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Function of the immunoregulatory CD4-CD8- T cells in the context of autoimmune diabetes

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Cette thèse intitulée:

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RÉSUMÉ

La tolérance immunitaire dépend de la distinction entre le soi et le non soi par le système immunitaire. Un bris dans la tolérance immunitaire mène à l'auto-immunité, qui peut provoquer la destruction des organes, des glandes, des articulations ou du système nerveux central. Le diabète auto-immun, également connu sous le nom diabète juvénile et diabète de type 1, résulte d'une attaque auto-immune sur les cellules β pancréatiques sécrétrices d'insuline, localisées au niveau des îlots de Langerhans du pancréas. Bien que le diabète auto-immun soit traitable par une combinaison d'injections quotidiennes d'insuline d'origine exogène, de régime et d'exercices, beaucoup de complications chroniques peuvent se manifester chez les patients, y compris, mais non limitées à, la cécité, les maladies cardiovasculaires, l'insuffisance rénale et l'amputation. En raison des nombreuses complications liées au diabète auto-immun à long terme, la recherche continue afin de mieux comprendre tous les facteurs impliqués dans la progression de la maladie dans le but de développer de nouvelles thérapies qui empêcheront, renverseront et/ou traiteront cette maladie.

Un rôle primordial dans la génération et l'entretien de la tolérance immunitaire a été attribué au nombre et à la fonction des sous-populations de cellules régulatrices. Une de ces populations est constituée de cellules T CD4-CD8- (double négatives, DN), qui ont été étudiées chez la souris et l'humain pour leur contribution à la tolérance périphérique, à la prévention des maladies et pour leur potentiel associé à la thérapie cellulaire. En effet, les cellules de T DN sont d'intérêt thérapeutique parce qu'elles montrent un potentiel immunorégulateur antigène-spécifique dans divers cadres expérimentaux, y compris la prévention du diabète auto-immun. D'ailleurs, en utilisant un système transgénique, nous avons démontré que les souris prédisposées au diabète auto-immun présentent peu de cellules T DN, et que ce phénotype contribue à la susceptibilité au diabète auto-immun. En outre, un transfert des cellules T DN est suffisant pour empêcher la progression vers le diabète chez les souris prédisposées au diabète auto-immun. Ces résultats suggèrent que les cellules T DN

puissent présenter un intérêt thérapeutique pour les patients diabétiques. Cependant, nous devons d'abord valider ces résultats en utilisant un modèle non-transgénique, qui est plus physiologiquement comparable à l'humain.

L'objectif principal de cette thèse est de définir la fonction immunorégulatrice des cellules T DN, ainsi que le potentiel thérapeutique de celles-ci dans la prévention du diabète auto-immun chez un modèle non-transgénique. Dans cette thèse, on démontre que les souris résistantes au diabète auto-immun présentent une proportion et nombre absolu plus élevés de cellules T DN non-transgéniques, lorsque comparées aux souris susceptibles. Cela confirme une association entre le faible nombre de cellules T DN et la susceptibilité à la maladie. On observe que les cellules T DN éliminent les cellules B activées in vitro par une voie dépendante de la voie perforine et granzyme, où la fonction des cellules T DN est équivalente entre les souris résistantes et prédisposées au diabète auto-immun. Ces résultats confirment que l'association au diabète auto-immun est due à une insuffisance en terme du nombre de cellules T DN, plutôt qu'à une déficience fonctionnelle. On démontre que les cellules T DN non-transgéniques éliminent des cellules B chargées avec des antigènes d'îlots, mais pas des cellules B chargées avec un antigène non reconnu, in vitro. Par ailleurs, on établit que le transfert des cellules T DN activées peut empêcher le développement du diabète auto-immun dans un modèle de souris non-transgénique. De plus, nous observons que les cellules T DN migrent aux îlots pancréatiques, et subissent une activation et une prolifération préférentielles au niveau des ganglions pancréatiques. D'ailleurs, le transfert des cellules T DN entraîne une diminution d'auto-anticorps spécifiques de l'insuline et de cellules B de centres germinatifs directement dans les îlots, ce qui corrèle avec les résultats décrits ci-dessus. Les résultats présentés dans cette thèse permettent de démontrer la fonction des cellules T DN in vitro et in vivo, ainsi que leur potentiel lié à la thérapie cellulaire pour le diabète auto-immun.

Mots-clés : Cellules T double négatives, diabète auto-immun, immunorégulation, souris transgénique, souris non-transgénique, antigène-spécifique.

ABSTRACT

Immune tolerance is dependent on the immune system discriminating between self and non-self. A break in immune tolerance results in autoimmunity, which can lead to the destruction of healthy organs, glands, joints or the central nervous system. Any disease that results from such an aberrant immune response is termed an autoimmune disease. Autoimmune diabetes, which is also referred to as juvenile diabetes and type 1 diabetes, results from an autoimmune attack on the insulin-producing β cells located within the islets of Langerhans of the pancreas. Although autoimmune diabetes is treatable through a combination of insulin therapy, diet and exercise, many chronic complications may arise in patients, including, but not limited to, blindness, cardiovascular disease, kidney failure and amputation. Due to the many complications associated with long-term autoimmune diabetes, research continues to better understand all the factors implicated in disease progression in order to develop new therapies that will prevent, reverse and/or cure this disease.

A prominent role in the generation and maintenance of immune tolerance has been attributed to the number and function of regulatory cell subsets. One of these regulatory cell populations, namely CD4-CD8- (double negative, DN) T cells, have been studied in both mice and humans for their contribution to peripheral tolerance, disease prevention and their potential for use in cellular therapy. DN T cells are of particular therapeutic interest because they exhibit an antigen-specific immunoregulatory potential in various experimental settings, including the prevention of autoimmune diabetes. Indeed, using a transgenic system, we have shown that autoimmune diabetes-prone mice carry fewer DN T cells and that this phenotype contributes to autoimmune diabetes susceptibility, where a single transfer of DN T cells is sufficient to prevent diabetes progression in otherwise autoimmune diabetes-prone mice. These results suggest that DN T cells may be of therapeutic interest for diabetic patients. However, we must first validate these results using a non-transgenic setting, which is more physiologically relevant to humans.

The main objective of this thesis is to determine the immunoregulatory function of the DN T cells as well as the therapeutic potential of these cells in the prevention of autoimmune diabetes in the non-transgenic setting. Here, we show that diabetes-resistant mice present with a higher proportion and cell number of DN T cells than diabetes-susceptible mice in the non-transgenic setting, which associates a deficiency in DN T cell number with disease susceptibility. We determine that DN T cells eliminate activated B cells in vitro via a perforin/granzyme-dependent pathway, where the function of DN T cells is equal between the diabetes-resistant and -susceptible mice, demonstrating that the association to autoimmune diabetes is due to a deficiency in DN T cell number rather than function. Interestingly, we show that non-transgenic DN T cells eliminate B cells loaded with islet antigen, but not B cells loaded with an irrelevant antigen, in vitro. Importantly, we establish that the transfer of activated DN T cells could prevent autoimmune diabetes development in the non-transgenic setting. Interestingly, we reveal that DN T cells migrate to the pancreatic islets and undergo preferential activation and proliferation within the pancreatic lymph nodes. Moreover, the transfer of DN T cells results in a decrease in both germinal center B cells directly within the pancreatic islets as well serum insulin autoantibody levels, which correlates with the aforementioned findings. Altogether, the results presented in this thesis have allowed us to enhance our understanding of the function of DN T cells both in vitro and in vivo as well as demonstrate the therapeutic potential for DN T cells as a novel cellular therapeutic for autoimmune diabetes.

Keywords : Double negative T cells, autoimmune diabetes, immunoregulation, transgenic mouse, non-transgenic mouse, antigen-specific.

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LIST OF ABBREVIATIONS

^{51}Cr: chromium-51

αGalCer: alpha-galactosylceramide

AHCT: allogeneic hematopoietic cell transplantation

ALPS: autoimmune lymphoproliferative syndrome

AML: acute myeloid leukemia

APC: antigen-presenting cell

B10: C57BL/10SnJ

B6: C57BL/6

CBV: coxsackie B virus

CFSE: carboxyfluorescein diacetate succinimidyl ester

C-peptide: connecting peptide

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

CTV: cell trace violet

CXCR5: chemokine (C-X-C motif) receptor 5

DC: dendritic cell

DLI: donor leukocyte infusion

DN: double negative, $CD4^-CD8^-$

DP: double positive, $CD4^+CD8^+$

EGTA: ethylene glycol tetraacetic acid

ELISA: enzyme-linked immunosorbent assay

EMCV: encephalomyocarditis virus

Fas: apoptosis stimulating fragment

FasL: apoptosis stimulating fragment ligand

FcRγ: Fc gamma receptor

FLT-3L: Fms-like tyrosine kinase 3 ligand

FOXP3: forkhead box P3

GAD65: 65-kDa isoform of glutamic acid decarboxylase

G-CSF: granulocyte colony-stimulating factor

gp: glycoprotein

GVHD: Graft-vs-host disease

HbA_{1c}: haemoglobin A_{1c}

HEL: hen egg lysozyme

HLA: human leukocyte antigen

IA-2: insulinoma-associated-2

IAA: insulin autoantibody

Idd: insulin-dependent diabetes

IFNγ: interferon gamma

Ig: immunoglobulin

iGb3: isoglobotrihexosylceramide

IL: interleukin

IL-10: interleukin-10

IL-10R: interleukin 10 receptor

IL2RA: interleukin 2 receptor, alpha

iNKT: invariant NKT

INS: insulin

IPEX: immunodysregulation, polyendocrinopathy, enteropathy, X-linked

LCMV: lymphocytic choriomeningitis virus

lpr: lymphoproliferation

LPS: lipopolysaccharide

MHC: major histocompatibility complex

MLR: mixed-lymphocyte reaction

NK: natural killer

NKT: natural killer-like

NOD: non-obese diabetic

OVA: ovalbumin

PLN: pancreatic lymph node

PMA: phorbol 12-myristate 13-acetate

PP: pancreatic polypeptide

PPI: preproinsulin

PTPN22: protein tyrosine phosphatase, non-receptor type 22

Rag: recombination activating gene

RIP: rat insulin promoter

SCID: severe combined immunodeficient

SD: standard of deviation

SIRP: signal regulatory protein

SLE: systemic lupus erythematosus

TCR: T cell receptor

TGFβ: transforming growth factor beta

Th2: T helper 2

TNF-α: tumor necrosis factor alpha

Treg: regulatory T cells

ZnT8: zinc transporter 8

β -GalCer : β-galactosylceramide

β-GlcCer: β-D-glucopyranosylceramide

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CHAPTER 1: INTRODUCTION

1.1 Autoimmunity

A low level of autoreactivity is physiologic and crucial to normal immune function, where autoantigens helps to form the repertoire of mature lymphocytes and promote the survival of naive T cells and B cells in the periphery[1]. Contrary to this physiological low level of autoreactivity, *autoimmunity* results from the failure of the immune system to discriminate self from non-self, leading to an immune response against cells and tissues. Any disease that results from such an aberrant immune response is termed an autoimmune disease. Autoimmune diseases are typically defined as either systemic, in which the autoimmune reaction occurs against various organs, tissues and cells of the body, such is the case for systemic lupus erythematosus, or organ-specific, in which the autoimmune reaction targets a single organ as the name implies, as in the case of autoimmune diabetes.

1.2 Autoimmune diabetes

Autoimmune diabetes, which is also referred to as juvenile diabetes and type 1 diabetes, results from the destruction of the insulin-producing β cells located within the islets of Langerhans of the pancreas.

1.2.1 The pancreas

The pancreas is made up of exocrine and endocrine tissues (**Figure 1**). The exocrine is made up of cellular clusters called acinus, which consist of acinar cells that secrete digestive enzymes into the intestine, including proteases, amylases, lipases and nucleases[2]. The endocrine cells, which secrete hormones, are contained within the islets of Langerhans, which are compact spheroidal clusters embedded in the exocrine tissue[2]. There are four major cell types of endocrine cells, namely α, β, δ and PP cells, where the β cells make up approximately 70% of the islet mass. Together the α and β cells regulate blood glucose levels, where the α cells secrete glucagon, which promotes the conversion of stored glycogen in the liver into glucose that can be released into the bloodstream, and the β cells secrete insulin, which is necessary for glucose uptake from the blood into cells. The δ cells secrete somatostatin, and the PP cells secrete pancreatic polypeptide (PP), both of which are implicated in modulating the secretory properties of the other cell types[2].

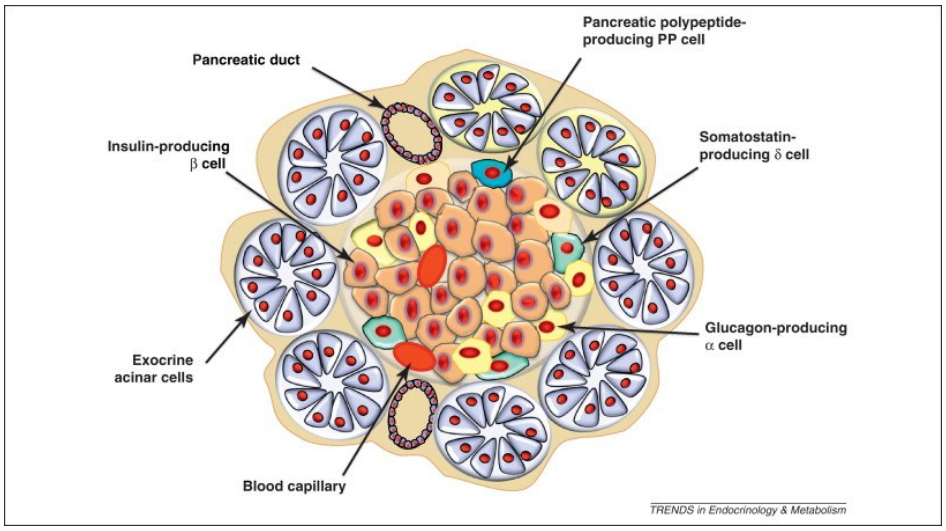

Figure 1. Schematic representation of islet and pancreas cell types. Adapted from [3].

1.2.2 Insulin

During insulin synthesis, preproinsulin is first secreted from the β cells of the pancreas with an A-chain, a connecting peptide (C-peptide), a B-chain, and a signal sequence, which, once cleaved, gives rise to proinsulin. Subsequently, the C-peptide is removed, leaving the A-chain and B-chain that constitute the mature insulin molecule. The mature insulin is stored in the β cells of the islets of Langerhans and its release is controlled by the level of glucose within the blood. Following insulin release, insulin binds to its receptor on the cell surface[2]. The signal transduction subsequently induces various physiological effects, including but not limited to the influx of glucose into cells, glycogen synthesis and lipid synthesis. As a result, insulin is a vital requirement for life[2].

Autoimmune diabetes was considered a fatal disease until the early 1920s when doctors Banting and Best, who were positioned in Toronto, demonstrated that the injection of concentrated pancreatic extracts, namely insulin, from dogs into human diabetic patients could maintain blood glucose homeostasis[4]. In 1923, this discovery would result in Dr. Banting being awarded the Nobel Prize at the age of 32[5]. Today, diabetic patients use human biosynthetic insulin to treat their disease.

1.2.3 Clinical signs and diagnosis

The classical symptoms associated with autoimmune diabetes include polyuria (excessive production of urine), polydipsia (excessive thirst), glucosuria (excretion of sugar in the urine) and tiredness, which are all connected to hyperglycemia, or high blood glucose/sugar levels. The average plasma glucose concentration over a 3-4 month period can be established by measuring haemoglobin A_{1c} (HbA_{1c}) levels, where HbA_{1c} is the glycated haemoglobin which forms due to the haemoglobin's exposure to plasma glucose.

At the time of the diagnosis, the mass of β cells is estimated to represent 10 to 33% of its initial value based on a glucose tolerance test that measures your blood glucose levels following glucose stimulation, which is insufficient to provide the insulin contribution necessary following glucose ingestion[6]. The insulin response continues to decline following diagnosis, where two years after diagnosis, the insulin response now represents 28 ± 8.4% of the response at diagnosis[7]. Indeed, there is a significant inverse relationship between the insulin secretory response and glucose control, which is reflected by the HbA_{1c} levels[7]. Therefore, increased HbA_{1c} levels are indicative of a decrease in the insulin secretory response.

The insulin secretory response following a glucose tolerance test assesses β cell function by measuring either insulin or C-peptide levels found in the plasma[6], where lower insulin levels would indicate a decrease in β cell function, thus the progression to diabetes. However, insulin is known to have a short half-life and an unpredictable hepatic extraction as well as peripheral clearance, making it difficult to assess insulin secretion based on insulin plasma levels following glucose stimulation[8]. On the contrary, C-peptide plasma levels are a more accurate measurement of insulin secretion as C-peptide, which is co-secreted with insulin from β cells at an equimolar concentration, does not undergo significant hepatic extraction, maintains a constant metabolic clearance rate and exhibits a longer half-life[6, 8]. Altogether, plasma C-peptide levels, and to a lesser extent plasma insulin levels, are

used to measure β cell function, where a decrease in β cell function is an indicator of autoimmune diabetes progression.

Another factor used to identify autoimmune diabetes development is the presence of autoantibodies to insulin (IAA) and other islet antigens, including autoantibodies targeting the 65-kDa isoform of glutamic acid decarboxylase, (GAD65) and autoantibodies targeting the phosphatase-related insulinoma-associated-2 (IA-2) molecule[9]. Moreover, the presence of these autoantibodies can also be used to predict the onset of disease[10, 11, 12, 13]. Indeed, IAA, GAD65 and IA-2 autoantibodies may arise before any signs of hyperglycemia, where the time interval between the emergence of autoantibodies and disease onset can range from a few months to several years[14]. Notably, not everyone who presents with autoantibodies progresses to autoimmune diabetes. However, as the number of autoantibody types increases, the risk of developing the disease is also amplified, where an individual who presents with three to four autoantibody types has a 60–100% risk of progressing to autoimmune diabetes[14]. Autoantibodies to the zinc transporter ZnT8, which is a member of the large cation efflux family, were recently discovered to be present in pre-diabetic and new-onset diabetic patients, where ZnT8 antibodies generally emerged later than IAA and GAD65 antibodies in pre-diabetic patients[11]. Indeed, the inclusion of ZnT8 autoantibody with IAA, GAD65 and IA2 autoantibody detection can increase the prognostic value to 98% rather than the previous 90%[11]. With the combination of these 4 autoantibodies, we are closer to detecting pre-diabetes in a general pediatric population with a family history of disease[11].

Altogether, the symptoms of autoimmune diabetes development include a decrease in insulin and C-peptide plasma levels that correlates with an increase in blood glucose levels and its associated symptoms as well as the potential presence of insulin and islet antigen autoantibodies.

1.2.4 Prevalence

Currently, there are 347 million people worldwide living with diabetes[i]. Of these diabetic patients, an estimated 5–10% constitute people living with autoimmune diabetes[15], which amounts to approximately 17-35 million people worldwide. In Canada alone, more than 300,000 people are living with this disease[ii]. Importantly, it is believed that the worldwide rate of diabetes incidence among children under the age of 14 will increase by three per cent annually[16]. Therefore, until a cure is discovered, this disease will continue to burden an increasing number of children and adults all over the world.

1.2.5 Risk factors

Importantly, both genetic and environmental factors have been implicated in disease susceptibility. Studies involving twins have helped to reveal the relative importance of environmental and genetic factors with regards to autoimmune diabetes. Indeed, in comparison to dizygous (non-identical) twins, who express a disease concordance of 6%–10%, monozygous twins (genetically identical) express an increased disease concordance of approximately 30-50%[17, 18] and a concordance of at least 66% for the presence of persistent islet cell autoantibodies and/or disease[17]. These results clearly demonstrate that genetic factors contribute to autoimmune diabetes development. However, as there is a significant fraction of monozygous twins that do not show disease concordance, it is also clear that environmental factors play an important role in disease development, as well. For instance, a study has revealed a higher prevalence of autoimmune diabetes ($\geq$ 20/ 100,000 per year) for many developed countries, such as the United Kingdom, Sweden, and Canada, whereas a very low prevalence (<1/100,000 per year) can be detected for various underdeveloped countries, such as Peru, Paraguay and Venezuela[19]. An additional indication of an environmental factor is the north–south gradient of disease frequency, where the incidence of disease decreases from north to south in the Northern Hemisphere[20]. Altogether, these results demonstrate that both genetic and

[i] WHO website
[ii] Canadian Diabetes Association. The prevalence and costs of diabetes. December 2009

environmental factors are implicated in autoimmune disease susceptibility. Although the genetic factors involved in disease development have been very compelling, the precise environmental causes have remained rather controversial.

1.2.5.1 Genetics

The major histocompatibility complex (MHC), also called human leukocyte antigen (HLA) in humans, remains the greatest genetic contributor to autoimmune diabetes susceptibility. Indeed, the genes located within the MHC region account for approximately 45% of the genetic component implicated in autoimmune diabetes[21]. The function of these genes is well known such that they are involved in the presentation of antigenic peptides to T lymphocytes. More specifically, MHC class I is expressed on all nucleated cells and presents peptides derived from cytosolic proteins, or extracellular proteins that have undergone cross-presentation, to $CD8^+$ T cells. On the other hand, the expression of MHC class II is limited to antigen-presenting cells (APCs) and presents peptides from the extracellular medium to $CD4^+$ T cells. The MHC class II region actually encodes the strongest genetic contribution to autoimmune diabetes[21]. More specifically, the HLA-DQ β chain in humans is directly associated with autoimmune diabetes susceptibility. Accordingly, the lack of the aspartic amino acid residue at position 57 of the β chain[22], which is responsible for the HLA-DQ conformational stability[23], affects antigen presentation. However, there exist HLA class II genes in humans that also protect from autoimmune diabetes, such as the DQB1*0602 haplotype, which confers dominant protection even in the presence of islet autoantibodies[24]. Overall, certain HLA haplotypes provide protection (ex. DQA1*0102, DQB1*0602, DRB1*1501) while others form susceptibility (ex. DQA1*0301, DQB1*0302, DRB1*0401) for disease development[25].

Multiple non-MHC genes also clearly contribute to disease susceptibility[26, 27, 28]. These include the insulin gene (*INS*) on chromosome 11p15[29], the cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) gene on chromosome 2q33[30], the protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) gene on chromosome 1p13[31],

and the interleukin 2 receptor, alpha (*IL2RA*, also known as *CD25*) gene on chromosome 10p15[28]. Moreover, a meta-analysis of 3 distinct genome-wide association studies uncovered over forty non-MHC candidate genes associated with autoimmune diabetes, which, in addition to those mentioned above, include CD69 and interleukin-10 (IL-10)[26, 28, 32]. Altogether, although the MHC, or HLA in humans, represents the greatest genetic contributor to autoimmune diabetes susceptibility, a multitude of non-MHC factors also play an important role in disease development.

1.2.5.2 Environment

Several environmental risk determinants, in addition to those mentioned above have been investigated for their potential role in autoimmune diabetes development, including early infant diet, vitamin D, social mixing and viral infections.

1.2.5.2.1 Dietary influence

As mentioned above, countries located further from the equator, such as the United Kingdom and Canada, express a higher incidence of autoimmune diabetes[20]. Due to the association between sun exposure and vitamin D synthesis, a lack of the daily requirement of vitamin D has also been implicated as a risk factor for disease development. Indeed, two studies have demonstrated that vitamin D supplementation during infancy was associated with a decreased risk of autoimmune diabetes[33, 34]. In addition to the role of vitamin D during infancy, various Finnish reports have suggested that breastfeeding during infancy and early exposure to cow's milk have a respective beneficial and negative outcome on autoimmune diabetes development in children[35, 36, 37]. In contrast, both the DAISY study performed in the United States of America[38] and the BABYDIAB study performed in Germany[39] have not documented any significant outcomes of the aforementioned factors. Therefore, a dietary influence on autoimmune diabetes development remains controversial.

1.2.5.2.2 Hygiene hypothesis

In 1989, David P. Strachan published an article demonstrating that both hay fever and eczema were less common in children from larger families, which would suggest a greater likelihood of exposure to more infectious agents through increased interactions with siblings[40]. This article was the first to corroborate the 'hygiene hypothesis', which suggests that a lack of early childhood exposure to infectious agents increases susceptibility to disease development by suppressing natural development of the immune system. The hygiene hypothesis can also be applied to autoimmune diabetes as studies have demonstrated that daycare attendance and social mixing during the first year of life, which facilitates the exposure to multiple infections, is associated with a significantly reduced risk of autoimmune diabetes[41, 42]. Moreover, both the number of children in the daycare setting and the number of sessions attended were significantly associated with protection from disease[41]. Altogether, these studies suggest that an increase in exposure to infections at an early age correlates with a reduced risk of developing autoimmune diabetes. Interestingly, an inverse association between the incidence of prototypical infectious diseases and the incidence of immune disorders has been observed between 1950 to 2000 in the United States[20]. Accordingly, the hygiene hypothesis may help to clarify why over the years, while standards of sanitation have continued to improve, disease incidence has continued to increase as well.

1.2.5.2.3 Viral infections

As explained by the hygiene hypothesis, exposure to viral infections may help to prevent the development of autoimmune diabetes. This hypothesis has been supported by studies using mouse models. Indeed, infecting young, diabetes-susceptible mice with either Mycobacteria avium [43] or lymphocytic choriomeningitis virus[44] results in autoimmune diabetes inhibition. The coxsackie B virus (CBV) is another example of this theory as the exposure to this virus can help to prevent autoimmune diabetes development in young diabetes-susceptible mice which have yet to undergo pancreatic islet infiltration[45]. However, in the presence of islet

infiltration, the CBV virus will not inhibit, but rather accelerate the onset of disease[45]. The association of CBV with autoimmune diabetes has been further suggested from studies involving diabetic patients. Indeed, CBV proteins could be found within the islets of patients[46, 47], while CBV RNA has been detected in the blood[48], of newly diagnosed autoimmune diabetes patients. In addition to CBV, a diabetogenic variant of the picornavirus encephalomyocarditis virus (EMCV) induces autoimmune diabetes in otherwise diabetes-resistant mice[49]. These studies demonstrate that viruses can either promote or inhibit autoimmune diabetes as well as suggest that the timing of viral exposure can have opposing consequences on disease development.

Although it remains controversial which environmental aspects play a clear role in autoimmune diabetes development, it is incontrovertible that both genetic and environmental factors contribute to disease susceptibility.

1.2.6 Pathogenesis

Autoimmune diabetes results from the destruction of the pancreatic β cells. As illustrated in **Figure 2**, β cell-derived antigens, such as preproinsulin (PPI), are taken up by APCs in the islets resulting in their maturation and subsequent migration to the draining pancreatic lymph nodes (PLNs). Within the PLN, the APCs will present the β cell-derived antigens to naive β cell-reactive T cells, thus activating them. Following their activation, the autoreactive T cells acquire the ability to migrate to the islets where they will encounter cognate antigen expressed by the β cells, leading to the destruction of the insulin-producing β cells[50].

The source of islet antigen is believed to be the wave of physiological pancreatic β cell death that occurs during pancreatic tissue remodelling. Indeed, in mice [51, 52], pigs[53] and humans[54], this wave of β cell death is evident early in life. In mice, this neonatal wave of β cell death peaks at about 2 weeks of age[51, 52], which correlates with the initiation of insulitis, which describes the infiltration and inflammation of the pancreatic islets, in diabetes-susceptible mice[50]. In humans, a

decrease in β cell proliferation is observed starting at 17-32 weeks of gestation up until 6 months of age, while the frequency of β cell apoptosis increases up to 6 months of age and subsequently decreases[54]. Evidence that this wave in β cell death is implicated in disease development is demonstrated through the removal of the PLNs from NOD mice prior to 3 weeks of age, when insulitis onset occurs, which results in protection from T1D[55]. Moreover, it has recently been shown that β cell death in young, female NOD mice induces the recruitment and activation of various innate immune cells to the pancreas[56]. This was not observed, however, when 1 week-old NOD mice were treated with a pan-caspase inhibitor to block β cell death, a treatment that also prevented T1D development up to 30 weeks of age[56]. Altogether, these results suggest that the onset of insulitis and subsequent progression of T1D is related to the wave of β cell death that occurs in the islets. However, as β cell death does not appear to be unique to the diabetes-susceptible NOD strain and is also apparent in diabetes-resistant strains [52, 57, 58], it cannot be said that this wave of β cell death is the trigger that leads to T1D onset despite its importance in the process.

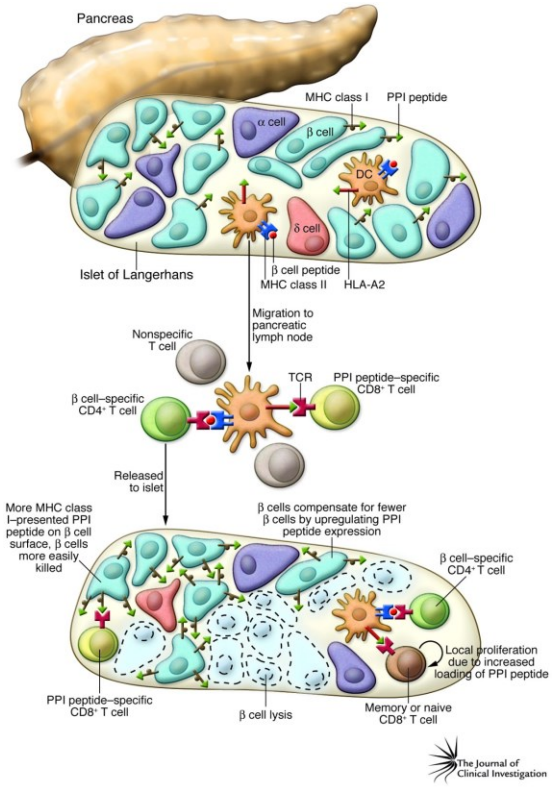

Figure 2. Model for the development of autoimmune diabetes. Adapted from [59]

During the first stage of autoimmune diabetes, the pancreatic islets are invaded by a mix of leukocytes, including dendritic cells, macrophages, NK cells, B cells, and T cells.

1.2.6.1 T cells

The utilization of mouse models has greatly enhanced our understanding of autoimmune diabetes pathogenesis. Accordingly, the non-obese diabetic (NOD) mouse model, which develops spontaneous autoimmune diabetes comparable to disease progression in humans (refer to Section 1.3), has become an important tool for researchers. Using this mouse model, it has been shown that T cell-enriched splenocytes from diabetic mice, contrary to T cell-depleted splenocytes, are able to transfer disease to young NOD mice[60], demonstrating that T cells are involved in disease development. Moreover, T cell-modulating therapies, such as anti-CD3 antibody[61] and cyclosporin[62], inhibit disease development. Altogether, these results demonstrate that autoimmune diabetes is a T cell-mediated disease.

The role of the diverse T cell subsets in disease development has been investigated. Accordingly, it was shown that a $CD4^+$ T cell clone required the co-transfer of $CD8^+$-enriched T cells in order to induce autoimmune diabetes in immunodeficient NOD.SCID (severe combined immunodeficient) mice, which lack both T cells and B cells[63]. Because of these results, some believed that $CD8^+$ T cells, and not CD4+ T cells, were essential for autoimmune diabetes development. However, it is now clear that both $CD4^+$ and $CD8^+$ T cells play a role in the development of disease. Indeed, autoimmune diabetes is impeded in the absence of $CD4^+$ T cells, as shown by the treatment of NOD mice with a monoclonal antibody targeting $CD4^+$ T cells[64, 65] as well as in mice that lack $CD4^+$ T cells[66], demonstrating that $CD4^+$ T cells are involved in disease progression. In addition, autoimmune diabetes is inhibited in mice that are deficient in $CD8^+$ T cells, either by anti-CD8 antibody injection into young mice[67] or of β2 microglobulin-deficient mice, in which few $CD8^+$ T cells develop[68]. Altogether, these studies demonstrate that autoimmune diabetes development is a function of the action of both $CD4^+$ and $CD8^+$ T cells.

On the contrary, another T cell population, the $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs), have been shown to play a role in autoimmune diabetes prevention rather than induction. Indeed, patients with IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, which is caused by mutations in the transcription factor FOXP3 (forkhead box P3), which is important for the development of Tregs, can also develop autoimmune diabetes[69]. Moreover, CD28-deficient NOD mice, which exhibit a drastic decrease in Tregs, develop accelerated disease[70]. In addition, the transfer of splenocytes from diabetic CD28-deficient mice, thus lacking Tregs, to NOD.SCID mice induced autoimmune diabetes, while the co-transfer of the Treg-deficient splenocytes and Tregs resulted in the prevention of disease transfer[70]. Therefore, these results demonstrate that Tregs play a role in autoimmune diabetes prevention.

Altogether, these results demonstrate that while $CD4^+$ and $CD8^+$ T cells are involved in the promotion of autoimmune diabetes, Tregs are involved in the prevention of disease.

1.2.6.2 B cells

While T cells are implicated in the destruction of pancreatic islets, B cells are also implicated in disease development. Indeed, diabetes incidence is much reduced in NOD mice lacking B cells [71, 72, 73, 74, 75], and similarly, the elimination of most B cells using anti-CD20 treatment reverses or delays diabetes progression in both mice and humans[76, 77]. Interestingly, diabetes susceptibility was restored in irradiated B cell-deficient NOD mice that were reconstituted with syngeneic bone marrow mixed with NOD B lymphocytes, but not in mice reconstituted with syngeneic bone marrow only[78]. Therefore, B cells contribute to disease progression.

Importantly, the antigen-presenting function of B cells is believed to play a role in autoimmune diabetes development. Accordingly, NOD mice for which the class II MHC molecule $I\text{-}A^{g7}$ expression was specifically deleted in B cells are

resistant to the development of disease, despite the presence of peri-insulitis, which describes infiltration surrounding the islets, of the pancreas[79]. Moreover, T cells from GAD65-primed B cell-deficient NOD mice failed to respond upon antigenic restimulation in vitro in the presence of either B cell-sufficient or -deficient APCs[78]. However, the reconstitution of irradiated B cell-deficient NOD mice with syngeneic bone marrow mixed with NOD B lymphocytes, but not with syngeneic marrow alone, restored T cell responses to GAD65 autoantigen[78]. These results suggest that antigen-presenting B cells are required for the initial in vivo priming of GAD65-reactive T cell responses in NOD mice. B cells are also activated in the presence of another autoantigen, the insulin antigen, where the costimulatory molecule, CD86, which is important for T cell crosstalk, is upregulated on autoreactive B cells following insulin autoantigen exposure[80]. When anti-insulin B cells are specifically eliminated by anti-insulin monoclonal antibody therapy, diabetes incidence is inhibited in NOD mice[80]. Altogether, B cells can present β cell autoantigens to autoreactive T cells, which overcomes a checkpoint in T cell tolerance to islet β cells and promotes autoimmune diabetes development.

H2-O (HLA-DO in humans) expression can influence the participation of B cells in the germinal centers, which are the site of B cell maturation [81]. More specifically, H2-O is a MHC class II-like protein that inhibits peptide loading onto MHC II by inhibiting the function of the MHC class II-like protein H2-M (HLA-DM in humans) [82]. Recent publications have demonstrated that germinal center B cells exhibit a decreased H2-O expression [83]. Moreover, it has been shown that H2-O-deficient antigen-specific B cells preferentially populate germinal centers in comparison to H2-O-sufficient B cells due to their enhanced ability to obtain antigen-specific T cell help, where T cell help is dependent upon antigen presentation by B cells [84]. Interestingly, H2-O expression is cell maturation-dependent as both mature B cells and DCs express H2-M and H2-O whereas developing immature B cells and DCs express very little H2-O [85, 86]. These results suggest that, as H2-O limits peptide exchange on MHC class II, loss of function of H2-O might lead to autoimmunity as presentation of self-peptides by antigen-

presenting cells such as B cells would not be attenuated. Although it has been shown that H2-O-deficient mice do produce higher titers of autoantibodies [87], H2-O expression on B cells and its direct association with autoimmune diabetes has not been evaluated.

B cells also contribute to autoimmune diabetes through the production of autoantibodies. Indeed, it has been demonstrated that maternally transmitted autoantibodies contribute to the disease onset in NOD offspring[88, 89]. Accordingly, the NOD progeny from B cell–deficient NOD mothers that were mated with a NOD males, as well as the NOD progeny of diabetes-resistant mothers that were implanted with the NOD embryos, displayed a reduction in autoimmune diabetes incidence[88]. Thus, the maternal transmission of antibodies is implicated in disease development.

Altogether, it is clear B cells play a role in autoimmune diabetes development.

1.2.6.3 Macrophages

Macrophages are present in the pancreatic islet infiltrates of NOD mice prior to T cell infiltration[64, 90], suggesting that macrophages are involved in autoimmune diabetes progression. Macrophages are believed to participate in disease pathogenesis through the production of various pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), which contribute to β cell dysfunction[90, 91], and interleukin-12 (IL-12), which promotes the differentiation of diabetogenic $CD8^+$ cytotoxic T cells in the diabetes-prone setting[92]. Interestingly, the injection of a monoclonal blocking antibody that is specific for the myelomonocytic adhesion-promoting type-3 complement receptor (CD11b/CD18), which targets macrophages but not T cells, prevented islet infiltration by both macrophages and T cells and inhibited disease onset[93]. Interestingly, TNF-α and IL-1β-producing macrophages have also been observed in pancreatic islet infiltrates from patients with recent-onset autoimmune diabetes in humans [94]. Therefore, these studies demonstrate a pathogenic role for macrophages in autoimmune diabetes.

1.2.6.4 Natural killer cells

Natural killer (NK) cells are composed of effector lymphocytes, known to play a crucial role in the control of viral infection and the prevention of cancer[95], as well as regulatory cells[95]. Indeed, while NK cells can kill immature DCs in humans and mice thereby influencing DC homeostasis [96, 97], the interaction between NK cells and immature monocyte-derived DCs in humans can also promote cytokine production by DCs [96]. Similarly, it has been shown that NK cells can both promote the priming of $CD4^+$ T cells through the secretion of IFN-γ[98] or kill activated T cells that express insufficient amounts of classical or non-classical MHC class I molecules [99]. Moreover, IL-10-secreting NK cells inhibit antigen-specific T cell proliferation while maintaining their natural cytotoxic activity [100]. Therefore, NK cells are not a homogenous subset and are composed of both effector and regulatory subsets.

NK cells are another cell population implicated in autoimmune diabetes. Indeed, similar to macrophages, NK cells also infiltrate the pancreas before T cells in NOD mice[101] and can be found directly within the islets of diabetic patients[47]. Moreover, the depletion of NK cells using an anti-asialo-Gm1 antibody prevented autoimmune diabetes in NOD mice [102]. Interestingly, NK cells from diabetes-susceptible NOD mice have impaired immune functions compared with NK cells from diabetes-resistant mouse strains[103, 104]. Accordingly, NK cells isolated from the pancreas of NOD mice are hyporesponsive such that they produce significantly less interferon gamma (IFNγ) and express less CD107a, which is a marker of degranulation[101]. Interestingly, NOD mice congenic for the NK cell surface marker NK1.1, which is expressed in the diabetes-resistant C57BL/6 strain but not in the NOD strain, exhibit a reduced disease incidence[105]. This protection from diabetes development also correlates with improved NK cell performance, as compared with wild-type NOD mice[105]. Thus, it is possible that the functional deficiency of NK cells from diabetes-susceptible mice, rather than an efficient NK cell function, contributes to autoimmune diabetes progression. Altogether, these results demonstrate that NK

cells infiltrate the pancreas and play a role in autoimmune diabetes. However, whether NK cells promote or protect from autoimmune diabetes remains controversial.

1.2.6.5 Dendritic cells

Antigen-presenting dendritic cells (DCs) play an important role in autoimmune diabetes pathogenesis. Accordingly, DCs are able to capture autoantigens released after β cell death, which occurs physiologically in NOD mice at 2 weeks of age during tissue remodelling[52], as described earlier in section 1.2.6. Subsequently, the autoantigen-specific DCs migrate to the PLN where they can present the autoantigen to islet antigen-specific T cells, which initiates the diabetogenic response[52]. Indeed, a large frequency of DCs presenting β cell antigens can be found within the PLNs of NOD mice[52]. Interestingly, in diabetes-susceptible NOD mice that constitutively overexpress human HLA-DO in DCs, where, as previously explained, the association of HLA-DO with HLA-DM modulates the peptide loading function of HLA-DM thus hampering the presentation of self-antigens and the inappropriate activation of T cells [82, 106], disease development is completely blocked[107]. This inhibition of autoimmune diabetes occurred despite a comparable diabetogenic T cell repertoire and level of peri-insulitis to control NOD mice [107], suggesting that HLA-DO expression dampens the presentation of islet-derived autoantigens by DCs to T cells and can consequently promote T cell tolerance. Altogether, it is clear that the antigen-presenting function of DCs plays a key role in disease initiation.

DC involvement in autoimmune diabetes has been demonstrated via the in vivo treatment using Fms-like tyrosine kinase 3 ligand (FLT-3L), which expands DC populations. Indeed, FLT-3L treatment can accelerate autoimmune diabetes development in NOD mice[108]. Nevertheless, the contribution of DCs to autoimmune diabetes development seems to depend on the timing of FLT-3L treatment and the presence of autoreactive T cells such that FLT-3L treatment can prevent autoimmune

diabetes in NOD mice at early stages of autoimmune diabetes progression[109], whereas it can also accelerate disease development at later stages when islet antigen-specific $CD8^+$ T cells are already detectable in the blood[108]. Altogether, the contribution of DCs to autoimmune diabetes development seems to depend on both the stage of disease development and the presence of autoreactive T cells.

1.3 The non-obese diabetic mouse model

Murine models are a useful tool for research as they allow for the characterization of the cellular mechanisms involved in the progression of autoimmune disease. Autoimmune diabetes can be experimentally induced with various chemicals, such as streptozotocin[110] and cyclophosphamide[111], or by genetic manipulation, which is observed for transgenic mice. However, spontaneous murine models of autoimmune diabetes also exist. In particular, the non-obese diabetic (NOD) mouse strain is a key tool for investigating the aetiology of human autoimmune diabetes[112]. Indeed, the NOD mouse strain spontaneously develops autoimmune diabetes, which shares many similarities to disease development in humans, such as the presence of autoreactive $CD4^+$ and $CD8^+$ T cells as well as pancreas-specific autoantibodies. Moreover, the broadly immunosuppressive anti-CD3 therapy, which has been successful in the treatment of new-onset type 1 diabetic patients during clinical trials, is a protocol initially developed in the NOD mouse [113, 114], further demonstrating a parallel in disease development between humans and mice.

Investigation of the genetic control of autoimmune diabetes in the NOD mouse has revealed an interesting parallel with genetic susceptibility to disease in humans[28, 32, 115, 116]. First, the MHC haplotype H-2^{g7} expressed by the NOD mouse does not express an MHC class II I-E molecule because of a defective Eα locus. In addition, similar to the human autoimmune diabetes-associated HLA-DQ β chain (HLA-DQB1*0302) [22, 117, 118], NOD mice have a unique MHC class II I-A molecule containing a non-aspartic acid substitution at position 57 of the β chain[119], which substantially alters the repertoire of MHC binding peptides presented by this allele[120]. Furthermore, autoimmune diabetes is multigenic in both mice[121, 122] and humans[123] with the MHC being the major locus contributing to disease development[123]. Accordingly, NOD.B10-*H-2*^{b} mice, for which the H-2^{g7} is replaced by the H-2 region from the diabetes-resistant C57BL/10SnJ (B10) strain, do not develop autoimmune diabetes[124]. However, in B10.NOD-*H-2*^{g7} mice, for which the H-2 region from the

diabetes-resistant B10 strain is replaced by the NOD H-2^{g7}, diabetes development does not ensue[105, 108], demonstrating that non-MHC genes are also implicated in disease susceptibility[122, 125]. Indeed, in the NOD mouse, over 20 insulin-dependent diabetes (Idd) loci have been identified with the MHC composing the Idd1 locus[126]. Accordingly, most of the human and mouse alleles conferring risk for autoimmune diabetes reveal common candidate genes that are concordant between humans and the NOD mouse, including the aforementioned *INS* (within the Idd2 locus) and *CTLA-4* (within the Idd12 locus) genes[115, 116, 127]. Hence, the NOD mouse represents a useful tool for the study of the genetic factors influencing autoimmune diabetes susceptibility.

In terms of autoimmune diabetes pathogenesis in the NOD mouse, infiltration begins around 3-4 weeks of age such that few immune cell infiltrates surround the islet (peri-insulitis). By 10 weeks of age, the cell infiltrates progress and invade the islets (insulitis). Overt diabetes is observed beginning at 12-14 weeks of age in females and slightly later in males. By 30 weeks of age, the incidence of disease observed in NOD mice is 80% in females and less than 20% in males[128]. Moreover, the pathological examination of the pancreas in NOD mice revealed a high frequency of lymphocyte infiltration around and/or into the pancreatic islets as well as a reduction in the number and size of pancreatic islets in the overt diabetic mice[128]. Finally, the NOD mouse also displays key diabetic symptoms that also arise in diabetic patients, such as polyuria, polydipsia, hyperglycemia, and glucosuria[128]. Because of these many similarities, the NOD mouse is a key tool for autoimmune diabetes research.

Nevertheless, it must be noted that there also exists various differences between NOD mice and humans. Firstly, there exists various differences between the innate and adaptive immunity of mice and humans, including variances in the balance of leucocyte subsets, $\gamma\delta$ T cells, cytokines and cytokine receptor expression, to name a few [129]. Moreover, additional differences have been noted between NOD mice and humans with respect to autoimmune diabetes manifestation. For instance, the islet infiltration in NOD mice is characterized by peri-insulitis followed by insultis. In

contrast to what is observed in NOD mice, few leukocytes are detectable within the inflamed islets of human patients [130]. Furthermore, while $CD4^+$ T cells are the predominant T cell subset found in the NOD mouse islet infiltrate[131], $CD8^+$ T cells are the most abundant cell population detected during insulitis in human patients [132]. In addition, the presence of maternal islet autoantibodies in the offspring of diabetic mice has been identified as a diabetogenic factor in mice [88], as described in Section 1.2.6.2. However, children of mothers with autoimmune diabetes who were autoantibody positive at birth had significantly lower risks for developing the disease than children of mothers with autoimmune diabetes who were islet autoantibody negative at birth [133], suggesting that fetal exposure to islet autoantibodies may be protective against disease development in humans. Lastly, the gender bias observed for autoimmune diabetes development in NOD mice, which is described above, is not observed in humans[134]. Therefore, despite many similarities, the NOD mouse does not fully mimic all aspects of autoimmune diabetes observed in humans.

Altogether, because of its many similarities to autoimmune diabetes in human patients, the NOD mouse has been used extensively in autoimmune diabetes research and as a pre-clinical tool for the development of new therapeutic strategies for the treatment of this disease.

1.4 Autoimmune diabetes therapy

Due to the many complications associated with long-term autoimmune diabetes, the life expectancy of people living with the disease may be shortened by as much as 15 years[iii]. As a result, research continues to better understand all the factors implicated in autoimmune diabetes progression in order to develop new therapies that will prevent, reverse and/or cure the disease. The main therapeutic avenues that are discussed in this section include the transplantation of pancreatic islets or of whole pancreas as well as immunotherapy.

1.4.1 Transplantation

Some patients who have experienced severe complications associated with autoimmune diabetes have seeked an alternative therapy, namely either pancreatic or islet transplantation, in an attempt to cure their disease and achieve glucose tolerance and an alleviation of their complications.

1.4.1.1 Pancreas transplantation

A pancreas transplant involves implanting a healthy pancreas from a recently deceased individual, or a partial pancreas from a living donor, into the diabetic patient. As mentioned above, the majority of pancreas transplant recipients have complicated autoimmune diabetes. Since 75% of diabetic patients with kidney failure do not survive longer than 5 years while receiving dialysis[135], approximately 90% of pancreas transplant recipients are either uremic (in kidney failure) or post-uremic. Importantly, mortality rate was decreased by approximately 50% when patients with autoimmune diabetes and end-stage renal failure received a simultaneous pancreas and kidney transplant[135].

[iii] Canadian Diabetes Association. An economic tsunami: the cost of diabetes in Canada. December 2009

In patients who receive a pancreas transplant alone, the actual patient and pancreas survivals at 5 years is 98.6% and 73.2%, respectively[136]. Long-term follow-up of transplant recipients has demonstrated that pancreas transplants are successful in returning normal glycemia to diabetic patients for periods as long as 1-2 decades[137]. Indeed, the half-life of the pancreas graft currently averages at 16.7 years[138]. Additional benefits of successful pancreatic transplant are improved retinopathy, blood pressure, as well as neuropathic and cardiac function[139]. The inability to maintain insulin-independence following transplantation is mostly explained by technical failure in the postoperative phase and rejection in the long-term period[138], where graft rejection remains the leading cause of pancreas loss[140]. Autoimmunity is also increasingly recognized as a cause of graft loss[141]. Nevertheless, improvements in graft preservation, surgical techniques, immunosuppression, and prophylactic treatments are expected to further improve the results of pancreas transplantation[138].

Altogether, pancreas transplantation consistently induces insulin-independence in diabetic patients, however this is at the cost of major surgery and its associated complications as well as life-long immunosuppression[138], hence the reason why only patients who have severe complications associated with the disease, and thus have no other options, consider this means of treatment.

1.4.1.2 Islet transplantation

Islet transplantation is similar to pancreas transplantation in that it entails the transplantation of isolated islets from a donor pancreas into a diabetic patient. However, this transplantation is considered to be a more attractive alternative to pancreas transplantation since the islets can simply be infused into the liver of the patient through a catheter. Once transplanted, the islets will begin to produce insulin leading to glucose regulation.

In 1988, Ricordi and colleagues first reported on the semi-automated method for human pancreatic islet isolation making it feasible to consistently reproduce the

difficult process of separating insulin-producing β cells from the rest of the pancreas[142]. In 2000, a group in Edmonton developed a steroid-free immunosuppressive regime termed the Edmonton protocol, which promoted long-term islet survival and function in patients[143]. With the development of the Edmonton protocol, 100% of patients, in comparison to the previous 8%, did not require insulin therapy 1 year after transplantation[143]. The initial success of the "Edmonton Protocol" quickly created an international passion and many centers adopted this technique. Unfortunately, 5 years after transplantation, more than 90% of the patients were again dependent on exogenic insulin[144]. Although much work is left to be done with regards to islet transplantation, researchers are still confident that this form of therapy will prove to be a success.

Indeed, both pancreas and islet transplantations result in the restoration of proper insulin production and, consequently, blood glucose regulation. Therefore, both methods have the potential to cure autoimmune diabetes, at least for a given amount of time. Nevertheless, both surgeries are accompanied by a lifelong regimen of immunosuppressive drugs to prevent graft rejection, where both the surgery itself and permanent immunosuppression is considered to be far more dangerous than continued insulin replacement therapy.

1.4.2 Immunotherapy

Several immunosuppressive agents have been tested for their ability to prevent/delay/reverse autoimmune diabetes development due to their ability to target either T cells or B cells, both of which are involved in disease progression.

1.4.2.1 Cyclosporine A

Cyclosporine A is an immunosuppressive agent that is widely used to prevent graft rejection due to its ability to interfere with T cell activity[145]. As T cells are directly involved in the destruction of pancreatic β cells, cyclosporine A was studied as a preventative therapy for autoimmune diabetes in mice and humans. NOD mice between the ages of 4 and 9 weeks that were treated with cyclosporine every 2 days

until 23 weeks of age demonstrated a significant reduction in disease incidence in comparison to control mice[62]. These results also correlated with a decrease in blood glucose levels, islet infiltration and islet destruction[62]. However, when cyclosporine treatment was started after development of glucose intolerance, the treatment appeared to have little therapeutic effect on disease development[62]. In humans, approximately 50% of patients who were treated with cyclosporine within 6 weeks of diagnosis and for a duration of 2 to 12 months became insulin-independent and showed C-peptide plasma concentrations in the normal range as well as decreasing titres of islet cell antibodies[146]. Nevertheless, this agent demonstrated multiple side effects, including nephrotoxicity, making it unsuitable for the treatment of disease[147].

1.4.2.2 Anti-CD3 monoclonal antibodies

Anti-CD3 antibodies, including teplizumab and otelixizumab, target T cells by binding to the CD3 molecule located on their cell surface. The ability of a CD3-specific antibody to reverse autoimmune diabetes onset in 64-80% of female NOD mice[61] paved the way for clinical trials in diabetic patients. Recent-onset diabetic patients who received the anti-CD3 antibody otelixizumab showed a sustained remission of the disease for up to four years[148]. Success of the treatment correlated with the initial residual β cell function where patients presenting with a higher functional β cell mass before starting the treatment responded better. Indeed, 18 months post-treatment, nearly 75% of patients in this subgroup were clinically insulin-independent[148]. Disease remission following anti-CD3 treatment is believed to be a result of the simultaneous action of the antibody on effector T cells, a fraction of which was deleted by apoptosis, and on Tregs, whose function was preserved and even increased[149, 150]. Therefore, anti-CD3 antibodies can be used to reverse and delay autoimmune diabetes progression.

1.4.2.3 Anti-CD20 monoclonal antibody

The anti-CD20 monoclonal antibody, rituximab, is a chimeric monoclonal antibody, incorporating human immunoglobulin heavy-chain sequences and murine immunoglobulin variable regions[151], that eliminates B cells by binding to the

extracellular portion of CD20 molecules, which are expressed on the cell surface of all B cells except for plasma cells. More specifically, rituximab depletes B cells via three mechanisms of action[152, 153] **(Figure 3).** The first mechanism is via the modulation of the apoptotic signal transduction pathway resulting in direct cytotoxicity. The second mechanism is via antibody-dependent cell-mediated cytotoxicity, in which NK cells and macrophages bind to the Fc portion of the anti-CD20 antibody by means of their Fcγ receptor and subsequently release effector molecules such as perforin leading to cell lysis. The third mechanism is via complement-dependent cytotoxicity, for which complement binds to the Fc portion of the anti-CD20 antibody thereby activating the complement cascade and formation of the membrane attack complex[154]. Although the mechanism by which B cell depletion ameliorates disease is not completely understood, the effect on antibody production and antigen presentation to T cells are thought to be important.

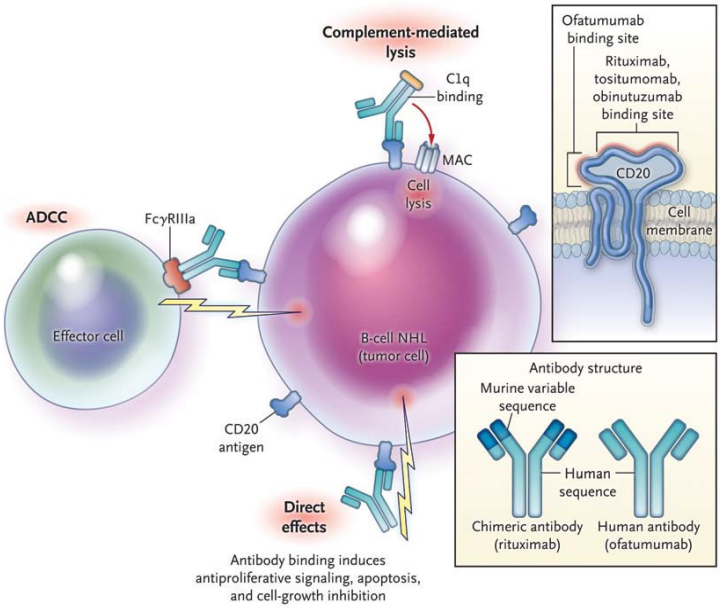

Figure 3. Mechanisms of action of anti-CD20 antibodies inducing B cell depletion. Adapted from [151].

As B cells are implicated in autoimmune diabetes development, rituximab treatment has been studied as a potential therapeutic agent for the disease in mice and humans. In mice, a 9 day cycle of 4 injections results in almost complete B cell depletion within 1 week after the last injection, where B cells begin to repopulate 3 weeks after the last injection and reach normal levels by 12 weeks after the last injection[77]. Using a transgenic NOD mouse expressing human CD20 on B cells, Hu and colleagues demonstrated that anti-CD20 treatment can significantly delay and reduce the onset of autoimmune diabetes in 4 week-old mice[77]. Furthermore, treatment of mice presenting with hyperglycemia resulted in a decrease in blood sugar levels in over one-third of diabetic mice[77]. In addition, the beneficial outcome of the anti-CD20 treatment was shown to be a result, at least in part, of a Treg expansion[77]. Altogether, anti-CD20 treatment was able to delay, inhibit and reverse autoimmune diabetes in mice.

In humans, anti-CD20 treatment leads to a transient depletion in B cells, which gradually recover over the course of 12 months[76]. New-onset diabetic patients that received a four-dose course of anti-CD20 demonstrated a significantly higher level of plasma C-peptide as well as a significantly lower level of HbA_{1c} over a period of 1 year in comparison to the placebo group[76]. These results correlated with a decrease in required insulin and, altogether, demonstrate a preserved β cell function in anti-CD20 treated patients[76]. Moreover, 40% of anti-CD20-treated patients who were IAA positive became IAA negative versus 0% of placebo-treated patients, where these results were ameliorated the sooner the patient begun treatment following diagnosis[155]. Therefore, anti-CD20 treatment in both mice and humans has a beneficial outcome for autoimmune diabetes.

Although these immunosuppressive agents have proven to impede further β cell destruction, thus preserving natural insulin production, the duration of this beneficial effect as well as the possible long-term negative effects have not been fully evaluated or completed for each of these therapies. Indeed, the most common side effect of immunosuppressive drugs is global immunodeficiency, resulting in increased susceptibility to infections and decreased cancer immunosurveillance. The

risk that accompanies global immunosuppression is the basis for the ongoing search for an antigen-specific cellular therapy for the treatment of autoimmune diabetes.

1.5 Immunoregulatory $CD4^-CD8^-$ (DN) T cells[iv]

Immune tolerance is dependent on the immune system discriminating between self and non-self. As explained earlier, a break in immune tolerance results in autoimmunity, which can lead to the destruction of healthy organs, glands, joints or the central nervous system. The triggers that cause T or B lymphocytes to aberrantly recognize and mount an immune response against self-antigens remain to be fully elucidated. However, a prominent role in the generation and maintenance of immune tolerance has been attributed to the number and function of regulatory cell subsets. Indeed, several populations belonging to the T cell or B cell compartment carry the ability to inhibit immune responses and promote peripheral tolerance, including $CD4^+CD25^+FoxP3^+$ naturally occurring T cells, IL-10-producing $CD4^+$ Tr1 cells, TGF-β-producing $CD4^+$ Th3 cells, $CD8^+CD28^-$ T cells, $CD4^-CD8^-$ (DN, double negative) T cells, $CD4^+CD8^+$ (DP, double positive) T cells, and Natural Killer-like T (NKT) cells [157, 158, 159, 160, 161, 162, 163, 164, 165, 166], as well as B10 regulatory B cells [167, 168]. These various regulatory cell populations express diverse mechanisms of action, which may also vary depending on the experimental model being studied [158], suggesting that the contribution to immune tolerance, as well as to disease prevention, differ amongst these various cell populations. Hence, there is a need to further examine the contribution of various T cell subsets to autoimmune predisposition. One of these regulatory T cell populations, namely $CD4^-CD8^-$ (double negative, DN) T cells, have been studied in both mice and humans for their contribution to peripheral tolerance, disease prevention and their potential for use in cellular therapy.

DN T cells are of particular interest because they have demonstrated the ability to inhibit immune responses in an antigen-specific manner[169, 170, 171]. This has major implications, since the use of DN T cells in immunotherapy should therefore

[iv] Most of the text in section 1.5, 1.5.1, 1.5.2 and 1.5.3 was recently accepted for publication as a review article: Reference 156. Hillhouse EE, Lesage S. A comprehensive review of the phenotype and function of antigen-specific immunoregulatory double negative T cells. *Journal of autoimmunity* 2012.. Hillhouse, E.E. & Lesage, S. A comprehensive review of the phenotype and function of antigen-specific immunoregulatory double negative T cells. *Journal of autoimmunity* (2012).

lead to fewer side-effects along with a decreased risk of infections, which remains a major concern in the application of broad immunosuppressive regimens[172].

1.5.1 Challenge associated with the specific identification of immunoregulatory DN T cells

$CD4^-CD8^-$ T cells are defined by exclusion (i.e., as $CD3^+$ non-CD4 non-CD8 T cells) and, as a consequence, the term refers to three T cell populations that are infrequent in the peripheral blood and lymphoid organs, including γδ T cells [173, 174], a subset of NKT cells [175], and DN T cells [171]. As a result, other markers besides CD4, CD8, and CD3 must be used in order to distinguish these three subsets. The expression of the γδ T cell receptor (TCR) separates γδ T cells from both NKT and DN T cell subsets, which both express an αβ TCR. Unfortunately, there are currently no known phenotypic markers which readily allow the discrimination between NKT and DN T cells, therefore their distinction is often ambiguous and is based on imprecise or incomplete characterization. To grasp the complexity of this challenge, the known phenotypic and functional characteristics of NKT and DN T cells must be understood (refer to **Table 1** for phenotypical comparison of $CD3^+CD4^-CD8^-$ T cell populations).

	DN T cells	**NKT I cells**	**NKT II cells**	**γδ T cells**
T cell receptor	αβ	αβ	αβ	γδ
Repertoire	Polyclonal [169, 176]	Oligoclonal [177]	Polyclonal [178]	Oligoclonal [179, 180, 181]
Restriction	MHC	CD1d [182, 183]	CD1d [184]	Rare MHC restriction [179, 185]
Antigen recognition	Peptides[186]	Glycolipids [187, 188, 189, 190]	Glycolipids [184, 191]	Protein and non-protein [179, 192]
NK1.1 expression	+/- *	+/- [193]	+/-[194]	+/- [195, 196]
CD49b expression	+/- [193]	+/- [193, 197]	+/- [159, 197]	N/A

Table 1. Properties of the various $CD3^+CD4^-CD8^-$ T cell populations. Adapted from [156]. *Manuscript in preparation, refer to Chapter 3, Supplementary Figure 1.

1.5.1.1 Distinguishing DN T cells from NKT cells

NKT cells can be categorized into two main subpopulations, namely type I and type II NKT cells, both of which express an αβ TCR and are either $CD4^+$ or $CD4^-$ $CD8^-$ in mice [175, 198, 199] or $CD4^+$, $CD8^+$ or $CD4^-CD8^-$ in humans [159, 200]. Thus, similar to DN T cells, a subset of both type I and type II NKT cells exhibit a $CD4^-CD8^-$ $TCR\alpha\beta^+$ phenotype, making other markers of NKT cells essential for their discrimination. Accordingly, NKT cells are known to express CD56 or CD161 in humans and CD49b or NK1.1 in mice [159, 200, 201, 202]. However, in mice, expression of the NK1.1 surface antigen is dependent on the expression of the C57BL/6 NK gene-complex haplotype as well as the genetic background. Consequently, NK1.1 expression on NKT cells is limited to just a few mouse strains, including C57BL/6, FVB/N, and NZB, whereas it is not expressed in other commonly used mouse strains, such as BALB/c, CBA/J, C3H, DBA/1, DBA/2, NOD, SJL, and 129. More importantly, CD49b and NK1.1 in mice, as well as CD56 and CD161 in humans, are neither exclusive to NKT cells, nor are they expressed by all NKT cells [159, 193, 200, 203, 204, 205]. Furthermore, a fraction of immunoregulatory DNT cells found in CD1d-deficient mice, which lack type I and II NKT cells (further discussed below), also express CD49b [193]. Therefore, these NK cell surface markers cannot be used to effectively distinguish NKT cell subsets from DN T cells.

Alternatively, type I NKT cells can be distinguished from both DN T and type II NKT cells by the distinct selectivity of their TCR repertoire. Indeed, while both DN T and type II NKT cells express a polyclonal TCR Vβ repertoire [169, 189, 199, 200], type I NKT cells, also referred to as invariant NKT (iNKT) cells, express an oligoclonal TCR repertoire. In mice, type I NKT cells express an invariant Vα chain (Vα14) and one of three possible Vβ chains (Vβ2, Vβ7, Vβ8.2) [198] while in humans, type I NKT cells are specified by the expression of the Vα24 chain and the Vβ11 chain, which are respectively homologous to mouse Vα14 and Vβ8.2 [159, 200, 206, 207]. Therefore, in both mice and humans, type I NKT cells can be specifically separated from both DN T and type II NKT cells based on their TCR repertoire.

With regards to $CD4^-CD8^-CD3^+$ T cell subsets, both γδ T cells and type I NKT cells can be specified through their TCR. However, type II NKT cells and DN T cells both express a polyclonal αβTCR repertoire [169, 189, 199, 200] and thus their specific identity must be resolved through other means. Type II NKT cells, as for type I NKT cells, differentiate in the thymus and are positively selected on an MHC class I-like, β2m-dependent, CD1d molecule [175, 183, 194]. Based on their TCR restriction to CD1d molecules, molecular tools have been developed to specifically identify both type I and type II NKT cells. More specifically, the invariant TCR of type I NKT cells recognizes the α-galactosyl ceramide (α-GalCer)-loaded CD1d tetramer [189, 190], while type II NKT cells bind to the sulfatide (I3SO3-GalCer)-loaded CD1d tetramer [184]. Similarly, human type II NK T cells recognize sulfatide presented by group I CD1 molecules [184, 208]. Nevertheless, not all type II NKT cells are reactive with sulfatide loaded CD1 molecules [209], further stressing the point that DN T and type II NK T cells cannot be readily distinguished. To circumvent this issue, one can resort to CD1d-deficient mice, which are fully devoid of both type I and type II NKT cells, as both subsets are fully dependent on CD1d for thymic positive selection [182, 183, 210, 211]. Therefore, using CD1d-deficient mice, immunoregulatory DN T cells, void of any contaminating NK T cells, may be successfully isolated.

1.5.2 DN T cell phenotype

Immunoregulatory DN T cells compose approximately 1-3% of total T cells in non-transgenic mice [212, 213, 214] and in humans [169] making them difficult to isolate and subsequently study. To circumvent these issues of scarcity, as well as their similarity in phenotype to NKT cells, DN T cells have been mostly investigated in TCR transgenic mice, where the insertion of various αβTCR transgenes increases the proportion of immunoregulatory DN T cells in the lymphoid organs of mice, as observed in the HY-TCR, the 4E3-TCR, the 1H3.1, the 2C-TCR and the 3A9 TCR transgenic mice. Moreover, these αβTCR transgenes are not restricted to CD1d and are thus incompatible with NKT cell differentiation. Similarly, most of these transgenes also preclude γδ T cell differentiation [171, 175, 214, 215, 216, 217, 218, 219, 220, 221]. In

other words, using an αβTCR transgene not only increases the number of DN T cells thereby facilitating the study of this otherwise rare T cell subset, the insertion of a given αβTCR transgene also facilitates the isolation of a pure DN T cell population void of other contaminating $CD4^-CD8^-$ T cell subsets, namely both NKT cells and γδ T cells.

1.5.2.1 Phenotype of TCR transgenic DN T cells

TCR transgenic DN T cells are a distinct regulatory T cell population as they do not express NKT cell markers, NK1.1 and CD49b [222] as well as the Treg marker, Foxp3 [214, 223]. Interestingly, DN T cells from TCR transgenic models generally exhibit a naive phenotype, in that they express low levels of CD44, are negative for CD25, CD28, CD69, CD122, and B220 expression and show high levels of Ly-6A [171, 214, 216, 218, 222, 224, 225, 226]. Additional phenotypic characterization demonstrates that TCR transgenic DN T cells express low levels of CD5 and express other T cell markers such as CD3, CD62L and CD45RB at levels comparable to naive CD4 T cells from TCR transgenic mice [214, 216, 222]. Altogether, TCR transgenic DN T cells express a phenotype comparable to that of naive T cells. However, in TCR transgenic mice where the cognate antigen is also expressed, TCR transgenic DN T cells express an activated phenotype such that they are $CD44^{hi}CD122^{hi}CD45RB^+$ [218, 226], but they do not express acute activation markers CD69 and CD25 [218].

In terms of cytokine production, 2C DN T cells have been shown to produce IFNγ [226] and perforin [222] whereas they do not produce IL-10, IL-2 or IL-4 [171]. In terms of anergy, in the three TCR transgenic models tested, namely 2C, IH3.1 and 3A9, DN T cells were shown to be anergic and, similar to $CD4^+FoxP3^+$ Tregs [214, 227, 228], this anergic phenotype was reversed following the addition of IL-2 [170, 214, 216, 218, 226, 229]. In summary, immunoregulatory DN T cells from αβTCR transgenic mice are anergic and exhibit a naive T cell phenotype.

1.5.2.2 Phenotype of non-transgenic DN T cells

With the goal to move towards a more clinical setting, the study of DN T cells using a non-transgenic system, which is more physiologically relevant to humans, is essential. Despite their scarcity in non-transgenic mice [212, 213, 214], as well as their lack of a specific marker, both of which present a challenge for their isolation, characterization and manipulation, various groups have studied DN T cells using non-transgenic mice.

Similar to TCR transgenic DN T cells, non-transgenic DN T cells express markers of T cells such as CD3 and CD5, but do not express Foxp3 or CD49b and do not stain with the CD1d-αGalCer tetramer [214, 230]. Contrary to TCR transgenic DN T cells, non-transgenic DN T cells do not exhibit a naive T cell phenotype, but rather show a $CD69^{low}CD25^{low}CTLA\text{-}4^{-}CD44^{+}$ profile [230]. However, a note of caution must be taken in the interpretation of these data, as neither γδ T cells nor NKT cells were specifically excluded in defining the phenotype of the non-transgenic DN T cells [230]. Nonetheless, and similarly to DN T cells from TCR transgenic mice expressing their cognate antigen, an activated DN T cell phenotype was also observed in other non-transgenic settings. Indeed, in CD4, CD8, MHC I and MHC II quadruple-deficient non-TCR transgenic mice (referred to as quad-deficient mice) DN T cells are found to exhibit a $CD5^{hi}CD69^{+}CD62L^{low}CD44^{hi}$ phenotype [231]. Interestingly, DN T cells from AND TCR transgenic quad-deficient mice express a more naive phenotype similar to other TCR transgenic models, such that they are $CD5^{low}CD69^{-}CD62L^{+}CD44^{-}$. From these observations, the authors were led to suggest that endogenous TCRs expressed by non-transgenic DN T cells in quad-deficient mice receive a signal from self-ligands whereas, in the absence of cognate antigen, TCR transgenic DN T cells do not[231]. Admittedly, the quad-deficient mouse model is a highly complex system and the phenotype of the resulting non-transgenic DN T cells from these mice must again be interpreted with caution, if only for the fact that the thymic selection process of these cells is limited to non-MHC molecules. Finally, non-TCR transgenic DN T cells have also been isolated from mice homozygous for the lymphoproliferation (lpr) spontaneous mutation, namely *lpr/lpr* mice, wherein a

mutation in Fas leads to an abnormal accumulation of DN T cells[232, 233, 234, 235, 236]. Interestingly, non-TCR transgenic DN T cells from *lpr/lpr* mice again exhibit a similar activated T cell phenotype, in addition to abnormally expressing the B220 B cell antigen[236]. Again, the severe lymphoproliferative disorder observed in the *lpr/lpr* mouse model must be noted as a limitation in defining the activation status of non-transgenic DN T cells. Although all of these models present with some limitations towards identifying the exact phenotype of immunoregulatory DN T cells, the phenotypic characterization of DN T cell in non-TCR transgenic mice, quad-deficient mice and *lpr/lpr* mice all suggest that immunoregulatory DN T cells exhibit an activated phenotype. If these findings consistently hold true in more physiological settings, the activated DN T cell phenotype may suggest that, similar to Tregs[237], the TCR repertoire of DNT cells is partial to the preferential recognition of self-antigens.

1.5.2.3 Phenotype of human DN T cells

Similar to non-transgenic DN T cells, human DN T cells compose approximately 1-3% of total T cells [169] making them difficult to isolate and subsequently study. Nevertheless, human DN T cells have been isolated from whole blood and, as for mouse DN T cells, have been shown to express a polyclonal αβTCR repertoire [176] and to lack the expression of the Treg marker, FoxP3, as well as the NKT cell marker, CD56 [169, 238]. In humans, DN T cells consist of both $CD45RA^{bright}CCR7^{+}$ naive cells and $CD45RA^{low}CCR7^{-}$ Ag-experienced cells [169, 238]. Moreover, human DN T cells are negative for CD28, CD25, CTLA-4 and CD16, but they do express Fas [169, 238]. However, as is observed for *lpr/lpr* mice, DN T cells isolated from patients presenting with a lymphoproliferative disorder, as a consequence of germline mutations in either Fas or Fas ligand (FasL), abnormally express the B cell antigen B220 [239]. In terms of cytokine production, human DN T cells mainly produce IFNγ [169], however, they also produce IL-4, IL-5, and IL-10, but not IL-2 [169]. Although they do not express granzyme B, they do express the cytolytic protein, perforin [169]. Altogether, human DN T cells are similar in phenotype to mouse DN T cells, suggesting that mouse models are a useful tool to study DN T cells.

1.5.3 Targets and mechanisms of action of DN T cells

The first evidence of an immunosuppressive function for DN T cells arose from a 25 year old study, when Strober and colleagues successfully cloned DN T cells from BALB/c mice and subsequently demonstrated that DN T cells mediate suppressor activity in a mixed-lymphocyte reaction (MLR)[213]. Although the DN T cells were not void of any contaminating NKT cells, these results still suggested an immunoregulatory potential for DN T cells and, consequently, prompted investigators to further explore the function of this rare T cell population.

In subsequent years, the immunoregulatory function of DN T cells has mainly been studied using the TCR transgenic system for several reasons. As explained above, using an αβTCR transgene not only increases the number of DN T cells thereby facilitating the study of this otherwise rare T cell subset, but the insertion of a given αβTCR transgene also facilitates the isolation of a pure DN T cell population void of other contaminating $CD4^-CD8^-$ T cell subsets. Importantly, DN T cells in humans and in non-transgenic mice exhibit a similar phenotype to DN T cells from TCR transgenic mice, demonstrating that the characteristics of DN T cells do not represent a transgenic artefact. Therefore, studies using the TCR transgenic system will undoubtedly give insight into the immunoregulatory function of DN T cells.

1.5.3.1 The immunoregulatory function of TCR transgenic DN T cells

The functional properties of DN T cells have been mainly studied using the 2C TCR transgenic mouse model, which is a MHC class I-restricted model where the DN T cells express the transgenic αβTCR heterodimer as detected by the 1B2 anti-2C clonotypic monoclonal antibody [240]. Using this model, 2C DN T cells were shown to suppress the proliferation and cytotoxic activity of 2C TCR transgenic $CD8^+$ T cells in vitro, but not of $CD8^+$ T cells carrying other antigen specificities [171, 218, 241, 242, 243]. In addition to blocking $CD8^+$ T cell proliferation and function, 2C DN T cells can

also directly lyse $CD8^+$ T cells [171, 186, 218, 225, 243, 244, 245]. The DN T cell-mediated suppression of $CD8^+$ T cells using the 2C TCR transgenic model requires the specific recognition of alloantigens through the TCR and can take place by three different mechanisms, as demonstrated by Dr. Li Zhang's group. The first of these mechanisms describes the direct recognition of an allogeneic-MHC expressed on target $CD8^+$ T cells by the TCR on the effector DN T cells[241]. The second mechanism describes the opposite, such that there is a direct recognition of an allogeneic-MHC expressed on the effector DN T cells by the TCR on the target $CD8^+$ T cells [241]. Accordingly, blocking the MHC on 2C DN T cells impedes their ability to suppress $CD8^+$ T cells [241]. The third mechanism involves a process known as trogocytosis [246]. Essentially, through trogocytosis, DN T cells acquire allogeneic-MHC from an antigen-presenting cell through their TCR and the acquired allogenic-MHC molecule is then presented on the surface of the DN T cell [171, 247]. Syngeneic $CD8^+$ T cells that carry the same TCR specificity as DN T cells can subsequently recognize the acquired allogeneic-MHC now expressed by DN T cells [171]. In all of the mechanisms described, in which an antigen-specific TCR-MHC interaction occurs between the target and effector cells, the target $CD8^+$ T cell will receive a death signal from the effector DN T cell due to a Fas-FasL interaction [171, 218]. Indeed, it has been demonstrated that Fas-deficient DN T cells are cytotoxic towards Fas-sufficient, but not Fas-deficient, $CD8^+$ and $CD4^+$ T cells, whereas FasL-deficient DN T cells are unable to carry out this function [171, 248]. Accordingly, 2C DN T cells obtained from FasL-deficient mice had a significantly reduced, but not completely abrogated, ability to kill allogeneic DCs (further discussed below) [249]. Therefore, it is believed that the Fas-FasL interaction between DN T cells and their target requires FasL expression on the DN T cell and Fas expression by the target cell (**Figure 4A**). Altogether, the immunoregulatory activity of 2C TCR transgenic DN T cells is cell contact-dependent, perforin-independent and is mediated through Fas-FasL interactions [171, 186, 218, 241]

Interestingly, 2C DN T cells were recently shown to down-regulate the expression of co-stimulatory molecules, namely CD80 and CD86, on mature DCs [249]. Furthermore, 2C DN T cells can lyse both immature and mature DCs in an antigen-

specific and Fas/FasL-dependent fashion in vitro [249]. Thus, in addition to their direct immunoregulation of $CD8^+$ T cells, DN T cells can also prevent naive T cell activation/proliferation indirectly through the inhibition of APCs. **Figure 4A** summarizes the targets and mechanism of action of TCR transgenic DN T cells.

With regards to the mechanism of action of DN T cells, the immunoregulatory function of TCR transgenic DN T cells is thus dependent on various factors, including cell contact, perforin and Fas-FasL interactions. Notably, various mechanisms are known to affect the efficacy of DN T cell immunoregulation. For instance, IL-10 has been shown to significantly reduce the cytotoxic function of 2C DN T cell clones towards $CD8^+$ T cells in vitro [224, 250]. Moreover, IL-10 was shown to induce the cellular apoptosis of 2C DN T cells [250]. Additionally, Ly-6A also contributes to 2C DN T cell function as the killing of activated $CD8^+$ T cells is significantly reduced in the presence of blocking antibodies to Ly-6A or when using Ly-6A-deficient 2C DN T cell clones [224]. Interestingly, Ly-6A expression on 2C DN T cells is reduced in the presence of IL-10 [224], suggesting that IL-10 also indirectly affects 2C DN T cell function by reducing Ly-6A expression. Lastly, the Fcγ receptor (FcRγ), which is expressed on 2C DN T cells [251], is also important for 2C DN T cell function as 2C.FcRγ-/- DN T cells are less efficient at inhibiting $CD8^+$ T cell function [251]. Altogether, IL-10, Ly-6A and FcRγ play a crucial role, directly or indirectly, on the immunoregulatory function of DN T cells (**Figure 4A**).

1.5.3.2 The immunoregulatory function of non- transgenic DN T cells

Similarly to TCR transgenic DN T cells, non-transgenic DN T cells maintain an antigen-specific suppressive activity towards $CD8^+$ T cells in vitro [171, 224, 241, 242, 245]. Interestingly, DN T cells from non-transgenic mice also suppress both $CD4^+$ and $CD8^+$ T cell responses [170, 229, 248, 252, 253]. Importantly, in contrast to 2C DN T cells, non-transgenic DN T cells do not require Fas-FasL interactions in order to mediate their cytotoxic function towards $CD8^+$ T cells **(Figure 4B)**. Rather, this cytotoxic function of non-transgenic DN T cells, which are free of most γδ T cells and NKT

cells, is perforin-dependent as perforin-deficient DN T cells are unable to efficiently lyse activated $CD8^+$ T cells as well as $CD4^+$T cells [170, 229]. However, two separate studies demonstrated that the cytotoxic effect towards $CD8^+$ T cells is in fact Fas-FasL dependent [171, 248]. Therefore, additional studies are necessary to clarify these diverging results.

Non-transgenic DN T cells have also shown cytotoxic activity towards activated B cells and DCs [170, 249]. Non-transgenic DN T cells directly lyse activated B cells in a contact- and perforin/granzyme-dependent manner[170], while DC elimination is Fas/FasL dependent[249]. Moreover, non-transgenic DN T cells can down-regulate the expression of co-stimulatory molecules, CD80 and CD86, expressed on antigen-presenting mature DCs[249]. Additional DN T cell targets were also identified using the non-transgenic setting that were not investigated using the TCR transgenic system, including plasma cells [170], macrophages [254], and NK cells [255], where the latter was shown to be lysed using a perforin-dependent mechanism [255]. **Figure 4B** summarizes the targets and mechanism of action of non-transgenic DN T cells.

Although the immunoregulatory function of non-transgenic DN T cells is mainly dependent on cell contact and granzyme/perforin, other mechanisms are known to affect the efficacy of non-transgenic DN T cell function. As was observed for 2C DN T cells, non-transgenic DN T cell expression of Ly-6A was demonstrated to be crucial for optimal DN T cell function, as Ly-6A-deficient DN T cells have impaired cytotoxicity towards $CD8^+$ T cells [224]. Furthermore, CTLA-$4^{-/-}$ DN T cells are unable to mediate the downregulation of costimulatory molecule expression on DCs [249]. Therefore, Ly-6A and CTLA-4 are critical for non-transgenic DN T cell function (**Figure 1B**).

Notably, the mode of action and immunoregulatory properties of TCR transgenic DN T cells appear to quite reliably reflect those of non-TCR transgenic DN T cells, attesting to the validity of using TCR transgenic settings to explore the phenotype and function of this cell type, which is found in low frequency thus

challenging to isolate in the non-transgenic setting (**Figure 4A and 4B**). Moreover, it is noteworthy that the non-TCR transgenic T cells used in the above experiments were either sorted by flow cytometry or purified using magnetic beads to exclude most, but not all, δγ T cells and NKT cells. Indeed, a pure population of DN T cells is difficult to achieve as a specific marker to discriminate DN T cells from type II NKT cells is lacking. Due to this challenge, we cannot disregard the possibility that any variation observed for the mechanism of action of DN T cells using a non-transgenic system is due to type II NK T cell contamination. However, both TCR transgenic and non-transgenic DN T cells exhibit comparable phenotypes and functions, suggesting that these isolation procedures reliably yield a sufficient number of immunoregulatory DN T cells. Nevertheless, additional studies using non-transgenic DN T cells isolated from CD1d-deficient mice are both necessary and crucial to eventually help resolve the exact function of immunoregulatory DN T cells in the absence of potentially contaminating NKT cells.

1.5.3.3 The immunoregulatory function of human DN T cells

The observations made using both TCR transgenic and non-transgenic systems have demonstrated that DN T cells may be of particular interest for cellular therapy due to their ability to eliminate various cellular targets in an antigen-specific manner. Although few studies have been performed using human DN T cells, a parallel can be made between the function of DN T cells in humans and mice (**Figure 4C**). First, DN T cells are cytotoxic towards $CD8^+$ T cells, where the trogocytosis of MHC molecules by human DN T cells enables them to lyse $CD8^+$ T cells in an antigen-specific manner [169]. Moreover, human DN T cells efficiently suppress both $CD4^+$ and $CD8^+$ T cell responses in vitro [238]. However, as opposed to murine DN T cells, Voelkl and colleagues have demonstrated that human DN T cell inhibit $CD4^+$ T cell proliferation independently of perforin/granzyme as well as transforming growth factor beta (TGFβ), IL-10 and Fas-FasL interactions [238]. Although the exact mechanism of action was not determined, DN T cell inhibition of $CD4^+$ T cell proliferation was shown to be dependent on cell contact, TCR signalling and

intracellular protein transport as the presence of a transwell, Lck inhibitor or monensin, respectively, blocked the $CD4^+$ T cell suppression [238]. Clearly, additional studies are needed to validate the cellular targets of human DN T cells as well as their mechanism of action.

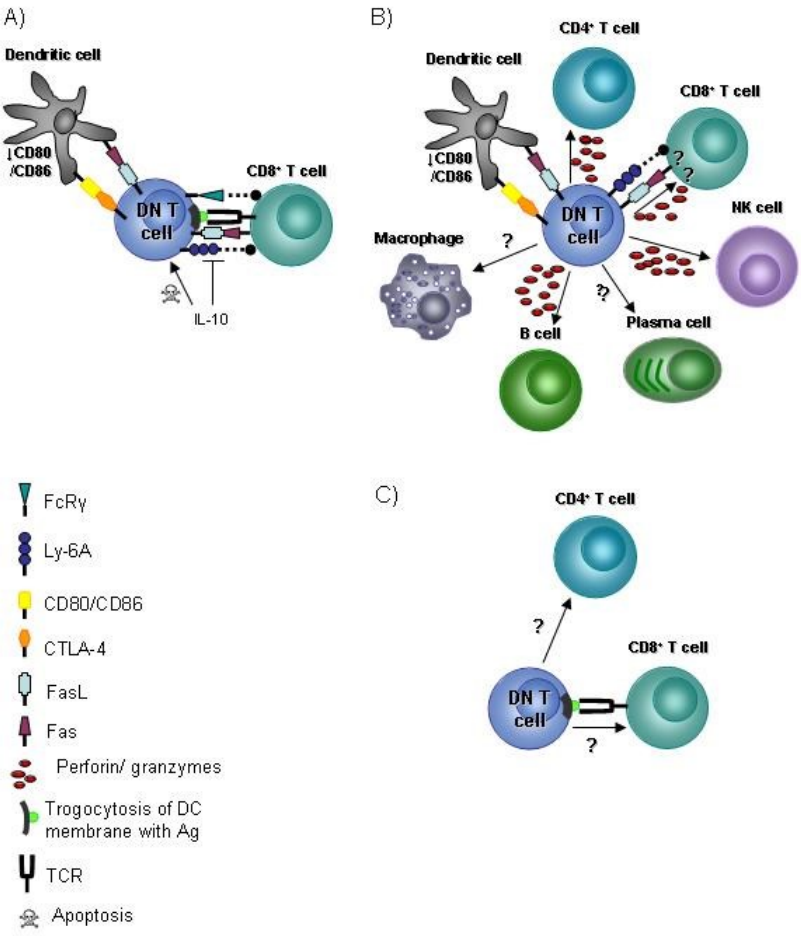

Figure 4. The targets and mechanism of action of DN T cells. TCR transgenic (A), human (B) and non-transgenic (C) DN T cells target various cell populations using different mechanisms of actions. Modified from [156] (Hillhouse, E.E. & Lesage, S. A (2012)).

1.5.3.4 In vivo function of immunoregulatory DN T cells

Although the literature for the in vitro function of DN T cells is vast, publications on the in vivo function of DN T cells are quite sparse. Indeed, using non-transgenic DN T cells, a single study has confirmed a targeted population in vivo, namely B lymphocytes. Ma, Y and colleagues [256] used a cardiac xenotransplantation model to verify whether DN T cell transfer, in addition to treatment with an immunosuppressive reagent, could ameliorate graft survival. Importantly, the transfer of perforin-sufficient, not perforin-deficient, DN T cells led to an increase in graft survival, which correlated with an increase in B cell apoptosis as well as a decrease in anti-donor total immunoglobulin (Ig) G and IgM antibody production and IgG graft deposition. Moreover, DN T cells from a third party also induced B cell apoptosis, indicating an antigen non-specific mechanism of action in vivo. Therefore, non-transgenic DN T cells eliminate B cells in vivo using perforin-dependent and antigen-independent mechanisms.

The in vivo function of DN T cells has also been studied using a TCR transgenic system, namely the 3A9 TCR transgenic model, which, as opposed to the 2C TCR model, is a MHC class II-restricted model [257] where the DN T cells express the transgenic αβTCR heterodimer as detected by the 1G12 anti-3A9 clonotypic mAb [258]. Following the studies performed by Ma, Y and colleagues [256], our lab has demonstrated that a single transfer of autologous TCR 3A9 transgenic DN T cells resulted in a significant reduction in antigen-specific IgG, but not IgM, autoantibody serum levels in diabetes-susceptible mice in comparison to mice that did not receive 3A9 DN T cells [214], further suggesting that DN T cells can eliminate autoreactive B cells in vivo via an antigen-specific mechanism. However, it remains to be demonstrated whether 3A9 DN T cells can directly lyse autoreactive B cells and via which mechanism of action this cytotoxic activity occurs.

Altogether, the observations made using both TCR transgenic and non-transgenic systems have demonstrated that DN T cells may be of particular interest

for cellular therapy due to their ability to eliminate various cellular targets in an antigen-specific manner and that a parallel can be made between the function of DN T cells in both humans and mice.

1.5.4 DN T cells in transplantation and disease models[v]

A central objective in organ transplantation and the treatment or prevention of autoimmune disease is the achievement of antigen-specific immune tolerance. As DN T cells exhibit a unique antigen-specific immunoregulatory potential towards multiple cellular targets, it suggests that DN T cells may serve as a solution to this challenge. Consequently, investigators were prompted to examine the role of DN T cells in various disease models, including graft-vs-host disease, graft tolerance, cancer and autoimmunity.

1.5.4.1 Graft tolerance

Although donor leukocyte infusion (DLI) is primarily used to prevent the relapse of cancer via its graft-vs-tumor effect, DLI treatments are also beneficial in solid organ transplants. In an attempt to understand why DLI has a positive outcome on allograft survival, Dr. Zhang's group took advantage of the antigen-specific 2C TCR transgenic model[260, 261], where the 2C TCR is alloreactive to the L^d MHC class I molecule[240]. Predictably, skin grafts bearing a single MHC mismatch at L^d are rapidly rejected by the 2C TCR recipient mice due to the expression of L^d MHC class I molecule on the donor skin cells[260, 261]. However, the injection of donor spleen cells to the 2C TCR recipient mice prior to the skin graft efficiently induced antigen-specific allograft tolerance[260, 261]. Interestingly, the antigen-specific tolerance to skin allografts induced by the transfer of donor T cells was proposed to be mediated by 2C

[v] Text similar to that in section 1.5.4 and its subsections was recently accepted for publication as a review article: Reference 259. Hillhouse EE, Delisle JS, Lesage S. Immunoregulatory CD4(-)CD8(-) T cells as a potential therapeutic tool for transplantation, autoimmunity, and cancer. *Frontiers in immunology* 2013, **4:** 6.. Hillhouse, E.E., Delisle, J.S. & Lesage, S. Immunoregulatory CD4(-)CD8(-) T cells as a potential therapeutic tool for transplantation, autoimmunity, and cancer. *Frontiers in immunology* **4**, 6 (2013).

TCR transgenic DN T cells as DN T cell numbers are increased directly within the skin allograft following adoptive transfer of donor lymphocytes[242] and this increase correlated with antigen-specific tolerance of both skin and cardiac allografts [171, 225, 243, 244]. Moreover, only the 2C TCR transgenic DN T cell subset, but not the 2C $CD4^+$ or 2C $CD8^+$ T cell subset, was able to suppress an MLR response in vitro[171]. Subsequently, Zhang's group went on to show that the injection of 2C TCR F1 DN T cell clones was sufficient to induce prolonged survival of both skin and cardiac allografts survival[171, 244], demonstrating that DN T cells do indeed promote allotolerance. Importantly, the allograft tolerance was antigen-specific, as full MHC-mismatched third party grafts were rapidly rejected[171]. Collectively, these data demonstrate that 2C DN T cells are sufficient to induce both skin and cardiac allograft survival, suggesting that immunoregulatory DN T cells contribute to the benefits of DLI on allograft survival.

To further understand the mechanism by which 2C DN T cells promote antigen-specific allograft tolerance, Young and colleagues undertook the examination of the leukocytes found within the tolerated skin grafts. Indeed, the 2C DN T cells isolated from mice which had received donor spleen cells prior to the skin graft demonstrate an enhanced suppressive function towards 2C $CD8^+$ T cells in vitro[242], demonstrating that 2C DN T cells promote graft tolerance, at least in part, through the elimination of pathogenic $CD8^+$ T cells. Furthermore, the comparison of transcriptome profiles between 2C TCR F1 DN T cells clones that are able or unable to confer cardiac allograft tolerance revealed FcRγ as a potential molecule involved in defining the tolerogenic potential of 2C DN T cells[225]. The importance of FcRγ expression on 2C DN T cells for the induction of allograft tolerance was confirmed as the adoptive transfer of FcRγ-sufficient, but not FcRγ-deficient, 2C DN T cells prior to transplantation increased skin allograft survival[251]. Altogether, these results demonstrate that 2C DN T cells participate in allograft tolerance, likely by inhibiting pathogenic 2C $CD8^+$ T cell responses, at least in the MHC class I-restricted graft tolerance model[156].

These observations were not limited to the 2C TCR transgenic setting. Accordingly, DLI treatment prior to transplantation in the non-transgenic setting resulted in long-term cardiac xenograft survival as well as an increase in the proportion of DN T cells in recipient mice[252], suggesting that non-transgenic DN T cells contribute to graft tolerance. Consistent with this, DN T cells isolated from DLI-treated mice are able to promote the survival of cardiac [170, 253] and skin grafts [248] while in vitro-generated DN T cells can also promote skin as well as pancreatic islet allograft tolerance[229, 262]. Importantly, the graft tolerance provided by non-transgenic DN T cells is antigen-specific as third-party skin allografts were rejected [248], further demonstrating a key characteristic of DN T cells, namely antigen-specificity.

The targets of non-transgenic DN T cells during the induction of tolerance are diverse, as is expected. Indeed, DLI-activated DN T cells efficiently suppressed the proliferation of both $CD4^+$ and $CD8^+$ T cell anti-donor reactive cells[252, 253], which may explain how the injection of non-transgenic rat donor spleen cells prevented $CD4^+$ T cell-mediated cardiac xenograft rejection in recipient mice[252, 253]. Additionally, it was also shown that non-transgenic DN T cells can eliminate B cells in vivo, leading to a reduction in anti-donor specific antibody levels and delayed cardiac xenograft rejection[256], suggesting that DN T cells can prevent antibody-mediated acute graft rejection, which is a major obstacle in xenograft survival. Taken together, these findings suggest that, as opposed to the MHC class I-restricted 2C TCR DN T cells, non-transgenic DN T cells promote antigen-specific graft tolerance via the suppression of $CD4^+$ and $CD8^+$ T cells as well as B cells.

The mechanism of action of DN T cells during graft tolerance has also been studied using the non-transgenic setting. As mentioned in Section 1.5.3.1, Fas-FasL interactions have been proposed as the molecular mechanism by which DN T cells eliminate anti-donor T cells, while the elimination of B cells is considered to be perforin-mediated[170, 171, 218, 256]. By taking advantage of Fas-deficient *lpr/lpr* mice, Ford et al. demonstrated that DN T cells can mediate their immunoregulatory function through the Fas pathway as long as the target T cells express a functional

Fas protein[248]. Indeed, DN T cells from lpr mice could effectively delay both single MHC class I- and single MHC class II-mismatched skin allograft rejection[248]. Moreover, mice that received perforin-sufficient, but not perforin-deficient, DN T cells ultimately accepted their cardiac xenograft transplant[256]. In summary, non-transgenic DN T cells can clearly induce an antigen-specific tolerance to both allografts and xenografts by eliminating or inhibiting the function of various target immune cells via perforin-mediated and/or Fas/FasL pathways. Altogether, these results validate the potential to use DN T cells as a cellular treatment to promote graft survival.

1.5.4.2 Graft-vs-host disease (GVHD)

In the treatment of hematological malignancies, allogeneic hematopoietic cell transplantation (AHCT) can eradicate several blood cancers that are reported to be incurable by chemotherapy alone. Despite indisputable successes, the efficacy of AHCT is still limited by cancer recurrence and the development of GVHD[263]. While the acute form of GVHD is triggered by direct T cell recognition of histocompatibility antigens, the pathophysiology of chronic GVHD remains more elusive and relies on several immune cell types[264]. Currently, 40 to 80% of patients develop chronic GVHD after AHCT, which brings substantial morbidity and mortality[265]. Several lines of evidence suggest that immunoregulatory immune cells, including immunoregulatory DN T cells, are paramount to GVHD prevention.

DN T cells exhibit an immunoregulatory function that would make them an ideal candidate for the prevention of GVHD. A potential role for DN T cells in the prevention of GVHD can be traced back to over two decades ago when Bruley-Rosset and colleagues demonstrated that the transfer of spleen cells from pre-immunized mice was able to inhibit GVHD only when DN T cells were included in the cellular preparations[266]. Although the DN T cells were not void of contaminating NKT cells, these results were the first to demonstrate a potential role for DN T cells in the prevention of GVHD. The combination of these findings along with functional

studies that demonstrated their prominent antigen-specific immunoregulatory potential, led investigators to further examine the contribution of DN T cells towards GVHD prevention using the 2C TCR single MHC mismatch model[243]. Specifically, mice bearing a single MHC-mismatch (L^d) injected with L^d-specific 2C TCR transgenic spleen cells survived for more than 150 days without any clinical or histological signs of acute or chronic GVHD[243, 267]. The tolerance of donor spleen cells may be explained, at least in part, by the accumulation of 2C TCR DN T cells[243, 267], which can effectively inhibit pathogenic $CD8^+$ T cell responses, in recipient mice[243]. Indeed, GVHD development caused by the transfer of 2C $CD8^+$ T cells was significantly decreased when recipient mice were co-infused with 2C DN T cell clones[243]. Based on these findings and what is known about the function of 2C DN T cells, Young and colleagues have proposed the following explanation for the role of DN T cells in the prevention of GVHD[267]; Following the transfer of allogeneic splenocytes to the recipient, DN T cells become activated and expand to become the major subset of T cells. DN T cells will then acquire allogeneic-MHC molecules from APCs, which can be recognized by anti-host T cells with the same TCR specificity. The anti-host T cells are subsequently killed by the DN T cells via the Fas/FasL pathway. Although it is not known whether the prevention of GVHD is caused directly through the elimination of alloreactive T cells, indirectly as a result of the elimination host DCs or B cells, or a combination of both, it is clear that DN T cells do indeed play a role in the prevention of GVHD.

Subsequently, the role of non-transgenic DN T cells in GVHD prevention was investigated using both parent to F1 and fully MHC-mismatched bone marrow transplantation following either a myeloablative[255] or non-myeloablative regimen[268]. In stark contrast to $CD4^+$ and $CD8^+$ T cells, the co-injection of non-transgenic DN T cells with T cell-depleted bone marrow into a sublethally irradiated host ensured prolonged recipient survival in the absence of clinical signs of GVHD[255, 268], indicating that DN T cells are not alloreactive nor pathogenic in this context. Moreover, this led to stable mixed chimerism and, as opposed to bone marrow transplantation alone, promoted donor allotolerance[255, 268]. Together, these findings

demonstrate that, as opposed to $CD4^+$ or $CD8^+$ T cells, the transfer of allogeneic DN T cells does not cause GVHD. Rather, DN T cells prevent GVHD, induce mixed chimerism and promote donor-specific allotolerance.

In support of Strober and colleagues' initial experiment in mouse in 1989, Kusnierz-Glaz and colleagues demonstrated that human DN T cells were also able to suppress a MLR in vitro, indicating that DN T cells may be able to prevent GVHD in humans[269]. Despite the lack of a pure DN T cell population due to the absence of the necessary NKT cell markers during DN T cell isolation, these results still paved the way for additional studies on human DN T cells and their role in the prevention of GVHD. More recently, two studies further suggested that human DN T cells are able to prevent GVHD as a clear correlation was observed between an elevated proportion of DN T cells and a low incidence of GVHD development in patients following AHCT[270, 271]. Indeed, the stem cells used for AHCT are currently most commonly obtained from the peripheral blood mononuclear cells of granulocyte colony-stimulating factor (G-CSF)-treated donors, where G-CSF is used to mobilize $CD34^+$ hematopoietic stem cells into the blood[264]. Interestingly, in addition to skewing towards a T helper 2 (Th2) phenotype, G-CSF treatment also results in an increase in the proportion of immunoregulatory DN T cells in the blood[269]. The combination of these two phenotypes may explain why the transfer of $CD34^+$ stem cells isolated following G-CSF treatment does not increase the incidence of acute GVHD relative to classical bone marrow transplants. Together, these observations support the view that human DN T cells may prevent GVHD.

1.5.4.3 Cancer

Although immunoregulatory T cells are known to promote tolerance, they typically have a negative impact on cancer prognosis [272, 273, 274, 275]. Indeed, the ability of Tregs to maintain the anti-tumor T cell responses remains controversial as Tregs have been demonstrated to also inhibit anti-tumor T cell responses, which could ultimately contribute to the progression of cancer[272, 273, 274, 275, 276]. However, DN T

cells exhibit a counter-intuitive anti-tumoral role, regardless of their immunoregulatory activity. Specifically, both 2C TCR transgenic and non-transgenic DN T cells isolated from the spleens of mice that were co-infused with a lethal dose of A20 lymphoma cells together with allogeneic splenocytes demonstrated cytotoxic activity towards A20 lymphoma cells in vitro[267]. Subsequently, Young and colleagues demonstrated that the injection of either 2C DN T cell clones or non-transgenic DN T cells was sufficient to prevent A20 lymphoma tumor growth[267]. Interestingly, Young and colleagues did not observe killing towards the P815 tumor cells and attributed this observation to the low expression of Fas by the P815 cell line, whereas the A20 tumor cell line expresses a high level of Fas[267]. Importantly, Young and colleagues were able to demonstrate that the lysing of the tumor cells was indeed Fas-dependent as 2C DN T cell clones that had been pre-incubated with a Fas-Fc fusion protein prior to the co-culture with A20 tumor cells had a decrease in their killing ability[267]. Thus, the elimination of tumor cells by DN T cells is Fas-dependent, at least in the 2C TCR transgenic system.

To further define the potential use of DN T cells in the treatment of hematological malignancies, Merims et al. performed a pre-clinical study in acute myeloid leukemia (AML) patients undergoing complete remission following standard chemotherapy[277]. In this study, they demonstrate that DN T cells can be readily isolated from the blood of AML patients. Moreover, the isolated DN T cells maintain their phenotype following a proficient cellular expansion reaching nearly 500-fold in two weeks[277]. Of interest, the DN T cells isolated and expanded in vitro demonstrated efficient cytolytic activity towards the autologous leukemic cells in 6 out of 7 patients[277]. Importantly, the anti-tumoral activity of DN T cells may extend beyond hematological malignancies, as melanoma-reactive DN T cells have also been successfully isolated from a previously immunized melanoma patient[278]. Altogether, these results demonstrate that DN T cells exhibit anti-tumoral activity all the while promoting tolerance.

The antigen-specific immunoregulatory potential of DN T cells in graft tolerance suggests that they may also participate in the induction of immune tolerance in various autoimmune settings. As a result, the role of DN T cells has been mostly explored in systemic lupus erythematosus (SLE), autoimmune lymphoproliferative syndrome (ALPS) and mouse models of autoimmune diabetes, as described below.

1.5.4.4 Systemic lupus erythematosus (SLE)

Although SLE patients present with greater numbers of DN T cells in their peripheral blood [279], these DN T cells are major producers of IL-17 [280], which is pathogenic in this disease [281, 282]. Moreover, IL-17-producing DN T cells can be found in the kidney sections of patients with lupus nephritis [279]. These results lead Crispin and colleagues to suggest that DN T cells themselves are pathogenic in SLE. However, it must be noted that, in these studies, DN T cells were defined as CD4-CD8-TCRαβ+ cells, which may contain contaminating NKT cells. NKT cells do indeed include a subset of $CD4^{-}NK1.1^{-}$ IL-17-producing cells [283]. Moreover, using a mouse model, the activation of NKT cells through the administration of α-GalCer exacerbated disease whereas the inhibition of NKT cell activation using an anti-CD1d blocking antibody resulted in disease amelioration, suggesting that NKT cells are implicated in SLE pathogenesis [284]. Thus, as for the response against infections, NKT cells cannot be ruled out as the true IL-17-producing cell subset involved in the pathogenesis of SLE. Altogether, an uncertain, yet possible, role for DN T cells in the pathogenesis of SLE remains to be defined.

1.5.4.5 Autoimmune lymphoproliferative syndrome (ALPS)

ALPS is a rare disorder characterized by mutations in either Fas or Fas ligand (FasL) [285, 286], which results in defective Fas-mediated apoptosis and, consequently, the abnormal proliferation of lymphocytes. Similar to patients with SLE, ALPS patients on average present with a tenfold increase in DN T cell proportion [176, 239, 287] and a sizeable proportion of $CD4^{-}CD8^{-}TCR\alpha\beta^{+}$ DN T cells can also be found in the

lymphoid organs of the murine models for ALPS, namely *lpr/lpr* (Fas-deficient) and *gld/gld* (FasL-deficient) mice [232, 233, 234]. Moreover, a greater proportion of DN T cells from ALPS patients, in comparison to healthy controls, express the B cell antigen, B220 [239], which is consistent with findings in *lpr/lpr* mice [236]. Accordingly, B220 expression is a general feature of proliferating T cells [288], which explains why healthy patients also express $B220^+$ DN T cells. Although it cannot be denied that a drastic increase in the proportion of DN T cells can be observed in *lpr/lpr* and *gld/gld* mice, as well as ALPS patients, there is no evidence to suggest that the accumulation of DN T cells is pathogenic. Indeed, Fas-deficient DN T cells found in *lpr/lpr* mice, but not FasL-deficient mice found in *gld/gld* mice, remain functional [248]. Hence, as DN T cell-mediated suppression depends on interactions between FasL on DN T cells and Fas on target T cells [248] it has been suggested that DN T cells accumulate in an attempt to compensate for their inability to suppress autoimmune T cells in *lpr/lpr* mice [248].

1.5.4.6 Autoimmune diabetes

The tolerogenic role of DN T cells in the prevention of autoimmune diabetes was first revealed using the P14/RIP-gp transgenic mouse model, where the MHC class I-restricted P14 TCR transgene recognizes the lymphocytic choriomeningitis virus (LCMV) protein, gp33–41 (gp33), which is expressed under the rat insulin promoter (RIP) in this model, forcing its expression in the pancreatic tissue. The resulting double transgenic P14/RIP-gp mice do not spontaneously develop diabetes[289]. However, when treated with gp33 peptide in combination with an anti-CD40 agonist antibody, P14/RIP-gp mice carry a very high percentage of gp33-reactive T cells and rapidly develop diabetes[290]. Indeed, P14/RIP-gp transgenic mice develop diabetes as a result of the infiltration of pancreatic islets by activated $CD8^+$ T cells and the subsequent destruction of the insulin-producing β cells. Using this model, it was shown that the transfer of gp-33 activated P14 DN T cells one day prior to diabetes induction can inhibit diabetes development[186]. These results were the first indication that DN T cells may participate in the prevention of a $CD8^+$ T cell-driven

autoimmune diabetes pathology, further lending support for a potential in vivo role of DN T cells towards the elimination of $CD8^+$ T cells in vivo.

The protective role of DN T cells in diabetes development was further investigated by our group using the 3A9 TCR:insHEL transgenic system, in which the MHC class II-restricted 3A9 TCR transgene recognizes a peptide from hen egg lysozyme (HEL) presented by I-A^k, while the insHEL transgene forces the expression of HEL in the pancreatic tissue. Although TCR:insHEL BALB.K mice are relatively resistant to autoimmune diabetes, CD47-deficient TCR:insHEL BALB.K mice have a high and spontaneous incidence of diabetes. Using this model of spontaneous autoimmune diabetes, a single transfer of 3A9 DN T cells in the TCR:insHEL CD47-deficient BALB.K transgenic model was able to significantly inhibit the development of autoimmune diabetes [214]. As noted previously, 3A9 DN T cell transfer lead to a significant reduction in antigen-specific autoantibody serum levels [214], suggesting the in vivo elimination of autoreactive B cells. Although this model is imperfect in that it is a CD47-deficient model, where CD47 is implicated in apoptosis, phagocytosis, cell migration and T cell responses [291, 292], it is nevertheless a spontaneous model of autoimmune diabetes and the results complement the findings of Ford and colleagues [186] suggesting that DN T cells may be of therapeutic interest for autoimmune diabetes.

Interestingly, the proportion of DN T cells is significantly reduced in diabetes-prone mice in comparison to diabetes-resistant mice in both the transgenic and non-transgenic systems [214]. It is of relevance that both the non-transgenic and TCR:insHEL transgenic diabetes-prone NOD mouse models exhibit a low number of DN T cells relative to other diabetes-resistant strains as it suggests that low DN T cell numbers are associated with diabetes susceptibility. Importantly, these results suggest that the restoration of DN T cell number can prevent autoimmune diabetes development in otherwise diabetes-susceptible mice.

The role of DN T cells in the prevention of autoimmune diabetes was also recently evaluated using non-transgenic DN T cells. Indeed, Duncan and colleagues have demonstrated that non-transgenic DN T cells can protect from autoimmune diabetes[230]. Specifically, diabetes induction was inhibited if DN T cells were transferred into recipient NOD.SCID mice 1 month prior to co-infusion with diabetogenic splenocytes, whereas the simultaneous co-infusion of diabetogenic splenocytes and DN T cells does not protect from disease[230]. However, as NKT cells were not fully excluded from the cellular preparation, additional studies using NKT cell-depleted non-transgenic DN T cells are warranted to firmly establish the biological function of these cells in the prevention of autoimmune diabetes in diabetes-susceptible NOD mice. Notably, further investigation is still needed to verify the role of DN T cells in autoimmune diabetes development using non-lymphopenic NOD mice, thus under more physiological and spontaneous conditions. Nevertheless, these results do provide useful information regarding the critical time of treatment initiation as well as evidence that non-transgenic DN T cells may exhibit the potential to inhibit autoimmune diabetes development in non-transgenic mice.

A most recent study further evaluated the role of non-transgenic DN T cells in autoimmune diabetes. Here, it was shown that 50% of $CD4^+$ T cells isolated from an MLR (with or without GAD65 peptide) had been converted to $CD4^-CD8^-$ T cells. These $CD4^+$ T cell-converted DN T cells sorted by flow cytometry were shown to delay autoimmune diabetes onset when adoptively transferred to NOD.SCID mice in combination with diabetogenic T cells[262]. This delay was further enhanced when using GAD65 antigen to stimulate the $CD4^+$ T cells, thereby likely generating a higher proportion of GAD65-specific DN T cells. Moreover, a single transfer of DN T cells enriched for GAD65 specificity was able to prevent diabetes development in 5-week-old NOD mice and decrease blood glucose levels in new-onset diabetic NOD mice. Lastly, GAD65-specific DN T cells in combination with rapamycin treatment, and not DN T cells or rapamycin alone, promoted islet allograft transplant survival in new-onset diabetic NOD mice. Therefore, GAD65-specific $CD4^+$ T cell-converted DN T cells can prevent and reverse the development of autoimmune diabetes as well

as promote islet allograft survival[262]. It remains to be determined whether DN T cells originating from down-regulation of CD4 expression upon in vitro stimulation exhibit the same characteristics as DN T cells present in vivo. Nevertheless, this study presents an interesting and potentially translatable therapeutic approach for the generation of antigen-specific tolerogenic $CD4^{-}CD8^{-}$ T cells in the prevention of autoimmune diabetes.

1.5.4.6.1 Comparing DN T cells and NK T cells and their role in autoimmune diabetes pathology

Indeed, type II NK T cells express a similar immunoregulatory function as DN T cells Not only do NKT cells share various phenotypical features with DN T cells, they also share a common immunoregulatory mechanism of action, which has been mostly investigated in the autoimmune diabetes setting. As is observed for DN T cells, diabetes-susceptible NOD mice also exhibit a marked deficiency in NKT cell number [293, 294] as well as NKT cell function, as observed by the decrease in IL-4 production [293, 294, 295, 296, 297]. However, in contrast to DN T cells, the reduction in NK T cell number is more pronounced in the thymus and, to a lesser extent, in the periphery [294]. Importantly, CD1d-deficient, thus NKT cell-deficient, NOD mice exhibit an accelerated onset of autoimmune diabetes [298, 299], suggesting that NKT cells, similar to DN T cells, play a role in the prevention of autoimmune diabetes progression. Accordingly, CD4-CD8- thymocytes, which are enriched for NKT cells, were shown to prevent the development of autoimmune diabetes when transferred into 4-week-old NOD recipient mice [294]. Moreover, the in vivo treatment of NOD mice with the α-GalCer ligand, which activates type I NKT cells, was shown to prevent diabetes in NOD mice [297, 300, 301], while TCR transgenic NOD mice carrying a CD1d-restricted TCR transgene that is specific for type II NKT cells also prevented autoimmune diabetes development [302]. Therefore, similar to DN T cells, both type I and type II NKT cells play a role in diabetes resistance.

As the discrimination between DN T cells and type II NK T cells remains a challenge (refer to Section 1.5.1), these results further demonstrate that the use of CD1d-deficient mice, which, by definition, lack both type I and type II NK T cells, is essential for the study of non-transgenic DN T cells devoid of any contaminating cells. Indeed, the lack of a specific marker to discriminate DN T cells from type II NKT cells renders it difficult to assure the purity of the DN T cells being studied, which may lead to ambiguous interpretations of results and the role DN T cells play in disease pathology. Accordingly, the study of non-transgenic DN T cells using CD1d-deficient mice would secure the integrity of the DN T cells and the corresponding results.

1.6 Future directions

Altogether, DN T cells may be of particular interest for cellular therapy due to their ability to eliminate various cellular targets in an antigen-specific manner. However, there is a clear lack of in vivo data to validate the various targets of DN T cells that were established using in vitro assays which will need to be resolved moving forward. Indeed, to our knowledge, only B cells have been confirmed as targets of DN T cells in vivo [256]. Moreover, the lack of a specific marker to identify DN T cells remains a central issue in this field of study. Indeed, as a specific marker to discriminate DN T cells from type II NKT cells is lacking, we cannot disregard the possibility that some of the variation that has been observed for the mechanism of action of DN T cells in both non-transgenic mice and human studies is due to type II NK T cell contamination. As a result, we propose that CD1d-deficient mice are both necessary and crucial for the study of non-transgenic DN T cells since, by definition, these mice lack both type I and type II NK T cells, thus allowing to specifically isolate DN T cells. Moreover, the isolation and validation of a specific marker able to differentiate DN T cells from type II NKT cells should remain a priority. This marker will not only facilitate studying DN T cells in the non-transgenic setting, but also in humans, including the translation to various clinical studies. Indeed, DN T cells demonstrate therapeutic potential in various disease settings, including autoimmune diabetes. We believe there is a need to further investigate the role of DN T cells in the prevention of autoimmune diabetes in the non-transgenic system, which is more physiologically relevant to humans. To do this, the implementation of CD1d-deficient mice becomes crucial for the isolation of DN T cells void of any contaminating NKT cells. As such, the study of their function in diabetes-susceptible mice as well as their ability to prevent autoimmune diabetes in the non-transgenic setting will help to reveal their therapeutic potential in the context of autoimmune diabetes in humans.

1.7 Rationale, hypothesis and objectives

DN T cells exhibit an antigen-specific immunoregulatory potential in various experimental settings, including the prevention of autoimmune diabetes. Indeed, by taking advantage of the 3A9 TCR:insHEL transgenic system, we have shown that low DN T cell proportion associates with autoimmune diabetes susceptibility and that a single transfer of TCR transgenic DN T cells is sufficient to prevent disease progression in otherwise autoimmune diabetes-prone mice [214]. Interestingly, the DN T cell-injected mice presented with a significant reduction in antigen-specific autoantibodies [214]. Altogether, these results suggest that DN T cell restoration leads to the elimination of autoreactive B cells *in vivo* and that DN T cells may be of therapeutic interest for diabetic patients. However, before moving towards a clinical setting, it is important to characterize DN T cells in the non-transgenic, TCR polyclonal setting, therefore under more physiologically relevant conditions, as well as determine whether DN T cells in the diabetes-susceptible NOD mouse are functional. This led us to hypothesize that:

Restoring DN T cell number in non-Tg autoimmune-prone mice prevents diabetes onset and may be of therapeutic interest for autoimmune diabetes in humans.

The main objectives of the thesis are to:

- Determine DN T cell phenotype and proportion using non-transgenic mice.
- Verify whether DN T cells from diabetes-susceptible mice are equally functional.
- Demonstrate whether non-transgenic DN T cells can delay/prevent diabetes development in vivo.
- Define the mechanism of action of non-transgenic DN T cells in vivo.

CHAPTER 2 : ARTICLE 1

2.1 IL-10 limits the expansion of immunoregulatory CD4-CD8- T cells in autoimmune-prone NOD mice

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I evaluate my contribution to this article at 85%. I participated in the experimental design alongside G. Chabot-Roy and S. Lesage. C. Beauchamp demonstrated the 3A9 DN T cell IL-10 production (Figure 3). G. Chabot-Roy determined the cellular target of 3A9 DN T cells by performing a cytotoxicity assay (Figure 1D). C. Beauchamp and V. Dugas contributed to the phenotyping of 3A9 DN T cells (Figure 1A,1C). Personally, I performed the experiments that produced the remaining figures. I set up the CD107a assay and determined both the cytotoxic potential and cytotoxic activity of 3A9 DN T cells, with the exception of Figure 1D. I performed all of the cellular expansions as well as the proliferation and apoptosis assays. I actively participated in the interpretation and analysis of the results, the generation of the figures, the writing of the manuscript as well as the response to the reviewers, which were accomplished with the help of S. Lesage.

The results presented in this article are essential to the thesis as they describe the target of 3A9 DN T cells and show the comparable cytotoxic activity of 3A9 DN T cells from the diabetes-resistant and -susceptible genetic backgrounds. Therefore, these results demonstrate that the association of DN T cells to T1D susceptibility is due to a deficiency in DN T cell number, not function. In addition, the findings presented in this article reveal that DN T cell expansion is regulated, at least in part, by IL-10, suggesting that the IL-10 pathway may contribute to the regulation of DN T cell number.

Title : IL-10 limits the expansion of immunoregulatory CD4-CD8- T cells in autoimmune-prone NOD mice

Running title: IL-10 limits DN T cell expansion

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2.2 Abstract

Regulatory T cells appear to show great potential for use in cellular therapy. In particular, CD4-CD8- (DN) T cells, which compose 1 to 3% of total T lymphocytes, exhibit prominent antigen-specific immune tolerance properties and confer immune tolerance in models of allografts and xenografts. We have recently demonstrated that autoimmune-diabetes prone mice carry fewer DN T cells and that this phenotype contributes to autoimmune-diabetes susceptibility, suggesting that increasing DN T cell number in autoimmune-prone individuals may be of therapeutic interest. To achieve this goal, we must first determine whether the remaining DN T cells in autoimmune-prone mice are functional. In addition, we must identify the parameters which regulate the numbers of DN T cells. Herein, we evaluate the immunoregulatory properties of DN T cells in the autoimmune-prone NOD genetic background. Using the 3A9 TCR transgenic mice, we demonstrate that DN T cells from both diabetes-resistant B10.Br and genetically autoimmune-prone NOD.$H2^k$ mice show an equivalent immuno-regulatory potential on a per cell basis. However, upon stimulation, there is a tenfold increase in the number of 3A9 TCR transgenic DN T cells that produce IL-10 from NOD.$H2^k$ mice in comparison to B10.Br mice. We further demonstrate that IL-10 facilitates DN T cell apoptosis and thus may regulate the number of DN T cells. Together, our results demonstrate that, although reduced in number, DN T cells from mice carrying an autoimmune-prone genetic background exhibit a potent cytotoxic potential and that DN T cell expansion is regulated at least in part by IL-10.

Keywords: autoimmune diabetes, IL-10, immune regulation, T cells, transgenic mice

2.3 Introduction

Many different T cell subsets have been shown to regulate immune responses and/or autoimmunity including $CD4^+CD25^+FoxP3^+$ naturally occurring T cells (nTregs), IL-10-producing $CD4^+$ Tr1 cells, TGF-β-producing $CD4^+$ Th3 cells, $CD8^+CD28^-$ T cells, $CD4^-CD8^-$ (DN, double negative) T cells, $CD4^+CD8^+$ (DP, double positive) T cells, and Natural Killer T (NKT) cells [1-3]. The contribution of these respective subtypes to the proper control of immune tolerance and prevention of autoimmune disease remains to be defined. Their diverse antigenic specificity and/or distinct mode of action hint that the contribution of each of these T cell subsets to specific peripheral tolerance requirements may vary [2]. Hence, there is a need to further examine the contribution of various T cell subsets to autoimmune predisposition.

Of these regulatory T cell subsets, CD4-CD8- (DN) T cells compose 1%–3% of peripheral T lymphocytes in both mice and humans [4, 5]. Importantly, the insertion of a TCR transgene has facilitated the identification and isolation of DN T cells, as well as their implication in antigen-specific immunosuppression [6-12]. Indeed, using various TCR transgenic mouse models, it has been shown that DN T cells carry a unique immunoregulatory potential, which resides in their ability to specifically suppress T cells in an antigen-specific manner [13-18]. DN T cells acquire antigen-specific peptide/MHC complexes through their interaction with antigen presenting cells and can subsequently present peptide/MHC complexes to CD8 T cells and eliminate the latter [13, 17]. Interestingly, these characteristics are equally shared by human DN T cells [4]. Moreover, non-transgenic DN T cells can also efficiently eliminate activated B cells presenting the cognate antigen [5]. Taken together, these data demonstrate that the immunosuppressive function of DN T cells is mediated, at least in part, through the antigen-specific elimination of activated T or B cells.

This antigen-specific immunoregulatory function of DN T cells was further revealed in the context of transplant tolerance mouse models [13, 14, 19]. Pre-transplant

treatment with donor–lymphocyte infusion (DLI) results in a significant increase in the proportion of antigen-specific DN T cells in accepted allografts and xenografts [13, 14, 19]. In addition, *in vivo* transfer of antigen-specific DN T cell clones prevents skin and cardiac allograft rejection [13, 15]. Activated DN T cells were also shown to protect from graft-vs-host disease and antigen-induced autoimmune diabetes [16, 18]. Therefore, the relatively rare DN T cell subset exhibits a remarkably potent immunosuppressive potential in multiple model systems, from transplantation to autoimmunity.

More recently, our group investigated the role of DN T cells in spontaneous models of autoimmune-diabetes. We took advantage of the 3A9 TCR:insHEL transgenic system, where the 3A9 TCR transgene recognizes a peptide from hen egg lysozyme (HEL) in the context of I-A^k [20], while the insHEL transgene enforces expression of HEL in the pancreatic tissue [21]. TCR:insHEL BALB.K mice are relatively resistant to autoimmune-diabetes progression and the deletion of CD47 in this strain is sufficient to break immune tolerance leading to a high diabetes incidence in TCR:insHEL CD47-deficient BALB.K mice [22]. Interestingly, the proportion of DN T cells was much reduced in the autoimmune-diabetes prone CD47-deficient mice and a single transfer of DN T cells in the CD47-deficient BALB.K transgenic model was sufficient to prevent autoimmune diabetes onset [22]. Of relevance, we also showed that both NOD non-transgenic and TCR transgenic autoimmune diabetes-prone mouse models exhibited a low number of DN T cells relative to other autoimmune diabetes-resistant strains [22]. Together, these results suggest that restoring DN T cell number in autoimmune-prone mice prevents diabetes onset and may be of therapeutic interest for type 1 diabetes.

The primary objective of this study was thus to determine whether the activity of DN T cells is naturally impaired in the spontaneous autoimmune diabetes-prone NOD genetic background. We opted to compare 3A9 TCR DN T cells from autoimmune resistant B10.Br and autoimmune-prone NOD.*H2*^{k} mice [9]. Surprisingly, DN T cells originating from both genetic backgrounds efficiently eliminate their target cells demonstrating that the cytotoxic function of DN T cells is not impeded in autoimmune-prone mice. However, we find that DN T cells derived from the NOD genetic background

produce more IL-10, which reduces the extent of DN T cell expansion. Altogether, the association of DN T cells to autoimmune diabetes susceptibility is most probably due to the homeostatic regulation of DN T cells.

2.4 Results

2.4.1 3A9 TCR transgenic DN T cells are cytotoxic towards B cells

Using non-transgenic and TCR transgenic models, it has been previously shown that DN T cells are cytotoxic towards T cells or B cells [5, 7, 13, 23]. We first aimed to determine whether DN T cells observed in 3A9 TCR transgenic mice carried a cytotoxic potential. To obtain purified DN T cells, we isolated 3A9 TCR transgenic T cells on the basis of $CD4^-CD8^-$ $1G12^+$ expression. Importantly, we show that $1G12^+$ DN T cells do not express DX5 or stain with aGal-Cer CD1d-tetramer (**Figure 1a**), suggesting that they are not $CD4^-CD8^-$ NKT cells. Moreover, following antigen-stimulation (anti-CD3, anti-CD28, IL-2 and IL-4), at least 90% of DN T cells remain $CD4^-CD8^-FoxP3^-$, with some experiments reaching purities above 99% (**Figure 1b** and data not shown). We subsequently demonstrate that these highly purified 3A9 DN T cells from both B10.Br and NOD.$H2^k$ mice express a high level of granzyme B upon antigenic stimulation in the presence of cytokines (**Figure 1c**). Moreover, 3A9 DN T cells from both strains efficiently stain with anti-CD107a, a degranulation marker.

As DN T cells in other systems are cytotoxic towards either T cells or B cells [5, 7, 13, 23], we next wanted to determine the specificity of 3A9 DN T cells. Using activated B and T cells as targets for activated 3A9 DN T cells from the B10.Br strain, we show that B10.Br 3A9 DN T cells efficiently eliminated HEL-antigen loaded B cells (**Figure 1d**). This killing is completely inhibited by the presence of EGTA suggesting a dependence on perforin/granzyme granules to mediate the cytotoxicity [24]. Moreover, the 3A9 DN T cells eliminated B cells in an antigen-specific manner, as they did not eliminate B cells in the absence of HEL (**Figure 2a, left**) Surprisingly, 3A9 DN T cells were unable to eliminate T cells (**Figure 1d**), as opposed to the DN T cells from 2C TCR and P14 TCR transgenic models [13, 18]. We are tempted to suggest that this difference is attributed to MHC-restriction, where MHC class II-restricted 3A9 DN T cells have a different specificity compared to MHC class I-restricted 2C TCR and P14 TCR DN T cells. Notwithstanding,

these data suggest that 3A9 DN T cells from the B10.Br background are directly cytotoxic towards B cells.

2.4.2 3A9 DN T cells from B10.Br and NOD.*H2*^{k} mice show comparable antigen-specific deletion of activated B cells

We have previously shown that a reduction in DN T cell number is associated with autoimmune disease predisposition [22]. These results do not preclude the fact that DN T cell function may also be altered in autoimmune-prone mice, further contributing to disease susceptibility. Therefore, we investigated whether the immuno-regulatory potential of DN T cells from a NOD genetic background was impaired. Surprisingly, NOD.*H2*^{k} 3A9 DN T cells were able to eliminate activated B cells loaded with HEL antigen (**Figure 2a, right**). As for the B10.Br 3A9 DN T cells, this response was both antigen-specific and inhibited by EGTA. These results suggest that DN T cells from either strain of mice exhibited cytotoxicity towards activated B cells. Interestingly, the suppressive activity of B10.Br 3A9 DN T cells appeared to exceed that of the NOD.*H2*^{k} 3A9 DN T cells based on the absolute value of their cytotoxicity (Figure 2a, left and right panels, respectively). However, it should be noted that the relative difference in the cytotoxic activity of B10.Br and NOD.*H2*^{k} 3A9 DN T cells is indeed equivalent as the background levels of cytotoxicity are increased for the control target cells from the B10.Br background. Therefore, variations between the spontaneous release of ^{51}Cr from target cells of the B10 and NOD backgrounds, rather than the DN T cells themselves, may account for the differences observed in the absolute values of the cytotoxicity.

To directly compare the cytotoxic efficiency of 3A9 DN T cells from both strains, we generated F1 (B10.Br x NOD.*H2*^{k}) mice, where F1 B cells could be used as common targets for 3A9 DN T cells from both genetic backgrounds. Our results clearly demonstrate that 3A9 TCR DN T cells from both B10.Br and NOD.*H2*^{k} background were equally cytotoxic towards B cells (**Figure 2b**). Based on these results, we conclude that, on a per cell basis, the DN T cell cytotoxic function is not impeded in the autoimmune-prone NOD genetic background.

2.4.3 3A9 DN T cells from NOD.*H2*^{k} mice produce more IL-10

Although the cytotoxic efficiency of 3A9 TCR DN T cell from either B10.Br or NOD.*H2*^{k} mice was comparable, we sought to investigate the existence of any other potential functional differences between DN T cells from these diabetes-resistant and -susceptible strains. Upon further phenotypic characterization of 3A9 DN T cells from B10.Br and NOD.*H2*^{k} mice, we found that DN T cells that had undergone antigenic activation in the presence of cytokines produced equivalent amounts of IFNγ (data not shown). Interestingly, however, the production of IL-10 was considerably elevated in NOD.*H2*^{k} 3A9 DN T cells (**Figure 3a**). Indeed, the culture supernatant of 3A9 DN T cells from NOD.*H2*^{k} mice contained tenfold more IL-10 when compared to those of B10.Br mice. This increased quantity of IL-10 in the supernatant reflects an increased proportion of NOD.*H2*^{k} 3A9 DN T cells efficiently producing IL-10 (**Figure 3b**). 3A9 DN T cells from the NOD genetic background are, thus, functionally different from those of the B10.Br strain in terms of IL-10 production.

2.4.4 IL-10 does not affect the cytotoxic potential of 3A9 DN T cells

IL-10 is primarily defined as an immunosuppressive cytokine and has been shown to impede different aspects of T cell responses [25]. More importantly, IL-10 had been previously shown to reduce the cytotoxic potential of 2C TCR DN T cells [26]. As a result, we verified the ability of IL-10 to hinder the cytotoxic function of 3A9 DN T cells. The cytotoxic potential of 3A9 DN T cells from B10.Br and NOD.*H2*^{k} mice was thus assessed in the presence or absence of IL-10. Interestingly, the expression of granzyme B and CD107a (**Figure 4a**) as well as the antigen-specific cytotoxic activity of 3A9 DN T cells from either mouse strain was not affected by the presence of IL-10 in the culture (**Figure 4b**).

2.4.5 IL-10 impedes 3A9 DN T cell *in vitro* expansion

Still, the activation of the 3A9 DN T cells in the presence of IL-10 revealed a different alteration in DN T cells. Indeed, we noted that the yield of 3A9 DN T cells cultured in the presence of IL-10 was repeatedly lower. We, thus, quantified the expansion of 3A9 DN T cells from B10.Br and NOD.$H2^k$ mice, in the presence or absence of IL-10. In each experiment, 3A9 DN T cells from B10.Br mice showed a significant reduction in cell expansion when cultured in the presence of IL-10 (**Figure 5a, left**). Notably, the expansion of 3A9 DN T cells from NOD.$H2^k$ mice, cultured in the absence of additional IL-10, was significantly lower than that of DN T cells from B10.Br mice. Moreover, although NOD.$H2^k$ 3A9 DN T cells efficiently produce IL-10, this response is not completely saturated as the addition of IL-10 to the cultures of 3A9 DN T cells from NOD.$H2^k$ mice slightly reduced their expansion **(Figure 5a, right**). These results demonstrate that IL-10 restrains DN T cell expansion and suggest that the reduced expansion potential of 3A9 DN T cells from NOD.$H2^k$ mice compared to that of B10.Br mice is the result of their elevated IL-10 production.

2.4.6 IL-10 facilitates DN T cell apoptosis.

Next, we wanted to determine the mechanism by which the presence of IL-10 influenced DN T cell expansion. Cellular expansion is a balance between the rate of proliferation and the rate of cell death. Therefore, we first verified the impact of IL-10 on DN T cell proliferation in vitro. Using CFSE-labelled 3A9 DN T cells, we demonstrate that IL-10 did not affect the proliferative response of DN T cells of either mouse strain (**Figure 5b**). These data indicate that the difference in DN T cell expansion potential between the two strains was not a consequence of a defect in cellular proliferation. In addition, these results demonstrate that IL-10 does not affect DN T cell proliferation.

Therefore, to explain the decrease in cellular expansion, we suspected that IL-10 facilitated DN T cell apoptosis. The percentage of apoptotic 3A9 DN T cells upon a 6 to 8 day culture was monitored by Annexin V staining (**Figure 6**). First, a higher proportion of 3A9 DN T cells from NOD.$H2^k$ mice, which spontaneously produce high levels of IL-

10, underwent apoptosis when compared to the B10.Br counterpart (compare **Figures 6a and 6b** without additional IL-10). Second, the percentage of apoptotic 3A9 DN T cells from B10.Br mice was elevated when IL-10 was added to the culture. Moreover, although this tendency was not significant overall, 3A9 DN T cells from NOD.$H2^k$ mice also showed a slight increase in apoptosis in some experiments when IL-10 was added to the culture, which is most probably attributed to the high level of endogenous IL-10 production by these cells. To further confirm the finding that IL-10 facilitated DN T cell apoptosis, we cultured 3A9 DN T cells from NOD.$H2^k$ mice, which produce high levels of endogenous IL-10, with 10μg/ml of an antibody targeting the IL-10 receptor. Indeed, the presence of this blocking antibody resulted in an increase in the cellular expansion of 3A9 DN T cells from NOD.$H2^k$ mice (**Figure 7a)**, as well as a decrease in their level of apoptosis (**Figure 7b**). Together, these results suggest that IL-10 impedes DN T cell expansion and correlates with an increase in DN T cell apoptosis.

2.5 Discussion

DN T cells are rare and potent immunosuppressive cells, which eliminate their target T cells or B cells in an antigen-specific manner [5, 13]. Importantly, the insertion of a TCR transgene facilitates the identification and isolation of DN T cells, as well as their implication in antigen-specific immunosuppression [6-12]. Indeed, the DN T cell subset exhibits a potent immunosuppressive potential in multiple model systems, including graft rejection[13-15, 19], graft-vs-host disease[16] and autoimmune diabetes [18]. We have recently demonstrated that autoimmune-diabetes prone mice exhibit fewer DN T cells than the autoimmune-resistant mouse strains [22]. In addition, we have shown that, in a syngenic 3A9 TCR BALB.K transgenic system, a single transfer of DN T cells is sufficient to confer resistance to diabetes progression [22]. These results suggest that modulation of DN T cell number may be of therapeutic interest, if DN T cell function is not affected in autoimmune diabetes-prone individuals. Herein, we examined the cytotoxic activity of DN T cells from both autoimmune diabetes-resistant and -prone mice. We further explored the role of IL-10 production by DN T cells from the NOD genetic background in the regulation of DN T cell expansion.

To compare the function of DN T cells in the autoimmune-resistant and -prone mice, we took advantage of the 3A9 TCR transgene, as it has been interbred to both the autoimmune-resistant B10.Br and autoimmune-prone NOD.$H2^k$ strains. Indeed, apart from the MHC locus, NOD.$H2^k$ mice are genetically identical to the NOD strain. Therefore, comparison of 3A9 DN T cells from B10.Br and NOD.$H2^k$ mice allows us to define whether the autoimmune-prone NOD genetic background influences DN T cell function. Using this model, we have provided compelling evidence that the cytotoxic ability was similar between the two strains. This result supports the view that autologous transfer of DN T cells in autoimmune-prone mice or individuals may be of therapeutic interest. We aim to further explore this novel antigen-specific therapeutic avenue.

DN T cells isolated from the non-transgenic system demonstrate antigen-specific cytotoxicity towards both T cells and B cells [5]. Interestingly, DN T cells from various

TCR transgenic models have been reported to specifically eliminate CD8 T cells [13-18], while we report that the 3A9 DN T cells target B cells. We believe that the difference in target cell specificity may be attributed to MHC-restriction where the 2C and P14 TCR transgenes, most commonly used to study DN T cell cytotoxicity, are both MHC class I-restricted, as opposed to the 3A9 TCR transgene, which is MHC class II-restricted. Notably, since the 3A9 TCR transgene is class II restricted, these transgenic mice hold a very limited number of HEL-specific CD8 T cells. Nevertheless, we extensively tested the cytotoxic potential of 3A9 DN T cells towards T cells by using total spleen, purified CD4 or purified CD8 T cells from 3A9 TCR transgenic mice (**Figure 1d** and data not shown). We have also attempted to pre-immunize the 3A9 TCR transgenic mice using HEL antigen with or without adjuvant. Yet, in all of these conditions, 3A9 DN T cells were unable to eliminate the 3A9 TCR transgenic CD4 or CD8 targets (data not shown). Consequently, we propose that MHC specificity dictates the cellular target of DN T cells, where the class II-restricted 3A9 DN T cells specifically recognize the HEL-peptide presented by I-A^k molecules at the surface of B cells. The study of DN T cell function in additional MHC class I and class II-restricted TCR transgenic models should help resolve this issue. Alternatively, isolation of DN T cells from non-transgenic mice may also help further unravel their function in immune tolerance.

We have previously shown that the proportion of DN T cells was reduced in autoimmune-prone mice. Our present results show that the cytotoxic function of DN T cells from autoimmune-prone mice is comparable to that of autoimmune-resistant mice. Upon further comparison of DN T cell functions between these strains, we went on to demonstrate that DN T cells from the NOD.*H2*^{k} background produce more IL-10, which limits their expansion potential. Although others have previously shown that IL-10 inhibits the cytotoxic function of DN T cell [27], we herein demonstrate that IL-10 has no direct effect on DN T cell cytotoxicity towards activated B cells in the 3A9 TCR transgenic setting. One possibility for this discrepancy is the nature of the DN T cells utilized in these assays. Indeed, IL-10 inhibited the cytotoxic potential of DN T cell clones [26], whereas we demonstrate that IL-10 does not influence the cytotoxic potential of primary cell cultures. Nonetheless, despite our observation that IL-10 had no direct effect

on DN T cell cytotoxicity on a per cell basis, it must be noted that IL-10 would ultimately have a secondary effect on DN T cell cytotoxicity since the presence of IL-10 results in a reduction in their cellular expansion, which will subsequently decrease the magnitude of the global cytotoxic response.

The pro-apoptotic role of IL-10 has also been reported for other cell types, including monocytes, Th1 cells, and dendritic cells [25]. It should be noted that the role of IL-10 in diabetes progression is controversial, and has been demonstrated to both protect from and accelerate disease development. For instance, daily IL-10 subcutaneous treatment of 9–10-week-old NOD mice delayed the onset of diabetes, reduced the severity of insulitis and significantly reduced disease incidence [28]. Furthermore, systemic administration with an IL-10/Fc fusion protein in female NOD mice from 5 to 25 weeks of age blocked diabetes development and provided lasting protection after treatment was terminated [29]. In contrast to these immunosuppressive effects of IL-10, the transgenic expression of IL-10 by insulin-producing pancreatic β cells or glucagon-producing pancreatic α cells led to an accelerated onset of diabetes in NOD mice [30-33]. These findings suggest that the IL-10 location plays a deciding role on the outcome of diabetes development where systemic IL-10 confers protection whereas islet-specific IL-10 exacerbates diabetes progression. It is tempting to speculate that the presence of IL-10 within the pancreatic islets impedes DN T cell expansion and thereby inhibits their immunoregulatory function, while systemic administration of IL-10 dominantly contributes to other immunosuppressive mechanisms.

Interestingly, IL-10 genetic polymorphisms have been associated with autoimmune diabetes susceptibility, as well as in the progression of various autoimmune diseases, such as lupus, rheumatoid arthritis, and colitis [25, 34, 35]. Clearly, IL-10 has an impact on a multitude of autoimmune disorders and extensive studies are required to define the mechanistic contribution of these genetic polymorphisms to the various pathologies. Although the *Il10* gene is not located within an insulin-dependent diabetes (*Idd*) susceptibility locus, IL-10 production by T cells is regulated at least in part by genetic polymorphisms encoded within the *Idd5.1* susceptibility locus [36]. As a result, we

are currently investigating whether the *Idd5* susceptibility locus plays a role on the regulation DN T cell number.

We have previously reported that a single transfer of DN T cells in the CD47-deficient BALB.K transgenic model was sufficient to prevent autoimmune diabetes onset [22]. Other regulatory T cell subsets have been shown to confer protection from diabetes progression, such as Foxp3$^+$ Tregs[37-39] and NKT cells [40, 41]. Interestingly, both of these cell types also produce IL-10[39, 41, 42]. Moreover, it has recently been shown that in the absence of IL-10 signalling there is a subsequent decrease in both expression of Foxp3 by Tregs as well as their suppressive activity [43]. This contrasts with our study where increased IL-10 signalling has no effect on DN T cell function yet facilitated DN T cell apoptosis resulting in decreased cell numbers. These observations highlight the interdependent cellular complexity of *in vivo* immune regulation.

Other than IL-10 production, DN T cells have an intriguing resemblance to NKT cells. They both carry a similar phenotype, where both DN T cells and a subset of NKT cells express TCRβ yet lack CD4 and CD8 co-receptor expression[22, 44, 45]; they both demonstrate cytotoxic activity as shown throughout this manuscript and [46, 47]; they are both present in lower numbers in NOD mice and they both confer protection from diabetes development [22, 48]. Moreover, we have recently shown that the CD47 pathway is implicated in the regulation of DN T cell numbers [22], while NKT cell numbers are regulated by *Idd13* [49, 50], a diabetes-susceptibility locus which encodes the highly polymorphic cellular ligand of CD47, namely CD172a [51, 52]. Together, these data strongly suggest that DN T cells carry an NKT cell-like phenotype. Notwithstanding, we have identified DN T cells in non-transgenic mice that are phenotypically similar to 3A9 TCR transgenic DN T cells. Indeed, as demonstrated herein for 3A9 TCR transgenic DN T cells **(Figure 1a**), the DN T cells isolated from non-transgenic mice lack DX5 expression and do not stain with CD1d-tetramer [22] .Taken together, these results suggest that although DN T cells exhibit similarities to NKT cells, they compose a unique subset.

In summary, evaluating the role of DN T cells in the 3A9 TCR transgenic setting has highlighted their antigen-specific mode of action towards activated B cells. Moreover, we have demonstrated that the cytotoxic potential of DN T cells on a per cell basis is not altered in autoimmune-prone mice or in the presence of IL-10. However, IL-10 facilitates DN T cell apoptosis resulting in an inefficient cellular expansion *in vitro*. We have previously shown that the CD47 pathway influences the proportion of DN T cells *in vivo* [22]. Future studies should decipher the contribution of both the IL-10 and the CD47 pathway in the homeostatic regulation of DN T cells. Altogether, as the cytotoxic function of DN T cells is normal in genetically autoimmune-prone mice, we propose that the association of DN T cells to diabetes resistance is most probably due to the regulation of DN T cell number. Therefore, with regards to new approaches in cellular therapy, our main focus should lie on the restoration and expansion of DN T cell numbers in an antigen-specific manner, which may contribute to immune-regulation and prevent autoimmune disease progression.

2.6 Methods

Mice

3A9 TCR [53] and insHEL (ILK-3) [21] transgenic mice on B10.Br, NOD.*H2*^{k} and F1 (B10.Br x NOD.*H2*^{k}) background have been previously described [9]. Studies were approved by the Maisonneuve-Rosemont Hospital ethics committee overseen by the Canadian Council for Animal Protection.

Flow cytometry

6–12-wk old non-diabetic mice (Diastix negative) were analyzed. Single cell suspensions of lymphoid organs were prepared by mechanical disruption through a 70 μm sterile cell strainer (BD Biosciences). NH_4Cl was used for erythrocyte lysis of single cell suspensions from spleens. αGal-Cer-loaded CD1d-tetramer is a generous gift from Dr. David Serreze. Intracellular staining for granzyme B (16G6, eBiosciences) and IL-10 (JES5-16E3, Biolegend) were performed using BD Biosciences Cytofix/Cytoperm Kit. The FITC Anti-Mouse/Rat Foxp3 Staining Set was used for Foxp3 staining according to the manufacturer's instructions (eBiosciences). Anti-clonotypic 1G12 antibody, specific to the 3A9 TCR [54], was detected using APC-labelled anti-mouse IgG1 (clone X56, BD Biosciences). PerCP-labelled CD8α (53-6.7) and PerCP-labelled 7-AAD were purchased from BD Pharmingen. All other antibodies and staining reagents were purchased from Biolegend. Data was collected on a FACSCalibur or FACS LSR II (BD Biosciences) and analyzed using FlowJo software (Treestar).

Cell purification

Prior to DN T cell sorting, cell suspensions were stained with biotin-labelled anti-mouse B220 antibody (RA3 6B2, Biolegend) and B220+ cells were removed using the EasySep™ biotin selection kit (Stem Cell). The B220 negative fraction was then stained with antibodies to CD4, CD8 and 3A9 TCR (using the 1G12 clone) and sorted using a FACS Vantage (BD Biosciences) for $CD4^-CD8^-1G12^+$ DN T cells (purity > 90%). For some experiments, DN T cells were obtained by removing CD4, CD8 and B220 positive cells using the EasySep™ biotin selection kit. This procedure efficiently removed $CD4^+$,

$CD8^+$ and $B220^+$ cells (< 10% contaminating cells). B and T cells were also selected using the EasySep™ PE selection kit, respectively staining for B220 or CD3 (purity > 85%). Similar results were obtained when B and T cells were sorted (purity > 95%).

Cellular expansion

Sorted DN T cells (250 000 cells/ml) were cultured in the presence of plate-bound anti-CD3 (1µg/ml, 145-2C11) and anti-CD28 (10µg/ml, 37.51) with IL-2 (30U/ml), IL-4 (30 U/ml) ± IL-10 (10ng/ml) or anti-IL-10R (10µg/ml, 1B1.3a) for 5 to 7 days, in RPMI supplemented with 10% fetal bovine serum (Invitrogen), 1% Penicillin Streptomycin (Invitrogen), 1% HEPES 1M (GIBCO) and 0.1% 2-mercaptoethanol 1000X (GIBCO). Cytokines were purchased at Feldan Bio. Cell cultures were monitored daily for confluence and cytokine supplemented media was added when cell density was > $1x10^6$ cells/ml. Cell number was quantified by trypan blue exclusion.

Cytotoxic potential

DN T cells were sorted or enriched using using the EasySep™ biotin selection kit as described above and cultured in the presence of either soluble anti-CD3 and irradiated APCs (T-depleted spleen cells, 2.5 Gy, 250 000 cells/ml) or plate-bound anti-CD3 and anti-CD28, with IL-2, IL-4 ± IL-10, at the same concentrations as indicated for cellular expansion. For granzyme B detection, cells were re-stimulated at day three for 4h with PMA (50ng/ml) (Sigma), ionomycin (0.5µg/ml) (Sigma), and Brefeldin A (10 µg/ml) (Sigma), prior to intracellular staining. For CD107a (LAMP1) staining, we replaced Brefeldin A with mononesin (2nM) (BD biosciences) and the anti-CD107a antibody was added during the 4h re-stimulation [55].

Cytotoxicity

Sorted or enriched DN T cells were stimulated 7 days in vitro with either soluble anti-CD3 and irradiated APCs or plate-bound anti-CD3 and anti-CD28, IL-2, IL-4 ± IL-10. LPS (1µg/ml) and anti-CD3 + irradiated APCs + IL-2 were used to stimulate B and T cells, respectively, in vitro for 2 days, and HEL antigen (1mg/ml) (Sigma) was added in the last 24h of culture. Target cells were washed and loaded with 50µCi ^{51}Cr (Perkin

Elmer) for 90 min. DN T cells and target cells were quantified by trypan blue exclusion prior to co-culture in order to obtain the desired DN T cell to target cell ratio. DN T cells and target cells were incubated for 3h in the presence or absence of EGTA (0.16mM) (J. T. Baker). %Cytotoxicity = 100*(E –S)/(M – S), where E = experiment, S = spontaneous and M = maximal release.

Measurement of cytokines in the supernatant of cultured DN T cells

DN T cells were sorted from TCR transgenic B10.Br and NOD.$H2^k$ mice and cultured for 3 days with soluble anti-CD3, irradiated APCs, IL-2 and IL-4. The amount of IL-10 from the supernatant of cultured DN T cells was measured by an ELISA kit (Biolegend) according to the manufacturer's protocol.

Proliferation assay

For proliferation assays, sorted DN T cells were labelled with CFSE (2μM) (Invitrogen) and washed twice in cold supplemented RPMI prior to culture. CFSE-labelled DN T cells were cultured in the presence of plate-bound anti-CD3, anti-CD28, with IL-2, IL-4 ± IL-10, as above. CFSE dilution was monitored by flow cytometry.

Apoptosis assay

Sorted DN T cells were cultured in the presence of plate-bound anti-CD3 and anti-CD28, with IL-2, IL-4 ± IL-10 for 6 to 8 days. Apoptosis was quantified by flow cytometry using Annexin-V.

Statistics

The paired student's *t* test or the Mann-Whitney U statistical test was applied where appropriate.

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2.9 Figures

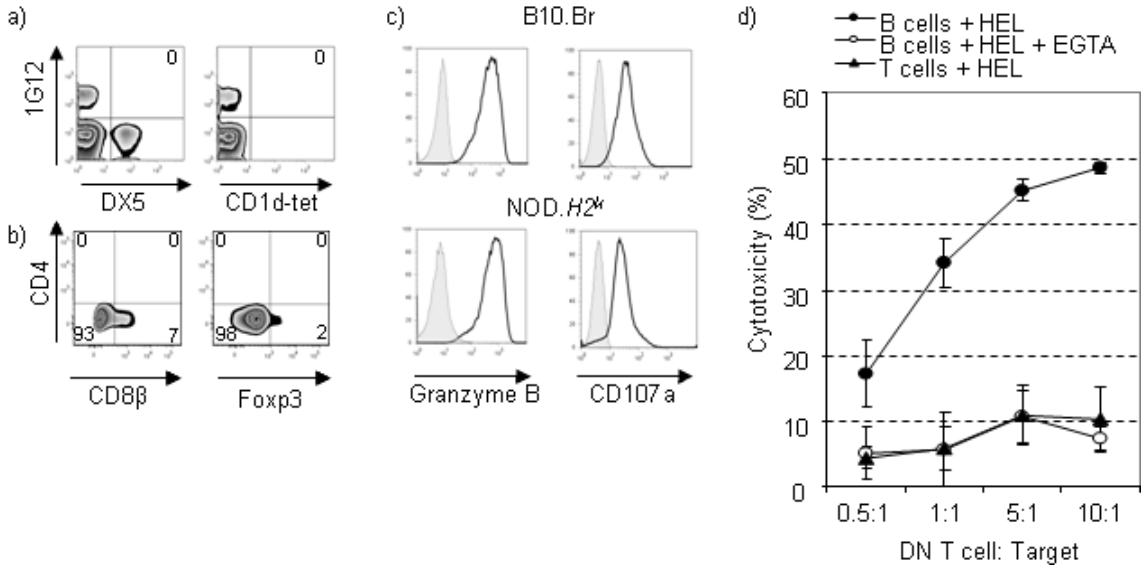

Figure 1: 3A9 DN T cells efficiently eliminate activated B cells. A) 3A9 TCR B10.Br splenocytes were stained for CD4, CD8, 1G12, DX5 and CD1d-tetramer. DX5 (left panel) and CD1d-tetramer (right panel) expression is shown for $CD4^{-}CD8^{-}$ gated cells relative to 1G12 expression. B) B10.Br 3A9 DN T cells were cultured with anti-CD3, anti-CD28, IL-2 and IL-4. After 7 days, DN T cells were stained CD4, CD8β, and Foxp3 expression. C) B10.Br and NOD.$H2^{k}$ 3A9 DN T cells cultured for 3 days with anti-CD3, APCs, IL-2 and IL-4. Left panels, Intracellular expression of granzyme B with (thick) or without cytokine stimulation (shaded). Right panels, CD107a expression with (thick) or without (shaded) PMA and ionomycin restimulation. D) The percent specific lysis is shown for 3A9 B10.Br DN T cells towards HEL-pulsed LPS-activated B cells or anti-CD3, APC and IL-2 activated T cells in the presence, or not, of EGTA. All data is representative of at least 3 independent experiments.

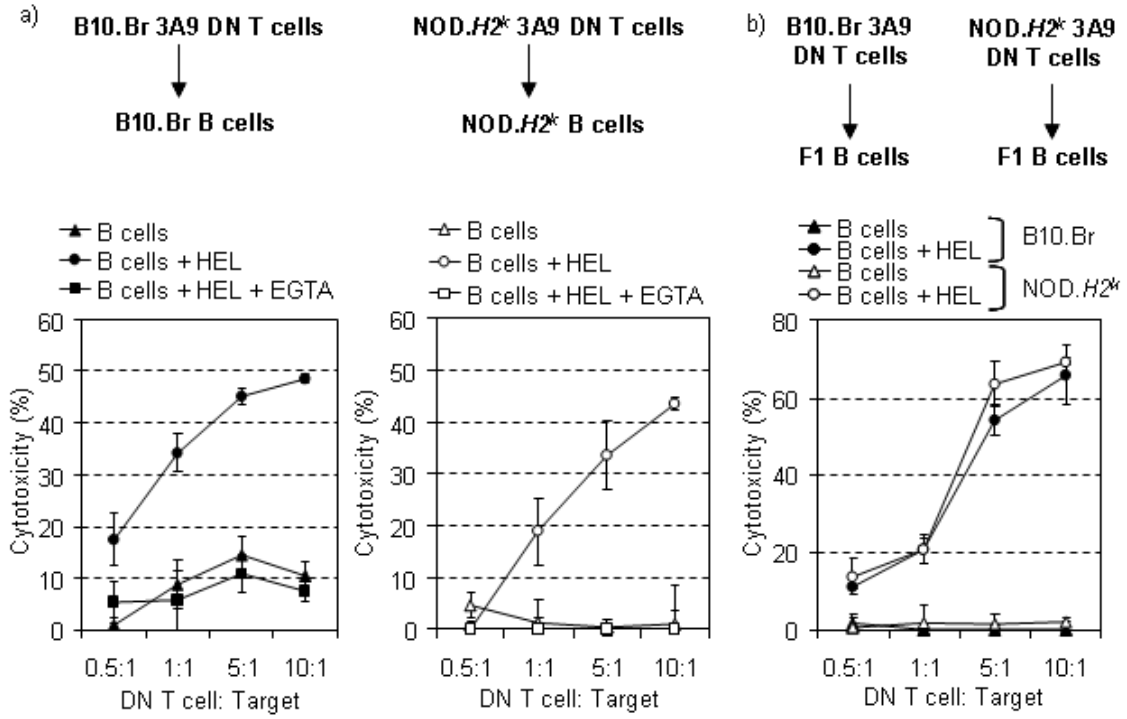

Figure 2: The cytotoxic function of 3A9 DN T cells from autoimmune-prone mice is not impaired. 3A9 DN T cells and B cells were isolated from the spleens of TCR transgenic B10.Br or NOD.$H2^k$ mice. A) The percent specific lysis is shown for B10.Br (left) and NOD.$H2^k$ (right) 3A9 DN T cells towards HEL-pulsed LPS-activated B cells from B10.Br and NOD.*H2k* mice, respectively. EGTA was added in some conditions, as indicated. B) The percent specific lysis is shown for B10.Br (filled shapes) and NOD.$H2^k$ (open shapes) 3A9 DN T cells towards a common target, HEL-pulsed LPS-activated F1 (B10.Br X NOD.$H2^k$) B cells. Representative of 4 independent experiments.

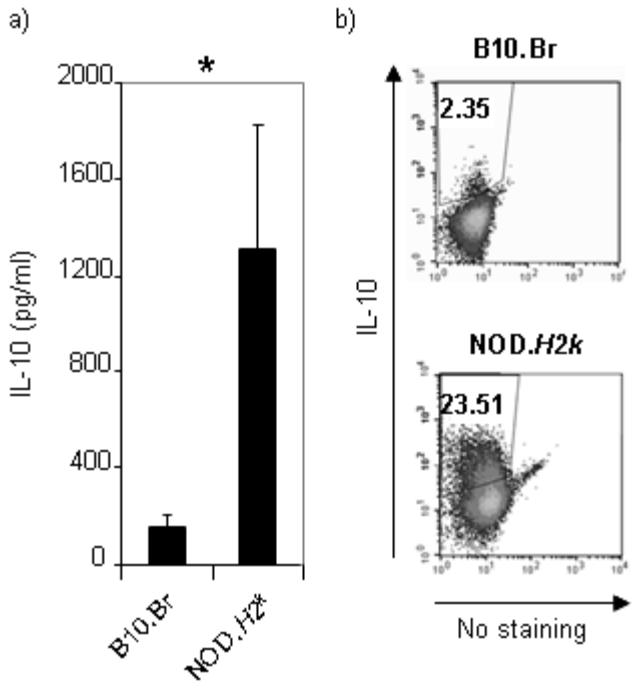

Figure 3. DN T cells from the diabetes-susceptible mice produce greater quantities of IL-10. 3A9 DN T cells were isolated from the spleen of TCR transgenic B10.Br or NOD.$H2^k$ mice and cultured in the presence of anti-CD3, APCs, IL-2 and IL-4 for 3 days. A) IL-10 levels were quantified by ELISA from the 3-day culture supernatant. Mean ± SD of at least 3 independent experiments. B) IL-10 was measured by intracellular staining. Representative of at least 3 independent experiments. *p <0.05 (Mann-Whitney U).

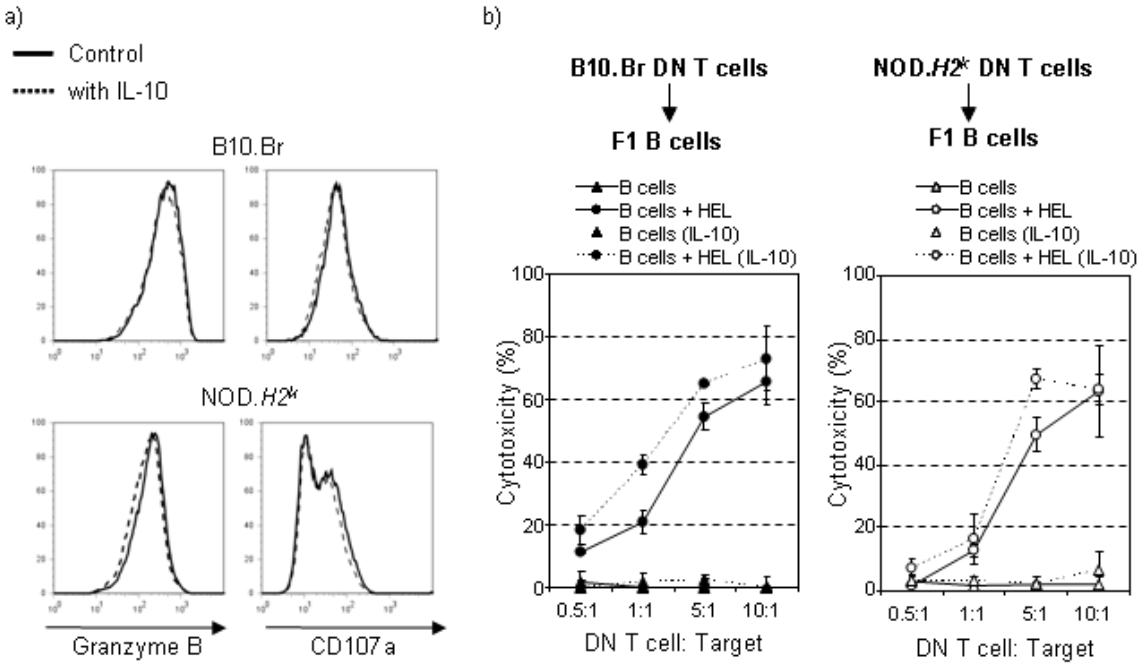

Figure 4. IL-10 does not impede DN T cell cytotoxic function. DN T cells and B cells were isolated from spleens of 3A9 TCR transgenic B10.Br or NOD.*H2*^{k} mice. A) Intracellular expression of granzyme B (left panels) and CD107a expression (right panels) are shown for DN T cells cultured in the presence of anti-CD3, anti-CD28, IL-2, IL-4 (solid) and IL-10 (dotted). B) The percent specific lysis is shown for 3A9 B10.Br and NOD.*H2*^{k} DN T cells that were cultured with (dotted) or without IL-10 (solid) towards HEL-pulsed LPS-activated F1 (B10.Br X NOD.*H2*^{k}) B cells. Representative of at least 3 independent experiments.

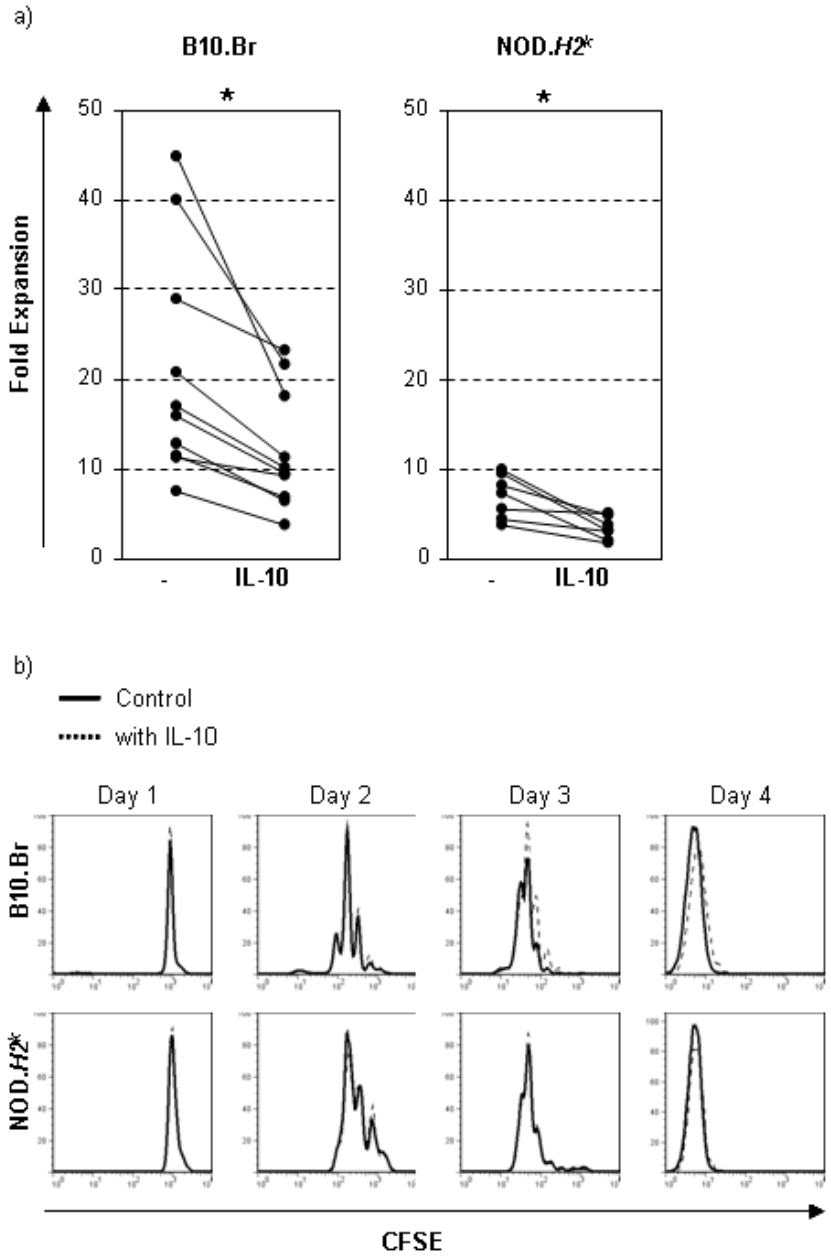

Figure 5. IL-10 impedes DN T cell expansion but not DN T cell proliferation. 3A9 DN T cells were sorted from B10.Br or NOD.$H2^k$ mice and activated using anti-CD3, anti-CD28, IL-2, and IL-4. A) Cellular expansion is shown for 3A9 DN T cells that were stimulated as described above ± IL-10 for 6 to 8 days. *p <0.05 (Paired student's *t* test). B) Cellular proliferation was measured using CFSE-labelled 3A9 DN T cells that were stimulated as described above with (dotted) or without (solid) IL-10. Representative of at least 3 independent experiments.

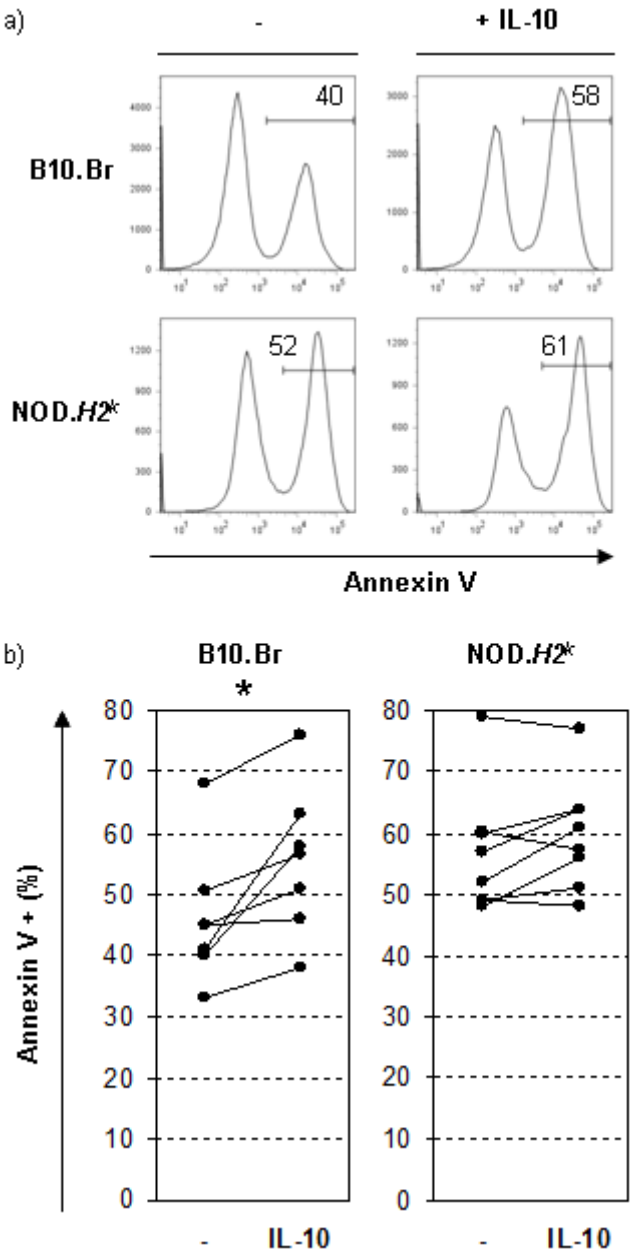

Figure 6. IL-10 increases cellular apoptosis. 3A9 DN T cells were isolated from the spleens of B10.Br and NOD.*H2*^{k} mice and stimulated with anti-CD3, anti-CD28, IL-2 and IL-4 ± IL-10 for 5 to 7 days. Amount of apoptotic cells was quantified by Annexin V staining. A) Representative results are shown. B) A compilation of all experiments are shown. *p <0.05 (Paired student's *t* test).

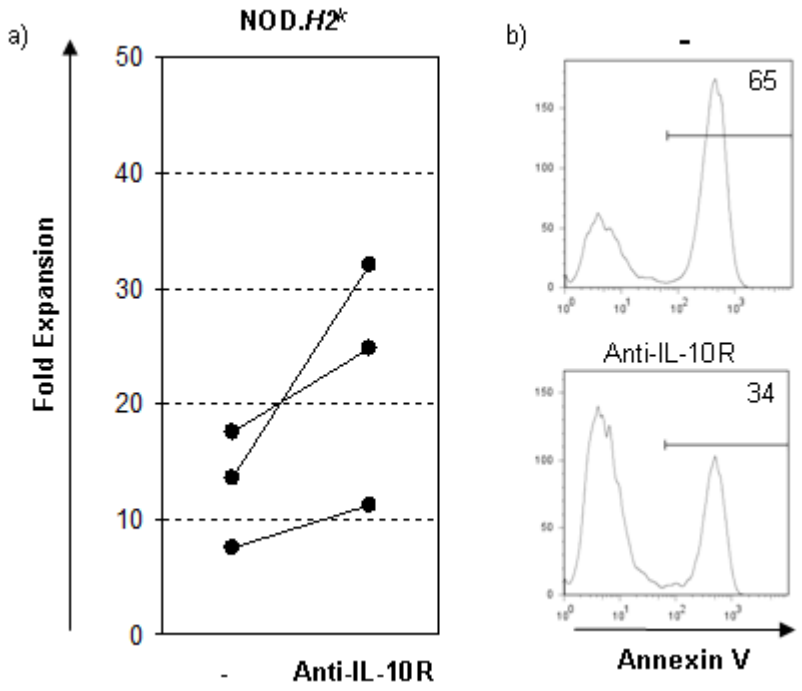

Figure 7. Blocking IL-10 signalling increases DN T cell expansion while decreasing DN T cell apoptosis. Sorted NOD.*H2*^{k} 3A9 DN T cells were stimulated with anti-CD3, anti-CD28, IL-2 and IL-4 ± anti-IL-10R for 7 days. A) Cellular expansion was quantified by cell count using trypan blue exclusion. B) The amount of apoptotic DN T cells was quantified by Annexin V staining.

CHAPTER 3 : ARTICLE 2

3.1 Immunoregulatory CD4-CD8- T cells, a novel cellular therapeutic preventing autoimmune diabetes

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This article is in the final stages of preparation for submission to *Nature Medicine*.

I evaluate my contribution to this article at 85%. I participated in the experimental design alongside S. Lesage. C. Beauchamp and V. Dugas participated in the phenotyping of TCR transgenic DN T cells (Supplementary Figure 2). G. Chabot-Roy set up the insulin autoantibody ELISA assay (Figure 5D) and assisted me in the experiments involving pancreatic islet isolations. M. Giroux determined the cytotoxic activity of human DN T cells (Figure 6C) and participated in the investigation of the cytotoxic potential of human DN T cells (Figure 6B). Personally, I performed all experiments pertaining to the study of non-transgenic DN T cells, including the phenotyping, cytotoxicity assays, insulin autoantibody ELISA assay, in vivo transfer experiments and the diabetes incidence study. Moreover, I participated in the investigation of the cytotoxic potential of human DN T cells. I actively participated in the interpretation and analysis of the results, with the help of S. Lesage. I personally generated the figures and wrote the manuscript taking S. Lesage's comments and suggestions into consideration.

The results presented in this article are central to the thesis as they demonstrate that DN T cells from diabetes-susceptible mice exhibit fewer DN T cells. We further show that non-transgenic DN T cells mediate their immunoregulatory function by eliminating B cells presenting islet antigens in vitro and that the transfer of non-transgenic DN T cells leads to a decrease in germinal center B cells directly within the pancreatic islets in vivo as well as a reduction in insulin autoantibody levels. Importantly, this article is the first to demonstrate that DN T cells can decrease diabetes incidence in non-transgenic, non-lymphopenic NOD mice.

Title: Immunoregulatory CD4-CD8- T cells, a novel cellular therapeutic preventing autoimmune diabetes

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3.2 Abstract

The design of treatments for autoimmune disease, such as type 1 diabetes, is geared towards the induction of antigen-specific of immune tolerance. CD4-CD8- immunoregulatory DN T cells exhibit antigen-specific immune tolerance and are thus gaining interest as a potential cellular therapeutic. We demonstrate that low DN T cell proportion is associated with diabetes susceptibility in NOD mice, where a single transfer of DN T cells is sufficient to decrease diabetes incidence. DN T cells mediate their immunoregulatory function by specifically eliminating B cells presenting islet antigens, leading to a decrease in germinal center B cells directly within the pancreatic islets and a concomitant decrease in insulin autoantibody levels. In addition, human DN T cells exhibit similar properties to mouse DN T cells. Altogether, these results demonstrate that DN T cells may be of therapeutic interest for autoimmune diabetes in humans.

3.3 Introduction

Autoimmunity can give rise to the destruction of healthy organs, glands, joints or the central nervous system due to a break in immune tolerance, which is dependent on the immune system discriminating between self and non-self. A prominent role in the prevention of autoimmunity has been attributed to the number and function of regulatory cell subsets, including $CD4^+CD25^+FoxP3^+$ naturally occurring T cells (Tregs), IL-10-producing $CD4^+$ Tr1 cells, natural killer-like T (NKT) cells[1-8], and B10 regulatory B cells[9-11]. One of these regulatory T cell populations, namely DN T cells that lack the expression of CD4 and CD8 T cell co-receptors, has been studied in both mice and humans for their contribution to peripheral tolerance, disease prevention and their potential for use in cellular therapy. Indeed, observations made using both TCR transgenic and non-transgenic systems have demonstrated that DN T cells may be of particular interest for cellular therapy due to their ability to eliminate various cellular targets, including T cells, B cells, NK cells, dendritic cells and macrophages, in an antigen-specific manner (reviewed in [12]). Consequently, DN T cells are gaining interest as a potential cellular therapeutic in various disease settings including graft-vs-host disease[13-15], cancer[16,17] and autoimmune diabetes [18-21].

Autoimmune diabetes results from the immune-mediated destruction of the insulin-secreting β cells present within the pancreatic islets. The NOD (non-obese diabetic) mouse spontaneously develops autoimmune diabetes and has been a key tool for investigating the aetiology of human type 1 diabetes[22,23]. Indeed, the broadly immunosuppressive anti-CD3 therapy, which has been successful in the treatment of new-onset type 1 diabetic patients during clinical trials, is a protocol initially developed in the NOD mouse [24,25], demonstrating a parallel in disease development between humans and mice. Therefore, mouse models, such as the NOD mouse strain, are a useful tool as they allow for the characterization of the cellular mechanisms involved in the progression of autoimmune diabetes.

Indeed, using mouse models, we have shown that, in comparison to autoimmune diabetes-resistant mice, the proportion of TCR transgenic DN T cells is significantly reduced in autoimmune diabetes-susceptible mice[18], which suggests an association between low DN T cell number and autoimmune diabetes susceptibility. Despite a reduction in cell number, we have also demonstrated that TCR transgenic DN T cells from autoimmune diabetes-resistant and -susceptible mice exhibit an equally potent cytotoxic function *in vitro*[26]. Together, these results propose that a deficiency in DN T cell number rather than function contributes to autoimmune diabetes susceptibility. Consequently, we demonstrated that a single transfer of DN T cells is sufficient to prevent diabetes progression in autoimmune diabetes-prone mice using a TCR transgenic setting[18]. Therefore, these results suggest that the restoration of DN T cell number can inhibit autoimmune diabetes development.

Although T cells are known to destroy pancreatic islets, B cells are also implicated in disease development. First, B cells contribute to disease progression, as the elimination of most B cells using anti-CD20 treatment reverses or delays diabetes progression in both mice and humans [27,28]. Moreover, both B cell-deficient NOD mice and NOD mice with an I-A^{g7} deficiency confined to the B cell compartment were protected from the development of autoimmune diabetes[29,30], suggesting that the antigen presentation function of B cells contributes to disease pathogenesis. Indeed, central to the development of autoimmune diabetes lies an antigen-specific response to islet antigens[31-33]. Another major function of B cells is the production of antibodies. Accordingly, autoantibodies to islet antigens, such as insulin autoantibodies (IAA), serve to predict the onset of diabetes[34,35] while maternally transmitted autoantibodies have been shown to contribute to the onset of disease in NOD offspring[36,37]. Altogether, B cells clearly contribute to autoimmune diabetes pathogenesis.

Here, we demonstrate that non-transgenic autoimmune diabetes-prone mice present with fewer DN T cells in comparison with autoimmune diabetes–resistant mice and that the restoration of DN T cell number in non-transgenic NOD mice significantly

decreases disease development. Moreover, we describe the phenotype and antigen-specific function of DN T cells as well as their role in vivo. Finally, we reveal the functional characteristics of human DN T cells and their potential for the development of therapeutic approaches.

3.4 Results

3.4.1 Non-transgenic DN T cells are reduced in autoimmune diabetes-prone mice

The specific isolation of immunoregulatory DN T cells from non-transgenic mice is complicated by the fact that DN T cells share the $CD4^{-}CD8^{-}TCR_{\alpha\beta}^{+}$ phenotype with a subset of NKT cells [12]. Indeed, a proportion of CD4-CD8-TCRβ+ cells that stain negative for the α-galactosyl ceramide-loaded CD1d tetramer, which is specific for type I NK T cells [38,39], still express NK1.1, which is expressed by natural killer (NK) and NKT cells[3] (**Supplementary Figure 1A**), but is also expressed by a fraction of DN T cells (**Supplementary Figure 1C**). Therefore, NKT cell surface markers cannot be used to effectively distinguish NKT cell subsets from DN T cells. However, immunoregulatory DN T cells differ from NKT cells, in that all NKT cells express a TCR restricted to CD1d molecules and are thus absent in CD1d-deficient mice[40]. As a result, we have chosen to use CD1d-deficient mice for the purification of DN T cells throughout these studies, where non-transgenic DN T cells can be easily isolated based on a CD4-CD8-CD19-TCRβ+ phenotype (**Supplementary Figure 1B**). Using this gating strategy, both the proportion (**Figure 1A**) and absolute cell number **(Figure 1B)** of DN T cells was determined for the spleen and lymph nodes of CD1d-deficient mice on the autoimmune diabetes-resistant C57BL/6 (B6) and BALB/c genetic backgrounds as well as the autoimmune diabetes-prone NOD genetic background. Indeed, the proportion and absolute number of DN T cells is significantly reduced in the secondary lymphoid organs of the autoimmune diabetes-prone mice. These results suggest an association between a reduced DN T cell number and autoimmune diabetes susceptibility in the non-transgenic setting.

3.4.2 Non-transgenic DN T cells express an activated phenotype

DN T cells have been mostly characterized in TCR transgenic settings where DN T cells exhibit an activated phenotype in the presence of their cognate antigen whereas

they express a naive phenotype in the absence of cognate antigen (reviewed in [12]). As a result, we chose to compare the phenotype of non-transgenic DN T cells to DN T cells from a TCR transgenic model. More specifically, to further characterize the non-transgenic DN T cells in the absence of any contaminating NKT cells, we compared the DN T cell surface phenotype of the non-transgenic CD1d-deficient mice alongside DN T cells from TCR transgenic mice, namely the 3A9 TCR:insHEL transgenic mouse model. In this model, the 3A9 TCR transgene, which can be detected using the 1G12 anti-3A9 clonotypic mAb [41], recognizes a HEL peptide in the context of I-A^k and the insHEL transgene is expressed under the rat insulin promoter [41,42]. As expected, DN T cells from either CD1d-deficient mice or 3A9 TCR:insHEL double transgenic mice all expressed the T cell marker, CD3, and expressed an intermediate level of another T cell marker, CD5 (**Supplementary Figure 2A**), analogous to 3A9 TCR single transgenic DN T cells [18]. Moreover, in comparison to 3A9 TCR DN T cells, which express a naive phenotype [18], DN T cells from 3A9 TCR:insHEL double transgenic mice, bearing the HEL cognate antigen for the TCR transgene, express the activation markers CD122, CD69 and CD44 (**Supplementary Figure 2B**). Interestingly, DN T cells from CD1d-deficient mice expressed similar levels of the various activation markers **(Supplementary Figure 2B**). Altogether, non-transgenic DN T cells express a similar activated phenotype as DN T cells from the 3A9 TCR:insHEL double transgenic system where DN T cells are activated by the presence of HEL, which represents a self-antigen.

3.4.3 Non-transgenic DN T cells exhibit antigen-specific cytotoxic activity

As the diabetes-susceptible NOD mice clearly demonstrate a deficiency in DN T cell number, we wanted to verify whether NOD mice also exhibit a deficiency in DN T cell function. However, due to the low number of DN T cells, in vitro cellular expansion is necessary in order to obtain a sufficient number of DN T cells to study. Indeed, we can readily expand DN T cells from non-transgenic mice by approximately 90-fold after 1 week in culture **(Supplementary Figure 3).** Subsequently, we examined whether non-transgenic DN T cells expressed cytotoxic potential. Accordingly, we demonstrate that expanded NOD CD1d-/- DN T cells do indeed express the cytolytic enzymes, granzyme

B and perforin, and, following re-stimulation, efficiently stain with the degranulation marker, anti-CD107a (**Figure 2A**). Therefore, DN T cells from diabetes-prone mice demonstrate cytotoxic potential.

As we have previously shown that 3A9 TCR transgenic DN T cells are cytotoxic towards B cells in an antigen-specific manner in vitro, we next wanted to validate these results in the non-transgenic setting. Due to the polyclonal nature of the TCR and the target antigens involved in diabetes progression, we decided to use a pool of islet antigens to study the function of non-transgenic DN T cells. Using activated B cells as targets, we show that B6.CD1d-/- DN T cells efficiently eliminate islet antigen-loaded B cells, but not HEL-loaded B cells, where HEL (hen egg lysozyme) is a non-relevant antigen (**Figure 2C**). Following the findings of **Figure 2B**, these results confirm that non-transgenic DN T cells do recognize self-antigens. Interestingly, this killing is completely inhibited by the presence of ethylene glycol tetraacetic acid (EGTA) (**Figure 2B**), suggesting a dependence on perforin/granzyme granules to mediate the cytotoxicity[43]. Therefore, non-transgenic DN T cells mediate an antigen-specific cytotoxic activity towards B cells using the perforin/granzyme pathway.

3.4.4 A single transfer of DN T cells is sufficient to reduce the diabetes incidence in NOD mice.

As NOD. CD1d-/- DN T cells exhibit a deficiency in number rather than function, we next wanted to determine whether the restoration of DN T cell number in autoimmune diabetes-prone mice prevented disease development in the non-transgenic setting. Due to the low number of DN T cells, in vitro cellular expansion is necessary. DN T cells were first sorted from non-transgenic NOD.CD1d-/- mice and expanded in vitro. $2x10^6$ activated DN T cells were transferred to 12 week-old non-diabetic female NOD mice. In comparison to non-injected or to activated $CD4^+$ T cell-injected controls, mice that were injected with activated DN T cells exhibited a significant reduction in the incidence of diabetes up to 32 weeks of age **(Figure 3** and **Supplementary Figure 4)**. Therefore, a

single autologous transfer of expanded DN T cells is sufficient to significantly decrease diabetes progression.

3.4.5 Transferred DN T cells migrate to, and proliferate within, pancreatic lymph nodes

As DN T cells impeded diabetes progression in NOD mice, we argued that they reached lymphoid organs, where they eliminated antigen-specific B cells. Indeed, the transferred DN T cells could be tracked to the spleen and peripheral lymph nodes as early as 24 hours, and for at least 4 weeks, post-transfer **(Supplementary Figure 5**). Moreover, the transferred DN T cells maintain their CD4-CD8- phenotype and do not upregulate B220 (**Figure 4A**), where B220 is expressed by DN T cells in Fas- or FasL-deficient mice [44,45]. Interestingly, a higher proportion of transferred DN T cells preferentially accumulate in the spleen and pancreatic lymph nodes in comparison to both skin-draining and mesenteric lymph nodes **(Supplementary Figure 5**). In addition, in both the spleen and the pancreatic lymph nodes, DN T cells express the activation marker CD69 **(Figure 5B**) and have entered cellular division (**Figure 5C**), suggesting that they recognize a self-antigen inducing their activation at these sites. Altogether, these results suggest that a preferential activation and proliferation of the transferred DN T cells occurs within the spleen and pancreatic lymph nodes.

3.4.6 DN T cell transfer leads to a reduction in germinal center B cells within pancreatic islets

Following these results, we postulated to that transferred DN T cells also migrated to the pancreatic islets, which is the site of beta cell destruction during diabetes development. Using CD45 allelic markers, we were able to track the transferred DN T cells to the pancreatic islets (**Figure 5B**). Accordingly, we find that a higher proportion of NOD.CD1d-/- DN T cells express the chemokine receptor CXCR5 in the pancreatic lymph nodes (**Figure 5A**), where CXCR5 is required for homing into germinal

centers[46,47]. Interestingly, the transfer of activated DN T cells leads to a decrease in the proportion of both GL7+CD95+ germinal center B cells as well as total GL7+ activated B cells within the pancreatic islets (**Figure 5C**) as well as a decrease in insulin autoantibody (IAA) serum levels **(Figure 5D**). In contrast, the transfer of CD4+ T cells leads to an increase in IAA serum levels **(Figure 5D).** Altogether, these results demonstrate that the reduction in germinal center B cells within the pancreatic islets and IAA serum levels in diabetes-susceptible NOD mice both correlate with the specific transfer of DN T cells.

3.4.7 Human DN T cells exhibit a therapeutic potential

As the transfer of expanded DN T cells results in a decrease in diabetes incidence in non-transgenic NOD mice, we wanted to verify whether human DN T cells exhibit similar properties. First, human DN T cells can be isolated from total PBMCs based on a $CD4^-CD8^-CD56^-V\alpha24\text{-}J\alpha18^-TCR\beta^+$ phenotype (**Figure 6A**). Following a 3 week expansion, DN T cells do indeed express the cytolytic enzymes, granzyme B and perforin (**Figure 6B**), demonstrating that human DN T cells exhibit cytotoxic potential. As murine DN T cells are cytotoxic towards B cells in vitro[26] **(Figure 2**) and DN T cell transfer leads to a reduction in activated B cells in vivo (**Figure 5C**), we wanted to verify whether human DN T cells exhibit cytotoxic activity towards B cells, as well. Here, irradiated allogeneic B cells were used as the target cells and cultured overnight in the presence of expanded human DN T cells. Indeed, in the presence of human DN T cells, a greater proportion of B cells undergo cell death in comparison to B cells cultured alone (**Figure 6C**). Therefore, similar to murine DN T cells, human DN T cells do exhibit cytotoxic activity towards B cells in vitro.

3.5 Discussion

This article is the first to demonstrate that DN T cells can inhibit diabetes development in non-transgenic, non-lymphopenic NOD mice. We further show that DN T cells mediate their immunoregulatory function by specifically eliminating B cells presenting islet antigens. This immunoregulatory function of non-transgenic DN T cells correlates with both a decrease in germinal center B cells directly within the pancreatic islets as well as a reduction in insulin autoantibody levels, where DN T cells preferentially migrate to the pancreas. Moreover, this article is the first to demonstrate that human DN T cells are cytotoxic towards B cells as they have previously only been shown to target T cells[48,49], demonstrating the similarity between murine and human DN T cells.

Importantly, we are not the only group to have demonstrated that DN T cells can inhibit autoimmune diabetes in the non-transgenic setting. Accordingly, Zhang and colleagues have shown that autoimmune diabetes induction was inhibited when islet-specific DN T cells were transferred to 5-week-old NOD mice[21]. However, this study used CD4-CD8- T cells that were converted from activated CD4+ T cells, which were not void of contaminating NK T cells, in vitro. Indeed, as a high proportion of NK T cells express CD4 and as NK1.1 is not expressed by the NOD mouse strain, where an NK1.1 antibody was used in the preparation of the CD4+ T cells, we cannot rule out the possibility that the observations made throughout this study were due to contaminating NK T cells. Conversely, our study uses CD1d-deficient mice, which are void of all NK T cells, as well as ex vivo isolated DN T cells followed by in vitro activation. Thus, our findings are the first to show that non-transgenic, uncontaminated DN T cells inhibit diabetes development in non-lymphopenic NOD mice. Furthermore, we have also revealed both the mechanism of action of non-transgenic DN T cells as well as the similarities between non-transgenic and human DN T cells. Therefore, our study is the first to clearly demonstrate the therapeutic potential of DN T cells for the treatment of autoimmune diabetes in humans.

DN T cells are of particular therapeutic interest for autoimmune diabetes because they inhibit immune responses in an antigen-specific manner[48,50]. Accordingly, the use of DN T cells in immunotherapy should lead to fewer side-effects along with a decreased risk of infections, which remains a major concern in the application of broad immunosuppressive regimens. With the ever growing evidence of the unique antigen-specific immunosuppressive properties of DN T cells, it is timely to address the *in vivo* impact of DN T cells on the immune response. The use of antigen-specific immunoregulatory DN T cells may eventually present itself as an alternative strategy to broad immunosuppressive regimens that lead to undesired side-effects, such as the rituximab treatment, which depletes all B cells as opposed to activated antigen-specific B cells[27,51].

Interestingly, Serreze and colleagues recently published an article demonstrating that B cell downregulate their expression of CD20 following their entry into pancreatic islets[52]. Consequently, anti-CD20 treatment, which has shown promising results in early clinical trials for autoimmune diabetes reversal[27], is unable to eliminate B cells found directly within the islets, where β cell destruction occurs[52]. Therefore, there are no current treatments that can efficiently target B cells within pancreatic islets. Our data in non-transgenic mice shows that DN T cell injection directly decreases the number of activated B cells within pancreatic lesions, suggesting that DN T cells could be more effective than anti-CD20 treatment at abrogating the local inflammatory autoimmune response. Moreover, as DN T cell transfer results in a decrease in antigen-specific autoantibody levels[18] (**Figure 5D**), this anticipates that autoantibodies can serve as a biomarker for the efficiency of DN T cell therapy.

In conclusion, we have demonstrated the impact of DN T cell proportion on autoimmune diabetes progression in non-transgenic NOD mice, elucidated the cellular mechanism by which DN T cells impede autoimmune responses *in vivo* and defined the characteristics of human DN T cells, all key aspects for the development of future therapeutic approaches. Altogether, these results demonstrate that DN T cells may be of therapeutic interest for autoimmune diabetes in humans.

3.6 Acknowledgments

The authors wish to thank Marie-Josée Guyon, Fany De Wilde and the animal house staff for curating the mouse colonies. EEH is a recipient of a CIHR PhD scholarship as well as a Diabète Québec scholarship, CB is a recipient of a Diabète Québec scholarship, VD is a recipient of a FRSQ scholarship, and SL holds a CIHR New Investigator scholarship. This project was funded by the Juvenile Diabetes Research Foundation and the Foundation of the Maisonneuve-Rosemont Hospital.

3.7 Methods

Mice

6 to 12 week-old mice were used for all the experiments. 3A9 TCR [53] and insHEL (ILK-3) [42] transgenic mice on the B10.Br background have been previously described [41] and were maintained at the Maisonneuve-Rosemont Hospital housing facility (Montreal, Canada). C57BL/6 and NOD^{g7} mice were purchased from the Jackson Laboratory. NOD^{g7,} NOD.*CD45*.2, B6.SJL, NOD.Rag1$^{-/-}$ mice, and CD1d-deficient mice on the NOD, C57BL/6, and BALB/c backgrounds were maintained at the Maisonneuve-Rosemont Hospital housing facility (Montreal, Canada). Studies were approved by the Maisonneuve-Rosemont Hospital ethics committee and overseen by the Canadian Council for Animal Protection.

Isolation of human cell populations

Heparinized blood samples were obtained from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Cells were then stained with antibodies against CD4, CD8, CD56, Vα14-Jα24, and TCRαβ. The human DN T cells (CD4$^-$ CD8$^-$ CD56$^-$ Vα14-Jα24$^-$ TCRαβ$^+$) or CD8$^+$ T cells (CD4$^-$ CD8$^+$ CD56$^-$ Vα14-Jα24$^-$ TCRαβ$^+$) were sorted by using a FACS Aria III (BD Biosciences). Cells were also stained using antibodies against CD19 and TCRαβ$^+$ and B cells (CD19$^+$TCRαβ$^-$) were sorted using a FACS Aria III (BD Biosciences). Sorted cells were reanalyzed after cell sorting and routinely showed more than 95% purity. Studies were approved by the Maisonneuve-Rosemont Hospital ethics committee. Informed consent was provided according to the Declaration of Helsinki.

Isolation of murine cell populations

Murine cell suspensions were stained with antibodies against CD4, CD8β, CD19 and TCRβ. CD4$^-$CD8$^-$CD19$^-$TCRβ$^+$ DN T cells and CD4$^+$CD8$^-$CD19$^-$TCRβ CD4$^+$ T cells were sorted using a FACS Aria III (BD Biosciences) (purity > 95%). B cells were purified using the EasySep PE selection kit, where B cells were first stained with CD19-biotin followed by streptavidin-PE. B cells were routinely enriched to >90% .

Cellular expansion

Sorted T cell populations from mice were cultured at 37˚C at a concentration of 250 000 cells/ml for 7 days in the presence of plate-bound anti-CD3 (1µg/ml, 145-2C11, Biolegend) and anti-CD28 (10µg/ml, 37.51, Biolegend) as well as IL-2 (30U/ml, Feldan Bio) and IL-7 (10ng/ml, generously provided by Dr. Martin Guimond) in RPMI supplemented with 10% fetal bovine serum (Invitrogen), Penicillin (100U/ml)-Streptomycin (100ug/ml) (Gibco), 0.01M HEPES (Gibco) and 2-mercaptoethanol 0.055mM (Gibco).

Sorted T cell populations from human PBMCs were cultured at 37˚C at a concentration of 250 000 cells/ml for 21-28 days in the presence of plate-bound anti-CD3 (10µg/ml, HIT3a, Biolegend) and anti-CD28 (5µg/ml, CD28.2, Biolegend) in RPMI supplemented with 10% human serum (Sigma) and Penicillin (100U/ml)-Streptomycin (100ug/ml)-L-Glutamine (29.2 mg/ml) (Gibco). 24 hours following initial culture, cytokines were added to the culture, including IL-2 (30U/ml, Feldan Bio), IL-7 (10ng/ml, generously provided by Dr. Martin Guimond) +/- IL-4 (30U/ml,Feldan Bio).

Cell cultures were monitored daily for confluence and cytokine supplemented media was added when cell density was > $1x10^6$ cells/ml. Cell number was quantified by trypan blue exclusion.

Flow cytometry

6 to 12 week-old non-diabetic mice (Diastix negative) were analyzed. Single cell suspensions of lymphoid organs were prepared by mechanical disruption through a 70 µm sterile cell strainer (BD Biosciences). NH_4Cl was used for erythrocyte lysis of single cell suspensions from spleens. Islets were isolated by ductal Collagenase (Sigma) injection to inflate the pancreas. The pancreas was then incubated 20 min at 37°C and washed with a solution containing FBS. Islets were handpicked and dissociated using a pipette tip. Human cells were prepared as described above. Cell counts were performed

by trypan blue exclusion using a hemacytometer. Intracellular stainings for granzyme B (anti-mouse: 16G6, eBioscience, anti-human: GB11, eBioscience) and perforin (anti-mouse: eBioOMAK-D, eBioscience, anti-human: dG9, eBioscience) were performed using BD Biosciences Cytofix/Cytoperm Kit. Anti-clonotypic 1G12 antibody, specific for the 3A9 TCR [54], was detected using APC-labelled anti-mouse IgG1 (clone X56, BD Biosciences). For anti-mouse antibodies, alpha GalCer:CD1d complex (clone L363) and GL7 (clone GL-7) were purchased from eBioscience, while CD95 (Fas) (clone Jo2) and CXCR5 (clone 2G8) was purchased from BD Biosciences. For anti-human antibodies, CD56 (clone CMSSB) and Valpha24-Jalpha18 TCR (clone 6B11) were purchased from eBioscience. All other antibodies and staining reagents were purchased from Biolegend. Data was collected on a FACSCalibur or FACS LSR II (BD Biosciences) and analyzed using FlowJo software (Treestar).

Diabetes monitoring

DN T cells and CD4 T cells were sorted from the spleen of NOD.CD1d-/- mice and expanded in vitro. 2 million expanded DN T cells or CD4 T cells were then injected intravenously in non-diabetic (diastix negative) 12 week-old female NOD^{g7} mice, where non-injected female NOD^{g7} mice served as a control. The diabetes incidence was monitored every two weeks using Diastix glucose test strips, which analyzes glucose levels in the urine. Diabetic mice showed 2 consecutive Diastix positive tests and blood glucose levels above 17mM.

Cytotoxic potential

Cells were sorted and cultured as indicated above. For granzyme B and perforin detection, cells were re-stimulated for 4h with phorbol 12-myristate 13-acetate (PMA) (50ng/ml) (Sigma), ionomycin (0.5μg/ml) (Sigma), and brefeldin A (10 μg/ml) (Sigma), prior to intracellular staining. For CD107a (LAMP1) staining, we replaced Brefeldin A with mononesin (2nM) (BD biosciences) and the anti-CD107a antibody was added during the 4h re-stimulation[55].

Cytotoxicity

Sorted murine DN T cells were stimulated 7 days in vitro with plate-bound anti-CD3 and anti-CD28, IL-2, and IL-7. Lipopolysaccharide (LPS) (1μg/ml, Sigma) was used to stimulate B cells in vitro for 2 days. HEL antigen (1mg/ml) (Sigma) or islet antigen was added in the last 24h of culture. Islet antigen was prepared by incubating isolated islet cells with DNase (Sigma) for 10 minutes at 37˚C. Islets were isolated from B6.CD1d-/- mice or NOD.Rag-/- mice, rather than NOD.CD1d-/- or NOD mice, because no lymphocytic infiltration can be found in NOD.Rag-/- mice. Target B cells were washed and loaded with 50μCi ^{51}Cr (Perkin Elmer) for 90 min. DN T cells and B cells were quantified by trypan blue exclusion prior to co-culture in order to obtain the desired effector to target cell ratio. DN T cells and B cells were incubated for 3h at 37˚C in the presence or absence of EGTA (0.16mM) (J. T. Baker). %Cytotoxicity = 100*(E –S)/(M – S), where E = experiment, S = spontaneous and M = maximal release.

For human cells, sorted B cells are incubated for 10 minutes at 37˚C with Cell Trace Violet (5 μM, Invitrogen). B cells are then incubated on ice for 5 minutes with cold FBS before washing with RPMI. B cells are distributed to the wells of a 96-well plate (round bottom). Subsequently, sorted and expanded human DN T cells (or CD8 T cells as a control) are washed and distributed to the wells yielding the appropriate effector to target cell ratio. Effector T cells and B cells were quantified by trypan blue exclusion prior to co-culture in order to obtain the desired effector to target cell ratio. Following an overnight co-culture, cells were washed and stained with propidium iodide (PI) and analyzed using flow cytometry. % Cytotoxicity = 100*(E–S), where E = experiment, S = spontaneous, where spontaneous cell death was calculated as the amount of B cells staining positive for PI in the absence of effector T cells.

Proliferation assay

For proliferation assays, $2x10^6$ expanded DN T cells were labelled with CFSE (2μM) (Invitrogen) and washed twice in cold supplemented RPMI prior to adoptive transfer. CFSE dilution was monitored by flow cytometry.

Insulin autoantibody ELISA

Insulin (Anaspec) was immobilized onto microwells. The diluted serum samples, added to the microwells. IgG specific antibodies to insulin in the serum sample bind to the insulin molecules on the microwells. Serum, which was previously incubated with insulin, will have already formed antibody-antigen complexes and be unable to bind to the insulin on the microwells, thus serving as a background control. After washing off unreacted serum materials, horseradish peroxidase-labeled polyclonal goat anti-mouse IgG (Poly4053, Biolegend) was added. After thorough washing to remove the unbound enzyme, TMB substrate solution was added and the color development was measured using spectrophotometry. The intensity of the color, namely the optical density (OD), is directly proportional to the concentration of insulin autoantibody in the sample. All samples were run in duplicate. The level of insulin autoantibody in the sample = (average of sample OD) - (average of background control OD).

Statistics

The paired student's T test and Logrank (Mantel-Cox) statistical tests were applied where appropriate.

3.8 Figures

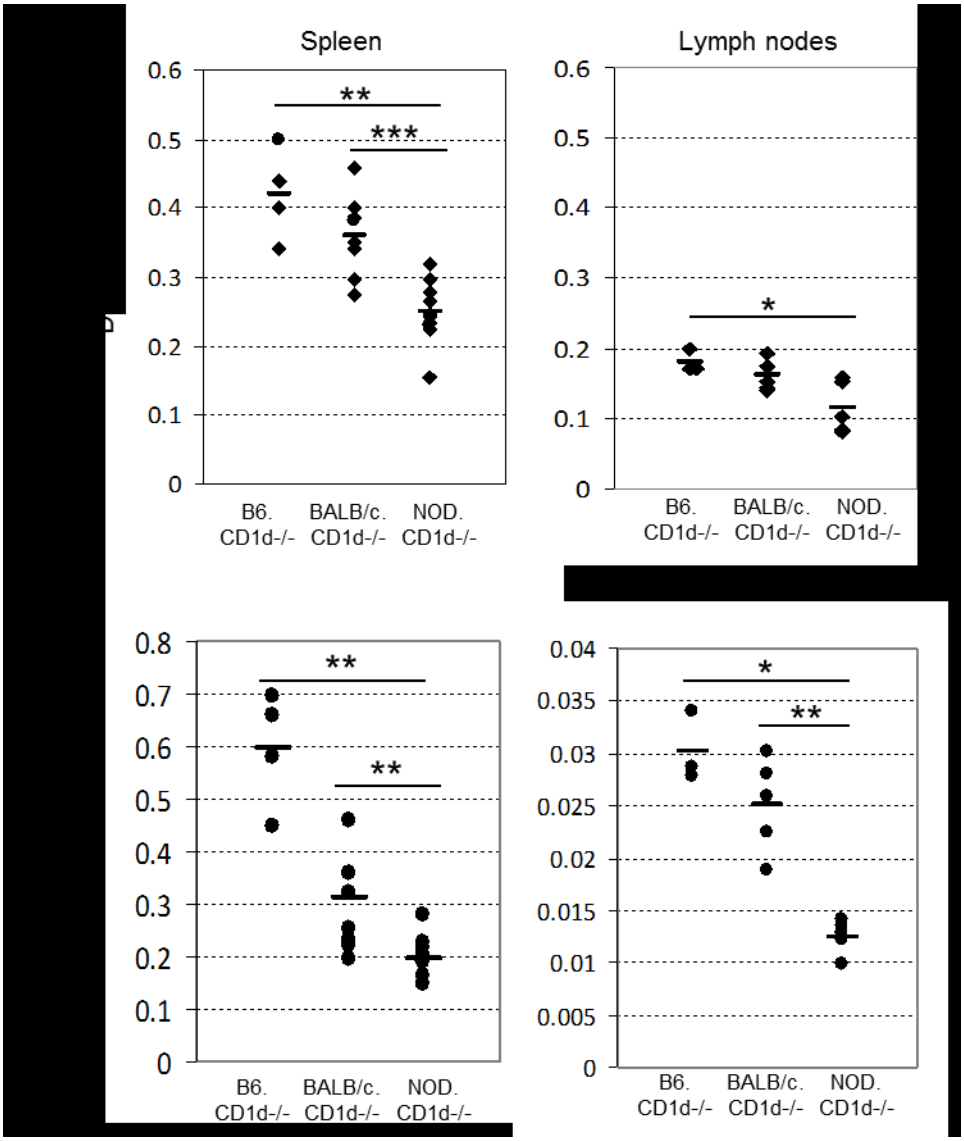


Figure 1. The proportion and absolute number of DN T cells is reduced in non-transgenic NOD.CD1d-/- mice. The spleen and lymph nodes were harvested from 8-12 week old non-transgenic CD1d-deficient mice of B6, BALB/c and NOD genetic backgrounds and stained with antibodies against CD19, CD4, CD8, CD5 and TCRβ to determine the proportion (A) and absolute number (B) of non-transgenic DN T cells (CD19- CD4- CD8- TCRβ+ CD5low). Mann Whitney test was performed. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

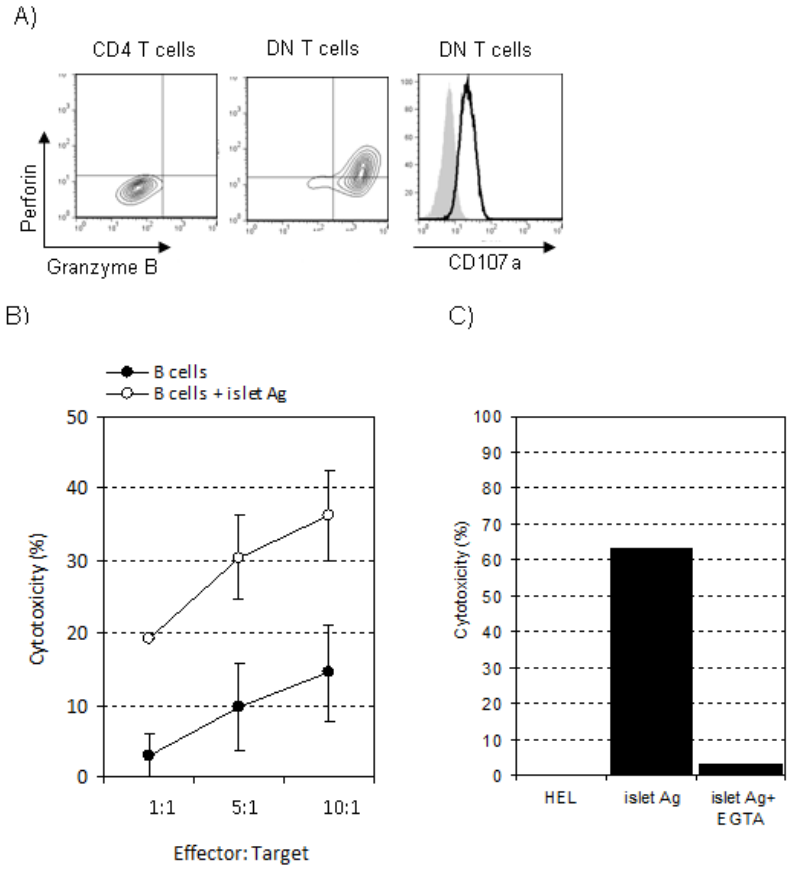


Figure 2. Non-transgenic DNT cells demonstrate an antigen-specific cytotoxic activity towards activated B cells. Non-transgenic B cells and DN T cells were sorted from the spleen of NOD.CD1d-/- mice. Sorted DN T cells were stimulated for 7 days in vitro with anti-CD3, anti-CD28, IL-2 and IL-7. A) In vitro expanded non-transgenic DN T cells were re-stimulated for 4 h with PMA, ionomycin and monensin along with the anti-CD107a antibody before intracellular staining. The level of perforin, granzyme B and CD107a is shown. Expanded DN T cells that were not re-stimulated were used as a control for CD107a expression (grey filled histogram in right panel). n≥3. (B) The percent-specific lysis is shown for NOD.CD1d-/- DN T cells toward target lipopolysaccharide-activated NOD.CD1d-/- B cells that were loaded, or not, with islet antigens. Representative of 2 experiments. (C) The percent-specific lysis is shown for B6.CD1d-/- DN T cells toward target LPS-activated B6.CD1d-/- B cells at a 10:1 effector to target ratio that were loaded with either islet antigens or the non-relevant HEL antigen, as indicated. EGTA was added in some conditions, as indicated. n=1.

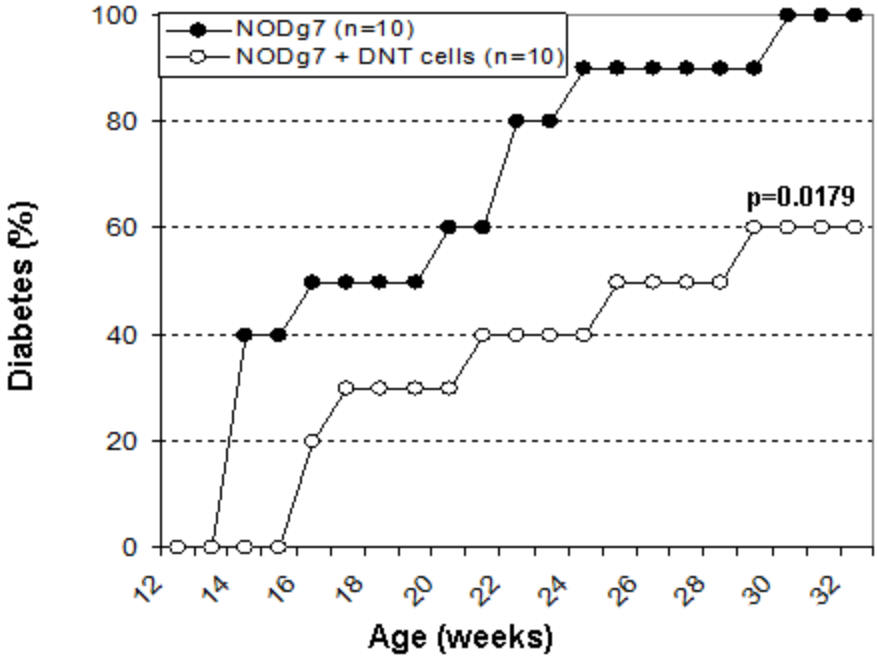


Figure 3. In vitro expanded non-transgenic DN T cells reduce the incidence of diabetes in NOD mice. DN T cells were sorted from the spleens of 8-12 week old NOD.CD1d-/- mice and activated in vitro for 7 days with anti-CD3, anti-CD28, IL-2 and IL-7. $2x10^6$ cells were injected intravenously into 12 week-old non-diabetic female NOD mice. Diabetes incidence is depicted for DN T cell-injected and non-injected mice up to 32 weeks of age. The Logrank (Mantel-Cox) test was performed for statistical analysis.

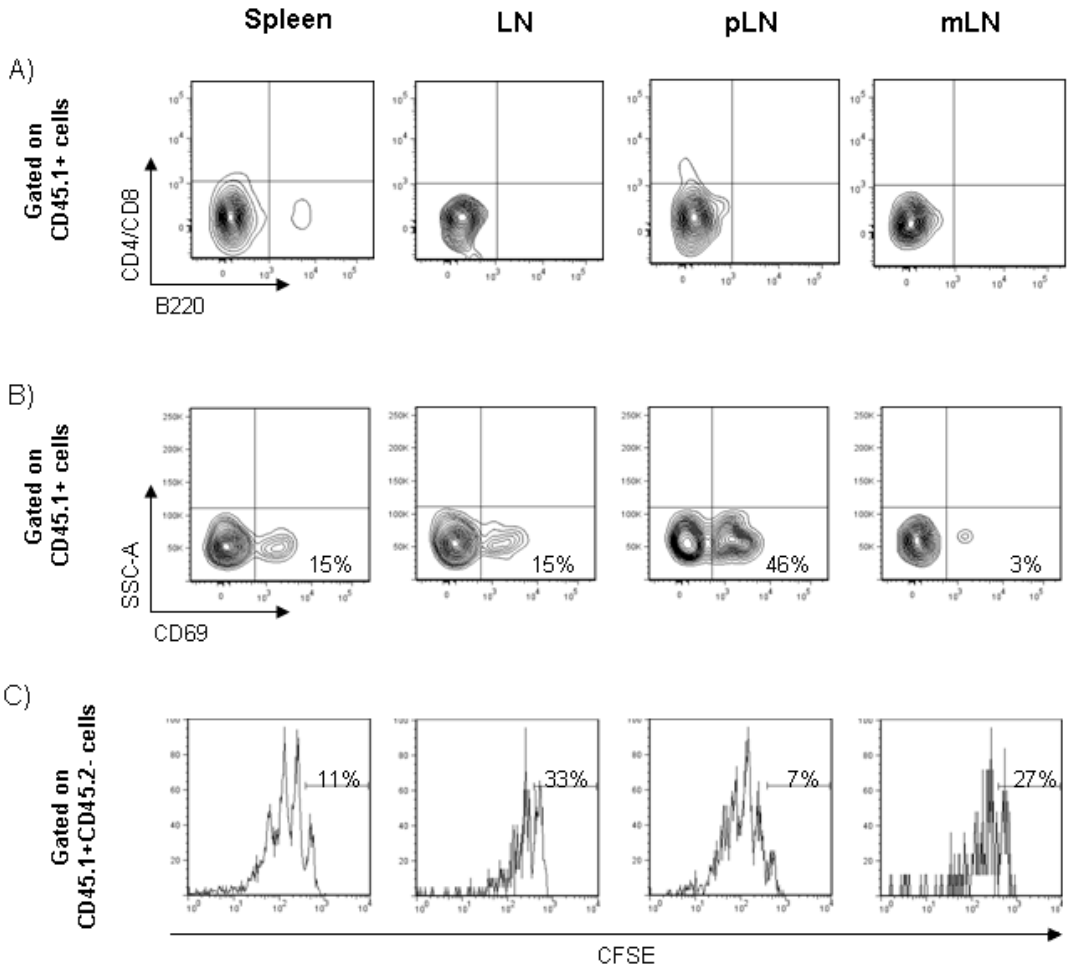


Figure 4. Preferential activation and proliferation of transferred DNT cells in the pancreatic lymph nodes. Non-transgenic DN T cells were sorted from the spleen of NOD.CD1d-/- (CD45.1) mice. Following a 7 day expansion *in vitro* with anti-CD3, anti-CD28, IL-2 and IL-7, 1-2 x 10^6 non-transgenic DN T cells were injected intravenously into non-diabetic 10-12 week-old recipient NOD.*CD45.2* mice. 4 weeks post-transfer, the spleen, skin-draining lymph nodes (LN), pancreatic lymph nodes (pLN) and mesenteric lymph nodes (mLN) were harvested. (A) The expression of CD4, CD8, B220 (B) and CD69 is shown for the transferred DN T cells ($CD45.1^+$). (C) Expanded DN T cells were stained with CFSE prior to their transfer. The histograms depict the level of expression of CFSE for the transferred DN T cells ($CD45.1^+CD45.2^-$). The percentage of cells that have not entered proliferation is shown. n≥3.

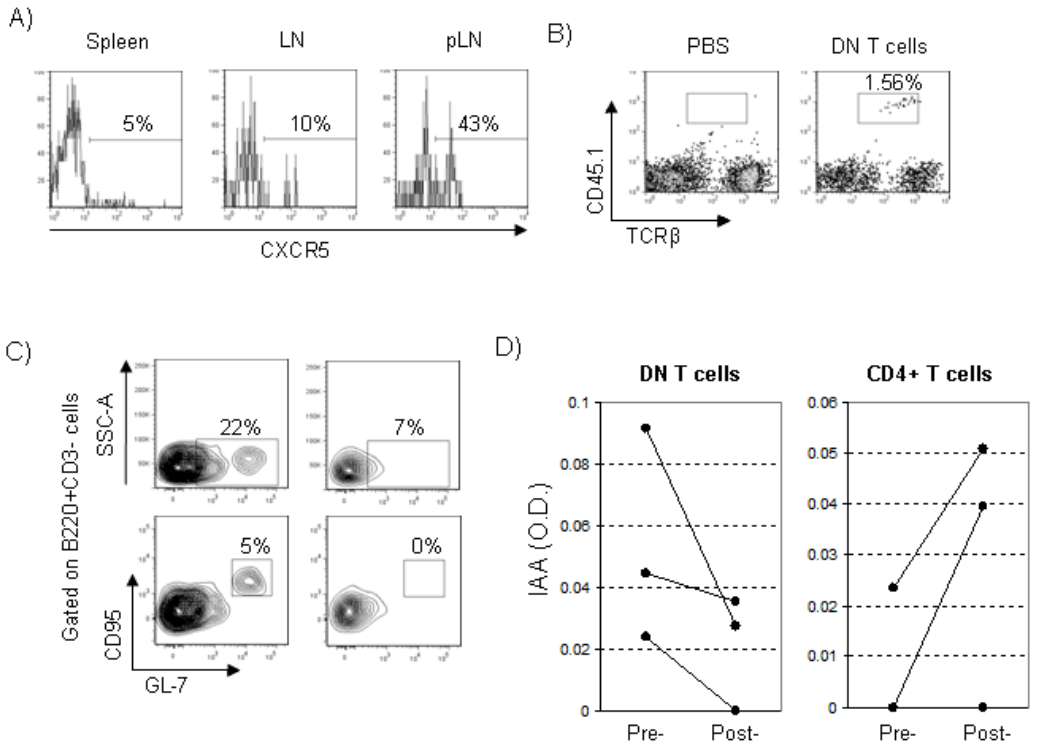


Figure 5. Transferred DN T cells migrate to the pancreatic islets and induce a reduction in germinal center B cells. (A) The spleen, skin-draining lymph nodes (LN) and pancreatic lymph nodes (pLN) were harvested from 8-12 week old NOD.CD1d-/- mice and stained with antibodies against CD19, CD4, CD8, and TCRβ for the isolation of non-transgenic DN T cells ($CD19^-$ $CD4^-$ $CD8^-$ $TCR\beta^+$). The histograms depict the level of expression of CXCR5 on DN T cells. n=3. (B-D) DN T cells were sorted from the spleens of 8-12 week old NOD.CD1d-/- (CD45.1) mice. Following a 7 day expansion in vitro with anti-CD3, anti-CD28, IL-2 and IL-7, 2 x 10^6 non-transgenic DN T cells, or PBS, were injected intravenously into non-diabetic 10-12 week-old recipient NOD.CD45.2 mice. 2 weeks post-transfer, (B) the proportion of transferred DN T cells ($CD45.1^+$ $TCR\beta^+$) and (C) the proportion of activated B cells (B220+ CD3- GL-7+, top panel) and germinal center B cells (B220+CD3-GL-7+CD95+, bottom panel) in pancreatic islets was quantified by flow cytometry, representative of at least 3 experiments. (D) Serum insulin autoantibody (IAA) levels were measured by ELISA before (pre-) and two weeks after (post-) DN T or CD4 T cell injection in NOD mice. These preliminary results are paired from individual mice.

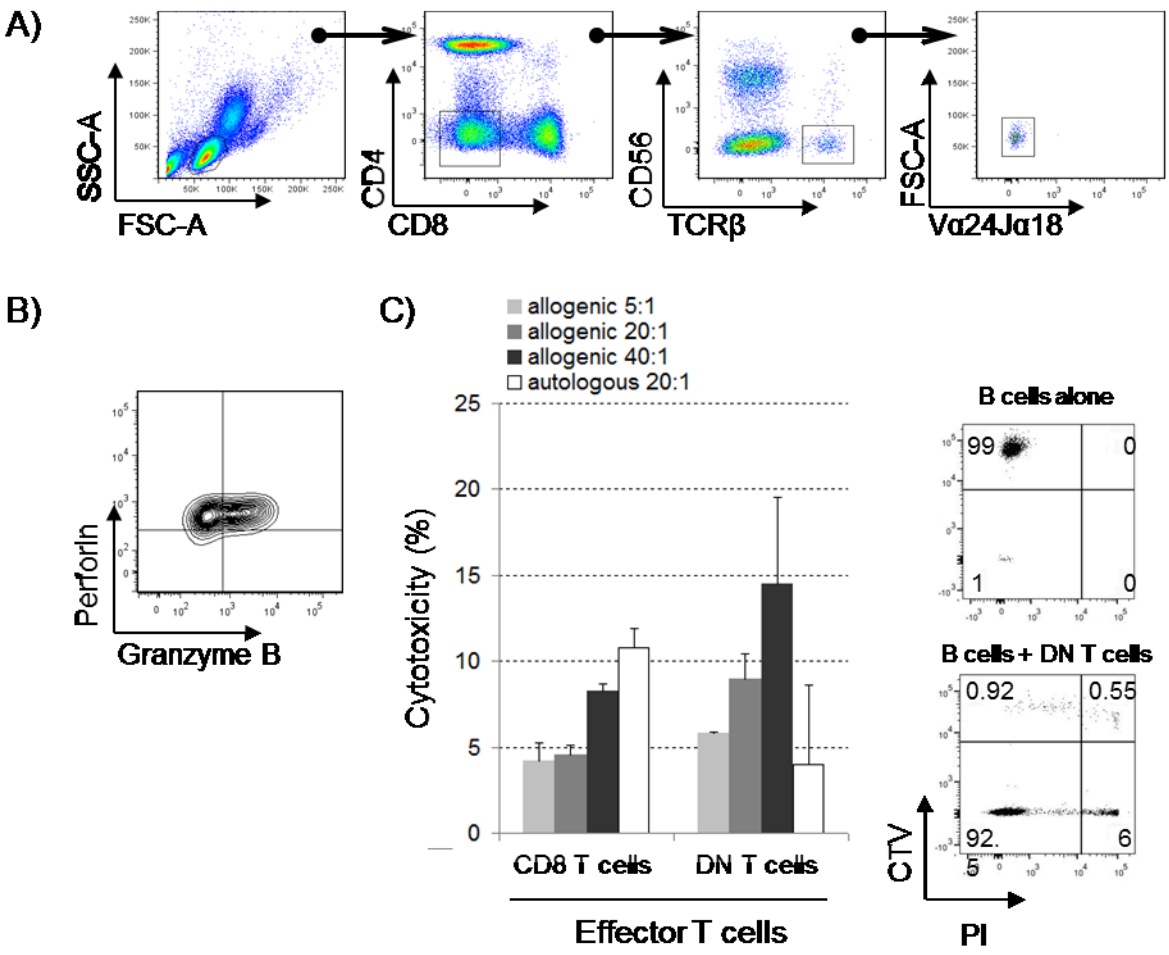
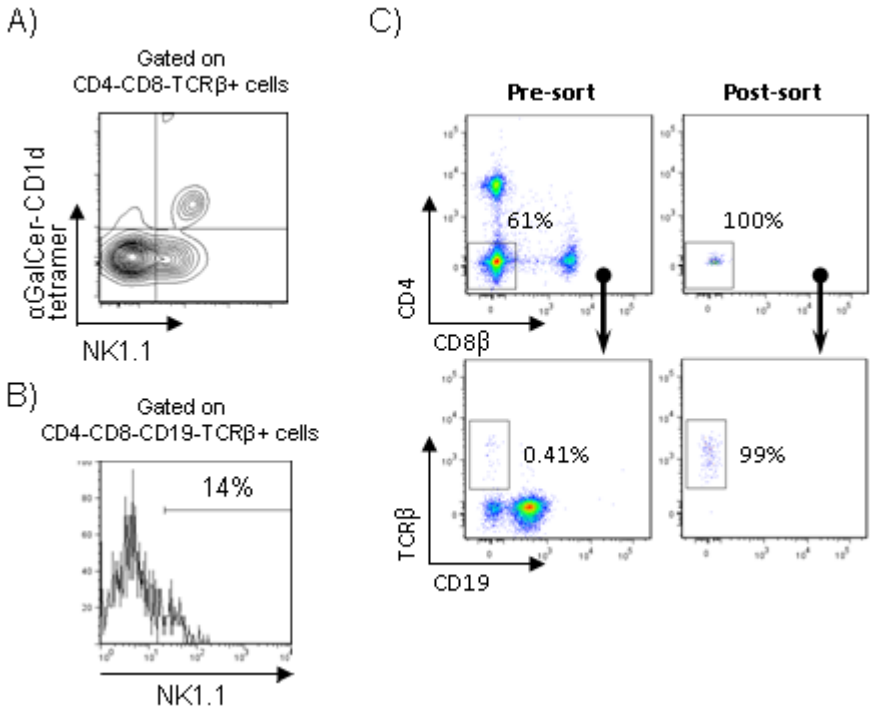
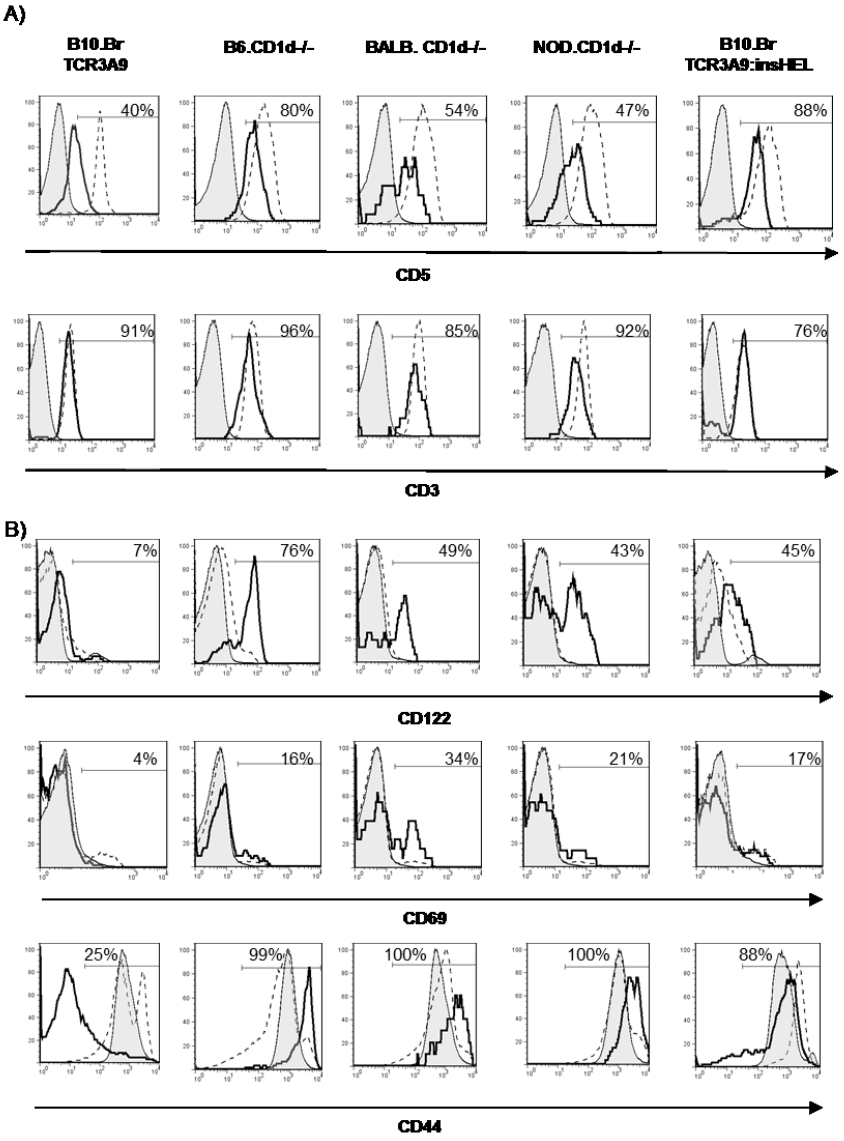


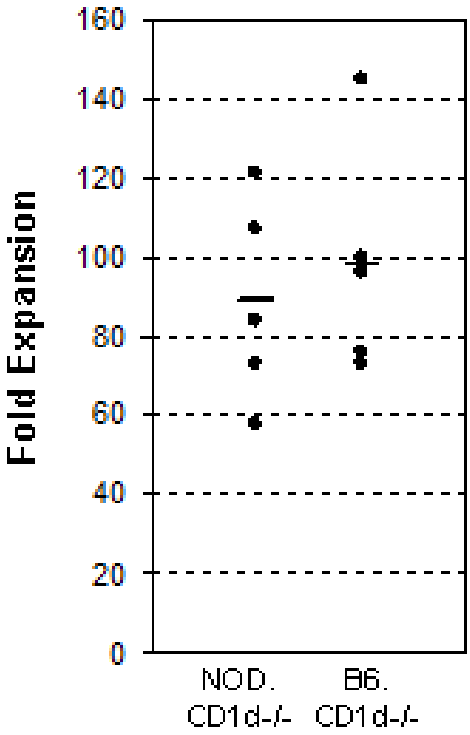
Figure 6. Human DN T cells exhibit phenotypic and functional properties reminiscent of mouse DN T cells. Human DNT cells ($CD4^{-}CD8^{-}CD56^{-}V\alpha24\text{-}J\alpha18^{-}TCR\alpha\beta^{+}$) were isolated from total PBMCs. A) Gating strategy for sorting and quantifying the proportion of DN T cells is shown. B) Human DN T cells were stained intracellularly for perforin and granzyme B expression following a 3 week expansion, Representative of at least 3 experiments. C) Expanded human DN T cells (or CD8 T cells as a control) were co-cultured overnight with Cell Trace Violet (CTV)+ autologous or allogeneic B cells. Following the overnight co-culture, cells were washed and stained with propidium iodide (PI) and analyzed using flow cytometry. The right panels illustrate a typical FACS dot plot for the negative control with CTV+ B cells alone (top) and in the presence of CTV- DN T cells (bottom). Percent specific lysis of target B cells is shown in the bar graph (left panel). n≥3.



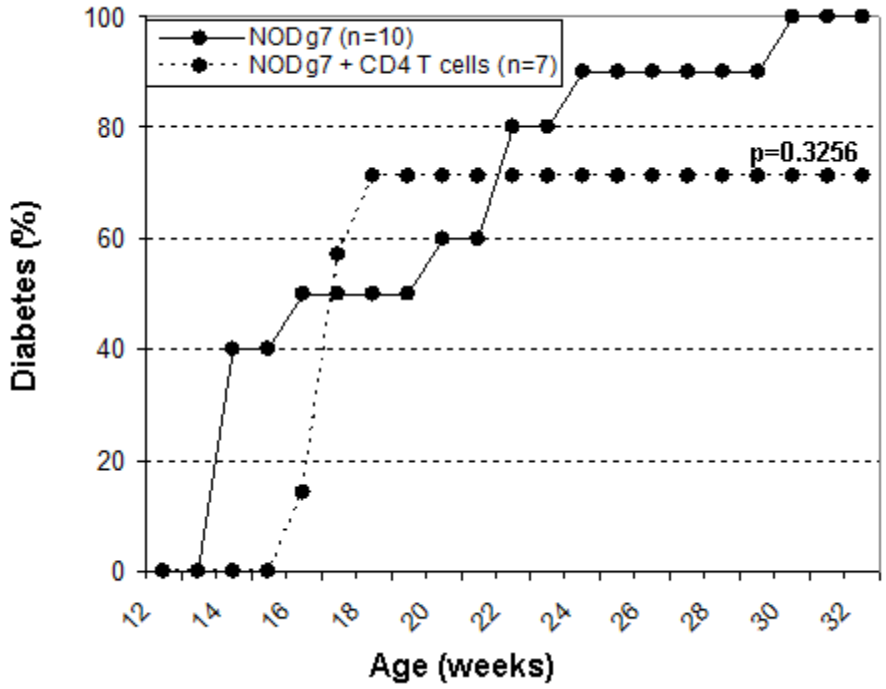
Supplementary Figure 1. Strategy for isolating DN T cells free of contaminating NKT cells. DN T cells and a subset of NKT cells share a common CD4- CD8- TCRβ+ phenotype. (A) The spleen was harvested from 8-12 week old B6 mice and stained with antibodies against CD4, CD8, TCRβ, NK1.1 and the α-GalCer:CD1d complex. (B) A proportion of DN T cells express NK1.1. The spleen was harvested from 8-12 week old B6.CD1d-/- mice and stained with antibodies against CD4, CD8, CD19, TCRβ and NK1.1. (C) Gating strategy used to sort DN T cells from the spleen is shown for NOD.CD1d-/- mice. Sorted cells are CD4- CD8- CD19- TCRβ+ (left panels). Cell purity was routinely above 95% (right panels). Results are representative of at least 3 independent experiments.



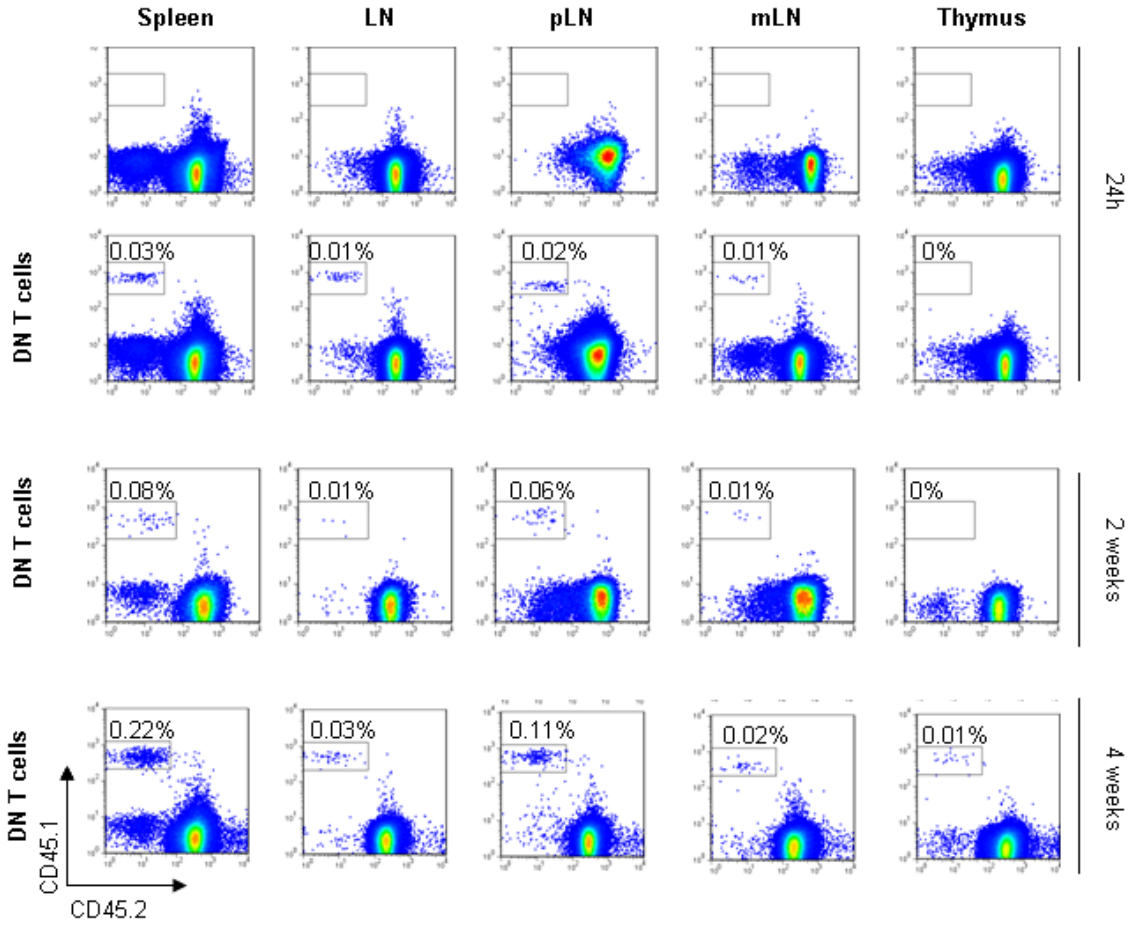
Supplementary Figure 2. Non-transgenic DN T cells express an activated phenotype. The spleen was harvested from 8-12 week old mice and stained with antibodies against CD4, CD8, and TCRβ or 1G12 and CD19 or B220 for the isolation of non-transgenic DN T cells (CD19- CD4- CD8- TCRβ+) or TCR transgenic DN T cells (B220- CD4- CD8- 1G12+). The histograms depict the level of expression of (A) pan-T cell markers, CD3 and CD5, as well as (B) activation markers, CD122, CD69 and CD44, on B220+ cells (shaded), CD4+ cells (dashed) and DN T cells (solid) from CD1d-deficient B6, BALB/c and NOD mice as well as TCR 3A9 and TCR 3A9:insHEL transgenic mice. The percentage of DN T cells that stained positive for the marker is shown in the upper right corner. Results are representative of at least 3 independent experiments.



Supplementary Figure 3. Expansion of non-transgenic DNT cells in vitro. Non-transgenic DN T cells were sorted from the spleen of CD1d-deficient B6 and NOD mice. Sorted cells were stimulated for 7 days in vitro with anti-CD3, anti-CD28, IL-2 and IL-7. Similar expansion efficiency was observed irrespective of the genetic background of the mice. Compilation of cellular expansion results is depicted.



Supplementary Figure 4. *In vitro* expanded non-transgenic CD4 T cells do not affect the incidence of diabetes in NOD mice. CD4 T cells were sorted from the spleens of 8-12 week old NOD.CD1d-/- mice and activated in vitro for 7 days with anti-CD3, anti-CD28, IL-2 and IL-7. $2x10^6$ cells were injected intravenously into 12 week-old non-diabetic female NOD mice. Diabetes incidence is depicted for CD4 T cell-injected and non-injected mice up to 32 weeks of age. The Logrank (Mantel-Cox) test was performed for statistical analysis.



Supplementary Figure 5. Transferred DNT cells migrate to various organs post-transfer. Non-transgenic DN T cells were sorted from the spleen of NOD.CD1d-/- (CD45.1) mice. Following a 7 day expansion in vitro with anti-CD3, anti-CD28, IL-2 and IL-7, 1-2 x 10^6 non-transgenic DN T cells were injected intravenously into non-diabetic 10-12 week-old recipient NOD.CD45.2 mice. At 24 hours, 2 and 4 weeks post-transfer, the spleen, skin-draining lymph nodes (LN), pancreatic lymph nodes (pLN), mesenteric lymph nodes (mLN) and thymus were harvested and cells were stained for CD45.1, CD45.2 and TCRβ. The transferred DN T cells ($CD45.1^+CD45.2^-$) have been gated and their proportion is shown. Representative of at least 3 experiments.

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CHAPTER 4: DISCUSSION

The results presented in this thesis provide insight into the function of DN T cells as well as the ability to prevent autoimmune diabetes through the restoration of DN T cell number in non-transgenic diabetes-susceptible mice. Accordingly, the findings in this thesis have revealed the therapeutic potential for DN T cells in the treatment of autoimmune diabetes in human patients.

4.1. Non-transgenic DN T cells: Phenotype and proportion

The specific isolation, and consequently investigation, of non-transgenic DN T cells is complicated by the fact that DN T cells share the $CD4^{-}CD8^{-}TCR_{\alpha\beta}^{+}$ phenotype with a subset of NKT cells (reviewed in [156], Hillhouse, E.E., Lesage, S., 2012). However, as NKT cells are known to express a TCR restricted to CD1d molecules and are thus, by definition, absent in CD1d-deficient mice[210], we chose to use CD1d-deficient mice for the purification of DN T cells throughout these studies to ensure a population of DN T cells void of any contaminating NKT cells.

Due to their scarcity in the non-transgenic setting, DN T cells have been mostly characterized using TCR transgenic settings. Indeed, DN T cells have been shown to exhibit a naive phenotype in the absence of cognate antigen, whereas they exhibit a more activated phenotype in the presence of cognate antigen (reviewed in [156], Hillhouse, E.E., Lesage, S., 2012). To characterize the phenotype of non-transgenic DN T cells in the absence of any contaminating NKT cells, we compared DN T cells from CD1d-deficient mice on the NOD, C57BL/6 (B6) and BALB/c genetic backgrounds alongside TCR transgenic DN T cells from both the 3A9 TCR and 3A9 TCR:insHEL transgenic mouse models. In this model, the 3A9 TCR transgene, which can be detected using the 1G12 anti-3A9 clonotypic mAb [258], recognizes a HEL peptide in the context of $I\text{-}A^{k}$ and the insHEL transgene is

expressed under the rat insulin promoter [258, 303]. As expected, DN T cells from the 3A9 TCR transgenic mice, thus in the absence of the cognate antigen, exhibit a naive phenotype whereas the DN T cells from the 3A9 TCR:insHEL transgenic mice, for which the HEL antigen is present, exhibit an activated phenotype **(Article 2, Supplementary Figure 2).** Interestingly, the phenotype of DN T cells from CD1d-deficient mice was analogous to that of DN T cells from the 3A9 TCR:insHEL transgenic mice, such that they expressed similar levels of the various activation markers **(Article 2, Supplementary Figure 2**). Furthermore, whereas DN T cells expressing the 3A9 TCR are monoclonal such that they express Vα3 and Vβ8.2 TCR chains[304], DN T cells from non-transgenic mice express a polyclonal TCR repertoire (**Appendix 2**). Because 3A9 TCR transgenic DN T cells are activated by the presence of HEL, which represents a self-antigen as it is expressed under the rat insulin promoter[258, 303], these results suggest that non-transgenic DN T cells also recognize self-antigens resulting in their activated phenotype ex vivo.

Other regulatory T cell populations, such as NKT cells and Tregs, express an activated phenotype[305, 306, 307] and are also known to recognize self-antigen. Indeed, NK T cells recognize lipid self-antigen, where β-galactosylceramide (β -GalCer), isoglobotrihexosylceramide (iGb3) [308] and β-D-glucopyranosylceramide (β-GlcCer)[309] have been identified for type I NK T cells and the sulfated galactosylceramide, sulfatide, has been identified for type II NKT cells[184]. Deficiency in NKT cells have also been implicated in autoimmune diseases in both mice and humans, such as gastritis, multiple sclerosis, and autoimmune diabetes [159]. Tregs also recognize self-antigens[310, 311], which is an important element to permit their thymic development [237]. Moreover, Treg deficiency is also associated with autoimmune disease development. For instance, the dysfunction of the transcriptional activator FOXP3, which is a master regulator for the development of Tregs, leads to the progression of IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome), which is a rare disease linked to the development of autoimmune diseases, including autoimmune diabetes [312]. Moreover, mice that are thymectomized on day 3 of life develop organ-specific autoimmune disease, such as gastritis, unless

the mice received a transfer of $CD4^+$ T cells within 2 weeks of birth, where the inhibitory activity was attributed to $CD4^+CD25^+$ Tregs [313]. Together, these results demonstrate that regulatory T cell populations such as NKT cells and Tregs recognize self-antigens and are consequently linked to the suppression of autoimmunity.

Our lab has previously demonstrated that diabetes-susceptible mice present with fewer DN T cells than diabetes-resistant mice in the TCR transgenic setting **(Appendix 1, Figure 4)**, which initiated our investigation into the difference in DN T cell proportion between diabetes-susceptible and -resistant mice in the non-transgenic setting. The use of CD1d-deficient mice facilitated the realization of this study as it ensured a DN T cell population void of any contaminating NKT cells and, thus, a true difference in proportion. Indeed, both the proportion and absolute number of non-transgenic DN T cells is significantly reduced in the secondary lymphoid organs of diabetes-susceptible NOD CD1d-deficient mice in comparison to diabetes-resistant B6 and BALB/c CD1d-deficient mice **(Article 2, Figure 1).** These results suggest an association between a low DN T cell number and autoimmune diabetes susceptibility in the non-transgenic setting. In addition, these results confirmed that the difference in DN T cell numbers observed in the TCR transgenic setting (**Appendix 1, Figure 4**) was not an artefact of the insertion of the transgene. More importantly, the validation of a reduction in DN T cell number in diabetes-susceptible mice supported our hypothesis that an increase in DN T cell number could prevent autoimmune diabetes development in the non-transgenic setting. Therefore, to validate that a reduced number in DN T cells is causal to autoimmune diabetes development in the non-transgenic setting, we must attempt to restore DN T cell number. However, without functional DN T cells, a restoration of DN T cell number would be ineffective regardless of the increase in number. Therefore, we subsequently verified whether DN T cells from the diabetes-susceptible genetic background were in fact functional.

4.2. The function of DN T cells

Before determining whether DN T cells from diabetes-susceptible mice were functional, we first needed to determine the target of our DN T cells. The function of DN T cells was determined using 3A9 TCR diabetes-resistant B10.Br mice for three main reasons. First, the insertion of the TCR transgene would increase DN T cell proportion, thus allowing for a sufficient number of cells to carry out functional assays. Second, we opted to use TCR transgenic mice from the B10.Br diabetes-resistant background, as these mice present with a greater number of DN T cells in comparison to diabetes-susceptible mice **(Appendix 1, Figure 4)**. Lastly, the utilization of the 3A9 TCR transgenic model would facilitate the study of the specificity of the DN T cells as the 3A9 TCR transgene is specific for the HEL antigen. As DN T cells from various TCR transgenic models have been reported to specifically eliminate $CD8^+$ T cells[171, 186, 218, 225, 243, 244, 245], we first verified whether 3A9 DN T cells also eliminate T cells. Using, 3A9 TCR transgenic B10.Br mice, we show that DN T cells are not cytotoxic towards T cells, but rather are cytotoxic towards activated B cells **(Article 1, Figure 1D).** Moreover, the cytotoxicity is both antigen-specific, as DN T cells specifically kill B cells that are loaded with HEL **(Article 1, Figure 1D),** and dependent on the release of the cytolytic granules, granzyme and perforin, as the cytotoxicity was blocked by the presence of ethylene glycol tetraacetic acid (EGTA)[314]. Therefore, 3A9 DN T cells are cytotoxic towards activated B cells in an antigen-specific and granzyme/perforin-dependent manner.

Since the publication of **Article 1** in 2010, 2C TCR transgenic DN T cells, which had previously only been shown to target $CD8^+$ T cells[171, 218, 225, 243, 244, 245], were shown to be also cytotoxic towards LPS-activated B cells in vitro[315]. This variation in broad vs B cell-limited target cell specificity between the 2C and 3A9 TCR transgenic models, respectively, may be explained by the fact that the 3A9 TCR model is MHC class II-restricted, whereas the 2C TCR model is MHC class I-restricted. Indeed, 3A9 DN T cells may be limited to exhibit cytotoxic activity only towards MHC class II expressing cells, including B cells, dendritic cells and

macrophages, while MHC class I-restricted 2C DN T cells do not exhibit such a limitation as MHC class I is expressed on all nucleated cells. The study of DN T cell function in additional MHC class I and class II-restricted TCR transgenic models should help resolve this issue.

Subsequently, we examined the cytotoxic activity of DN T cells from both autoimmune diabetes-resistant and -susceptible mice towards B cells to verify whether the cytotoxic activity from both strains was comparable. To do so, we compared the function of 3A9 DN T cells from B10.Br and NOD.$H2^k$ mice, where NOD.$H2^k$ mice are genetically identical to the NOD strain apart from the MHC locus. Therefore, as both B10.Br and NOD.$H2^k$ mice express the same MHC locus, this study would allow us to define whether the autoimmune-prone NOD genetic background influences DN T cell function. Using a common target cell, namely B cells from B10.Br x NOD. $H2^k$ F1 mice, we demonstrated that the cytotoxic activity of 3A9 DN T cells is comparable between diabetes-resistant and -susceptible strains **(Article 1, Figure 2B).** Moreover, the expression of the cytolytic granules, granzyme B and perforin, as well as the degranulation marker, CD107a, were equal between B10.Br and NOD. $H2^k$ 3A9 DN T cells **(Article 1, Figure 1C).** As the diabetes-susceptible genetic background does not influence DN T cell function, these results demonstrate that the 3A9 DN T cells are associated with autoimmune diabetes due to a deficiency in DN T cell number rather than function. Interestingly, non-transgenic DN T cells from both the diabetes-susceptible genetic background express granzyme B, perforin, and CD107a **(Article 2, Figure 2A)**, thus exhibit a comparable cytotoxic potential. Therefore, to validate that a reduced number in DN T cells, rather than a deficiency in their function, is causal to autoimmune diabetes development in the non-transgenic setting, we must attempt to restore DN T cell number in diabetes-susceptible NOD mice.

Interestingly, upon further comparison of DN T cell functions between the diabetes-resistant and -susceptible strains, we revealed that 3A9 DN T cells from the NOD.$H2^k$ background produce more IL-10, which is characterized as an

immunosuppressive cytokine, on a per cell basis **(Article 1, Figure 3).** Two previous studies have shown that IL-10 inhibits the cytotoxic function of 2C DN T cell clones towards $CD8^+$ T cells in vitro[224, 250]. More, specifically, it was demonstrated that incubation with IL-10 significantly reduced Ly-6A expression on 2C DN T cells, where deficiency in Ly-6A did not affect the activation and proliferation of DN T cells but rather it impeded the ability of DN T cells to kill activated $CD8^+$ T cells[224]. Moreover, Marra and colleagues demonstrated that IL-10 abrogates the suppressive function of 2C DN T cells by enhancing their susceptibility to cell death[250]. Therefore, we decided to verify whether IL-10 similarly impeded the function of 3A9 DN T cells. Accordingly, we showed that IL-10 did not inhibit DN T cell cytotoxicity towards activated B cells in the 3A9 TCR transgenic setting on a per cell basis **(Article 1, figure 4)**. Interestingly, although IL-10 did not affect the proliferation of 3A9 DN T cells (**Article 1, Figure 5B**), the presence of IL-10 led to an increase in the cellular apoptosis of 3A9 DN T cells **(Article 1, Figure 6).** Therefore, in comparison to 2C DN T cells, we did not observe a reduction in 3A9 DN T cell function in the presence of IL-10. This difference might be attributed to various differences between the models. For instance, 3A9 DN T cells are cytotoxic towards activated B cells via a granzyme/perforin-dependent pathway (**Article 1, Figure 2A**), whereas 2C DN T cells are cytotoxic towards $CD8^+$ T cells via the Fas/FasL pathway[171]. As a result, perhaps IL-10 impairs the Fas/FasL pathway, but not the granzyme/perforin pathway. As 2C DN T cells were recently shown to eliminate activated B cells in a perforin-dependent fashion[315], it would be interesting to determine whether IL-10 inhibits 2C DN T cell cytotoxicity towards activated B cells. Indeed, this experiment would exclude differences in both the target cell and mechanism of action implicated. Another possibility pertains to the effect on Ly6A expression. Although 3A9 B10.Br DN T cells do express high levels of Ly6A[214], it is possible that IL-10 does not induce a reduction in its expression in the 3A9 model. As a result, demonstrating whether IL-10 leads to a decrease in Ly6A expression on 3A9 DN T cells would help to resolve this issue. Clearly, additional studies are needed in order to better explain the differences observed between the modulation of 2C and 3A9 DN T cell function in the presence of IL-10. Nevertheless, we can conclude that

while we did not observe an impaired cytotoxic function of 3A9 DN T cells towards their target cell population on a per cell basis, IL-10 would ultimately decrease the degree of the global cytotoxic response as it induced DN T cell apoptosis.

Interestingly, as 3A9 DN T cells from the diabetes-susceptible genetic background, which have fewer DN T cells, produce more IL-10 **(Article 1, Figure 3)**, which induces cellular apoptosis of DN T cells **(Article 1, Figure 6),** these results also suggest that IL-10 may play a role, at least in part, on the regulation of DN T cell number. Furthermore, activated 3A9 DN T cells from NOD.$H2^k$ mice express greater amounts of IL-10 receptor (IL-10R) than 3A9 DN T cells from B10.Br mice (**Appendix 3**), suggesting that 3A9 DN T cells from NOD.$H2^k$ mice may be more sensitive to IL-10-induced apoptosis. Indeed, the addition of an antagonistic IL-10R antibody led to an increase in the cellular expansion of 3A9 DN T cells from NOD.$H2^k$ mice **(Article 1, Figure 7).** This should be kept in mind for future studies on DN T cells in both mice and humans, such that a lower expansion of DN T cells from diabetes-susceptible individuals may be due to an increase in IL-10 production and thus cellular apoptosis in vitro, where the addition of an IL-10R blocking antibody can promote a better expansion of DN T cells. Moreover, results generated by my colleagues, Véronique Dugas and Roxanne Collin, demonstrate that the regulation of DN T cell number is multigenic, where chromosome 9 is linked to this regulation (unpublished data). Interestingly, the alpha chain of IL-10R [316], is indeed located within chromosome 9. Moreover, my colleagues have generated mice congenic for the *Idd2* locus, which encompasses *Il10ra*, and have subsequently determined that the *Idd2* locus partially restores DN T cell number (unpublished data). These results suggest the IL-10/IL-10R pathway may play a role in the regulation of DN T cell number in vivo for which *Il10ra* is a likely candidate gene. Altogether, it is tempting to speculate that the presence of IL-10 within the pancreatic islets of diabetes-susceptible mice impedes DN T cell expansion and thereby inhibits their immunoregulatory function, leading to the development of autoimmune diabetes.

Determining whether *Il10ra* does indeed influence DN T cell number in vivo is yet to be determined. IL-10-deficient mice may seem like an appropriate tool to validate that the IL-10 pathway influences DN T cell numbers in vivo. However, the absence of the immunoregulatory cytokine IL-10 in these mice will likely influence many other cell populations and is known to lead to the development of an inflammatory disease, namely colitis[317]. Thus, the inflammatory setting present in IL-10-deficient mice will preclude the analysis of the direct impact of IL-10 on the proportion of DN T cells. Alternatively, we could take advantage of IL-10RA-knockdown bone marrow chimeras. Indeed, supposing that the in vivo number of DN T cells is regulated, at least in part, by the ability of DN T cells to respond to IL-10, we would target Il10ra by shRNA in bone marrow cells from a 3A9 TCR transgenic NOD.$H2^k$ mouse, which would subsequently be transferred to a lethally irradiated recipient F1 (B10.Br x NOD.$H2^k$) mouse at a 1:1 ratio with bone marrow from a 3A9 TCR transgenic B10.Br mouse. 8 weeks following bone marrow transfer, we would isolate the various secondary lymphoid organs and determine the proportion of 3A9 DN T cells from the donor mice. This experiment would allow us to verify whether the inhibition of the IL-10/IL-10R pathway promotes the restoration of 3A9 DN T cells from the diabetes-susceptible NOD background. The results from this experiment would help to clarify whether the IL-10/IL-10R pathway contributes, at least in part, to the regulation of DN T cell number in vivo.

Given that 3A9 DN T cells are cytotoxic towards activated B cells, we decided to determine whether non-transgenic DN T cells were also cytotoxic towards B cells. Similar to 3A9 TCR transgenic DN T cells, non-transgenic DN T cells mediate their immunoregulatory function by eliminating activated B cells in vitro (**Article 2, Figure 2**), where this cytotoxic function is dependent on granzyme and perforin, as shown by the inhibition of the cytotoxic activity in the presence of EGTA **(Article 2, Figure 2C)**. Therefore, non-transgenic DN T cells are cytotoxic towards activated B cells in a granzyme/perforin-dependent manner.

Interestingly, non-transgenic DN T cells eliminate activated B cells presenting islet antigens, but not B cells presenting a non-relevant antigen such as ovalbumin **(Article 2, Figure 2B)**, demonstrating that the function of DN T cells is self-antigen-specific. These results further corroborate our findings that non-transgenic DN T cells exhibit an activated phenotype suggesting that they recognize self-antigens. These results are important as they suggest that activated DN T cells do not eliminate activated B cells presenting foreign antigen, as is the case during infection. Therefore, DN T cells do not induce global immunosuppression, which is associated with an increased risk of infection and is an undesired consequence of various types of immunotherapy. It would be of interest to verify whether this holds true for B cells presenting other self-antigens. As a result, DN T cell immunotherapy may present itself as an alternative strategy to broad immunosuppressive regimens that lead to undesired side-effects.

Altogether, as the cytotoxic function of DN T cells is normal in mice with a diabetes-susceptible genetic background, we propose that DN T cells are associated with autoimmune diabetes due to a deficiency in DN T cell number rather than function. Consequently, with regards to a cellular therapeutic approach for the treatment of autoimmune diabetes, our main focus should lie on the restoration and expansion of DN T cell numbers.

4.3. The in vivo role of non-transgenic DN T cells in autoimmune diabetes

We and others have demonstrated the therapeutic potential of DN T cells for the prevention of autoimmune diabetes development using the TCR transgenic setting[186, 214]. First, we showed that the proportion of 3A9 DN T cells was significantly decreased in the diabetes-susceptible CD47-deficient BALB.K mice, in comparison to the CD47-sufficient BALB.K mice **(Appendix 1, Figure 3B).** In order to verify whether the restoration of DN T cell number could impede autoimmune diabetes development, we performed a single transfer of 3A9 DN T cells from CD47-sufficient BALB.K mice into diabetes-susceptible CD47-deficient 3A9 TCR:insHEL BALB.K transgenic mice. Indeed, a single transfer of DN T cells was sufficient to inhibit autoimmune diabetes development **(Appendix 1, Figure 8).** These results paved the way for the investigation into the therapeutic potential of DN T cells in non-transgenic mice.

As diabetes-susceptible CD1d-deficient NOD mice exhibit a deficiency in DN T cell number in comparison to diabetes-resistant mice **(Article 2, Figure 1)**, we next wanted to determine whether the restoration of DN T cell number in diabetes-susceptible mice prevented disease development in the non-transgenic setting. Due to the low number of DN T cells, in vitro cellular expansion is necessary in order to obtain a sufficient number of DN T cells. Furthermore, the expansion of DN T cells in vitro prior to their transfer is in line with the therapeutic protocol envisioned for the treatment of autoimmune diabetes in patients using DN T cells. Indeed, in human diabetic patients, the use of autologous DN T cells, thus cells originating from the recipient, would circumvent the need for immunosuppressive agents as the DN T cells would not undergo rejection. However, the transfer of isolated DN T cells back to the patient would not result in an increase in the proportion of DN T cells within this particular individual without an intermediate step of cellular expansion to increase their number. Essentially, the therapeutic protocol envisioned for DN T cells

in the treatment of autoimmune diabetes involves the isolation of DN T cells from the blood of the affected individual, the expansion of the DN T cells in vitro, followed by the transfer of the expanded DN T cells back to the patient **(Figure 5).**

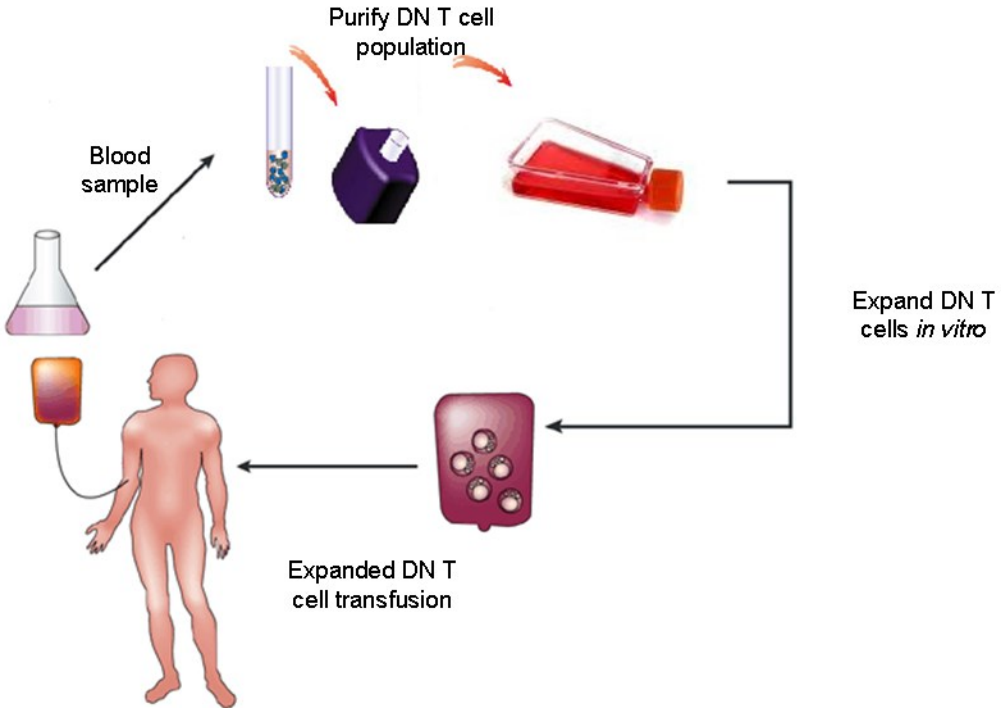

Figure 5. Illustration of the potential adoptive DN T cell therapy protocol for the treatment of autoimmune diabetes in humans. Modified from [318].

Therefore, the in vitro expansion of DN T cells from diabetes-susceptible mice is a crucial factor in the investigation into the therapeutic potential of DN T cells. Accordingly, DN T cells sorted from non-transgenic CD1d-deficient mice can be readily expanded in vitro with an average cellular expansion of approximately 90-fold in 7 days **(Article 2, Supplementary Figure 3**). Furthermore, transferred DN T cells survive in vivo post-transfer for at least 4 weeks, as we can track the transferred DN T cells to the spleen, skin-draining lymph nodes, pancreatic lymph nodes, and mesenteric lymph nodes at 24 hours, 2 weeks and 4 weeks post-transfer **(Article 2, Supplementary Figure 5).** Based on their survival post-transfer, these results further demonstrate the potential for activated DN T cells in adoptive cell therapy. Importantly, in comparison to non-injected or activated $CD4^+$ T cell-injected control

mice **(Article 2, Supplementary Figure 4)** the transfer of expanded DN T cells from CD1d-deficient NOD mice to 12 week-old non-diabetic female NOD mice results in a significant reduction in the incidence of diabetes up to 32 weeks of age, **(Article 2, Figure 3)**. Therefore, a single autologous transfer of expanded DN T cells is sufficient to significantly decrease diabetes progression.

Modifications to the protocol for DN T cell transfer may reveal an alternative method that results in an improved, or possible complete, inhibition in autoimmune diabetes development. Indeed, adjustments in the quantity of DN T cells injected as well as the timing of injection may prove to be beneficial. Moreover, female NOD mice do not all progress to autoimmune diabetes at the same age such that overt diabetes is observed beginning at 12-14 weeks of age with approximately 80% of mice developing the disease by 30 weeks of age[128], therefore it is possible that a single injection at 12 weeks of age is either too early or too late with regards to DN T cell impact on autoimmune diabetes initiation. Therefore, modifying the frequency of DN T cell transfers from a single transfer to multiple transfers may improve the inhibition of disease development as multiple transfers would cover a greater span of time/age resulting in an enhanced window of opportunity for the immunotherapeutic action of DN T cells. Furthermore, as non-transgenic DN T cells express a polyclonal TCR repertoire[169] (and **Appendix 2**) and thus only a fraction are potentially specific for islet antigens, the use of islet-antigen specific, rather than polyclonal activation, DN T cells could also prove to be favourable. Indeed, we could expand DN T cells in vitro in the presence of irradiated antigen-presenting cells and pancreatic islet lysates, which would serve as the source of islet antigen. As such, only DN T cells that recognize and are specific for islet antigens would be activated and proliferate. As a result, we would obtain a population of DN T cells that have the potential to be more effective as they are specific to antigens expressed in the pancreas. Altogether, the sum of these factors merit further investigation.

Interestingly, two other groups[230, 262] have investigated the ability of DN T cells to prevent autoimmune diabetes in non-transgenic mice, as mentioned in

Section 1.6.4.6. However, for both studies, either the DN T cells were not void of contaminating NK T cells[230, 262] or the therapeutic potential of DN T cells was tested using a lymphopenic system [230]. Conversely, our study uses CD1d-deficient mice, which are void of all NK T cells, as well as non-lymphopenic NOD mice. Therefore, our findings are the first to show that non-transgenic, uncontaminated DN T cells inhibit autoimmune diabetes development in non-lymphopenic NOD mice.

4.4. The in vivo mechanism of action of non-transgenic DN T cells

To our knowledge, there exists only one published study to date which has investigated the cellular target of non-transgenic DN T cells in vivo. Accordingly, using a cardiac xenotransplantation model, Ma, Y and colleagues demonstrated that the transfer of perforin-sufficient, not perforin-deficient, DN T cells led to an increase in graft survival, which correlated with an increase in B cell apoptosis as well as a decrease in anti-donor total IgG and IgM antibody production and IgG graft deposition [256]. Together, this study demonstrated that the transfer of non-transgenic DN T cells leads to an increase in B cell apoptosis in vivo, which is dependent on the perforin pathway, as well as an associated decrease in anti-donor IgM and IgG antibody levels.

Our findings using the 3A9 TCR:insHEL transgenic model have further corroborated these results as we have shown that diabetes-susceptible CD47-deficient BALB.K TCR: insHEL mice that received a single transfer of 3A9 DN T cells from CD47-sufficient BALB.K 3A9 TCR mice exhibited a significant reduction in antigen-specific anti-HEL IgG, but not IgM, autoantibody serum levels in comparison to mice that did not receive DN T cell transfer **(Appendix 1, Figure 8B,C).** These results further suggest that DN T cells eliminate B cells in vivo via an antigen-specific mechanism, which is analogous with our in vitro observations **(Article 1, Figure 2 and Article 2, Figure 2)**. In order to validate the cytotoxic function of 3A9 DN T cells towards B cells in vivo, immunodeficient Rag-/- mice, that lack both B cells and T cells, were first injected with a 1:1 ratio of activated B cells loaded with HEL or ovalbumin (OVA) protein, then, 24 hours later were injected with 3A9 DN T cells. Mice that did not receive a transfer of DN T cells still demonstrated a 1:1 ratio of cells loaded with HEL or OVA in the spleen, however, mice that did receive a transfer of 3A9 DN T cells exhibited a drastic decrease in B cells presenting HEL but not of those presenting OVA, a protein which is not recognized by the TCR 3A9 (results generated by Véronique Dugas, unpublished data). These results further

validate that 3A9 DN T cells eliminate B cells in an antigen-specific manner in vivo. Altogether, B cells remain the only target of DN T cells to be validated in vivo.

As DN T cells impeded autoimmune diabetes in otherwise diabetes-susceptible NOD mice and have been shown to target B cells in vivo ([256] and **Appendix 1, Figure 8**), we hypothesized that DN T cells migrated to the pancreas where they could eliminate autoreactive B cells. Indeed, autoreactive B cells are defined by their ability to produce autoantibodies and present self-antigens to autoreactive T cells thereby activating them **(Figure 6**), both of which are implicated in autoimmune diabetes development. Therefore, the elimination of such autoreactive B cells could explain the inhibition of autoimmune diabetes following DN T cell transfer. Indeed, the transferred DN T cells could be tracked to the pancreatic lymph nodes as early as 24 hours, and for at least 4 weeks, post-transfer where we observed a preferential accumulation of the transferred DN T cells in comparison to both the skin-draining and mesenteric lymph nodes **(Article 2, Supplementary Figure 5**). This accumulation correlated with a higher proportion of transferred DN T cells expressing the activation marker CD69 **(Article 2, Figure 4B**) and having entered cellular division, demonstrated by carboxyfluorescein succinimidyl ester (CFSE) staining (**Article 2, Figure 4C**). Together with our findings that non-transgenic DN T cells exhibit an activated phenotype ex vivo (**Article 2, Supplementary Figure 2**), which is representative of an ability to recognize self-antigens, and their ability to kill B cells presenting islet antigen (**Article 2, Figure 2B**), these results suggest that DN T cells recognize islet antigens presented in the pancreatic lymph node thus inducing their preferential activation, proliferation and accumulation at this site.

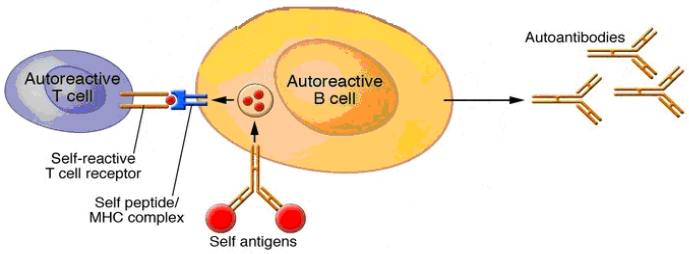

Figure 6. Representation of the roles of autoreactive B cells in autoimmune disease, such as autoimmune diabetes. Modified from [319].

As autoimmune diabetes is caused by the destruction of pancreatic insulin-producing β cells, we next wanted to determine whether transferred DN T cells could impede the autoreactive response directly within the pancreatic islets. Indeed, we can track DN T cells to the pancreatic islets post-transfer (**Article 2, Figure 5B**). Moreover, a higher proportion of CD1d-deficient NOD DN T cells express the chemokine receptor CXCR5 in the pancreatic lymph nodes in comparison to the spleen and skin-draining lymph nodes **(Article 2, Figure 5A**), where CXCR5 is required for homing into germinal centers[320, 321]. Accordingly, the transfer of activated DN T cells leads to a decrease in the proportion of germinal center B cells directly within the pancreatic islets, which are represented by the expression of GL7, which is a marker of T and B cell activation but is mostly used as a marker of germinal center B cells, and Fas antigen (CD95) (**Article 2, Figure 5C**). As germinal centers are the site of the generation of antibody-producing plasma cells (further discussed below), we decided to investigate the serum levels of IAA. Indeed, we observed that mice that had received a transfer of DN T cells exhibited a decrease in serum IAA levels whereas mice that received a transfer of $CD4^+$ T cells exhibited an increase in serum IAA levels (**Article 2, Figure 5D**), demonstrating that the decrease in IAA levels is specific to DN T cells. Therefore, the decrease in germinal center B cells correlates with a decrease in serum IAA levels, suggesting a decrease in autoreactive germinal center B cells recognizing islet antigen such as insulin. Although it is tempting to conclude that DN T cells eliminate germinal center B cells in vivo, a true appreciation of the complexity of germinal center formation makes this assumption less definite.

Germinal centers develop within the B cell follicles and are the site of B cell maturation (Reviewed in [81], **Figure 7**). Indeed, B cells are activated following their encounter with cognate antigen, which usually occurs within the T cell zone of lymphoid organs by interaction with T helper cells and dendritic cells. Activated B cells then migrate into B cell follicles, start to proliferate and differentiate into centroblasts, thereby establishing germinal centres. The proliferating centroblasts activate the process of somatic hypermutation thus generating antibody variants.

Once the centroblasts have stopped proliferating, they are known as centrocytes, which are selected for based on the high affinity of their B cell receptor for the cognate antigen. Germinal center B cells will then compete for survival signals from follicular DCs and T cells in order to escape the default apoptosis pathway of germinal centre B cells. Selected germinal center B cells will undergo repeated rounds of proliferation, mutation and selection. The immunoglobulin genes of many germinal center B cells also undergo remodelled by isotype switching in order to increase their affinity for the cognate antigen. Finally, the B cells will differentiate into either plasma cells able to secrete large quantities of antibody or memory B cells that can be reactivated in subsequent contacts with the same antigen, both of which can now exit the germinal center.

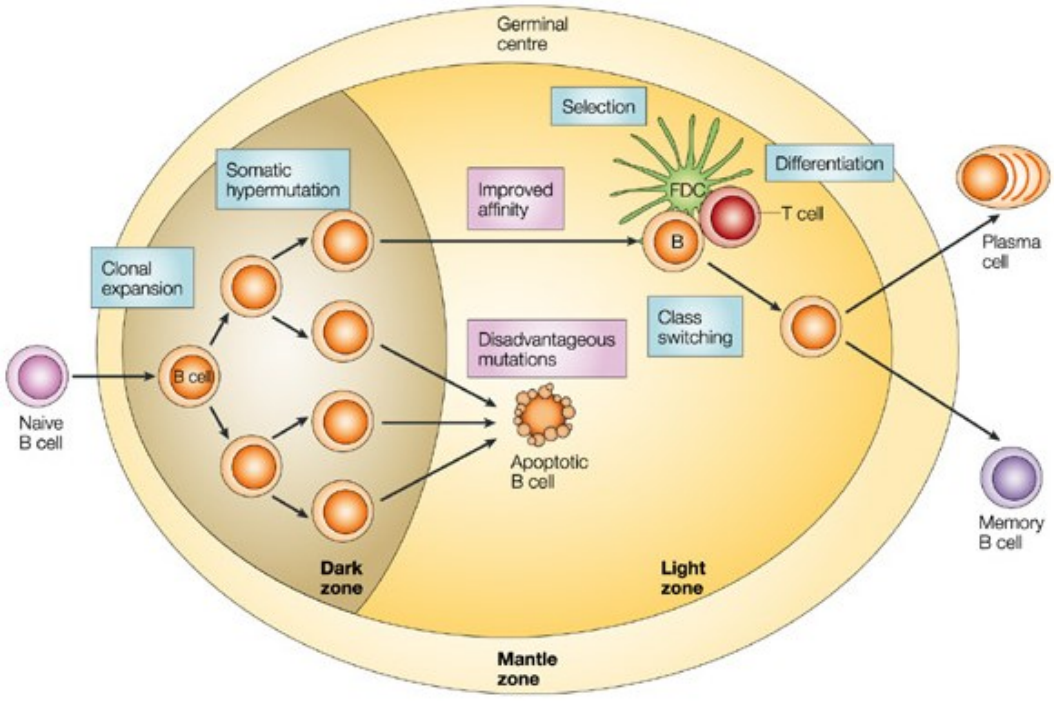

Figure 7. Representation of the B cell developmental pathways in the germinal center. Adapted from [322].

Therefore, these results demonstrate that the transfer of DN T cells to diabetes-susceptible NOD mice correlates with a reduction in germinal center B cells as well as autoantibody-producing plasma cells directly within the pancreatic islets, which could be explained by a direct elimination of plasma cells and/or germinal center B cells. Indeed, the elimination of autoreactive germinal center B cells would lead to a consequential decrease in autoantibody-producing plasma cells, which draws a parallel to our findings. However, as illustrated in **Figure 8**, the elimination of either follicular DCs or T cells that provide survival signals to germinal center B cells would also ultimately result in the decrease in both germinal center B cells and differentiated plasma cells. Therefore, we cannot conclude on whether the reduction in germinal center B cells is a direct or indirect consequence of DN T cell transfer. The investigation into the exact target of DN T cells in vivo as well as their mechanism of action merits further investigation. The possible cellular targets of DN T cells whose elimination would result in a decrease in autoantibody levels is illustrated in **Figure 8.**

Altogether, the transfer of DN T cells leads a preferential activation and accumulation of transferred DN T cells within the pancreatic lymph nodes suggesting that DN T cells recognize self-antigen presented within the pancreatic lymph node, a proposition that corresponds with our findings that DN T cells eliminate B cells presenting islet antigens and express an activated phenotype ex vivo. Transferred DN T cells can also migrate to the pancreatic islets, which results in a decrease in both the proportion of germinal center B cells as well as serum IAA levels. These results correlate with our finding that DN T cells within the pancreatic lymph nodes express the germinal center homing receptor, CXCR5, demonstrating that DN T cells can indeed migrate to the germinal center, the site of both germinal center B cells and the generation of antibody-producing plasma cells.

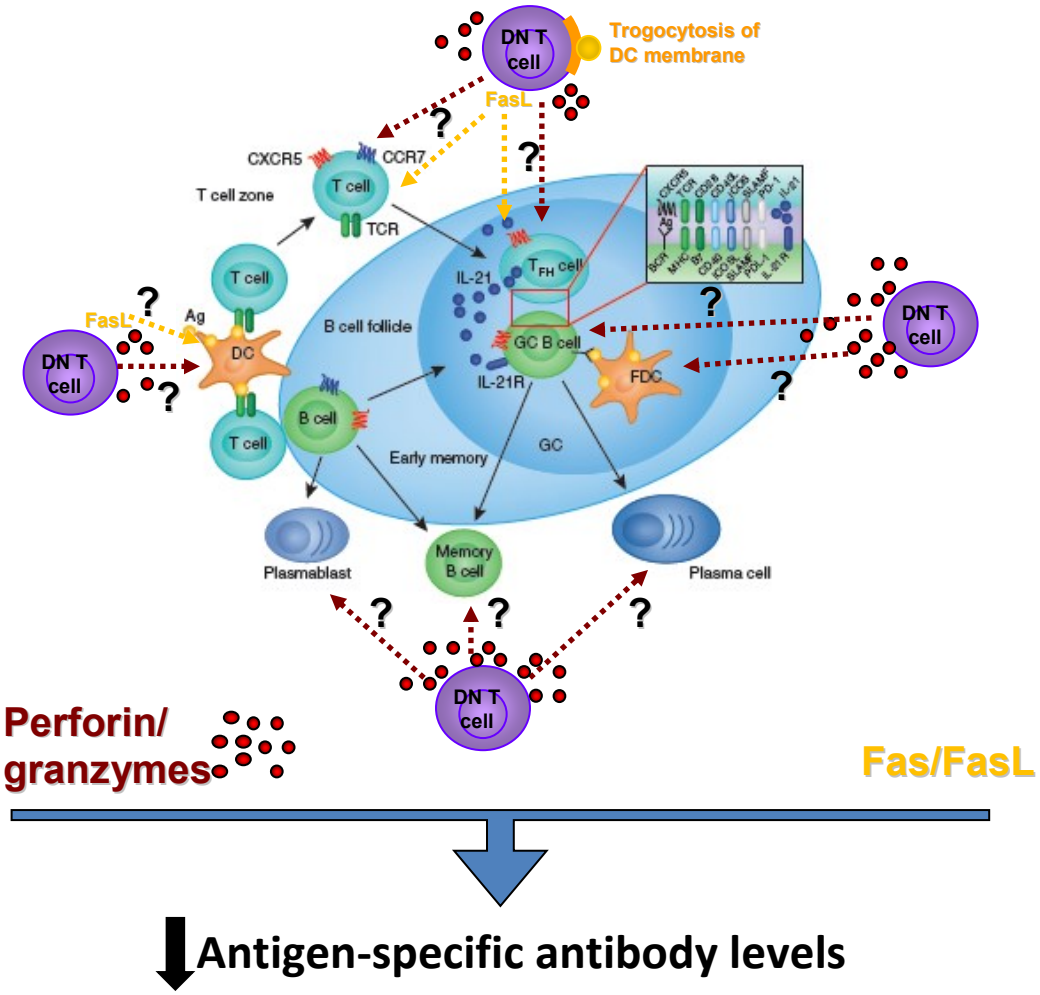

Figure 8. Working hypothesis of possible cellular targets of DN T cells leading to a decrease of antigen-specific antibody levels in vivo. The above diagram represents the possible targets of DN T cells in vivo, any of which would ultimately lead to a decrease in antigen-specific antibody levels. As is seen above, possible targets of DN T cells include dendritic cells (DC), plasmablasts, memory B cells, plasma cells and T cells as well as follicular DC (FDC), germinal center (GC) B cells and follicular helper T (T_{FH}) cells within the germinal center. The DN T cells may lyse target cells through the release of perforin and granzymes, illustrated by the red arrows, or induce apoptosis through the Fas pathway, indicated by the yellow arrows. Modified from [323].

4.5 Conclusion and future perspectives

The main objective of this thesis project was to determine whether the restoration of DN T cell number in non-transgenic mice prevents diabetes onset in otherwise diabetes-susceptible mice and, thus, may be of therapeutic interest for autoimmune diabetes in humans. Here, we showed that diabetes-resistant mice present with a higher proportion and cell number of DN T cells than diabetes-susceptible mice in the non-transgenic setting, which associates a deficiency in DN T cell number with autoimmune diabetes susceptibility. We also determined that DN T cells eliminate activated B cells in vitro via a perforin/granzyme-dependent pathway. Importantly, the function of DN T cells is equal between the diabetes-resistant and -susceptible mice, demonstrating that the association to autoimmune diabetes is due to a deficiency in DN T cell number rather than function. We established that the transfer of activated DN T cells could prevent autoimmune diabetes development in the non-transgenic setting, where the transfer of DN T cells correlates with a decrease in germinal center B cells and serum IAA levels. These results draw a parallel with our finding that DN T cells can migrate to both pancreatic lymph nodes and islets and that DN T cells can eliminate B cells presenting islet antigen in vitro. We thus propose that DN T cells either directly eliminate germinal center B cells presenting islet antigen in vivo or other cell populations involved in the maturation of autoreactive B cells in the germinal center, such as follicular dendritic cells or T cells.

DN T cells are of particular therapeutic interest for autoimmune diabetes because they inhibit immune responses in an antigen-specific manner (**Article 1, Figure 2** and reviewed in [156], Hillhouse, E.E., Lesage, S., 2012). Interestingly, Tregs can also inhibit Ag-specific proliferation of target cells that are specific for the same antigen[227, 324, 325].Moreover, this immunoregulatory function of Tregs is hindered in the absence of their cognate antigen. However, Tregs are also capable of mediating suppression in an antigen non-specific manner[227, 324, 325]. Indeed, antigen-specific Tregs are able to suppress target T cells of a different antigen specificity, but only in the presence of the cognate antigen of the Tregs[325]. Accordingly, once the Tregs are

activated by their cognate antigen, their suppressive function becomes antigen non-specific[325]. Therefore, Treg suppression is dependent upon the presence of its cognate antigen, however, once activated, Tregs can mediate their suppressive function in both an antigen-specific and -non-specific fashion. In addition, Tregs can also inhibit tumor-specific effector cells, where tumor immunity can be enhanced in the absence of Tregs[272, 274], while DN T cells have been demonstrated to promote tumor immunity[267, 277]. Altogether, DN T cells are a better option as a cellular therapeutic agent as they inhibit immune responses in an antigen-specific manner while maintaining tumor immunity.

Due to their antigen-specific immunoregulatory function, the use of DN T cells in immunotherapy should lead to fewer side-effects along with a decreased risk of infections, which remains a major concern in the application of broad immunosuppressive regimens. The use of antigen-specific immunoregulatory DN T cells may eventually present itself as an alternative strategy to broad immunosuppressive regimens that lead to undesired side-effects, such as the rituximab treatment, which depletes all B cells as opposed to activated antigen-specific B cells[76, 326]. Moreover, the ability to readily expand DN T cells in vitro prior to their autologous transfer back to the patient evades the need for immunosuppressive drugs, which can dampen the immune response against infection and cancer. Therefore, DN T cells demonstrate key aspects for a successful cellular therapy.

Interestingly, Serreze and colleagues recently published an article demonstrating that B cells downregulate their expression of CD20 following their entry into pancreatic islets[327]. Consequently, anti-CD20 treatment, also known as rituximab, which has shown promising results in early clinical trials for autoimmune diabetes reversal [76], is unable to eliminate B cells found directly within the islets[327]. Therefore, there are currently no treatments that can efficiently eliminate B cells directly within pancreatic islets where pancreatic β cell destruction occurs. The findings from this thesis demonstrate that the transfer of DN T cells decreases the

proportion of germinal center B cells directly within pancreatic islets, suggesting that DN T cells could be more effective than anti-CD20 treatment at abrogating the local inflammatory autoimmune response. Therefore, DN T cells exhibit the potential to become a novel cellular therapeutic agent for the treatment of autoimmune diabetes or, alternatively, can be combined with current therapies, such as anti-CD20 treatment, to generate optimal outcomes.

Overall, DN T cells exhibit the potential to be used for various facets of autoimmune diabetes in humans. Based on our results, DN T cells demonstrate the therapeutic potential to be used for prevention and/or delay of autoimmune diabetes development in at-risk patients. Future studies investigating whether the transfer of DN T cells can reverse autoimmune diabetes in new-onset diabetes-susceptible mice will allow us to determine whether DN T cells also demonstrate a potential for the treatment of recently diagnosed autoimmune diabetes patients. Due to their immunoregulatory and antigen-specific function, it is also possible that DN T cells may also serve to replace the immunosuppressive agents that are used to promote islet cell and pancreas transplant survival. Importantly, DN T cell transfer results in a decrease in antigen-specific autoantibody levels[214] (**Article 2, Figure 5D**), suggesting that autoantibodies can serve as a biomarker for the efficiency of DN T cell therapy. Indeed, the evaluation of autoantibody levels following treatment would allow for the modification in the course of the treatment, such as additional transfers or an increase in the amount of DN T cells injected. If the DN T cell treatment showed no signs of being effective for a particular patient, which is possible as all patients are different, the ability to evaluate the efficiency of DN T cell treatment via autoantibody levels would allow for a rapid change in the patient's form of treatment. Lastly, as a low proportion of DN T cells is associated with autoimmune diabetes susceptibility in non-transgenic mice (**Article 2, Figure 1**) which are more physiologically relevant to humans, we propose that DN T cell proportion can serve as a biomarker to facilitate the identification of individuals at risk of developing autoimmune diabetes. Altogether, DN T cells exhibit the potential to be used for the treatment and diagnosis of diabetic patients.

Following from the results presented in this thesis, we believe that it is timely to begin the translation to human DN T cell studies. Although human DN T cells have been investigated by other groups[169, 238], they have not been investigated in the context of autoimmune diabetes. Indeed, it will be important to validate our results and to verify whether human DN T cells exhibit similar properties as murine DN T cells. Accordingly, it will be interesting to verify whether diabetic patients present with fewer DN T cells than otherwise healthy individuals and whether these DN T cells are indeed functional. Importantly, we have already commenced the study of human DN T cells. Indeed, we have demonstrated that we are able to efficiently isolate human DN T cells **(Article 2, Figure 6A).** Following their expansion in vitro, we have shown that human DN T cell both exhibit cytotoxic potential (**Article 2, Figure 6B)** as well as demonstrate cytotoxic activity towards B cells in vitro (**Article 2, Figure 6C**). Importantly, we are the first to demonstrate that human DN T cells can target B cells in vitro, demonstrating a functional similarity between murine and human DN T cells. Nevertheless, due to a lack of a specific marker for the discrimination of DN T cells from type II NKT cells, we believe that it is crucial to invest into the identification of a cell marker for human DN T cells. This will not only ensure the purity of the human DN T cell population but will also facilitate their isolation and subsequent study.

In conclusion, we have demonstrated the impact of DN T cell proportion on autoimmune diabetes progression in non-transgenic NOD mice, elucidated the cellular mechanism by which DN T cells impede autoimmune responses *in vivo* and commenced the characterization of human DN T cells, all key aspects for the development of future therapeutic approaches. Therefore, the results presented in this thesis have paved the way for additional studies on the therapeutic potential of DN T cells for the treatment of autoimmune diabetes as well as other diseases of the immune system, such as GVHD and cancer (reviewed in [259], Hillhouse, E.E., Delisle, J.S. & Lesage, S., 2013).

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APPENDIX 1

Title: Implication of the CD47 pathway in autoimmune diabetes.

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Abstract

CD47 and signal regulatory protein (SIRP) interactions have been proposed to take part in autoimmune disease susceptibility. Importantly, a recent genome wide association study for type 1 diabetes susceptibility highlighted the association of the 20p13 region comprising the *SIRP* cluster, where some of the SIRP proteins encode functional ligands to CD47. Using a TCR transgenic mouse model at the brink of autoimmune disease, we demonstrate that CD47-deficiency is sufficient to break the immune tolerance and provoke the onset of autoimmune diabetes. Interestingly, CD47-deficient mice show a severe reduction in the number of mature CD4-CD8- T cells, and passive transfer of these CD4-CD8- T cells is sufficient to restore immune tolerance and prevent diabetes progression. Together, these findings constitute an *in vivo* demonstration that CD47 is involved in diabetes susceptibility and controls the homeostatic regulation of CD4-CD8- T cells.

Keywords: T cells, tolerance, autoimmune diabetes, transgenic mice, CD47, signal regulatory protein

Introduction

CD47 is a ubiquitously expressed atypical protein which encodes for five transmembrane domains that interact in *cis* with integrins to form a complete seven transmembrane complex, which signals through G-proteins [1]. CD47 is involved in various immunological processes, including apoptosis, phagocytosis, cell migration as well as T cell responses [1-12]. Particularly, CD47 ligation with either antibodies or its physiological ligand potentiates T cell activation [5-7]. The cellular ligands for CD47 include members of the signal-regulatory protein (SIRP) family, SIRPα and SIRPγ [13, 14]. CD47 also interacts with thrombospondin, although this latter interaction is currently the subject of debate [14, 15].

SIRP compose a family of genes, including SIRPα, SIRPβ and SIRPγ proteins, which have most probably arisen as a consequence of gene duplication [16]. Their extracellular domains are highly homologous and are characteristic of the immunoglobulin superfamily, while their intracellular domains, and consequently their signaling, are very divergent [17]. Indeed, the SIRP family is defined as "paired receptors", having both activating and inhibitory receptors [13, 18]. Of interest, the cellular expression and function of these CD47 ligands also differs. The biological function of the highly polymorphic SIRPα protein has been most extensively studied in myeloid cells, where it is expressed [2, 7, 17]. Alternatively, SIRPγ expressed on T cells and NK cells, has been revealed as an important modulator of T cell responses [3, 19-21]. Thus, both CD47 and SIRP are known to influence T cell responses.

More recently, CD47-SIRP interactions have been proposed to take part in T cell-mediated autoimmune disease susceptibility. For instance, animals lacking the intracellular domain of SIRPα become resistant to the development of a myelin oligodendrocyte glycoprotein peptide-induced EAE and collagen-induced arthritis [22, 23], whereas CD47-deficiency facilitates the progression of autoimmune hemolytic anemia in mice [24, 25]. In addition, autoimmune diabetes linkage studies in rat and mice have revealed respective associations with *Iddm27* and *Idd13*, two

genetic loci encompassing the highly polymorphic *Sirp*α gene [17, 26-28]. Moreover, a recent genome-wide association study for type 1 diabetes susceptibility in humans highlighted the association of the 20p13 region comprising the *SIRP* cluster [29]. Importantly, based on linkage disequilibrium (LD), the SIRP cluster contains three genes, namely SIRPD, SIRPB1, SIRPG some of which are known to encode functional ligands to CD47 [16, 29]. Therefore, we aimed to define the contribution of the CD47 pathway in autoimmune diabetes.

For our studies, we use a TCR transgenic mouse model at the brink of autoimmune disease, allowing us to reveal the impact of subtle genetic differences in the regulation of T cell tolerance. In this transgenic model, deletion of CD47 is sufficient to break the immune tolerance, provoking the onset of autoimmune diabetes. We previously demonstrated that T cell central tolerance mechanisms were not altered in TCR transgenic CD47-deficient mice [30], therefore suggesting that defects in peripheral tolerance were at play. Accordingly, we observe a reduction in the number of mature CD4-CD8- T cells in association with disease susceptibility in different genetic backgrounds. Interestingly, these CD4-CD8- T cells are phenotypically similar to immunoregulatory T cells identified by others [31-34]. Moreover, we show that increasing the number of CD4-CD8- T cells is sufficient to confer immune tolerance and impede autoimmune diabetes development. Together, these findings lend support to the view that the CD47-SIRP pathway is implicated in susceptibility to autoimmune diabetes.

Materials and Methods:

Mice

3A9 TCR [35] and insHEL (Hen egg lysozyme (HEL)) under the rat-insulin promoter a.k.a ILK-3) [36] transgenic mice on B10.Br, NOD.$H2^k$ and BALB.K backgrounds have been previously described [30, 37]. CD47-deficient BALB/c mice [38] were bred to TCR:insHEL B10.Br mice as previously described [30]. Non-transgenic B10.Br (#000465) and NOD (#001976) mice were purchased from The Jackson Laboratory. The Maisonneuve-Rosemont Hospital ethics committee overseen by the Canadian Council for Animal Protection approved all experimental procedures.

Flow cytometry

6–12-wk old nondiabetic mice (Diascreen negative) were analyzed. All lymphoid organs were passed through a sterile cell strainer. Cell counts were performed by trypan blue exclusion using a hemacytometer. The FITC Anti-Mouse/Rat Foxp3 Staining Set was used for Foxp3 staining according to the manufacturer's instructions (eBiosciences). Anti-clonotypic 1G12 antibody recognizing the expression of the 3A9 TCR [39] was used as a culture supernatant and detected using APC-labelled X56 from BD. Anti-CD25 antibody was purchased from Caltag. All other antibodies were purchased from Biolegend. CD1d tetramer is a generous gift from Dr. David Serreze. Data was collected on a FACSCalibur (BD Biosciences), cells were sorted on a FACSVantage (BD Biosciences) and all data was analyzed with FlowJo software (Treestar).

Proliferation and suppression assays

To measure the extent of T cell proliferation, sorted CD4 and DN T cells were labeled with 2μM CFSE (Invitrogen, [40]) and washed twice in cold RPMI containing 10% FCS prior to culture. They were stimulated *in vitro* (250 000 cells/ml) for three days in the presence of irradiated T-depleted splenocytes (2.5 Gy, 250 000 cells/ml) and anti-CD3 (1μg/ml), with or without IL-2 (30 U/ml). To quantify the potential of Tregs at suppressing CD4 T cell proliferation, CD4+CD25-

effectors cells were sorted and labeled with CFSE as above, and cultured for 3 days in presence of the indicated ratio of CD4+CD25hi Tregs or CD4+CD25- control effector cells.

Diabetes monitoring

The diabetes incidence was monitored every two weeks using Diascreen, where diabetic mice were positive for two consecutive tests and showed blood glucose levels above 17mM. To evaluate the role of CD4-CD8- (double negative, DN) T cells, spleen and peripheral lymph nodes cells were obtained from BALB.K TCR mice. Where mentioned, DN T cells were enriched using EasySep^{TM} biotin selection kit, efficiently removing >95% of CD4+ (RM4-5), CD8+ (53-6.7) and B cells (CD45R, RA3-6B2). More than 50% of the remaining cells were positive for 1G12 expression. 10 million enriched DN T cells or 10 million selected CD4+ T cells (purity 95%) were then injected i.v. in non-diabetic (diastix negative) 9 week-old CD47$^{-/-}$ BALB.K TCR:insHEL mice. Alternatively, 6 week-old non-diabetic CD47$^{-/-}$ BALB.K TCR:insHEL mice were injected with 2 million sorted DN T cells. Sorted 1G12+ DN T cells purity was above 90%. Serum was collected from diabetic mice (showing two consecutive Diascreen positive tests and blood glucose levels above 17mM) or when mice reached 24 weeks of age. Anti-HEL IgM and IgG serum levels were measured by ELISA using HEL-coated plates and polyclonal goat anti-mouse IgM and IgG peroxidase conjugate antibodies (Calbiochem).

Phenotyping and quantifying DN T cells in non-transgenic mice

Phenotyping of non-transgenic DN T cells from the spleen and lymph nodes of B10.Br and NOD mice was performed by depleting CD4, CD8, B220, CD49b and TCRδ positive cells using EasySep™ biotin selection kit. For determining the number of DN T cells, cell separation was not performed to avoid cell loss. The proportion of DN T cells was obtained by electronic gating on TCRβ^{+}CD5^{low}CD4^{-} CD8^{-}B220^{-}CD1d tetramer^{-} cells and multiplied by the absolute cell number in each organ.

Statistics

Mann-Whitney U test was performed where appropriate unless otherwise specified.

Results:

CD47-deficient mice are prone to autoimmune diabetes.

CD47-SIRP interactions regulate T cell responses [1-3, 5-7, 17] and the *SIRP* cluster has been recently associated with autoimmune diabetes progression [29]. We therefore elected to evaluate the impact of the CD47-SIRP pathway on diabetes onset in a T cell dependent model. As such, we took advantage of the TCR:insHEL transgenic mouse model, where the 3A9 TCR transgene recognizes a HEL peptide in the context of I-A^k and the insHEL transgene is expressed under the rat-insulin promoter [36, 37]. Accordingly, TCR:insHEL transgenic mice carry a significant number of autoreactive T cells [36, 37]. This model should thus help reveal the impact of subtle genetic differences in the regulation of T cell tolerance.

In humans, both SIRPα and SIRPγ bind to CD47, while the ligand for SIRPβ has not been defined [13, 17]. Murine SIRPα also binds to CD47; however mice do not express SIRPγ [3]. To effectively disrupt the CD47-SIRP pathway and determine its impact on autoimmune disease progression, we monitored the diabetes incidence in TCR:insHEL CD47-deficient, which had been previously generated on the BALB.K background [30]. In this antigen-specific context, less than 20% of the TCR:insHEL CD47-sufficient mice developed autoimmune diabetes within 24 weeks (Figure 1). In contrast, within this same time frame, the incidence of diabetes reached 90% in TCR:insHEL CD47-deficient mice. Moreover, the onset of disease occurred much earlier in CD47-deficient mice, where the first diabetic mice appeared at 6 weeks as opposed to 15 weeks of age in CD47-sufficient mice (Figure 1). Together, these data highlight an important role for the CD47 pathway in the regulation of immune tolerance in a T-cell model of autoimmune diabetes.

Tregs, CD4 and CD8 T cells are not influenced by the absence of CD47

Both central and peripheral T cell tolerance defects may contribute to the pathogenesis of autoimmune diabetes. Although we have previously shown a defect in central tolerance using this TCR transgenic model on a different genetic

background [37], we have recently reported that CD47-deficiency does not affect thymic selection events in the TCR:insHEL BALB.K model [30]. Therefore, it is unlikely that defects in central tolerance describe the increased susceptibility to autoimmune diabetes in CD47-deficient mice.

Subsequently, to evaluate the peripheral regulation of T cells independently of the previously ascribed effects of thymic negative selection processes [37], we examined the T cell populations in the spleen and lymph nodes of single TCR transgenic mice, i.e. in the absence of the insHEL transgene which causes negative selection of potentially autoreactive TCR transgenic T cells. As for the B10.Br and NOD.$H2^k$ genetic backgrounds [37], single TCR transgenic mice do not develop diabetes on the BALB.K genetic background (data not shown); consequently, any cellular differences observed by comparing CD47-deficient and –sufficient mice will reveal phenotypes associated with disease predisposition in the complete absence of islet inflammation. Moreover, the insertion of both TCR and insHEL transgenes is needed to allow diabetes progression, demonstrating that a sufficient number of HEL-specific T cells, in the presence of HEL expression in the pancreas, must be present to initiate the pathology.

Peripheral T cell regulation is mediated at least in part by Foxp3-expressing CD4 regulatory T cells (Tregs) known to limit auto-reactive T cell activation [41]. As a result, we first evaluated whether the absence of CD47 modulated the number or function of Tregs. By comparing 3A9 TCR clonotype $(1G12)^+$ cells in the spleen and lymph nodes of TCR single transgenic CD47-deficient and –sufficient mice, we observed no significant difference in the proportion of Tregs or in their potential to inhibit *in vitro* CD4 T cell proliferation (Figure 2a and 2b). Indeed, CD47-deficient Tregs from 3A9 TCR transgenic mice were at least as efficient as those from CD47-sufficient mice at inhibiting CD4 T cell proliferation, monitored by the extent of CFSE dilution. These results support previous findings demonstrating that neither the quantity nor the function of Tregs is reduced in CD47-deficient non-transgenic mice [42]. Rather, Tregs appear to slowly accumulate with age in CD47-deficient mice

[42]. Together, these data suggest that the increased susceptibility to autoimmune diabetes in CD47-deficient mice cannot be attributed to a defect in Treg homeostasis or function.

Secondly, we showed that the proportion, the absolute number and activation markers of either CD4 or CD8 T cells were not altered in peripheral lymphoid organs of TCR transgenic CD47-deficient mice when compared with CD47-sufficient mice (Figure 2c, Table I and data not shown). Together, these data suggest that the T cell pool is not affected by the absence of the CD47 molecule.

Decreased proportion of CD4-CD8- T cells in CD47 deficient mice.

Yet, while analyzing the CD4 and CD8 T cell subsets, we observed a high number of TCR clonotype 1G12+ cells lacking both CD4 and CD8 expression (Figure 3a). Indeed, approximately 50% of TCR clonotype $1G12^+$ cells were $CD4^-CD8^-$, revealing the presence of non-conventional T cells (Figure 3a). Almost 5% of total spleen cells and an astounding 15% of total lymph nodes cells were $CD4^-CD8^-1G12^+$ (Figure 3b). We hereafter refer to these cells as double negative T (DN T) cells. More importantly, DN T cells were severely reduced in TCR CD47-deficient mice when compared with CD47-sufficient mice (Figure 3b). Finally, similar results were obtained in TCR:insHEL mice, where the number of DN T cells was also significantly lower in the absence of CD47 (data not shown). These results suggest that CD47 is associated with the homeostatic regulation of DN T cells.

Elevated DN T cell numbers are associated with diabetes resistance.

Hitherto, our results show that CD47-deficiency accelerates autoimmune diabetes progression and is associated with a reduced number of DN T cells in pre-diabetic mice. To determine whether elevated DN T cell number is associated with diabetes resistance, we investigated the proportion of DN T cells in two other genetic backgrounds. The proportion and number of DN T cells was comparable for both diabetes-resistant TCR B10.Br and BALB.K mice in the spleen and lymph nodes (Figure 4a, Table II). Moreover, both the diabetes-prone TCR NOD.$H2^k$ and CD47-

deficient BALB.K mice show a severe reduction in DN T cell proportion and number (Figure 4a, Table II). Interestingly, similar to CD47-deficient and -sufficient mice, the TCR transgene on B10.Br and NOD.$H2^k$ backgrounds did not impact the proportion of CD4 and CD8 T cell subsets (Figure 4b). Finally, the discrepancy in DN T cell number between the B10.Br and NOD.$H2^k$ strains was also apparent in TCR:insHEL transgenic mice (Figure 4c). Thus, we find elevated DN T cell numbers in both diabetes-resistant TCR B10.Br and BALB.K CD47-sufficient mouse models and low DN T cell numbers in both diabetes susceptible TCR NOD.$H2^k$ and BALB.K CD47-deficient mouse models. Taken together, these results suggest that elevated DN T cell number is associated with autoimmune diabetes resistance.

Phenotypic characterization of DN T cells.

Immunoregulatory DN T cells have been previously described by others [31, 32]. These cells are rare in non-transgenic mice [43, 44] and humans [34]. Interestingly, the insertion of TCR transgenes has been shown to increase the proportion of DN T cells in many different models, including the HY-TCR, the 4E3-TCR, the 1H3.1 and the 2C-TCR transgenic mice [31, 32, 45-48]. Similarly, we have found that the proportion of DN T cells is considerably increased in the context of the 3A9 TCR transgene (Figure 3). As a sizeable proportion of DN T cells can be found in TCR transgenic mice, it is not surprising to find that the most extensive characterization of this cell type has been performed using the TCR transgenic settings [31, 32, 46]. We compared the phenotype of the 3A9 TCR transgenic T cells to these immunoregulatory DN T cells.

Similar to the immunoregulatory 2C TCR transgenic DN T cells as well as the IH3.1 DN T cells, those obtained in the 3A9 TCR transgenic mice are anergic and the anergy can be reversed upon addition of IL-2 [32, 46, 49] (Figure 5). Moreover, DN T cells from 2C, IH3.1 and 3A9 TCR transgenic mice exhibit a comparable phenotype, in that they express low levels of CD44, are negative for CD25, CD69, CD122 and Foxp3 expression and show high levels of Ly6A [31, 32, 46, 50, 51] (Figure 6a). We performed additional phenotypic characterization and demonstrate

that 3A9 TCR transgenic DN T cells express many naïve T cell markers such as CD3, CD62L and CD45RB at levels comparable to CD4 T cells from TCR transgenic mice and low levels of CD5 [46, 52] (Figure 6b). Therefore, the CD4-CD8-1G12+ subset found in the 3A9 TCR transgenic mice defines a naïve T cell type and carries a similar phenotype to the previously defined immunoregulatory DN T cell subset.

DN T cells are reduced in number in non-transgenic autoimmune prone NOD mice. Insertion of TCR transgenes are known to alter the homeostatic regulation of various T cell subsets [53]. We thus aimed to verify whether the increased number of DN T cells is associated with autoimmune diabetes resistance in a non-transgenic setting. We took advantage of the phenotypic characterization performed on TCR transgenic DN T cells to identify the rare DN T cells in non-transgenic mice. Spleen and lymph node cells from B10.Br and NOD mice were selected for lack of CD4, CD8, DX5, B220, and TCRδ expression (purity >95%). Up to 25% of this enriched cellular population are shown to express high levels of TCRβ, the majority of which do not stain for the CD1d-tetramer, thereby excluding potential contaminating $CD4^-$ $CD8^-$ NKT cells from the analysis (Figure 7a and data not shown). Finally, similar to the observations made for TCR transgenic DN T cells, these non-transgenic DNT cells showed a low level of CD5 expression (Figure 7a). Therefore, DN T cells can clearly be identified in a non-transgenic setting.

As autoimmune-prone TCR transgenic mice show a severe reduction in the proportion of DN T cells, we quantified the proportion of DN T cells in H-2^{g7} non-transgenic NOD mice. Our data demonstrate at least a three-fold reduction in DN T cell number in non-transgenic NOD mice when compared to diabetes-resistant B10.Br (Figure 7b). This result further supports the view that low DN T cell proportion is associated with diabetes susceptibility.

DN T cells are sufficient to restore diabetes resistance.

We next aimed to determine whether DN T cells were directly implicated in resistance to diabetes progression. We selected a group of 23 TCR:insHEL transgenic

CD47-deficient BALB.K mice, which tested negative for urine glucose (Diascreen) at 9 weeks of age. Nine of these mice received 10 million DN T cells and the cohort of 23 mice was monitored for diabetes incidence. At 24 weeks of age, only 1/9 mice which had received DN T cells was hyperglycemic, as compared to 11/14 in non-injected animals (Figure 8a). As a control, 2 mice were injected with 10 million CD4+ T cells from TCR transgenic BALB.K mice and 1 of these became diabetic before 24 weeks (Table III). Moreover, to exclude the possibility that contaminating cells found in the DN T cell preparation were responsible for conferring protection from diabetes progression, we generated an additional cohort of mice, which were instead injected with 2 million DN T cells sorted to high purity. All of the four mice, injected at 6 weeks of age, have maintained low urine and blood glucose levels (Table III).

Furthermore, we have previously shown that, in the TCR:insHEL model, anti-HEL IgG antibody levels correlate with diabetes susceptibility in other genetic backgrounds [37]. We thus quantified the HEL specific auto-antibodies in TCR:insHEL CD47-deficient mice, which had, or had not, received DN T cells. Levels of serum anti-HEL IgM auto-antibodies were similar between both groups (Figure 8b). Interestingly, DN T cell transfer in TCR:insHEL CD47-deficient mice not only protected the mice from diabetes progression but these recipients also showed a lower level of serum anti-HEL IgG auto-antibodies (Figure 8c). Taken together, these results suggest that restoring DN T cell number in diabetes-prone mice prevents diabetes progression.

Discussion:

T cell-mediated autoimmune diabetes progresses similarly in humans and in NOD mice [54]. In support of this view, genetic susceptibility to autoimmune diabetes is quite concordant between the two species, where the *MHC*, *PTPN22* and *CTLA-4* genetic polymorphisms associate with disease [55]. Moreover, insulin has been suggested as the initiating autoantigen in both humans and mice [56-59]. Finally, the mouse *Il2* and human *CD25* genes are also both associated with increased risk, suggesting that the IL-2 pathway is involved in disease susceptibility [55]. Herein, we lend support for the implication of yet another pathway which is involved in autoimmune diabetes progression in both species: the CD47-SIRP pathway.

CD47-SIRP interactions modulate T cell responses and have been suggested to contribute to T cell-mediated autoimmune pathogenesis [17, 22, 23, 29]. Using a TCR transgenic mouse model, we demonstrated that CD47-deficiency is sufficient to break the immune tolerance and promote autoimmune diabetes progression. Transgenic mouse models have been commonly used to validate the implication of other biological pathways in the progression of autoimmune diabetes. A TCR transgenic approach was used to verify the association of the IL-2 candidate gene, located within the *Idd3* locus, with autoimmune diabetes susceptibility [60]. In addition, the role of CTLA-4 in autoimmune diabetes was evaluated using a transgenic mouse model for a specific CTLA-4 isoform [61]. These studies highlight the importance of murine transgenic systems to reveal the *in vivo* role of candidate genes and/or pathways implicated in immune tolerance and associated with human autoimmune diseases.

The introduction of the CD47-deficiency on non-transgenic C57BL/6 or BALB/c background, genetically resistant to autoimmune diseases, is not sufficient to cause autoimmune diabetes. This is not surprising, considering the multigenic character of autoimmune diseases, and certainly does not preclude a role for the CD47 pathway in autoimmunity. Autoimmune disease predisposition is a complex genetic trait, where

multiple genes are associated with disease progression [62]. By definition, one gene is thus not sufficient to confer disease progression. For example, the MHC I-A^{g7}, the most important factor contributing to autoimmune diabetes susceptibility in NOD mice, is not sufficient to confer diabetes susceptibility in autoimmune resistant C57BL/6 mice when these mice are made congenic for the $H2^{g7}$allele (a.k.a. B6.g7) [63]. Furthermore, in support of a role for the CD47 pathway in autoimmunity, CD47-deficient autoimmune-prone NOD mice were shown to develop hemolytic anemia [25]. CD47 expression on erythrocytes is of critical importance [24, 64], and CD47-deletion may embrittle the red blood cells, increasing the predisposition to autoimmune hemolytic anemia in a strain already highly susceptible to autoimmune diseases. Thus, we propose that, as for other factors associated with autoimmune diabetes predisposition, the CD47 pathway is one of many parameters contributing to autoimmune disease susceptibility.

Within the CD47 pathway, it is tempting to propose *SIRPα* as the candidate gene. First, CD47 is only known to bind SIRPα and SIRPγ [14]. Second, SIRPγ is not expressed in mice [16]. Third, *SIRPα* is located within the *Idd13* and *Iddm27* diabetes susceptibility locus in mice and rats, respectively [26, 27]. Finally, *SIRPα* is highly polymorphic [28]. However, it must be noted that the polymorphisms in the human *SIRPα* gene are unlikely to affect binding with CD47 [13, 14]. Moreover, the human genetic association to type 1 diabetes was more strongly linked to *SIRPγ* and appeared to exclude *SIRPα* as a candidate gene [29]. Thus, although we have demonstrated that ablating CD47 is sufficient to contribute to autoimmune disease progression, more studies are required to resolve the exact polymorphism and protein interactions increasing autoimmune diabetes susceptibility.

In this study, we demonstrate that the CD47 pathway influences the homeostatic regulation of an unconventional T cell subset (the DN T cells), where DN T cell transfer is sufficient to prevent diabetes progression. Immunoregulatory DN T cells have been previously characterized by others [31, 32], where they must be activated by antigen to efficiently eliminate CD8 T cells carrying the same antigenic specificity

[33]. Indeed, these cells can prevent skin and heart allograft as well as heart xenograft rejection [31, 65, 66]. Using a different TCR transgenic model, where diabetes is induced upon peptide and anti-CD40 challenge, DN T cells were also shown to confer protection from autoimmune diabetes progression [67]. Our data thus support and add to the findings that DN T cells protect from diabetes progression. For one, we demonstrate that DN T cells can prevent diabetes onset in a spontaneous model of diabetes. Secondly, we perform a more extensive phenotypic characterization of this T cell subset and show that they exhibit a similar profile to naïve CD4 T cells. Thirdly, we associate low DN T cell number with diabetes susceptibility in TCR transgenic CD47-deficient BALB.K mice and NOD.$H2^k$ mice, as well as in non-transgenic NOD mice. Finally, we demonstrate that DN T cell transfer associates with lower autoantibody levels.

In summary, our observations have permitted the association of a defect in the CD47 pathway with autoimmune diabetes progression and identify at least part of the mechanism by which the disruption of the CD47 pathway accelerates disease onset; through the regulation of DN T cell number. As TCR:insHEL NOD.$H2^k$ mice carry a low number of DN T cells and are highly susceptible to autoimmune diabetes, it would be expected that introgression of the *Idd13* resistance allele, which contains SIRPα, in this transgenic model would restore DN T cell number and confer protection from diabetes progression. Alternatively, DN T cell proportion could be monitored in humans carrying either the resistance or susceptibility allele for the *SIRP* locus genetic marker rs2281808 [29], to determine the association between the genotype and phenotype. Such an association would identify a novel therapeutic target and lead the way for a potential cellular therapy approach, where expansion of DN T cell numbers may protect from autoimmune disease progression.

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Figures and Tables:

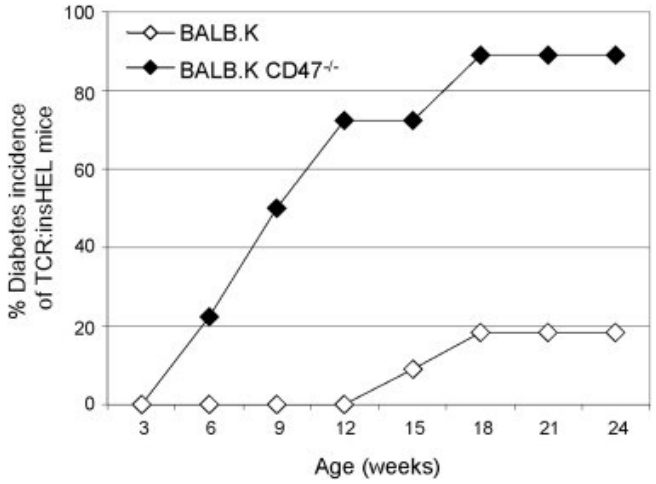


Figure 1: Diabetes incidence is increased in TCR:insHEL BALB.K CD47-deficient mice. Diabetes incidence was monitored in CD47-sufficient (n = 11) and CD47-deficient (n = 18) TCR:insHEL BALB.K mice.

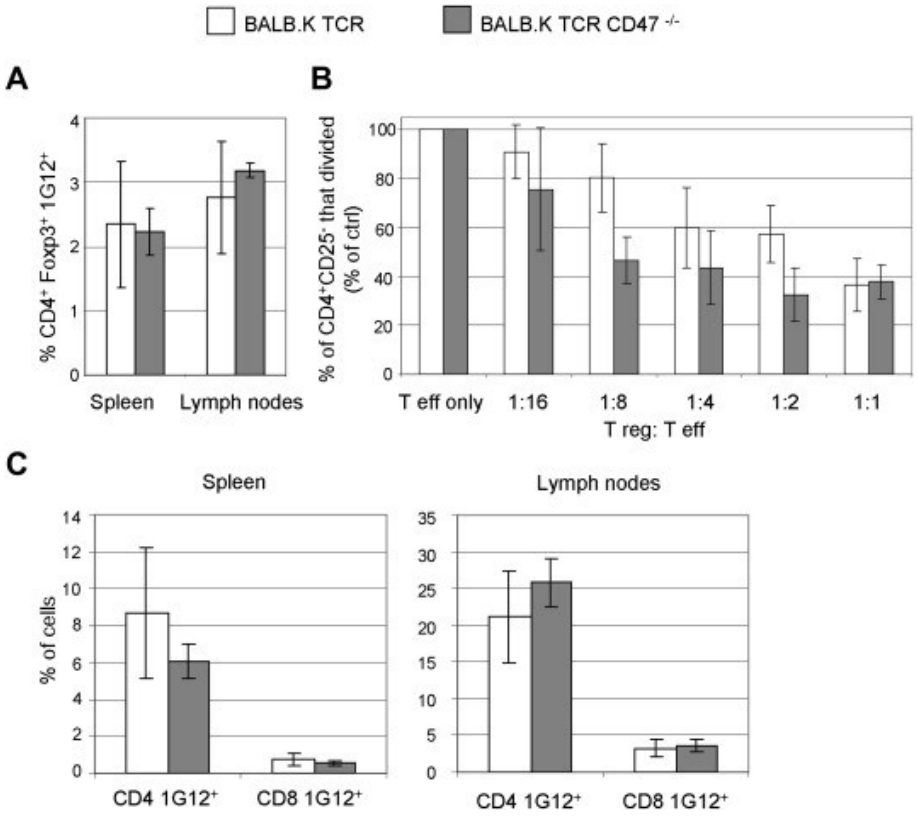


Figure 2: Tregs, CD4 and CD8 T cells are not influenced by the absence of CD47
The proportion of T cells from the spleen and lymph nodes of TCR transgenic CD47-sufficient and -deficient BALB.K mice was determined by staining for CD4, CD8, 1G12 and Foxp3. A) The proportion of TCR transgenic CD4+ Foxp3+ T cells ($n \geq 5$) and B) the ability of sorted $CD4^{+}CD25^{hi}$ Tregs to suppress the proliferation of CFSE-labeled $CD4^{+}CD25^{-}$ effector T cells *in vitro* is shown for CD47-sufficient (white) and –deficient (grey) TCR transgenic BALB.K mice ($n = 3$). C) The proportion of TCR transgenic CD4 and CD8 T cells in the spleen and lymph nodes are shown for CD47-sufficient (white) and –deficient (grey) BALB.K mice ($n \geq 5$). * $p < 0.01$, ** $p < 0.001$

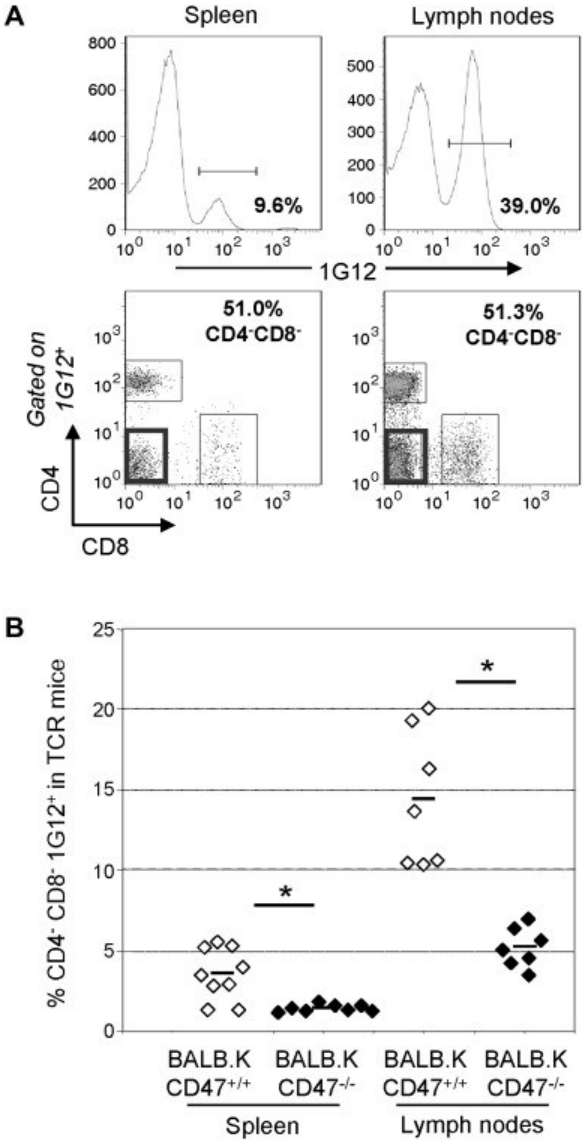


Figure 3: Decreased proportion of an unconventional cell-type in CD47-deficient mice. Cells were prepared as in Figure 2. A) The gating strategy used to detect CD4-CD8-1G12+ T cells is shown for spleen (left) and lymph nodes (right). $n \geq 5$. B). The proportion of CD4-CD8-1G12+ is presented for the spleen and lymph nodes of each mouse from the indicated strains. DN T cell proportion is significantly decreased in CD47-deficient TCR transgenic BALB.K mice ($n \geq 5$). Student's T test * $p < 0.05$.

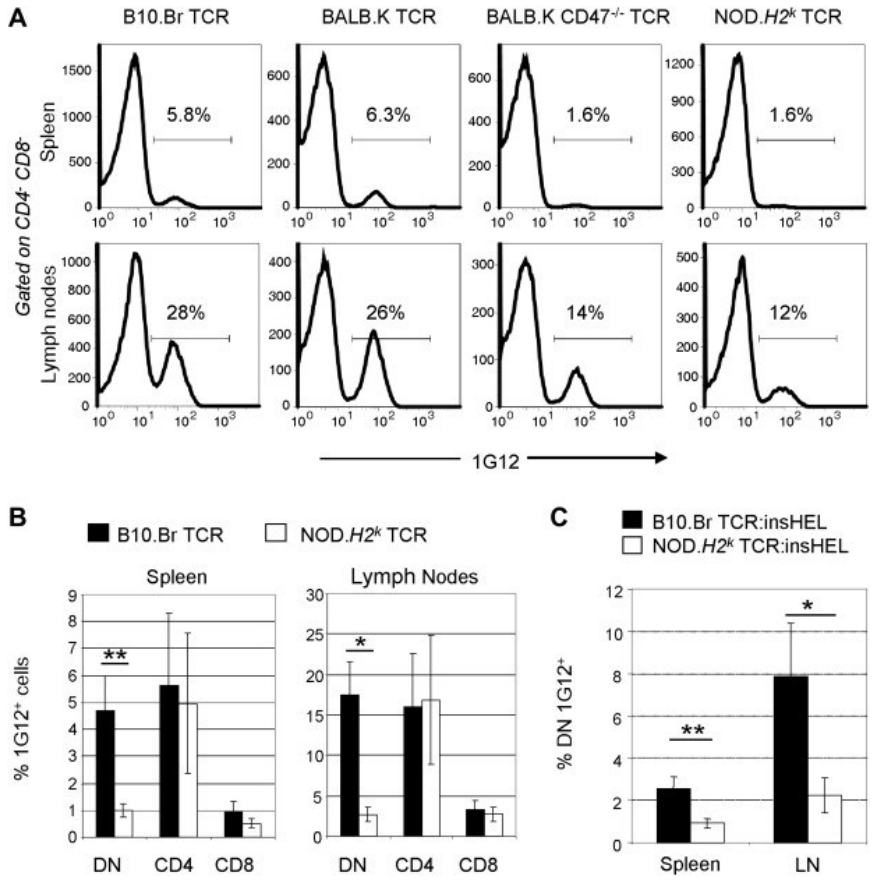


Figure 4: Elevated DN T cell numbers are associated with diabetes resistance. Cells from lymphoid organs of transgenic BALB.K, B10.Br and NOD.*H2*^{k} mice were harvested and stained for CD4, CD8 and 1G12 expression. A) DN T cell proportion in TCR transgenic CD47-deficient and -sufficient BALB.K compared to TCR transgenic diabetes-resistant B10.Br and –susceptible NOD.*H2*^{k} mice. The data is representative of at least three independent experiments. The bar charts demonstrate B) the proportion of CD4+1G12+, CD8+1G12+ and CD4-CD8-1G12+ (DN) T cells in the spleen and lymph nodes of TCR transgenic B10.Br and NOD.*H2*^{k} mice and C) the proportion of CD4-CD8-1G12+ (DN) T cells in the spleen and skin-draining lymph nodes (LN) of TCR:insHEL transgenic B10.Br and NOD.*H2*^{k} mice. The mean and SD of more than five mice per group is shown. * $p < 0.01$, ** $p < 0.001$

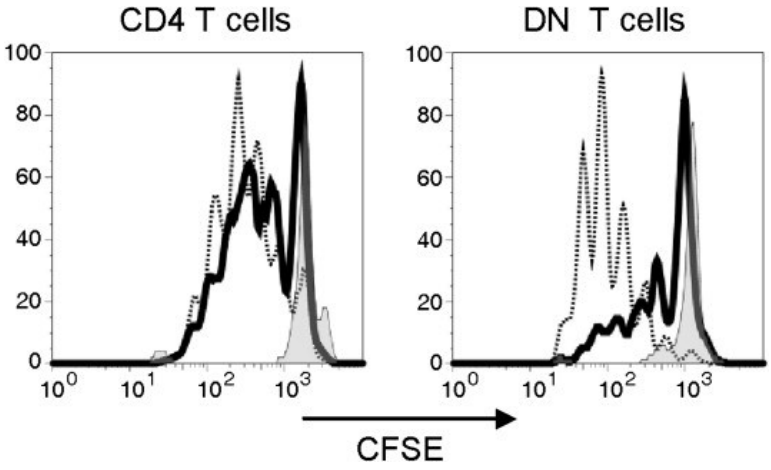


Figure 5: DN T cells are anergic. CFSE-labeled CD4 and DN T cells were cultured for 3 days with APCs (shaded); APCs and anti-CD3 (thick); or APCs, anti-CD3 and IL-2 (dotted). The histograms indicate the level of CFSE dilution corresponding to cellular proliferation. Representative of 3 independent experiments.

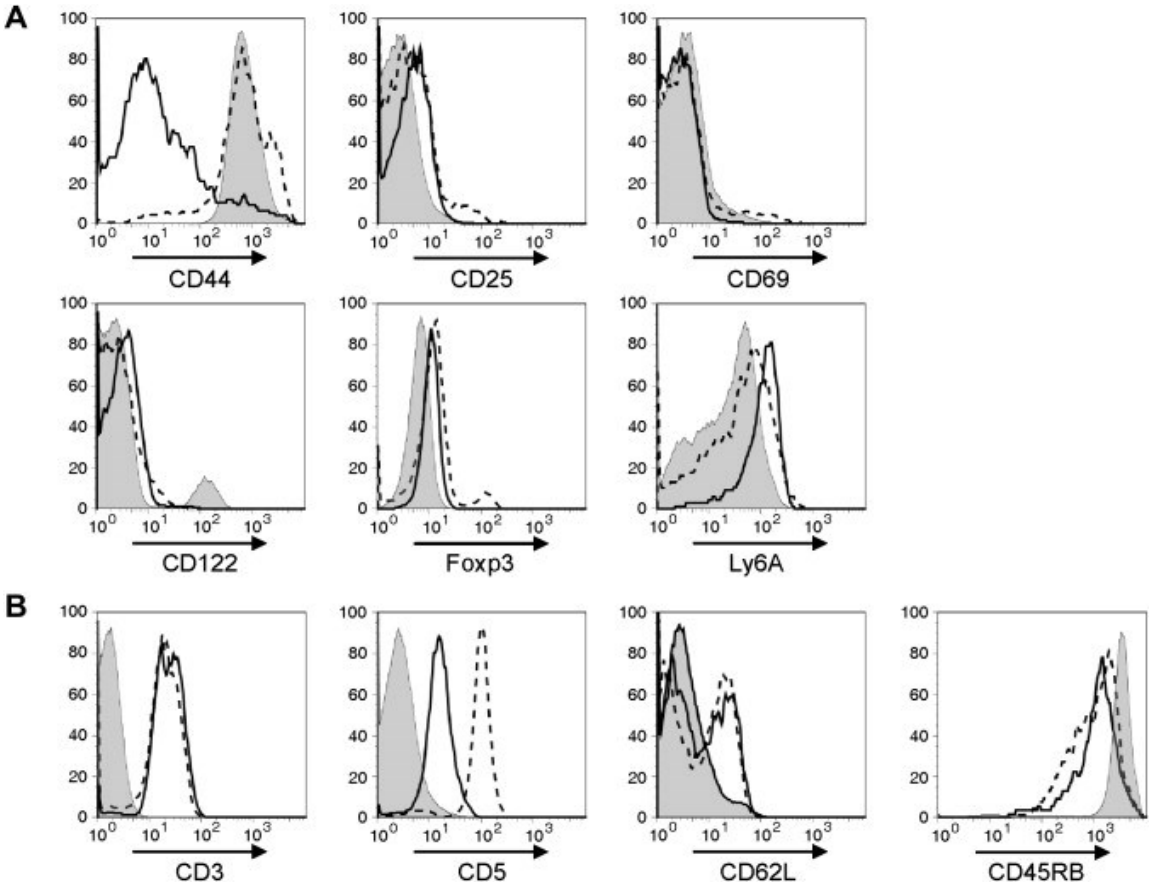


Figure 6: Phenotypic characterization of DN T cells. Spleen cells of TCR transgenic B10.Br mice were stained with CD4, CD8, 1G12 and various other phenotypic markers, as indicated. The expression of cell surface markers is indicated for DN T cells (CD4-CD8-1G12+, thick), CD4 T cells (CD4+CD8-, dashed) and non T cells (CD4-CD8-1G12-), comprising mostly B cells, shaded) were analysed for various cell surface markers. Histograms show expression level of A) surface markers characterizing immunoregulatory DN T cells and B) naïve T cell markers. Representative of at least three independent experiments.

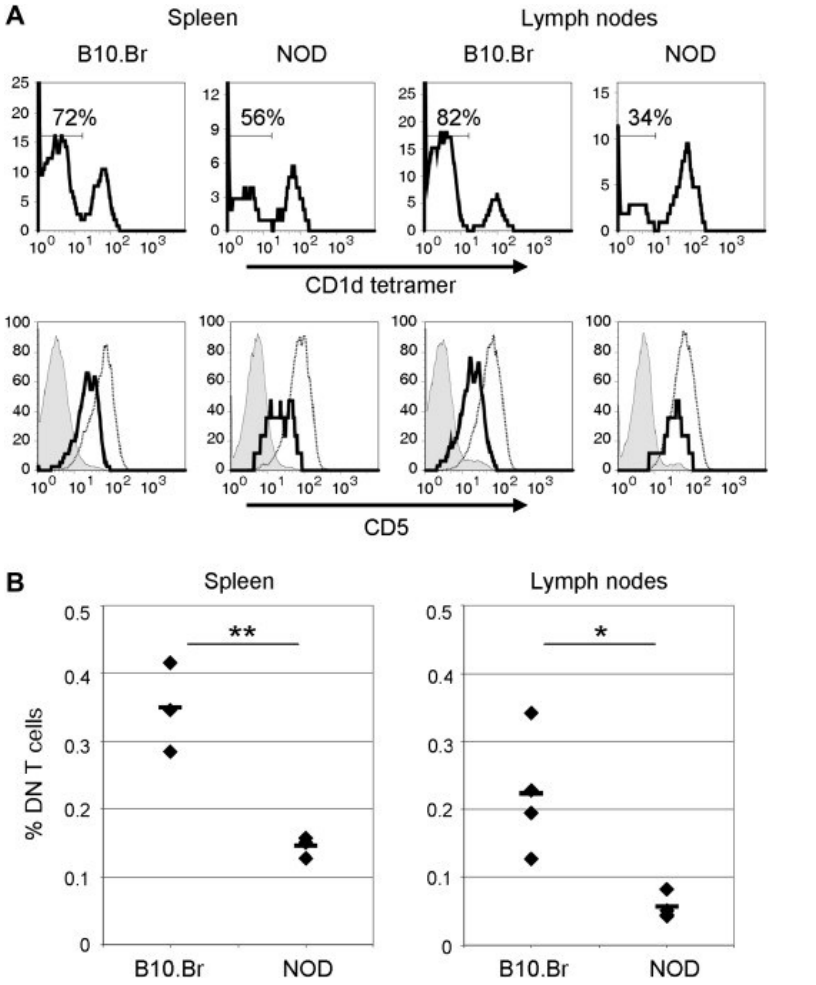


Figure 7: Non-transgenic autoimmune-prone NOD mice show a reduction in the number of DN T cells. A) Cells from spleen and lymph nodes of non-transgenic B10.Br and NOD (I-Ag7) mice were negatively selected for CD4, CD8, CD49b, B220 and TCRδ expression using magnetic separation. *Top panels:* Cells are gated for TCRβ+ expression. The percentage of CD1d tetramer negative cells is shown. *Bottom panels*: The histograms show the level of CD5 expression on B220+ (shaded), CD4+ (dotted) and DN (solid) T cells. Representative of at least three experiments. B) Proportion of DN T cells ($B220^-$ $CD4^-$ $CD8^-$ CD1d-$tetramer^-$ $TCR\beta^+$ $CD5^{low}$) in the spleen and lymph nodes of non-transgenic B10.Br and NOD mice. Student's T test. * $p< 0.05$, ** $p < 0.01$

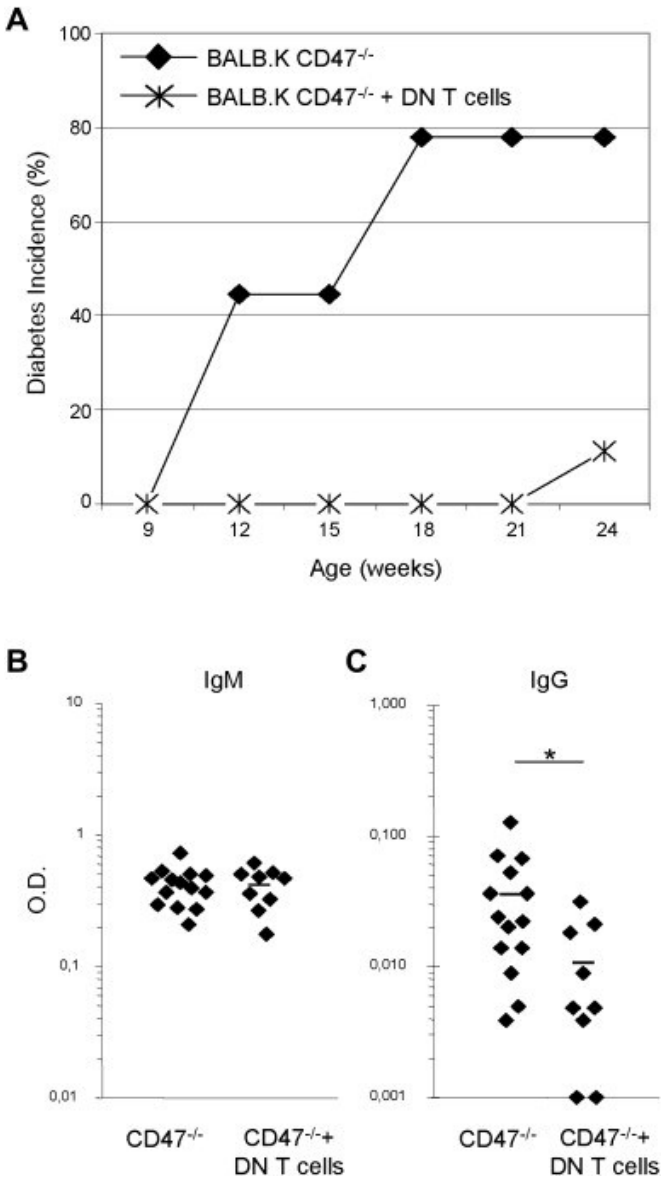


Figure 8: DN T cells are sufficient to restore diabetes resistance. A) Diabetes incidence and B) HEL-specific antibody serum levels of IgM and C) IgG are shown for TCR:insHEL BALB.K CD47$^{-/-}$ mice injected (n = 9) or not (n = 14) with DN T cells. * $p < 0.05$.

Table 1: Absolute numbers of CD4 1G12+ and CD8 1G12+ cells found in TCR transgenic CD47-sufficient and -deficient BALB.K mice. Data represents mean ±SD $n \geq 5$

T cell subset	Mice	Spleen ($\times 10^6$ cells)	Lymph nodes ($\times 10^6$ cells)
CD4 1G12$^+$	BALB.K	9.15 ± 4.46	3.46 ± 2.05
	BALB.K CD47$^{-/-}$	7.30 ± 4.76	3.52 ± 1.52
CD8 1G12$^+$	BALB.K	0.82 ± 0.47	0.55 ± 0.39
	BALB.K CD47$^{-/-}$	0.70 ± 0.54	0.51 ± 0.25

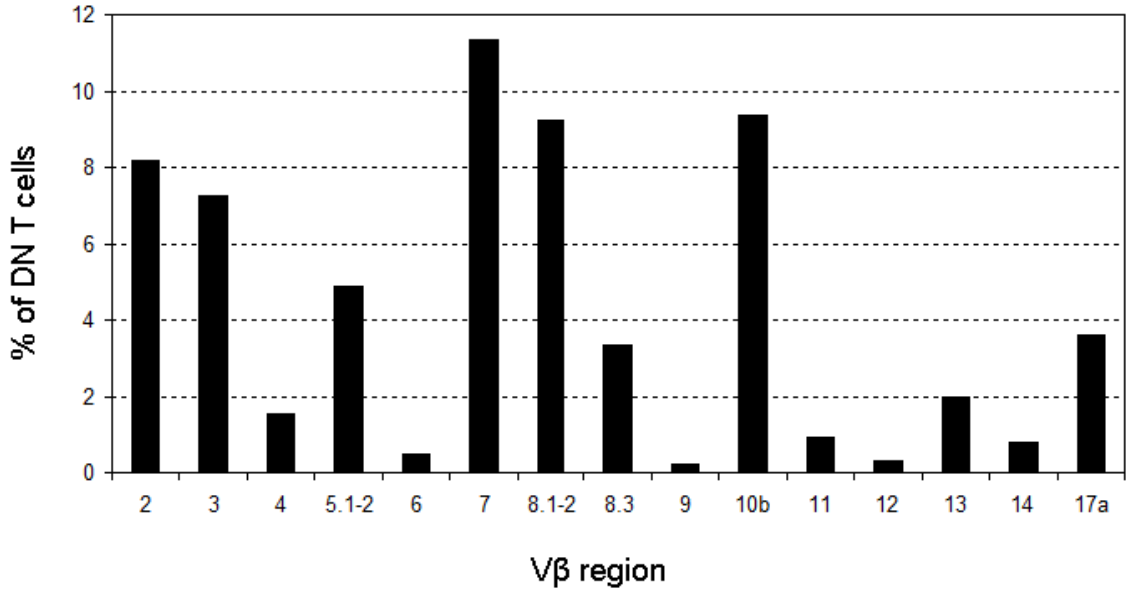
Table 2: Total cellularity and absolute numbers of DN T cells found in TCR transgenic B10.Br, NOD.$H2^k$ and CD47-sufficient and -deficient BALB.K mice. Data represents mean ± SD n ≥ 4

Mice	Spleen		Skin-draining Lymph nodes [6]	
	Total cellularity ($\times 10^7$ cells)	Nb DN T cells ($\times 10^6$ cells)	Total cellularity ($\times 10^7$ cells)	Nb DN T cells ($\times 10^6$ cells)
B10.Br	7.52 ± 3.12	3.73 ± 1.57	1.30 ± 0.56	2.17 ± 1.18
BALB.K	9.72 ± 2.89	3.71 ± 1.99	1.56 ± 0.48	2.37 ± 0.38
BALB.K $CD47^{-/-}$	8.75 ± 3.05	1.82 ± 1.27	1.20 ± 0.46	0.71 ± 0.29
NOD.$H2^k$	7.48 ± 0.40	0.78 ± 0.19	1.10 ± 0.33	0.21 ± 0.11

Table 3: Description of diabetes incidence cohorts injected either with sorted DN T cells or with CD4 T cells.

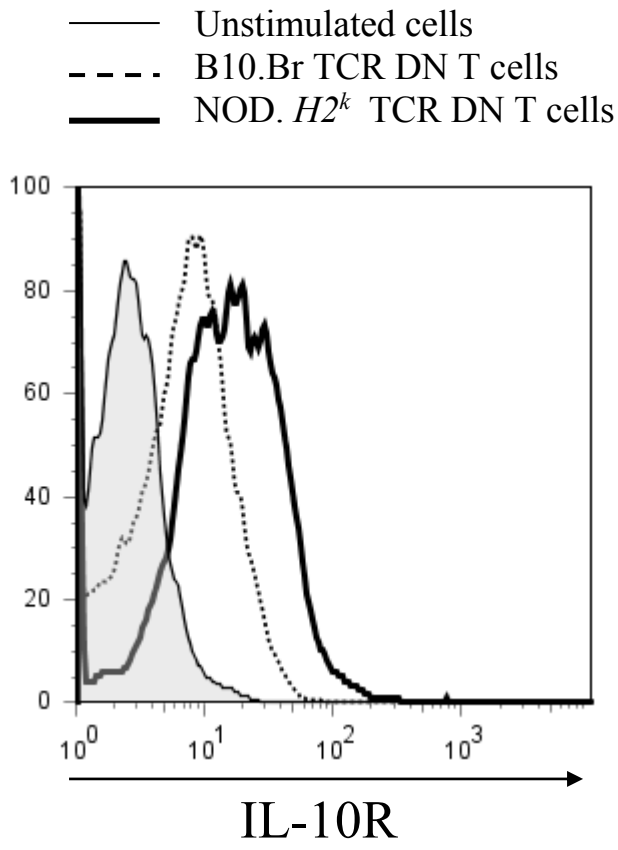
Mouse Nb	Age at transfer (weeks)	Age at final assessment (weeks)	Type of cell transferred	Purification	Nb of cells (millions)	Blood glucose	Age diabetic (weeks)
1	5	17	1G12$^+$ DN T cells	Sorted	2	10.9	–
2	5	17	1G12$^+$ DN T cells	Sorted	2	11.7	–
3	6	15	1G12$^+$ DN T cells	Sorted	2	8.6	–
4	5	9	1G12$^+$ DN T cells	Sorted	2	8.9	–
5	9	24	CD4$^+$ T cells	Enrichment (91%)	10	8.5	–
6	9	13	CD4$^+$ T cells	Enrichment (91%)	10	**30.1**	11

APPENDIX 2



Non-transgenic DN T cells exhibit a polyclonal Vβ TCR repertoire. The spleen was isolated from CD1d-deficient NOD mice. DN T cells were gated based on a CD4-CD8-CD19-TCRβ+ phenotype. The Vβ TCR repertoire of DN T cells from CD1d-deficient NOD mice was assessed by flow cytometry. Representative of two independent experiments.

APPENDIX 3



DN T cells from diabetes-susceptible mice express higher levels of IL-10R. DN T cells were enriched from the spleens of 3A9 TCR B10.Br and NOD. *H2*^{k} mice and activated in vitro for 3 days. The histograms depict the level of expression of IL-10R. n=3.

OTHER CONTRIBUTIONS

FIRST AUTHOR ARTICLES

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CO-AUTHOR ARTICLES

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E.E. Hillhouse. 2010. *Chapter 3: B cells From Immunogenetics: Tolerance and Autoimmunity.* Sylvie Lesage (Ed). Nova Novinka Publishers. ISBN : 978-1-61761-478