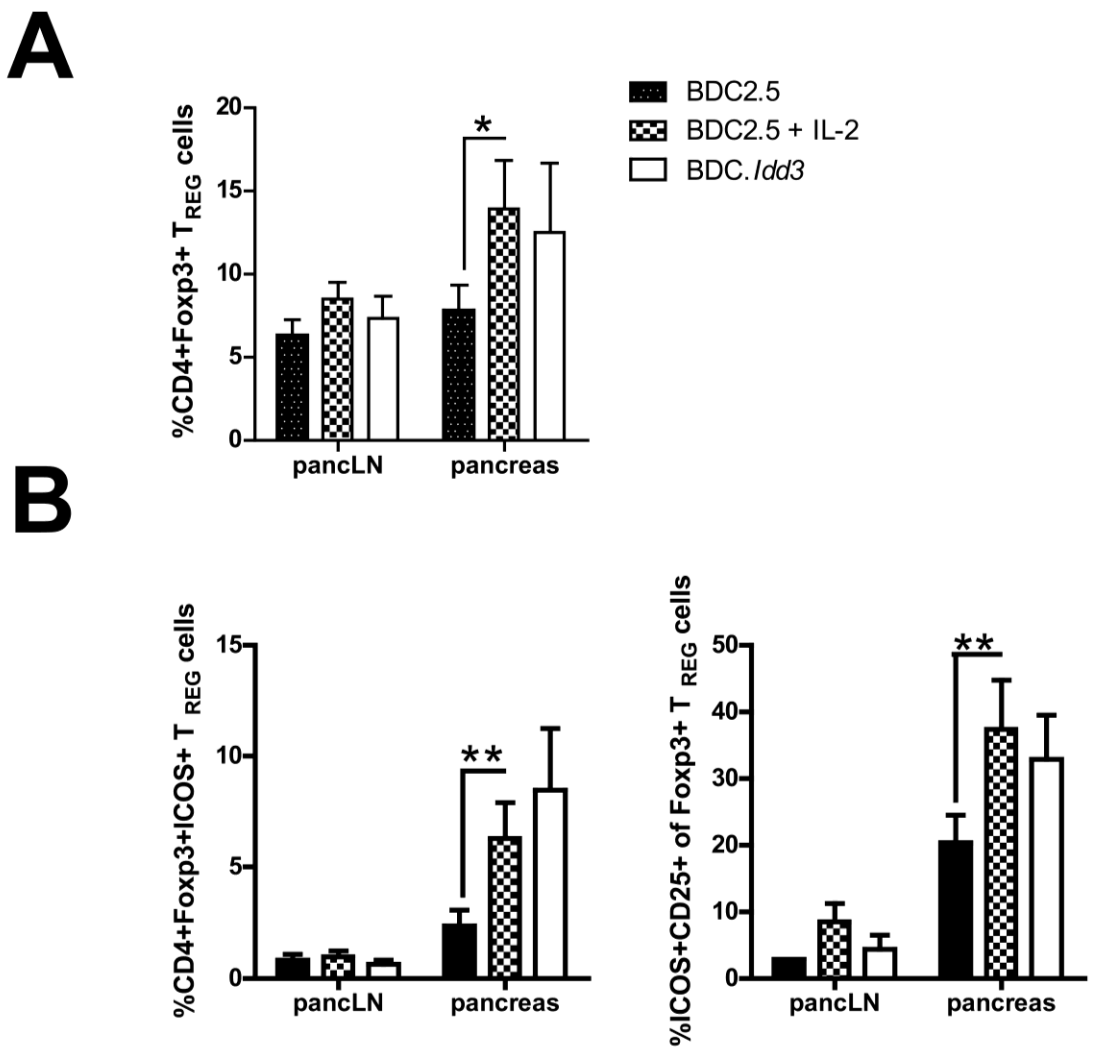
**FIGURE 6. T1D-protective Il2 allelic variants restore ICOS expression on $CD4^+Foxp3^+$ $T_{reg}$ cells and ICOS-L expression on pancreatic $CD11c^+$ DC.**

(A-B) Cell suspensions of draining pancLN and pancreas of neonatal and adult BDC.*Idd3* and BDC2.5 mice were stained using anti-CD4, anti-Foxp3, anti-ICOS and anti-Ki-67 and analyzed by flow cytometry. (C) Neonatal or adult NOD and NOD.B6 *Idd3* mice were stained with anti-CD11c and anti-ICOS-L. Profiles depict $CD11c^+$ gated cells. Data are representative of at least three separate experiments. *$p<0.05$


A
BDC.Idd3
pancLN
pancreas
2.41 1.46
85.6 10.5
19.4 19.2
53.9 7.44
4wks
4.64 1.00
87.4 6.97
9.61 10.4
74.3 5.76
12wks
ICOS
Foxp3
Pancreas
BDC2.5
BDC.Idd3
% CD4+Foxp3+ICOS+ T REG cells
4wks
12wks
B
pancreas
4wks
12wks
29.9 38.9
23.7 7.42
26.4 23.8
41.5 8.25
ICOS
Ki-67
%Ki-67+ICOS+ of Foxp3+ T REG cells
C
NOD 4wks
NOD 12wks
Idd3 4wks
Idd3 12wks
% ICOS-L+ DC
ICOS-L MFI

## FIGURE 7. Low-dose IL-2 therapy restores ICOS expression in Foxp3$^{+}$ $T_{reg}$ cells within the pancreatic lesion.

Three-week-old female BDC2.5 mice (n=8) were treated with daily injections of rhIL-2 (5μg/mL) for five consecutive days. On day 7 following the initial injection, pancLN and pancreas cell suspensions were stained for anti-CD4, anti-ICOS, anti-CD25 and anti-Foxp3. Results are representative of at least three independent experiments. *$p<0.05$, **$p\leq0.03$.


A
B
BDC2.5
BDC2.5 + IL-2
BDC.Idd3
%CD4+Foxp3+ TREG cells
20
15
10
5
0
*
pancLN
pancreas
%CD4+Foxp3+ICOS+ TREG cells
15
10
5
0
**
pancLN
pancreas
%ICOS+CD25+ of Foxp3+ TREG cells
50
40
30
20
10
0
**
pancLN
pancreas

# CHAPTER V *IL-2 production by dendritic cells augments Foxp3$^{+}$ regulatory T cell function in autoimmune resistant NOD mice.*

## Bridging statement from Chapter IV to V

The data generated in chapter IV suggested that in the autoimmune-prone BDC2.5 strain, an IL-2 deficit leads to a substantial net loss in the expression of ICOS, which is an active participant in $T_{reg}$ cell expansion and suppressive functions. Indeed, age-related loss of ICOS signals in the $T_{reg}$ cell pool coincides with diabetes progression. Consistently, in T1D-protected BDC.*Idd3*, IL-2 and ICOS costimulation act in concert to maintain $T_{reg}$ cell functions within the peripheral compartment. Interestingly, the decline in ICOS levels correlated with attenuation of ICOS-L expression within the intra-islet DC compartment. The observation that both ICOS and ICOS-L decline with age in $T_{reg}$ cells and DC respectively, strongly pointed to a defective feedback mechanism. This data led us to hypothesize that the bidirectional signalling along the ICOS/ICOS-L pathway enabling reciprocal conditioning of $T_{reg}$ cells and DC is somehow disrupted. Two possibilities can be envisioned: the age-related waning of $T_{reg}$ cells results in defective DC functions or defective DC are not optimally promoting $T_{reg}$ cell functions.

Since DC represent the exclusive APC subset capable of activating $T_{reg}$ cell functions and many documented DC defects exist in the NOD mouse, we focused our analysis on the DC compartment. In light of the existence of a $T_{reg}$ cell/DC cross-talk, we sought to further assess the tolerogenic role of DC in diabetes. In order to assess the relative contribution of DC, the priming capabilities of DC in the context of $T_{reg}$ cell functions originating from the congenic T1D-protected NOD.B6 *Idd3* mouse were

compared to those from T1D-susceptible NOD mouse. Interest was also cast on the overall costimulatory load of DC. The NOD.B6 *Idd3* mouse model was also used to assess whether the *Il2* allelic variants within the *Idd3*$^{B6}$ genetic interval endowed DC with the capacity to express IL-2. We made the unanticipated finding that indeed DC express IL-2 within a narrow window of time post-activation, suggesting that DC may provide $T_{reg}$ cells with the initial IL-2 to launch the signature suppressive phenotype.

# IL-2 production by dendritic cells augments Foxp3$^+$ regulatory T cell function in autoimmune-resistant NOD mice.

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**Keywords:** CD4$^+$CD25$^+$ regulatory T cells, Foxp3, diabetes, tolerance, IL-2, DC

**Running title:** IL-2 gene variation imprints DC to preferentially activate $T_{reg}$ cells.

Manuscript to be submitted

## 1. Abstract.

NOD mice congenic for the *Idd3*$^{B6}$ locus show a marked resistance to type 1 diabetes (T1D). *Il2* is the primary gene within the *Idd3*$^{B6}$ locus responsible for T1D-protection. IL-2 is pivotal for the fitness and homeostasis of Foxp3$^{+}$ regulatory T ($T_{reg}$) cells, and recent studies have shown that the *Idd3*$^{B6}$ locus augments IL-2 production by effector T ($T_{eff}$) cells, which in turn enhances the potency of $T_{reg}$ cell functions. Given the important role dendritic cells (DC) play in $T_{reg}$ cell mediated tolerance induction, we hypothesized that DC from *Idd3*$^{B6}$ congenic mice contribute to increased $T_{reg}$ cell activity. In this study, we observe that CD11c$^{+}$ DC, harboring the protective *Idd3*$^{B6}$ genes, are endowed with the capacity to secrete IL-2, enabling them to preferentially promote $T_{reg}$ cell functions. Collectively, our results show that *Il2* gene variation may imprint DC to favor T cell regulation of autoimmunity.

## 2. Introduction.

Non-obese diabetic (NOD) mice spontaneously develop type 1 diabetes (T1D), a T cell-mediated autoimmune disease resulting in the destruction of the insulin-producing β-islets of Langerhan's, and hyperglycemia [1, 2]. Genomic mapping studies of congenic NOD strains show that T1D susceptibility is inherited by over 20 T1D-linked insulin-dependent diabetes (*Idd*) loci, collectively predisposing to autoimmune responses to β islet cells. The onset and progression of T1D in NOD mice is tightly controlled by regulatory T ($T_{reg}$) cells constitutively expressing CD25 and Foxp3 [3]. Depletion of CD25-expressing T cells, genetic disruption of *Foxp3*, IL-2 neutralization or blockade of B7/CD28 or CD40/CD40L pathways in NOD mice abrogates $T_{reg}$ cell function, and leads to an accelerated T1D onset compared to wild-type (WT) NOD mice [4-8]. Moreover, reconstitution of $CD4^+CD25^+$ $T_{reg}$ cells restores tolerance to β-islet antigens, underpinning the essential role of $T_{reg}$ cells in T1D protection. However, it is unclear whether T1D progression is due to a decline in $T_{reg}$ cell frequency and/or functions or from the overriding of such regulation by $T_{reg}$ cell-resistant diabetogenic T cells [9, 10].

Many reports indicate a progressive waning in $T_{reg}$ cell functions, despite a stable cellular frequency of $T_{reg}$ cells, as a potential trigger of T1D [11-14]. We and others have shown that T1D progression is associated with a progressive loss in the capacity of $Foxp3^+$ $T_{reg}$ cells to expand in β-islets, which in turn perturbs the $T_{reg}/T_{eff}$ cell balance and unleashes anti-islet immune responses [11, 13-15]. A study by Tang *et al.* showed that an IL-2 deficiency within islets of NOD mice triggered the defective survival/function

of $T_{reg}$ cells, provoking a $T_{reg}/T_{eff}$ cell imbalance and progressive loss of self-tolerance, a condition restored by IL-2 treatment [15]. Consistently, congenic NOD mice, introgressed with the *Idd3*$^{B6}$ genetic interval, show significant resistance to T1D [12, 16, 17]. The *Il2* gene has been identified as the major candidate gene in the *Idd3*$^{B6}$ locus and the strongest contributor for protection against T1D [17, 18]. Several lines of evidence show that IL-2 is critical for Foxp3$^{+}$ $T_{reg}$ cell functions [8, 19]. Moreover, we have recently shown that *Idd3*$^{B6}$ bolsters IL-2 production by $T_{eff}$ cells, which in turn enhances the functional activity of $T_{reg}$ cells in pancreatic lesions [12]. Currently, the mechanisms that reverse IL-2 deficiency and support the functional stability of $T_{reg}$ cells in T1D remain to be elucidated.

DC can orchestrate the balance between immunity and self-tolerance [20] by modulating $T_{reg}$ cell functions [21]. DC-expanded $T_{reg}$ cells blocked the induction and even reverse established T1D in NOD mice, demonstrating that DC can promote the suppressive activity in $T_{reg}$ cells [22, 23]. Numerous studies showed that functional abnormalities of DC underlie the risk of T1D in humans and in NOD mice [24, 25]. It is thought that the inability of DC to efficiently expand $T_{reg}$ cells may directly impact $T_{reg}$ cell homeostasis, and lead to a loss in $\beta$ cell tolerance [26].

Here, we hypothesized that DC from *Idd3*$^{B6}$ congenic mice may contribute to the increased $T_{reg}$ cell activity seen in these mice. We show that the expression of *Idd3*$^{B6}$ in DC enables them to enhance $T_{reg}$ cell functions more efficiently than WT DC. Interestingly, we found that *Idd3*$^{B6}$ DC are imprinted with the unique ability of expressing IL-2 mRNA briefly after DC activation, which in turn enabled $T_{reg}$ cells to expand preferentially. Consistently, IL-2 blockade *in vitro* completely abrogated the proliferative

advantage conferred by *Idd3*$^{B6}$ DC on $T_{reg}$ cells. Overall, our results suggest that IL-2 gene variation in DC may condition them to become an initial non-T cell source of IL-2 required to maintain $T_{reg}$ cell fitness, and may represent an important checkpoint in the control of $T_{eff}$ cells in T1D.

## 3. Materials and Methods.

***Mice*.** All mice were maintained in SPF conditions at McGill University. BDC2.5 $CD4^+$ transgenic (Tg) mice were a generous gift from Christophe Benoist (Harvard Univ., Boston, USA). BDC2.5/$Foxp3^{gfp}$ mice were generated by backcrossing BDC2.5 mice to C57BL/6 $Foxp3^{gfp}$ reporter mice, kindly provided by Alexander Rudensky (Univ. Wash., Seattle, USA) [27]. NOD.B6 *Idd3* congenic mice (line#1098) were obtained from Taconic Farms, and BDC.*Idd3* mice were generated by in-house breeding.

***Bone marrow-derived dendritic cells (BMDC) cultures.*** BMDC were produced according to a technique adapted from Inaba *et al.* [28] and Lutz *et al.* [29]. Non-adherent cells were collected on day 8 and used as immature BMDC for the experiments. In certain instances, day 8 BMDC were matured with LPS (1μg/mL) and pulsed with the BDC2.5 mimetope peptide (RVRPLWVRME) (Sigma) (100ng/mL) overnight and used in proliferation assays on day 9. The purity was typically 90-95% $CD11c^+$ cells, as determined by FACS analysis.

***Cell purification.*** $CD4^+CD25^{+/-}$ or $Foxp3^{gfp+/-}$ T cell subsets were purified from LN or spleens using the autoMACS cell sorter (Miltenyi Biotech, San Diego, CA) or FACSAria cell sorter (BD Bioscience), as described previously [30]. The purity was typically 95-97% for CD25 or $Foxp3^{gfp}$ expression.

***Flow cytometry.*** Stainings were done with the following fluorochrome-conjugated or biotinylated monoclonal antibodies (mAb): anti-CD4 (clone RM5), anti-CD25 (clone PC61), anti-Vβ4 (CTVB4), anti-CD80 (16-10A1), anti-CD86 (GL-1), anti-CD11c (p150/90), anti-CD40 (3/23), anti-RT1b (OX-6), (eBioscience, San Diego, CA). Anti-

Foxp3 (FJK-16s) intranuclear staining was performed according to the manufacturer's protocol (eBioscience, San Diego, CA). Stained cells were acquired on a FACSCalibur (BD Bioscience, Mississauga, Ontario) and analyzed with Flowjo software.

***Adoptive transfers.*** Purified $CD4^+CD25^{+/-}$ T cells were CFSE-labeled (Invitrogen) and transferred intravenously, either alone or in combination with BDC2.5 mimetope-pulsed mature BMDC, into NOD recipient mice (4:1 ratio; $7x10^6$/mouse). Expansion of donor T cells was evaluated 4 days post-transfer pancLN, as previously described [12].

***In vitro proliferation assays***. Proliferation assays were performed by culturing FACS purified $CD4^+$, $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells ($7.5x10^3$) from BDC2.5 or BDC.*Idd3* mice in 96-well flat-bottom microtiter plates with irradiated BMDC, FACS-sorted pancreatic LN (pancLN) or splenic $CD11c^+$MHC $II^+$ DC ($2.5x10^3$) and BDC2.5 mimetope (10 or 100ng/mL) for 72h at $37^oC$. In some instances, neutralizing anti-IL-2 mAb (10μg/mL) (JES6-5H4) (BD Bioscience, Mississauga, Ontario) were added to the cultures. Cell cultures were pulsed with 1μCi $^3$H-TdR for the last 8-12h and analyzed as previously shown [12]. All experiments were repeated at least 3 times.

***In vitro Foxp3 conversion assays.*** FACS purified BDC2.5 $CD4^+Foxp3^{gfp-}$ T cells ($2.0x10^5$) were primed with BMDC ($5x10^4$) of either genotype in the presence or absence of BDC2.5 mimetope (300ng/mL) and rhIL-2 (100 units/mL) for 4-6 days. Conversion was determined as the frequency of newly-induced $Foxp3^{gfp+}$ cells assessed by flow cytometry.

***Intracellular cytokine staining.*** BDC2.5 or BDC.*Idd3* $CD4^+Foxp3^{gfp-}$ T cells were stimulated with NOD or $Idd3^{B6}$ BMDC (4:1 ratio) and BDC2.5 mimetope (100ng/mL) for

72h, and treated with Golgi-Stop (BD Bioscience, Mississauga, Ontario) for the last 3-4h of culture. Intracytoplasmic staining was performed using anti-IL-2 mAb (JES6-5H4) or appropriate isotype controls (BD Bioscience, Mississauga, Ontario) as previously shown [13].

***Reverse-transcription polymerase chain reaction (RT-PCR).*** *Il2* gene expression in resting and LPS-activated NOD or *Idd3*$^{B6}$ BMDC at time-points ranging from 2h-48h was achieved by normalizing the IL-2 densitometric value with the intensity of the G3PDH amplicon for each sample, and reported as arbitrary IL-2/G3PDH ratios, as previously described [12].

**Statistical analysis**. All statistical analysis was performed with a Student's *t* test. Values of $p<0.05$ were considered significant.

## 4. Results.

**Protective *Idd3*$^{B6}$ alleles promote the capacity of DC to prime and expand $T_{reg}$ cells.**

We and others have shown that NOD.B6 *Idd3* mice display delayed onset, incidence and severity of T1D relative to WT NOD mice due to an enhanced regulatory feedback loop initiated by IL-2-producing self-reactive *Idd3*$^{B6}$ $CD4^+$ $T_{eff}$ cells [12, 16, 17]. The enhanced production of IL-2 by $CD4^+$ $T_{eff}$ cells of NOD.B6 *Idd3* mice favors the preferential expansion and function of $CD4^+Foxp3^+$ $T_{reg}$ cells, and a concomitant dampening of the autoreactive $T_{eff}$ cell response directly within the pancreatic lesion [31]. These observations compelled us to consider the potential contribution of DC in this regulatory feedback loop. Thus, we hypothesized that the *Idd3*$^{B6}$ locus may imprint DC with the capacity to augment the priming of antigen-specific $T_{reg}$ cells *in vitro*. To test this, BDC2.5 or BDC.*Idd3* $CD4^+CD25^+$ $T_{reg}$ cells were activated in an antigen-specific manner in the presence of BMDC of either genotype. Our results show that BDC.*Idd3* $CD4^+CD25^+$ $T_{reg}$ cells exhibited greater proliferation when activated with *Idd3*$^{B6}$ BMDC than with NOD BMDC (Fig.1A, left panel). In contrast, BMDC of either genotype were equally potent in their ability to prime $T_{eff}$ cells, suggesting that *Idd3*$^{B6}$ DC preferentially impact $T_{reg}$ cell functions (Fig.1A, right panel). Strikingly, *Idd3*$^{B6}$ BMDC were more potent at expanding BDC.*Idd3* $T_{reg}$ cells relative to BDC2.5 $T_{reg}$ cells, suggesting that the *Idd3*$^{B6}$ genetic interval is operative in both DC and T cells to ensure optimal proliferation of $T_{reg}$ cells.

**_Idd3_$^{B6}$ licenses DC in pancreatic lymph nodes to enhance $T_{reg}$ cell functions.**

DC integrate signals from the inflammatory environment and may influence T cell responses accordingly. To address the possibility that DC within inflammatory pancreatic sites are licensed by the _Idd3_$^{B6}$ locus to dampen the priming of diabetogenic $CD4^+$ T cells, $CD11c^+$MHC $II^+$ DC were purified from draining pancLN and spleen of mice of either genotype, and tested for their ability to induce antigen-specific proliferation of BDC2.5 or BDC._Idd3_ $T_{reg}$ cells _in vitro_. $T_{reg}$ cell proliferation was comparable in the presence of splenic DC, irrespective of the genotype of the responding T cell or activating DC population. In stark contrast, $CD11c^+$MHC $II^+$ DC isolated from the pancLN of NOD.B6 _Idd3_ mice promoted BDC._Idd3_ $T_{reg}$ cell expansion more efficiently than with WT DC from similar sites (Fig.1B), suggesting that _Idd3_$^{B6}$ DC are more tolerogenic within pancreatic sites than WT DC. In order for pancreatic _Idd3_$^{B6}$ DC to ensure optimal $T_{reg}$ cell activation/expansion, the presence of the _Idd3_$^{B6}$ locus was also required in responding $T_{reg}$ cells (Fig.1B). Interestingly, these _Idd3_$^{B6}$-mediated effects were not noted in $T_{eff}$ cells (data not shown), suggesting that the _Idd3_$^{B6}$ locus can contribute to self-tolerance by favoring DC-mediated $T_{reg}$ cell activity and dampening $T_{eff}$ cell activity.

We then sought to determine whether _Idd3_$^{B6}$ DC favored the expansion of pre-existing, naturally-occurring $T_{reg}$ cells or the conversion of newly-generated $Foxp3^+$ $T_{reg}$ cells from $Foxp3^-$ T cell precursors. To this end, $CD4^+Foxp3^{gfp-}$ $T_{eff}$ cells were isolated from BDC2.5 $Foxp3^{gfp}$ reporter mice, and activated in an antigen-specific manner in the presence of BMDC of either genotype, as described above. Our results show that no significant induction of $Foxp3^{gfp+}$ $T_{reg}$ cells could be discerned in any stimulatory

condition, suggesting that the *Idd3*$^{B6}$ locus does not confer DC with the potential of preferentially promoting the conversion of $T_{eff}$ cells to $T_{reg}$ cells (Fig.1C). Overall, this result indicates that *Idd3*$^{B6}$ DC selectively promote the priming/expansion of pre-existing Foxp3$^{+}$ $T_{reg}$ cells circulating in the NOD repertoire.

**Phenotypic characterization of CD11c$^{+}$ DC in pancreatic sites.**

Foxp3$^{+}$ $T_{reg}$ cells have a greater requirement for B7-CD28 costimulation compared to $T_{eff}$ cells. This is best illustrated by CD28 or B7.1/B7.2 deficiency in NOD mice, which disturbs the peripheral homeostasis and competency of Foxp3$^{+}$ $T_{reg}$ cells, and consequently provokes an increased T1D onset compared to WT NOD mice [4, 6]. We then asked whether the costimulatory phenotype of CD11c$^{+}$ DC differed between NOD and T1D-resistant NOD.B6 *Idd3* mice, and whether these differences could account for the $T_{reg}$ cell priming observed *in vitro*. We phenotyped CD11c$^{+}$ DC from different sites for various costimulation markers at the onset of insulitis, a time-point during which $T_{reg}$ cells are actively expanding and prominently affected by *Il2* allelic variation, as we previously showed [12]. The frequency of CD8$^{+}$ lymphoid, CD8$^{-}$ myeloid and overall CD11c$^{+}$ DC in spleen, distal, draining pancLN and pancreas was comparable between NOD and NOD.B6 *Idd3* mice (data not shown). Although MHC II expression levels were modestly elevated in the pancreas relative to draining pancLN of both genotypes (Fig.2A), this expression did not differ between genotypes within the pancreatic lesion (Fig.2B), suggesting that the *Idd3*$^{B6}$ locus does not impact antigen presentation, a finding consistent with our previously published study [12]. The levels of CD80, and to a greater extent CD86 and CD40, were significantly increased on CD11c$^{+}$

DC within the pancreas of both NOD and NOD.B6 *Idd3* mice relative to their counterparts in draining pancLN (Fig.2A). Interestingly, a substantial decrease in CD80 and CD86 expression on $CD11c^+$ DC was observed in the pancreas of NOD relative to NOD.B6 *Idd3* mice (Fig.2B), in contrast to CD40 expression, which was modestly increased in the pancreas of WT relative to NOD.B6 *Idd3* mice. The expression of PDL-1 and OX-40L, associated with tolerogenic DC phenotypes, on $CD11c^+$ DC from pancreatic sites of both genotypes were also evaluated, but were undetectable in both cases (data not shown). Overall, the increased CD80 (B7.1) and CD86 (B7.2) expression on pancreas-resident $CD11c^+$ DC of T1D-resistant NOD.B6 *Idd3* mice correlates with the increased expansion of intra-islet $Foxp3^+$ $T_{reg}$ cells and resistance to T1D observed in these mice.

**Protective *Idd3*$^{B6}$ alleles promote DC-mediated expansion of $T_{reg}$ cells *in vivo*.**

We then assessed the impact of the protective *Idd3*$^{B6}$ locus in DC-mediated priming of $T_{reg}$ and $T_{eff}$ cells *in vivo*. To this end, $CD4^+CD25^+$ or $CD4^+CD25^-$ T cell subsets from BDC2.5 mice were CFSE-labeled, adoptively transferred alone or in combination with BDC2.5 mimetope-pulsed, SNARF-labeled NOD or *Idd3*$^{B6}$ BMDC into immunocompetent NOD recipients, and antigen-specific expansion of donor T cells was monitored by CFSE dilution. The *Idd3*$^{B6}$ locus did not impact the influx of $CD11c^+$ DC into the draining pancreatic sites, as the proportion of SNARF-labeled $CD11c^+$ DC that accumulated in draining pancreatic sites did not vary based on genotype (data not shown). Despite a modest enhancement in the accumulation of $Foxp3^+$ $T_{reg}$ cells in the draining pancreatic sites of mice receiving NOD BMDC relative to control NOD mice (Fig.3C, left panel), the extent of $T_{reg}$ cell proliferation remained unaffected (Fig.3A, top

panel). In stark contrast to $T_{eff}$ cells, a more prominent enhancement in $T_{reg}$ cell proliferation (72.1%±0.4 vs 51.7%±3.5; $p \leq 0.01$) (Fig.3A, top panel) and accumulation (43,229 cells ± 2,604 vs 15,697 cells ±172; $p < 0.005$) (Fig.3C, left panel) was observed in the presence of *Idd3*$^{B6}$ BMDC relative to NOD BMDC. More specifically, the proportion and frequency of dividing Foxp3$^{+}$ $T_{reg}$ cells exposed to *Idd3*$^{B6}$ BMDC was significantly greater compared to $T_{reg}$ cells activated by WT BMDC (Fig.3B). Although $T_{eff}$ cell proliferation was readily detectable in the presence of WT BMDC, $T_{eff}$ cell expansion was not affected to the same degree as $T_{reg}$ cells when exposed to *Idd3*$^{B6}$ BMDC *in vivo* (Fig.3A and B). No difference was observed in the accumulation of $T_{eff}$ cells within pancreatic sites, suggesting that the infusion of exogenous DC neither affected the migration or expansion of $T_{eff}$ cells within draining LN (Fig.3C, right panel). Overall, these data show that *Idd3*$^{B6}$ DC are intrinsically more potent at inducing antigen-specific expansion of $T_{reg}$ cell proliferation *in vivo*.

**CD11c$^{+}$ DC from pancreatic LN of NOD.B6 *Idd3* mice express IL-2 and promote $T_{reg}$ cell functions.**

Our previous data showed that the *Idd3*$^{B6}$ genetic interval enhanced IL-2 production by BDC.*Idd3* $T_{eff}$ cells relative to their WT counterparts when expanded by WT BMDC [12]. We wondered whether *Idd3*$^{B6}$ DC had the capacity to enhance IL-2 production in antigen-specific $T_{eff}$ cells. To this end, BDC2.5 CD4$^{+}$Foxp3$^{gfp-}$ $T_{eff}$ cells were activated by WT or *Idd3*$^{B6}$ BMDC in the presence of BDC mimetope, and IL-2 secretion by responding T cells was assessed by FACS. Our results show that $T_{eff}$ cells primed by *Idd3*$^{B6}$ BMDC produced substantially greater levels of IL-2 relative to WT

BMDC (29.7% vs 20.1%) (Fig.4A, left panel), suggesting that *Il2* allelic variation in $CD11c^+$ DC influences their capacity to prime IL-2 secretion by autoreactive T cells.

Granucci *et al.* showed that DC, under certain inflammatory conditions, might secrete IL-2 and potentially influence the activation of T cells [32]. As the *Il2* gene is the strongest candidate for diabetes protection in the $Idd3^{B6}$ locus [17], we tested whether the $Idd3^{B6}$ locus enabled BMDC to express IL-2. To this end, BMDC of either genotype were matured by LPS, and *Il2* gene expression was assessed at various time-points by RT-PCR. Interestingly, while *Il2* gene expression was modestly detectable in matured WT BMDC, IL-2 mRNA levels were significantly more elevated in $Idd3^{B6}$ BMDC within a few hours post-activation, suggesting that a short burst of DC-derived IL-2 under homeostatic conditions may contribute to the promotion of $T_{reg}$ cell functions (Fig.4B), a finding consistent with recent observations [33].

These results begged the question of whether DC-derived IL-2 could potentially influence $T_{reg}$ cell functions. To this end, BDC2.5 or BDC.*Idd3* $T_{reg}$ cells were activated by peptide-pulsed NOD or $Idd3^{B6}$ BMDC in the presence or absence of a blocking anti-IL-2 antibody. In contrast to NOD BMDC, $Idd3^{B6}$ BMDC-dependent proliferation was abolished when IL-2 was neutralized, suggesting that DC-derived IL-2 is an important factor in augmenting the expansion of BDC.*Idd3* $T_{reg}$ cells. Consistently, $Idd3^{B6}$ BMDC-derived IL-2 also impacted, albeit to a lesser extent, the proliferation of WT BDC2.5 $T_{reg}$ cells, as IL-2 blockade led to a marked decrease in their proliferation (Fig.4C).

In order to address whether $CD11c^+$ DC from draining pancreatic sites promote $T_{reg}$ cell expansion in an IL-2 dependent fashion, $CD4^+CD25^+$ $T_{reg}$ cells of either

genotype were activated by WT or *Idd3*$^{B6}$ DC originating from pancLN or spleen, and in the presence or absence of IL-2 neutralizing conditions. Splenic DC did not impact the proliferative capacity of $T_{reg}$ cells, irrespective of their genotype (Fig.4D, left panel). In contrast to WT CD11c$^{+}$ DC, which induced a modest proliferation of WT and *Idd3*$^{B6}$ $T_{reg}$ cells, the robust proliferation of *Idd3*$^{B6}$ $T_{reg}$ cells expanded by pancLN *Idd3*$^{B6}$ CD11c$^{+}$ DC was abrogated in the presence of blocking anti-IL-2 antibodies, a situation not observed with WT $T_{reg}$ cells (Fig.4D, right panel). Taken together, gene and protein expression of *Il2* allelic variants in CD11c$^{+}$ DC may provide the initial bolus of IL-2 early in an autoimmune response necessary for the fitness of $T_{reg}$ cells in the intra-pancreatic environment. These results also suggest that this IL-2 secretion by CD11c$^{+}$ DC harboring *Idd3*$^{B6}$ protective genes may be induced by tissue-specific or inflammatory factors, which imprint tissue-resident CD11c$^{+}$ DC with this capacity.

## 5. Discussion.

IL-2 is an important signal for $CD4^+Foxp3^+$ $T_{reg}$ cell function, peripheral homeostasis and competitive fitness *in vivo*, and alterations in IL-2 signaling may attenuate $T_{reg}$ cell function and provoke autoimmunity [12, 17, 19]. Consistently, IL-2 neutralization or genetic ablation of B7 or CD28 in NOD mice reduces $CD4^+Foxp3^+$ $T_{reg}$ cell numbers, resulting in a more aggressive form of T1D than control littermates [4, 6, 8]. Moreover, T cells from prediabetic NOD mice have reduced T cell proliferative and IL-2 production capabilities, hallmark features, which coincide with a skewing towards pathogenic, β islet antigen-specific T effector function [34]. While T1D progression is not attributed to systemic fluctuations in $CD4^+Foxp3^+$ $T_{reg}$ cell numbers, there is a paradoxical increase of $T_{reg}$ cells in the pancLN at the time of T1D onset [35]. Recently, we and others have shown that T1D progression is associated with a progressive loss in the capacity of $CD4^+Foxp3^+$ $T_{reg}$ cells to expand in pancreatic sites, which in turn perturbs the equilibrium between $T_{eff}$ cells and $T_{reg}$ cells and unleashes the diabetogenic potential of $T_{eff}$ cells in inflamed islets [11-14]. Moreover, intra-islet $T_{reg}$ cells expressed reduced amounts of CD25 and Bcl-2 relative to the $T_{reg}$ cells in the pancLN, suggesting that the $T_{reg}/T_{eff}$ cell imbalance was due to a defect in intra-islet $T_{reg}$ survival, reversed by low-dose IL-2 treatment [15]**.** Thus, IL-2 deficiency contributes to intra-islet $T_{reg}$ cell dysfunction and progressive loss of peripheral self-tolerance in the islets.

Defining the mechanisms underlying the protective effects of T1D gene variants is important to understand how genetic variation may affect natural checkpoints in T1D

onset and progression. Fine mapping studies of the *Idd3*$^{B6}$ locus have previously identified the *Il2* gene as the strongest candidate for protection in NOD.B6 *Idd3* mice [18]. We showed that T1D-resistance in NOD.B6 *Idd3* congenic mice correlates with increased IL-2 mRNA and protein production in islet-reactive CD4$^+$ T cells, and that the *Idd3*$^{B6}$ allele favors the proliferative and suppressive functions of $T_{reg}$ cells *in vitro* [12]. Moreover, this increased $T_{reg}$ cell function, in contrast to controls, restrains the expansion, effector functions, and pathogenicity of autoreactive CD4$^+$ T cells more efficiently *in vivo* [12]. Interestingly, resistance to T1D in NOD.B6 *Idd3* congenic mice correlates with the ability of protective *Il2* allelic variants alleles to promote the expansion and function of $T_{reg}$ cells locally in islets [12]. Collectively, T1D-protective *Il2* allelic variants impinge the development of organ-specific autoimmunity by bolstering the IL-2 production of diabetogenic CD4$^+$ $T_{eff}$ cells, initiating a regulatory feedback loop driving the functional homeostasis of CD4$^+$Foxp3$^+$ $T_{reg}$ cells in the target organ.

It is well established that DC may induce a state of tolerance via the maintenance of $T_{reg}$ cell functions. A temporal or functional alteration in DC-mediated costimulation may attenuate $T_{reg}$ cell function and provoke autoimmunity, conditions believed to occur in NOD mice [33]. Recent evidence elegantly demonstrated that $T_{reg}$ cells form lasting and stable contacts with DC [36], suggesting two possible outcomes from these interactions: DC-mediated activation of $T_{reg}$ cell suppressive activity or $T_{reg}$ cell-mediated dampening of DC immunogenic functions. Many DC anomalies have been documented in NOD mice, which may contribute greatly to T1D pathogenesis, ranging from enhanced NF-κB function, defective maturation and indoleamine 2,3 dioxygenase (IDO) activity resulting in defective function [25, 37, 38].

In the current study, we investigated whether T cell-extrinsic variables, such as DC, played an important role in T1D protection in NOD.B6 *Idd3* mice. We discovered that DC from NOD.B6 *Idd3* mice are more potent activators of $T_{reg}$ cell functions *in vitro* and *in vivo*, and this increased capacity of congenic DC to prime $T_{reg}$ cells is attributed to their ability to produce IL-2. These results show that *Il2* allelic variants can drive IL-2 mRNA expression and protein secretion by DC which, in conjunction with the action of the *Idd3*$^{B6}$ variant in T cells, conditions more potent Foxp3$^{+}$ $T_{reg}$ cells for the control of T1D progression.

We observed that the *Idd3*$^{B6}$ interval licences DC within draining pancreatic sites to potentiate $T_{reg}$ cell functions. The CD11c$^{+}$ DC were found to have a costimulatory load that was different within the target organ of NOD.B6 *Idd3* relative to NOD mice. We showed that the *Idd3*$^{B6}$ interval promoted changes in DC phenotype, illustrated by the higher CD80, CD86 and decreased CD40 levels directly within the pancreatic lesion, which correlates with T1D protection, although the impact of this difference on T1D pathogenesis remains to be elucidated. These findings are in accordance with studies demonstrating that B7 or CD28 deficient NOD animals develop exacerbated diabetes due to a marked decrease in $T_{reg}$ cell frequency, underpinning the importance of the CD28/B7 axis in $T_{reg}$ cell homeostasis [4, 6, 39]. Our findings of elevated CD40 levels in the NOD mouse model are also consistent with one study that elegantly demonstrated that CD40 ligation releases DC from $T_{reg}$ cell suppression [7].

Recent evidence points to the predominant role of IL-2 in the maintenance of peripheral $T_{reg}$ cell fitness and function in inflammatory sites [19]. The defective IL-2 production by activated T cells in the NOD mouse is restored by the protective *Idd3*$^{B6}$

genetic interval [12, 17]. Given the capacity of DC to secrete IL-2, we hypothesized that the *Idd3*$^{B6}$ locus confers an IL-2-producing capability to DC enabling them to promote $T_{reg}$ cell functions [32, 40]. In addition to the impact of the *Idd3*$^{B6}$ locus on IL-2 production by $T_{eff}$ cells, this genetic interval also conditions tissue-resident DC to deliver a swift bolus of IL-2, in turn fueling $T_{reg}$ cell expansion and disease protection.

A recent study by Anderson *et al.* indicated that $CD11c^{-}CD11b^{+}$ macrophages, and not $CD11c^{+}$ DC, in NOD.B6 *Idd3* mice were responsible for the increased activity of $CD4^{+}CD25^{+}$ $T_{reg}$ cells *in vitro* [41]. While $CD11c^{-}CD11b^{+}$ macrophages may potentially have the capacity to prime $T_{reg}$ cell functions in NOD mice, this study did not consider the impact of IL-2 derived from $T_{eff}$ cells or purified $CD11c^{+}$ DC, particularly from pancreatic sites, on $T_{reg}$ cell potency. Furthermore, these findings need to be corroborated *in vivo.* Overall, we make the striking observation that $CD11c^{+}$ DC from NOD.B6 *Idd3* mice are more potent activators of $T_{reg}$ cell proliferation and suppressive function *in vitro* and *in vivo*, a process attributed to the ability of $CD11c^{+}$ DC to produce IL-2 and to drive $T_{reg}$ cell proliferation in an IL-2-dependent fashion. Thus, T1D genes such as the *Il2* allelic variants, may directly impinge the functional capacity of DC and, in addition to T cells, modulate the homeostasis and activity of $CD4^{+}Foxp3^{+}$ $T_{reg}$ cells, and in turn, contribute to T1D susceptibility.

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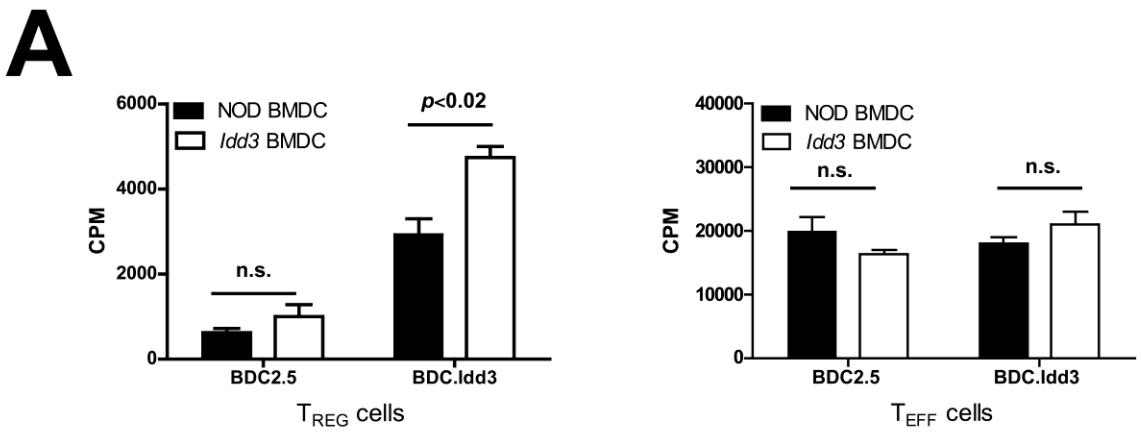
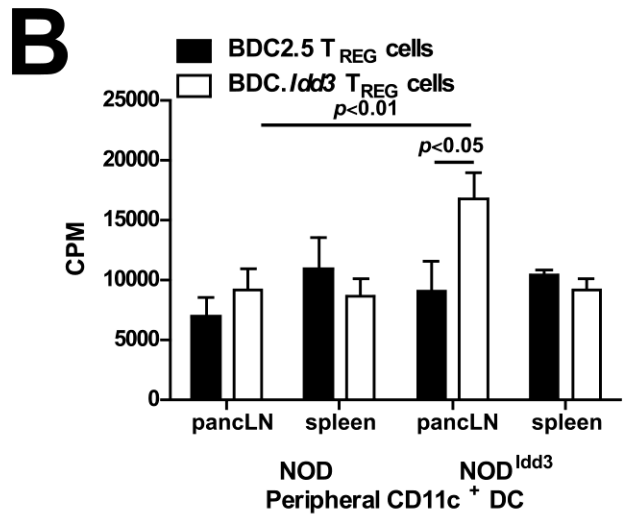
## 8. Figure Legends

### FIGURE 1. The *Idd3*$^{B6}$ locus conditions DC to preferentially promote proliferation of $T_{reg}$ cells.

(A) BDC2.5 and BDC.*Idd3* $CD4^+CD25^+$ $T_{reg}$ cells (left panel) or $CD4^+CD25^-$ $T_{eff}$ cells ($7.5x10^3$) (right panel) were activated in round-bottom 96-well plates in the presence of NOD or *Idd3*$^{B6}$ BMDC ($2.5x10^3$) and BDC2.5 mimetope (100ng/mL) respectively. (B) The proliferation assay was repeated as in (A) using pancreatic LN or splenic highly-purified $CD11c^+$MHC $II^+$ DC. Proliferation was assessed by thymidine incorporation at 72h post-activation. Results represent the mean ± SD. Data are representative of at least three experiments. (C) The *Idd3*$^{B6}$ locus does not confer to DC the ability to convert $T_{eff}$ cells to $T_{reg}$ cells. Purified BDC2.5 Foxp3$^{gfp-}$ $T_{eff}$ cells were expanded by NOD or *Idd3*$^{B6}$ BMDC in an antigen-specific manner for a period of 2-6 days. Cells were collected at indicated time-points and GFP expression was used as an indicator for Foxp3 induction. The profile on the left panel is representative of Foxp3$^{gfp}$ induction.


A
NOD BMDC
Idd3 BMDC
p<0.02
n.s.
CPM
6000
4000
2000
0
BDC2.5
BDC.Idd3
T_REG cells
40000
30000
20000
10000
n.s.
n.s.
T_EFF cells


B
BDC2.5 T_REG cells
BDC.Idd3 T_REG cells
p<0.01
p<0.05
CPM
25000
20000
15000
10000
5000
0
pancLN
spleen
pancLN
spleen
NOD
NOD^Idd3
Peripheral CD11c+ DC

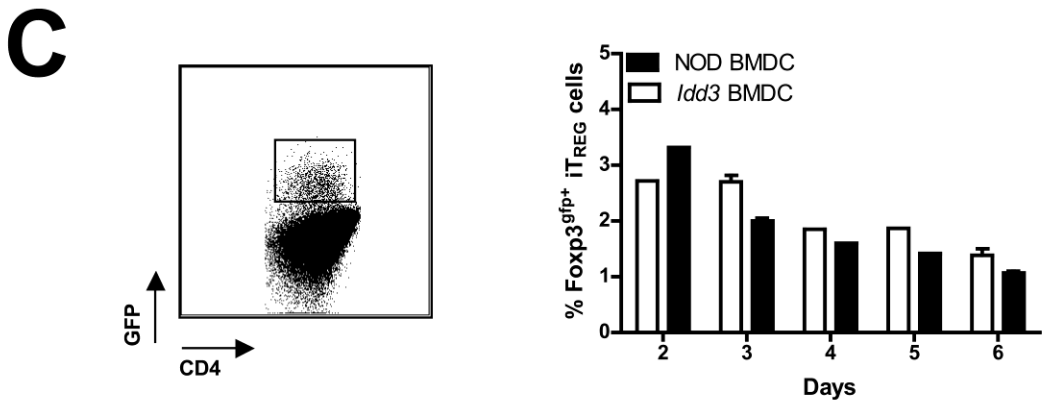

C
GFP
CD4
NOD BMDC
Idd3 BMDC
% Foxp3^gfp+ iT_REG cells
5
4
3
2
1
0
2
3
4
5
6
Days

**FIGURE 2. Phenotypic characterization of CD11c$^+$ DC from WT NOD and T1D-protected NOD.B6 *Idd3* mice.**

Cell suspensions of pancreatic LN and pancreas of 4week-old NOD and NOD.B6 *Idd3* female mice were stained with anti-CD11c, MHC II, CD80, CD86 and CD40. Analysis was performed using FlowJo software on CD11c$^+$ cells. Data is representative of at least five independent experiments.

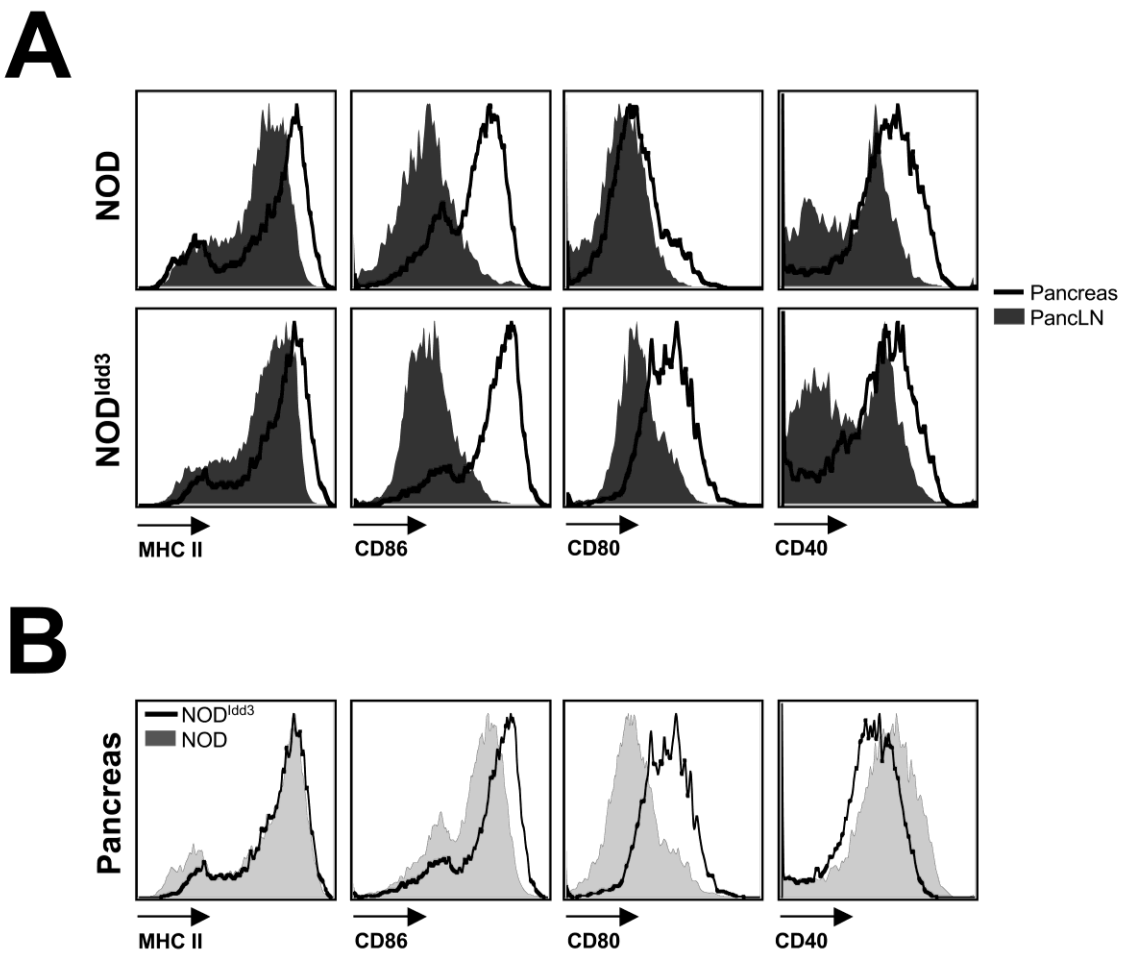
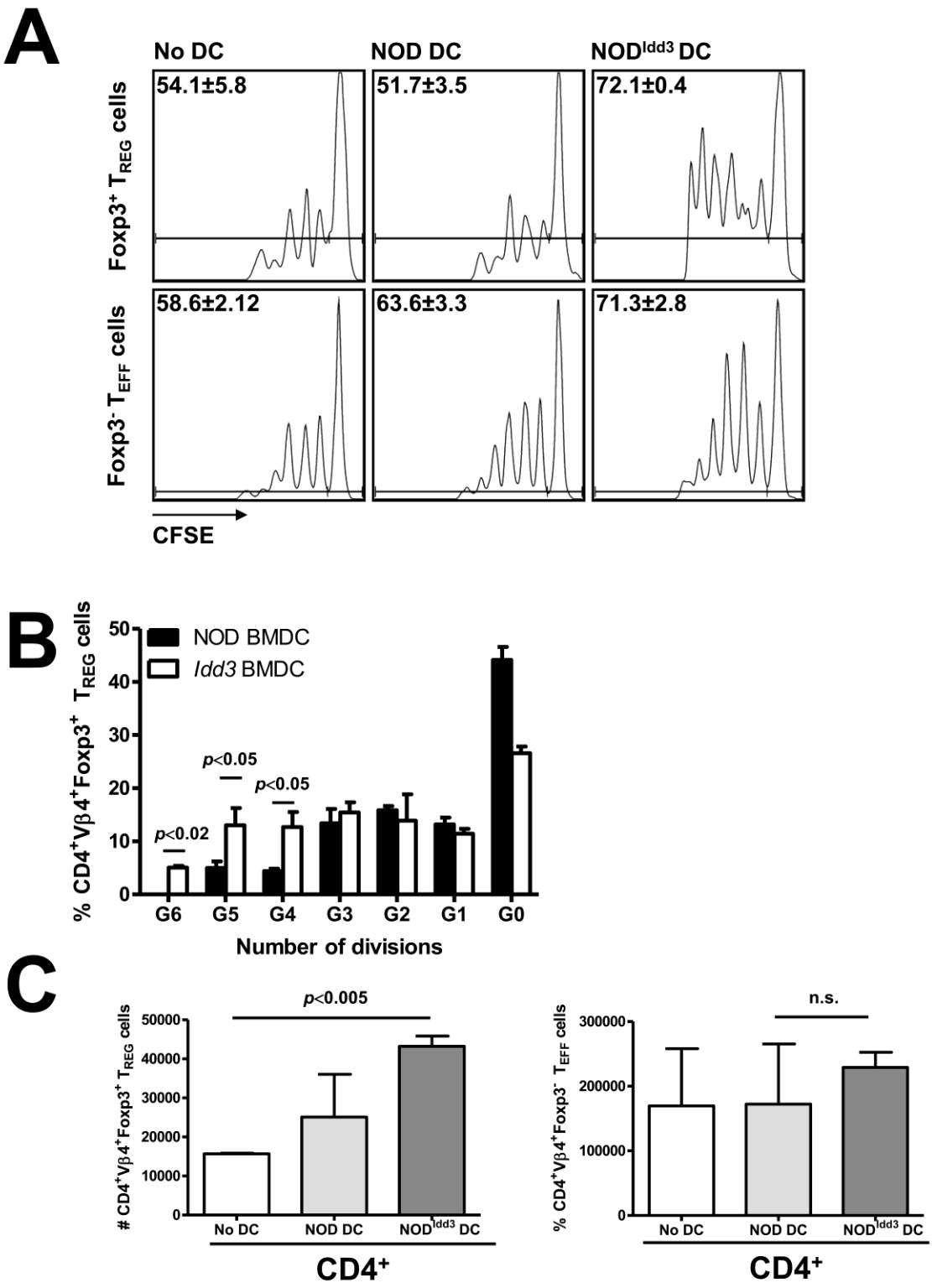

A
NOD
NOD^Idd3
Pancreas
PancLN
MHC II
CD86
CD80
CD40
B
NOD^Idd3
NOD
Pancreas
MHC II
CD86
CD80
CD40

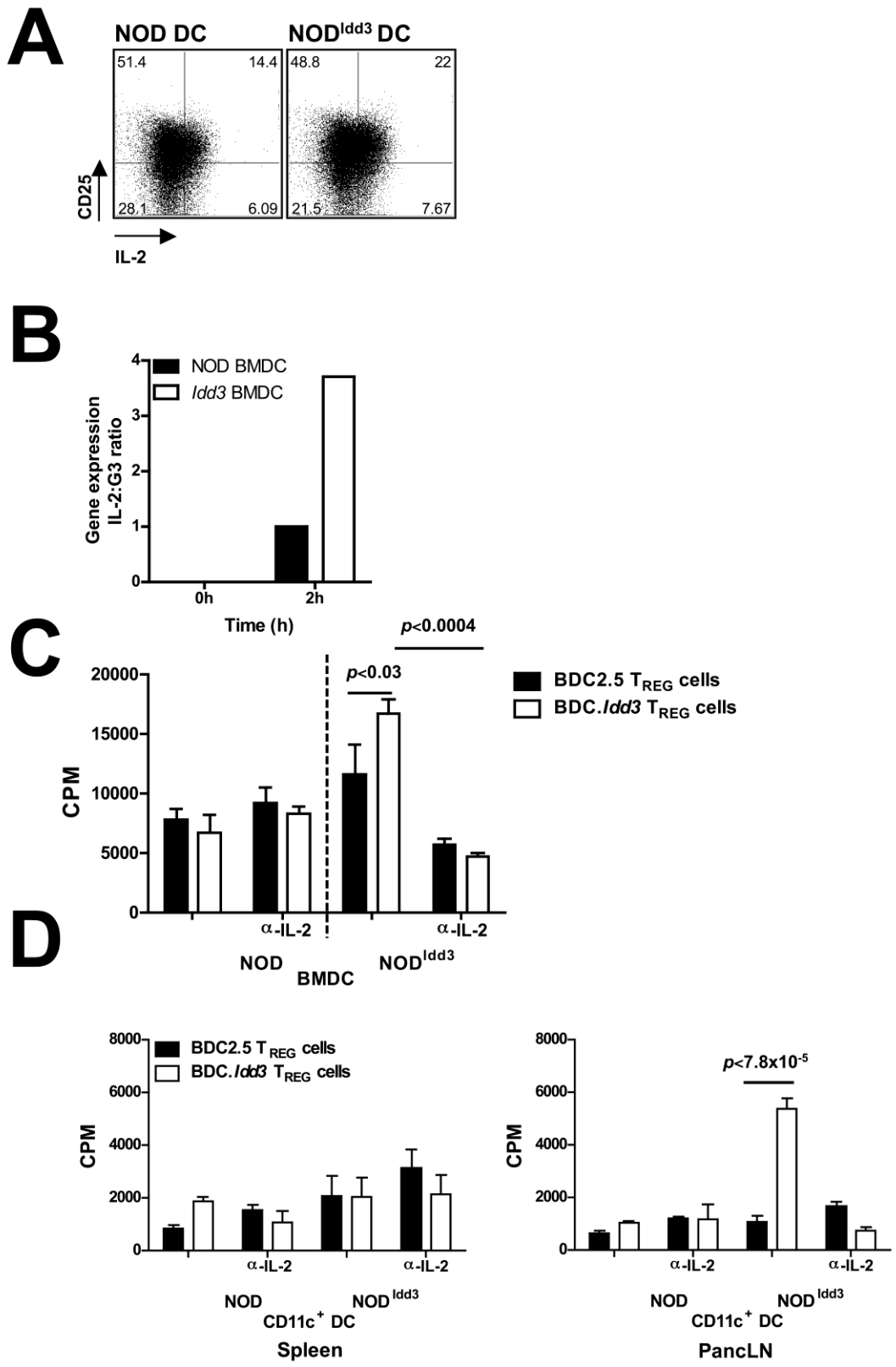
**FIGURE 3. *Il2* allelic variation in DC augments $T_{reg}$ cell expansion *in vivo*.**

(A) CFSE-labeled BDC2.5 $CD4^+$ T cells ($1x10^6$) were injected alone or in combination with SNARF-labeled NOD or $Idd3^{B6}$ BMDC ($2.5x10^5$) at a 4:1 ratio. Four days post-transfer, pancLN were harvested and CFSE dilution analysis was performed by flow cytometry. Data are representative of at least three separate experiments. Results represent the mean ± SD. $p \leq 0.01$ difference with cycling $CD4^+Foxp3^+$ $T_{reg}$ cells in the presence of WT NOD BMDC. (B) Cell division analysis of $T_{reg}$ cells expanded in the presence of NOD or $Idd3^{B6}$ BMDC. (C) The $Idd3^{B6}$ locus in DC mediates a greater accumulation of $T_{reg}$ cells in draining pancreatic sites. Absolute numbers of $T_{reg}$ cell (left panel) and $T_{eff}$ cell (right panel) accumulation in the pancLN 4 days post-transfer.


A
No DC
NOD DC
NOD^Idd3 DC
54.1±5.8
51.7±3.5
72.1±0.4
58.6±2.12
63.6±3.3
71.3±2.8
Foxp3+ TREG cells
Foxp3- TEFF cells
CFSE
B
NOD BMDC
Idd3 BMDC
% CD4+Vβ4+Foxp3+ TREG cells
p<0.02
p<0.05
p<0.05
G6
G5
G4
G3
G2
G1
G0
Number of divisions
C
p<0.005
# CD4+Vβ4+Foxp3+ TREG cells
No DC
NOD DC
NOD^Idd3 DC
CD4+
n.s.
% CD4+Vβ4+Foxp3- TEFF cells
No DC
NOD DC
NOD^Idd3 DC
CD4+

## FIGURE 4. CD11c$^{+}$ DC from the pancLN of *Idd3*$^{B6}$ congenic mice express IL-2 and promote $T_{reg}$ cell functions.

(A) $T_{eff}$ cells were expanded by WT or *Idd3*$^{B6}$ BMDC for 48h and IL-2 production was assessed by intracellular staining. (B) BMDC of both genotypes were activated using LPS (1μg/mL) on day 8 of culture and harvested at different time-points, ranging from 2h to 48h. RNA was extracted and RT-PCR was performed to detect IL-2 expression. (C) In order to evaluate the impact of *Idd3*$^{B6}$-mediated DC-derived IL-2 on $T_{reg}$ cell proliferation, highly-purified CD4$^{+}$CD25$^{+}$ $T_{reg}$ cells (7.5x10$^{3}$) were activated with LPS-matured BMDC (C), pancLN (D, left panel) or splenic CD11c$^{+}$MHC II$^{+}$ DC (D, right panel) (2.5x10$^{3}$) and BDC2.5 mimetope (10ng/mL) in the presence or absence of neutralizing IL-2 antibodies. Proliferation was assessed by thymidine incorporation at 72h post-activation. Data are representative of at least three separate experiments. Results represent the mean ± SD.


A
NOD DC
NOD^Idd3 DC
51.4
14.4
28.1
6.09
48.8
22
21.5
7.67
CD25
IL-2
B
NOD BMDC
Idd3 BMDC
Gene expression IL-2:G3 ratio
0h
2h
Time (h)
C
p<0.0004
p<0.03
BDC2.5 T_REG cells
BDC.Idd3 T_REG cells
CPM
α-IL-2
NOD
NOD^Idd3
BMDC
D
BDC2.5 T_REG cells
BDC.Idd3 T_REG cells
CPM
α-IL-2
NOD
NOD^Idd3
CD11c+ DC
Spleen
p<7.8x10-5
PancLN

# CHAPTER VI *Conclusions and Discussion*

## CONCLUSIONS

The overarching rationale and hypothesis for this study was that developmental or functional deficiencies in $T_{reg}$ cells provoke autoimmunity. We and others [302, 304] have not detected any developmental defects for the $nT_{reg}$ cell pool, as the NOD genetic background does not alter the frequency or suppressive activity of $nT_{reg}$ cells in thymic [302, 304] or peripheral compartments [309, 319, 321, 323]. Therefore, we focused our efforts on the assessment of the functional dynamics of peripheral $Foxp3^+$ $T_{reg}$ cells corresponding with T1D onset and progression. We and others have shown that susceptibility to pancreatic islet autoimmunity has been attributed to an age-related decline in the suppressive activity of $T_{reg}$ cells relative to non-autoimmune prone strains [305, 306, 322, 370]. Our results demonstrate that $T_{reg}$ cells of pre-diabetic mice exist in normal numbers and are functionally operative, preventing the onset of disease. However, as disease progresses, $Foxp3^+$ $T_{reg}$ cells are no longer capable of restraining the autodestructive potential of $T_{eff}$ cells. Our data is corroborated by various groups [309, 319, 321, 323]. As a second step, we wondered at which stage of T cell priming $T_{reg}$ cells exerted their suppressive activity. Our observations suggested that, although $T_{reg}$ cells do not impact the expansion of $T_{reg}$ cells in pancreatic sites, they hamper the differentiation of $T_{eff}$ cells, as per their capacity to secrete TNF-$\alpha$ and IL-17 (chapter II).

We then sought to dissect the events that led to the waning of $T_{reg}$ cell functions over time. Given the importance of IL-2 in $T_{reg}$ cell fitness and thus peripheral tolerance [235, 237], the congenic T1D-resistant NOD.B6 *Idd3* mouse, which is introgressed with protective *Il2* alleles from a genetically resistant strain [76], was an excellent starting point to identify the faulty mechanism of $T_{reg}$ cell functions. We hypothesized that the

*Idd3*$^{B6}$ locus confers T1D protection by enhancing the survival, function or recruitment of $T_{reg}$ cells. We showed that *Idd3*$^{B6}$ controls IL-2 secretion by islet-reactive $T_{eff}$ cells, favoring the cycling and function of $T_{reg}$ cells locally in the pancreas. The more potent $T_{reg}$ cell suppressors in turn dampened the expansion of $T_{eff}$ cells within the lesion of *Idd3*$^{B6}$ relative to WT NOD counterparts, which ultimately afforded resistance to T1D, underpinning IL-2 as a crucial regulator of $T_{reg}$ cells (chapter III). IL-2 deficiency early in the disease course of WT NOD or BDC2.5 mice is paradoxical with the outcome, which is a progressive inflammation and eventual T1D development. This phenomenon may be explained by exhausted memory T cell responses, defective APC presentation, competition for IL-2 by activated $T_{eff}$ cells or cleavage of CD25 by matrix metalloproteases induced by local inflammation, impairing the positive feedback loop between CD25 expression and IL-2 secretion [341]. Earlier work demonstrated that the constitutive expression of IL-2 by β-islets in a transgenic mouse model resulted in an accelerated form of diabetes in an antigen-independent fashion [343]. This demonstrates that IL-2 is such a potent inflammatory mediator that, in large enough doses, can bypass the necessity to activate β-islet specific autoreactive$T_{eff}$ cells to induce the destruction of the insulin-producing islets [343]. This old study highlights the importance of tight regulation of IL-2 signalling such that $T_{reg}$ cell fitness requirements are met and $T_{eff}$ cell activation can be dampened. Interestingly, similar observations were made in a murine model of systemic lupus, whereby diminished $T_{eff}$ cell derived IL-2 production impacted $T_{reg}$ cell fitness, provoked a $T_{reg}/T_{eff}$ cell imbalance and in turn strongly accelerated disease progression, corrected by IL-2 therapy [340]. Thus, IL-2

shortage seems to be one of the critical underlying factors in the attenuated competitive fitness of $T_{reg}$ cells observed in autoimmune disorders.

Furthermore, we hypothesized that an IL-2 shortage in pancreatic islets was an initial insult that impacted the functional potency and stability of $T_{reg}$ cells. Since ICOS blockade in BDC2.5 mice results in a marked decrease in $T_{reg}$ cells [360] and ICOS expression is IL-2 dependent [357], we wondered whether ICOS is an active participant in $T_{reg}$ cell functions. We showed that islet-reactive $Foxp3^+$ $T_{reg}$ cells predominantly express ICOS in the antigen-bearing tissue, unlike $Foxp3^-$ $T_{eff}$ cells or $Foxp3^+$ $T_{reg}$ cells in draining pancLN. Moreover, ICOS expression on $Foxp3^+$ $T_{reg}$ cells endows them with a greater suppressive function and the specific capacity to secrete IL-10, an important modulatory cytokine in T1D. Although a substantial frequency of expanding $CD25^+ICOS^+Foxp3^+$ $T_{reg}$ cells was found at the time of insulitis, there was a nearly two-fold decline of this regulatory population at a more advanced stage of insulitis in the prediabetic BDC2.5 mice. Consistently, $T_{reg}$ cells from diabetic mice sustain a drastic loss in ICOS signals and in turn their ability to differentiate into IL-10 producing cells is dampened. Prophylactic recombinant IL-2 therapy or *Il2* allelic variation (*Idd3*$^{B6}$ genetic interval) bolstered the expansion of $CD25^+ICOS^+Foxp3^+$ $T_{reg}$ cells *in vivo*, establishing the IL-2/ICOS signalling pathways as critical components in the functional stability of $Foxp3^+$ $T_{reg}$ cells in inflamed target organs. In accordance with this finding, we showed that ICOS promotes IL-2 production by $T_{eff}$ cells, as demonstrated by *in vitro* ICOS blockade studies. Thus, a positive feedback loop initiated by $T_{eff}$ cell-derived IL-2 may potentiate $T_{reg}$ cell functions through the upregulation of ICOS and CD25, which in turn enables $T_{reg}$ cells to shut down the autoreactive $T_{eff}$ cell pool.

Although our data shows that ICOS plays an important role in $T_{reg}$ cell stability and effector functions, the mechanism of action remains to be elucidated. Does ICOS exert its functions in order to enhance survival, fitness or to program a specific immunomodulatory cytokine profile? In order to determine whether ICOS confers a competitive advantage to $T_{reg}$ cells, bone marrow chimera reconstitution experiments may be performed. A mixture of Thy1.1$^{+}$ wild-type NOD$^{gfp}$ and Thy1.2$^{+}$ NOD.ICOS$^{-/-}$ bone marrow cells may be transferred to irradiated syngeneic recipients in order to evaluate the development, survival and competitive fitness of $T_{reg}$ cells. WT Foxp3$^{+}$ $T_{reg}$ cells may be tracked based on GFP expression and Thy1.1 congenic markerdistinguishing them from Thy1.2$^{+}$ ICOS$^{-/-}$ Foxp3$^{+}$ $T_{reg}$ cells.

Our results cannot exclude the possibility that ICOS participates in the lineage commitment and development of Foxp3$^{+}$ $T_{reg}$ cells. ICOS expression during thymic development may imprint the ICOS$^{+}$ $T_{reg}$ cell subset with the capacity to dampen the autoimmune responses in inflamed target organs. Thus, defective thymic selection may result in an increased deletion of this subset of $T_{reg}$ cells, resulting in an imbalance in the $T_{reg}/T_{eff}$ cell ratio, leading to T1D. In order to investigate the impact of ICOS on development of $T_{reg}$ cells, we compared the phenotype of thymic $T_{reg}$ cells in NOD.ICOS$^{-/-}$ mice relative to their WT NOD counterparts. The thymus of NOD.ICOS$^{-/-}$ mice was normal in size, and the thymocytes did not exhibit any abnormalities in their differentiation, based on the frequencies of CD4$^{+}$CD8$^{-}$, CD4$^{-}$CD8$^{+}$ SP and CD4$^{+}$CD8$^{+}$ double-positive (DP) thymocytes (unpublished findings). As in normal mice, Foxp3$^{+}$ $T_{reg}$ cells were mainly found in the CD4 SP thymocyte gate and the expression of CD4$^{+}$Foxp3$^{+}$ SP thymocytes was comparable in adult NOD and NOD.ICOS$^{-/-}$ mice

(unpublished observations). These preliminary results excluded a role for ICOS in the overall thymic development of $T_{reg}$ cells. However, the role of ICOS on development of β-islet specific $T_{reg}$ cells remains to be elucidated. In order to elucidate the role of ICOS in positive selection of $T_{reg}$ cells specific for pancreatic islet antigens, which will contribute to self-tolerance in the periphery, the CDR3 region of the TCR of thymic and pancreatic $T_{reg}$ cells may be sequenced and compared between NOD and NOD.ICOS$^{-/-}$ mice. Conversely, ICOS may promote the negative selection of self-reactive $T_{eff}$ cells and in this manner contribute to self-tolerance. Therefore, it would be worthwhile to also sequence the CDR3 region of $T_{eff}$ cells from both sites in both genotypes.

The ICOS$^{-/-}$ NOD mouse model represents an invaluable tool for the dissection of the role of ICOS in $T_{eff}$ and $T_{reg}$ cell functions in the context of spontaneous diabetes, although it is not without its shortcomings. A recent study reported that NOD.ICOS$^{-/-}$ mice are protected from T1D, due to improper activation of $T_{eff}$ cells and thus absence of pancreatic inflammation, suggesting that ICOS participates in early events of $T_{eff}$ cell priming [358]. This, in theory, precludes the examination of the impact of ICOS signalling on $T_{reg}$ cell suppressive activity, which is activated only in the context of inflammatory stimuli. However, our preliminary data demonstrated that polyclonal *in vitro* stimulation resulted in robust activation of ICOS$^{-/-}$ $T_{eff}$ cells similar to that of WT $T_{eff}$ cells (unpublished observations). In accordance with this observation, the pancreatic infiltrate did not differ between the two genotypes, suggesting that ICOS$^{-/-}$ $T_{eff}$ cells are activated in priming sites and migrate to the antigen-bearing tissue as efficiently as WT $T_{eff}$ cells (unpublished observations).

In order to circumvent the shortcomings of the NOD.ICOS$^{-/-}$ mouse model, ICOS conditional knock-out mice may be generated using the Cre-Lox system. This will enable the dissection of the temporal requirements of ICOS-mediated costimulation on $T_{reg}$ cell and $T_{eff}$ cell functions. Moreover, temporal genetic deletion of ICOS in T cells at important checkpoints during disease progression (insulitis, progressive insulitis, overt diabetes) will shed light on whether ICOS is dispensable in the priming, effector or suppression phases of the autoimmune response. Accordingly, in order to evaluate whether the ICOS expression can be potentiated and in turn reverse the temporal waning of $T_{reg}$ cell functions, administration of a monoclonal agonistic ICOS-specific antibody WT mice may be undertaken.

As I mentioned in chapter I, ICOS signalling promotes the induction of IL-10 secretion by CD4$^{+}$ T cells [361]. IL-10 has an established role in dampening the inflammatory responses to intestinal flora and also limiting immune-mediated pathology during pathogen clearance, underpinning the importance of this cytokine in immune responses [284]. This is best exemplified in IL-10$^{-/-}$ mice, which develop spontaneous IBD [403]. Specific genetic ablation of IL-10 in Foxp3$^{+}$ $T_{reg}$ cells or CD4$^{+}$CD25$^{+}$ $T_{reg}$ cells from IL-10$^{-/-}$ mice were incapable of preventing IBD [282], although a recent finding states the contrary [299]. CD4$^{+}$CD25$^{+}$ $T_{reg}$ cell-derived IL-10 is also important for the persistence (leishmaniasis) [404] and protection of tissue-damaging effects of polarized immune responses (schistosomiasis) [405] of parasitic infections. Therefore, the mechanism of IL-10-mediated immunosuppression depends on the inflammatory context. In most animal models of autoimmunity such as CIA, EAE and IBD, the

absence of IL-10 exacerbates disease, demonstrating the power of this immunosuppressive cytokine in restraining exaggerated immune responses [406].

The immunodulatory role of IL-10 in diabetogenesis is controversial, and may reflect the context-dependent nature of IL-10 production in disease onset and protection. Early exposure to IL-10 accelerates diseases [367, 407] whereas systemic exposure during the effector phase of diabetes inhibits disease [364, 365]. The genetic ablation of IL-10 on the NOD background did not alter the kinetics of diabetogenesis [109]. The administration of IL-10 mAb in NOD mice prevented insulitis [408]. Thus, the impact of IL-10 is context-dependent and complex in nature.

In contrast to $ICOS^-$ $Foxp3^+$ $T_{reg}$ cells, $ICOS^+$ $T_{reg}$ cells secrete IL-10, linking ICOS costimulation to IL-10 production. However, we did not address which cell types are targeted by this immunomodulatory cytokine. A recent study suggested that $T_{reg}$ cell-derived IL-10 may be used in an autocrine fashion to stabilize Foxp3 expression [299]. In light of these findings, it would be interesting to examine whether ICOS signalling mediates IL-10 secretion by $T_{reg}$ cells in order to stabilize their expression of Foxp3 in an autocrine fashion. Using a Foxp3 lineage reporter system, Zhou *et al.* showed that there is spontaneous loss of Foxp3 expression *in vivo* in NOD diabetic mice [300]. In our study, the age-related waning of actively expanding $ICOS^+$ $T_{reg}$ cells in the inflamed pancreas may be due to the instability of Foxp3 as a consequence of inadequate IL-10 secretion. We showed that pancreatic $Foxp3^+$ $T_{reg}$ cells sustained a drastic loss in ICOS MFI and IL-10 secretion in diabetic relative to T1D-protected mice, suggesting that ICOS mediates the IL-10-dependent stabilization of Foxp3 expression

(chapter IV). Conversely, ICOS-driven IL-10 production may contribute to the maintenance of suppressive function of $T_{reg}$ cells. The decline in the frequency of the cycling $ICOS^+$ $T_{reg}$ cell frequencies may result in a prominent drop in IL-10 levels, ultimately leading to defective Treg cell suppressive activity and thus T1D development. In order to distinguish between these two possibilities, Foxp3 expression may be tracked by taking advantage of the $Foxp3^{gfp}$ reporter system. As a first step, Foxp3 expression of $ICOS^+$ or $ICOS^-$ $T_{reg}$ cells, which cannot respond to IL-10 signalling ($IL\text{-}10^{-/-}$ or $IL\text{-}10R^{-/-}$) may be compared to WT $ICOS^+$ or $ICOS^-$ $T_{reg}$ cells under homeostatic conditions or T1D induction using $Foxp3^-$ $T_{eff}$ cells. In order to exclude paracrine sources of IL-10 from non-T myeloid cells [299], T cell transfer studies in $IL\text{-}10^{-/-}$ recipients should be used. Therefore, if IL-10 is essential for the stabilization of Foxp3 expression in $T_{reg}$ cells, we expect to observe an attenuation of Foxp3 expression in $IL\text{-}10^{-/-}$ or $IL\text{-}10R^{-/-}$ $T_{reg}$ cells, irrespective of ICOS expression relative to WT $ICOS^+$ $T_{reg}$ cells under homeostatic conditions or T1D induction. As a result, loss of Foxp3 expression in $T_{reg}$ cells incapable of producing or responding to IL-10 would precipitate diabetes. Conversely, if $IL\text{-}10^{-/-}$ or $IL\text{-}10R^{-/-}$ $T_{reg}$ cells maintain similar Foxp3 expression relative to WT $T_{reg}$ cells, but are still incapable of restraining $T_{eff}$ cells, we can infer that IL-10 secretion promotes $T_{reg}$ cell suppression and not Foxp3 expression. This would be a novel mechanism of $T_{reg}$ cell suppression in the NOD mouse model.

Since the functional contribution of DC in $T_{reg}$ cell-mediated resistance to organ-specific autoimmunity was ill-defined, we sought to investigate this aspect of the regulatory arm of the adaptive immune response. Our observation that tissue-resident $CD11c^+$ DC, in contrast to their counterparts from distal and draining sites, preferentially

upregulated ICOS-L suggested that the pancreatic inflammatory milieu promotes DC-specific ICOS-L expression. In addition, the fact that $T_{reg}$ cells predominantly expressed ICOS within the pancreas suggested a possible, reciprocal conditioning between $T_{reg}$ cells and DC. In order to determine the impact of ICOS/ICOS-L on DC functions, it would be interesting to characterize the phenotype and the priming capacity of pancLN and intra-islet resident $CD11c^+$ DC of $ICOS^{-/-}$ and ICOS-$L^{-/-}$ NOD mice. Our preliminary findings demonstrated that $ICOS^{-/-}$ APCs from distal sites were as efficient at expanding T cells as WT APCs, suggesting that the absence of ICOS expression on T cells does not impact the priming potency of APC. The inflammatory factors that modulate ICOS and ICOS-L expression on $T_{reg}$ cells and DC respectively are currently ill-defined and would represent an important area for future investigation. Also, the cytokines induced by ICOS-L expression are also unknown and merit further studies.

We also made the interesting finding that ICOS-L expression on intra-islet DC declines with age, which coincides with loss of ICOS expression on $T_{reg}$ cells and T1D progression. In light of our findings, it stands to reason that if $T_{reg}$ cell/DC cross-talk is disrupted via diminished ICOS/ICOS-L signalling, DC may become less amenable to $T_{reg}$ cell suppression and thus more potent primers of $T_{eff}$ cells, ultimately upsetting the delicate $T_{eff}$ cell/$T_{reg}$ cell balance and resulting in T1D. Conversely, ICOS-L deficiency in DC may render them less efficient at maintaining $T_{reg}$ cell homeostasis. In order to evaluate the impact of ICOS-L on T1D progression, we propose to determine the onset, incidence and severity of ICOS-$L^{-/-}$ NOD mice. Accordingly, the insulitic lesion and phenotype of WT and ICOS-$L^{-/-}$ mice may be compared, and ICOS-$L^{-/-}$ DC from the pancreatic sites may be used in functional assays in order to gauge the impact of ICOS-

L in $T_{reg}$ cell-mediated conditioning of DC or their capacity to prime and maintain $T_{reg}$ cell functions *in vivo*.

In order to extend our findings concerning the impact of DC defects on T1D progression, we sought to characterize the phenotype and functions of DC in the context of T1D susceptibility and protection. We hypothesized that the protective *Il2* allelic variants conferred T1D protection by supporting $T_{reg}$ cell function and expansion *in vivo* by enhancing the tolerogenic properties of DC. Our data demonstrated that the *Idd3*$^{B6}$ locus was operative in CD11c$^{+}$ DC, in turn mediating the preferential enhancement of $T_{reg}$ cell expansion *in vitro* and *in vivo*. Our findings show that the costimulatory load of DC was drastically different only within the pancreatic lesion of NOD mice relative to the T1D-resistant counterparts NOD.B6 *Idd3*, as illustrated by decreased CD80 and CD86 levels and concomitant elevated CD40 levels. Although these findings are merely correlative with the progression to T1D, one can speculate that these differences represent one among many defects in the NOD that precipitates T1D. However, in order to draw decisive conclusions concerning the role of each individual costimulatory molecule in T1D development, functional studies must accompany the phenotypic data reported in chapter V.

The issue of recruitment of $T_{reg}$ cells and $T_{eff}$ cells to the target organ from the pancLN where priming takes place, has not been addressed in this project, although it is of utmost interest. We have not explored the chemokine receptor profile and the respective ligands of the active participants of the inflammatory environment. Interestingly, in a TCR transgenic model of diabetes, autoreactive T cells under $T_{reg}$ cell suppression failed to upregulate IFN-$\gamma$-dependent CXCR3 and in turn infiltrate the

pancreas [297]. Therefore, $T_{reg}$ cells in an indirect manner, control migration of autoreactive T cells into the antigen-bearing tissue by blocking Th1 differentiation. Interestingly, $T_{eff}$ cells of $ICOS^{-/-}$ NOD mice exhibit impaired differentiation as per their capacity to produce IFN-γ [358]. Since IFN-γ induces the expression of CXCR3 and $ICOS^{-/-}$ $T_{eff}$ cells are impaired in their capacity to secrete IFN-γ, it would be interesting to examine the impact of this defect on the CXCR3 profile of $T_{eff}$ cells and $T_{reg}$ cells of these animals. Also, we have not examined the players involved in the chemoattraction of these immune mediators in pancreatic sites. Preliminary data from our laboratory demonstrates that $T_{reg}$ cells selectively express CXCR3 in response to the ligand, CXCL10, which β-islet cells have been shown to express in response to inflammation [409]. Interestingly, T1D progression correlates with a decline in $CXCR3^{+}$ $T_{reg}$ cells, suggesting that survival/functions of this subset wane with age (our unpublished observations). The fact that $T_{eff}$ cells and $T_{reg}$ cells present a distinct chemokine receptor profile in T1D-susceptible mice suggests that the inflammatory environment promotes an address code, which favours the autoreactive pool.

Our studies have shown strong evidence that functional and phenotypic differences within the $T_{reg}$ cell compartment of T1D-resistant versus susceptible NOD mice occur directly within the target organ, and not in other peripheral tissues including draining pancLN (chapter III). However, our conclusions have important ramifications for the analysis of $T_{reg}$ cell function in human autoimmune disorders. Thus, the experimental dissection of human autoimmune disorders is rather limited for technical and ethical reasons. Indeed, for most autoimmune diseases such as T1D and MS, we are limited to peripheral blood, which gives a very limited scope for understanding the

breakdown in tolerance. In contrast to T1D and MS, samples can be extracted from the tissue undergoing autoimmune attack (synovial fluid) from patients suffering from rheumatoid arthritis. Interestingly, collagen-specific T cells can only be detected in the synovial fluid, not the blood [410]. An enrichment of $T_{reg}$ cells has been documented in the synovial fluid of RA patients, irrespective of disease duration, severity or type of anti-inflammatory drug treatment [410]. However, in the peripheral blood compartment, no difference, increase or decrease in the frequency of $T_{reg}$ cells relative to control healthy controls have been reported, illustrating major differences between the peripheral blood compartment and tissue.

Methods have been invented to circumvent this issue and recapitulate the human immune system to determine what occurs directly within the target organ undergoing autoimmune attack [411]. Thus, the humanized mouse model was created, which consists of the substitution of murine genes by their human counterparts to better reproduce a human milieu [411]. The most widely used approach was to introduce the HLA class II transgenes onto a murine genetic background [411]. These humanized mouse models have been established for MS, RA, and T1D, since HLA is a susceptibility gene for all three organ-specific autoimmune disorders [411]. However, the HLA transgenic humanized mouse must be crossed onto susceptible backgrounds such as the NOD [411]. Humanized mouse models are mostly used to test therapeutic interventions. However, the autoimmune reaction obviously cannot be mimicked completely using this approach due to many species differences [412, 413]. In recent years, this problem has been circumvented by the reconstitution of immunodeficient mice with human hematopoietic stem cells (HSC) to endow them with a complete

human immune system [412, 413]. The NOD has been successfully reconstituted with human HSC, as they generate human lymphoid and myeloid lineages and exhibit normal T and B cell development [412, 413].

This project may provide insight into the cellular basis of T1D, which can be transposed in human studies with the ultimate goal of developing novel approaches to potentiate $T_{reg}$ cell activity and prevent the onset of autoimmunity. Overall, our work provides a contribution as to the mechanism of $T_{reg}$ cell suppression in the context of autoimmunity and may provide novel therapeutic avenues. In our study we proved that $T_{reg}$ cell homeostasis and more importantly metabolic fitness have an absolute requirement for IL-2. Since IL-2 is such an important factor in $T_{reg}$ cell fitness, it was suggested that IL-2 therapy would be a feasible avenue in the reversal of disease by potentiating $T_{reg}$ cell functions. Low-dose IL-2 administration has proven to protect NOD mice by enhancing the frequency of $T_{reg}$ cells [305]. An IL-2 regimen has reached clinical trials in diabetes patients [414]. However, IL-2 is also used as treatment in cancer patients to promote immunity, suggesting that IL-2 therapy must be combined with $T_{eff}$ cell depleting treatments to prevent potential disease exacerbation [401]. In light of our findings, agonistic ICOS-specific antibody treatment to boost the potency of $T_{reg}$ cell-mediated immunosuppression may also be a feasible therapeutic tool. However, the use of agonists as therapeutic agents has proven to be dangerous in past clinical trials. The best-documented case came from the disastrous outcome of CD28 superagonist treatment in phase I of clinical trials [415]. This CD28 superagonist was developed for use in humans to treat autoimmunity, based on mouse studies showing specific potentiation of $T_{reg}$ cell functions by CD28 superagonist [415]. A single injection

of the CD28 mAb resulted in a systemic cytokine storm in volunteers, likely due to the ability of the CD28 superagonist to activate T cells [415]. The trial was interrupted immediately [415]. Although there is a certain danger to transpose results from murine data to human clinical trials, manipulating $T_{reg}$ cells as a therapeutic tool holds promise. We hope that our study moved this possibility one small step forward.

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