Induction of Antigen-Specific Tolerance and Development of Autoreactive T cells in an Experimental Model of Autoimmune Diabetes

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ABSTRACT

Immune responses to proinsulin initiate anti-islet autoimmunity in non-obese diabetic (NOD) mice and possibly in humans. This results in autoimmune destruction of insulin secreting beta cells leading to type 1 diabetes (T1D). Therapies that bolster immune tolerance to islet antigens are highly desirable, however such approaches have failed to prevent clinical T1D. The major aim of this thesis was to determine a stage of life when antigen-specific tolerance is most effective in preventing anti-islet immune responses.

Chapter 2 describes generation and validation of transgenic NOD mice engineered to express islet antigens proinsulin (TIP mice) and IGRP (TII mice) in the antigen presenting cells (APCs) in a tetracycline dependent manner. MHC class II IEα promoter in combination with tet-OFF transactivator induced robust, doxycycline dependent and APC specific expression of proinsulin and IGRP in TIP and TII mice respectively. TIP mice expressing proinsulin did not develop insulitis and were protected from cyclophosphamide-induced diabetes, suggesting that proinsulin expression in TIP mice was sufficient to induce functional antigen-specific tolerance.

In chapter 3, we examined the impact of antigen-specific tolerance on the development of autoreactive T cells and spontaneous diabetes by expressing islet antigens proinsulin and IGRP in the APCs during defined periods of life in TIP and TII mice. Our results indicate that tolerance to proinsulin in early life until the weaning period is sufficient to prevent diabetes development in TIP mice. The protection from diabetes was not due to dominant tolerance, but mainly due to a significant reduction in the insulin reactive T cells. Although insulin reactive T cells were not completely absent, the residual autoreactive T cells lacked pathogenic potential. By tracking IGRP reactive T cells in TII mice we demonstrate that IGRP T cells also emerge during early life. These data suggest that early life is a vulnerable period for escape of islet reactive T cells, and that

boosting immune tolerance to islet antigens during this time imparts durable protection from islet autoimmunity.

Immune tolerance to proinsulin-2 imparts robust protection from autoimmune diabetes in the NOD mice. Whether dampening immune responses to proinsulin-1 would influence diabetes development in NOD mice remains to be investigated. Chapter 4 describes the generation of transgenic NOD mice that express proinsulin-1 in the APCs (TIP-1 mice) in a tetracycline dependent manner. TIP-1 mice displayed a significantly reduced incidence of spontaneous diabetes, which was associated with reduced severity of insulitis and insulin autoantibody development. Antigen experienced proinsulin specific T cells were significantly reduced in number in TIP-1 mice indicating immune tolerance. Although immune response to downstream antigen IGRP was reduced in TIP-1 mice, tolerance to proinsulin-1 was unable to prevent diabetes in NOD 8.3 mice with a pre-existing repertoire of IGRP reactive T cells. Thus, despite being highly conserved to proinsulin-2, tolerance to proinsulin-1 only partially prevents islet-autoimmunity in NOD mice, which suggests an ongoing residual immune response to proinsulin-2 epitopes in TIP-1 mice.

DECLARATION

This is to certify that:

i. This thesis comprises only my original work towards the PhD except where indicated in the Preface;

ii. Due acknowledgement has been made in the text to all other material used;

iii. This thesis is fewer than 100, 000 words in length, exclusive of tables, figures, bibliographies and appendices.

GAURANG JHALA

PREFACE

I assess my contribution to the results described in each chapter to be:

Chapter 2: 90%

Chapter 3: 80%

Chapter 4: 80%

The following list outlines the experiments performed by others that have been included in the results section of this thesis:

Chapter 2:

DNA microinjection to generate transgenic TIP and TII mice was performed by the technicians at the Central Microinjection Services Centre of the Walter and Eliza Hall Institute, Melbourne.

- Prerak Trivedi performed some experiments to characterize rtTA-GFP and TA-GFP mice.

Chapter 3:

- Jonathan Chee performed IGRP-tetramer staining in TIP mice
- Esteban Gurzov did the western-blot analysis for insulin expression

Chapter 4:

- Jonathan Chee performed IGRP-tetramer staining in TIP-1 mice
- Claudia Selck performed some of the insulitis scoring and insulin autoantibody ELISA assays.

The following list is the publications and manuscripts in review or preparation that contain work performed during my PhD:

Published

Perinatal tolerance to proinsulin is sufficient to prevent autoimmune diabetes.
Jhala G, Chee J, Trivedi PM, Selck C, Gurzov EN, Graham KL, Thomas HE, Kay TW, Krishnamurthy B.
JCI Insight. 2016 Jul 7;1(10):e86065.

Analysis of antigen specific T cells in diabetes - Lessons from pre-clinical studies and early clinical trials.
Krishnamurthy B, Selck C, Chee J, **Jhala G**, Kay TW.
J Autoimmun. 2016 Jul;71:35-43. doi: 10.1016/j.jaut.2016.03.018. **Review.**

BIM Deficiency Protects NOD Mice From Diabetes by Diverting Thymocytes to Regulatory T Cells.
Krishnamurthy B, Chee J, **Jhala G**, Trivedi P, Catterall T, Selck C, Gurzov EN, Brodnicki TC, Graham KL, Wali JA, Zhan Y, Gray D, Strasser A, Allison J, Thomas HE, Kay TW.
Diabetes. 2015 Sep;64(9):3229-38. doi: 10.2337/db14-1851.

Effector-memory T cells develop in islets and report islet pathology in type 1 diabetes.
Chee J, Ko HJ, Skowera A, **Jhala G**, Catterall T, Graham KL, Sutherland RM, Thomas HE, Lew AM, Peakman M, Kay TW, Krishnamurthy B.
J Immunol. 2014 Jan 15;192(2):572-80. doi: 10.4049/jimmunol.1302100.

Multicenter Australian trial of islet transplantation: improving accessibility and outcomes.
O'Connell PJ, Holmes-Walker DJ, Goodman D, Hawthorne WJ, Loudovaris T, Gunton JE, Thomas HE, Grey ST, Drogemuller CJ, Ward GM, Torpy DJ, Coates PT, Kay TW; **Australian Islet Transplant Consortium.**
Am J Transplant. 2013 Jul;13(7):1850-8. doi: 10.1111/ajt.12250.

Complete diabetes protection despite delayed thymic tolerance in NOD8.3 TCR transgenic mice due to antigen-induced extrathymic deletion of T cells.
Krishnamurthy B, Chee J, **Jhala G**, Fynch S, Graham KL, Santamaria P, Morahan G, Allison J, Izon D, Thomas HE, Kay TW.
Diabetes. 2012 Feb;61(2):425-35. doi: 10.2337/db11-0948.

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Tolerance to proinsulin-1 partially prevents autoimmune diabetes in NOD mice.
Manuscript in preparation

Mollah Z*, Quah HS*, Graham KL, **Jhala G**, Krishnamurthy B, Chee J, Trivedi PM, Pappas EG, Mackin L, Fynch S, Trapani JA, I Bird PI, Chong MMW, Brodnicki TC, Kay TWH, and Thomas HE. Granzyme A-deficiency breaks immune tolerance and promotes autoimmune disease through a type I interferon-dependent pathway. **Manuscript submitted**.

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ABBREVIATIONS

AIRE Autoimmune Regulator

APCs Antigen Presenting Cells

APC Allophycocyanin

BCR B cell receptor

CFA Complete Freund's Adjuvant

CFSE 5,6-carboxylfluorescein diacetate succinimidyl ester

CMV Cytomegalovirus

ChgA Chromogranin A

Ct Cycle threshold

CTL Cytotoxic T Lymphocyte

DC Dendritic Cells

Dox Doxycycline

FACS Fluorescence activated cell sorter

FoxP3 Foxhead/wing-helix P3

FITC Fluorescein isothiocyanate

GAD Glutamic Acid Decarboxylase

GFP Green fluorescent protien

HEL Hen Egg Lysozyme

HLA Human Leukocyte Antigen

IAA Insulin autoantibodies

IA-2 Insulinoma-associated protein

ICA Islet Cell Autoantigen

IFN Interferon

IGRP Islet-specific glucose-6-phosphatase catalytic subunit related protien

IL	Interleukin
ILN	Inguinal lymph node
INS	Insulin
MACS	Magnetic activated cell sorting
MHC	Major Histocompatiblity Complex
mTEC	Medullary Thymic Epithelial Cells
NK	Natural Killer
NOD	Non-obese diabetic
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI	Propidium iodide
PI	Proinsulin
PLN	Pancreatic lymph node
RAG	Recombinase-Activating Gene
rtTA	Reverse tetracycline transactivator
SCID	Severe combined immune deficiency
SEM	Standard error of the mean
T1D	Type 1 diabetes
TCR	T cell receptor
Teff	Effector T cells
Tet	Tetracycline
TGFβ	Transforming growth factor beta
Th	T helper cell subset
TNF	Tumour Necrosis Factor
Treg	Regulatory T cells

tTA Tetracycline transactivator

ZnT8 Zinc Transporter 8

LITERATURE REVIEW

1.1 Type 1 Diabetes

Type 1 diabetes (T1D) is a chronic autoimmune disease commonly diagnosed in children and therefore is also termed juvenile diabetes, however it can occur at any age. Currently there are over 130,000 Australians with T1D, with 7 new cases diagnosed every day (Australian Institute of Health and Welfare AIHW estimates 2014). The global incidence of T1D is increasing by 3 % every year and is predicted to double by the year 2020 in children under the age of 5 years in developed nations (1, 2).

In individuals with T1D, autoreactive T cells specific for multiple islet autoantigens selectively destroy insulin producing beta cells in the pancreas. This leads to progressive loss of insulin production and life-long reliance on exogenous insulin to maintain glucose homeostasis.

Improvements in insulin formulations, insulin delivery devices and glucose monitoring devices over the years have contributed to T1D patients having a normal life expectancy. Despite insulin therapy being the standard of care in T1D a vast majority of subjects still fail to achieve euglycemia and they are at risk for development of life-threatening complications such as ketoacidosis and hypoglycemia in addition to chronic microvascular disease (nephropathy, retinopathy and neuropathy) and macrovascular disease (cardiovascular, cerebrovascular and peripheral vascular disease) (3),(4). Currently, there is no other treatment available to preserve or restore endogenous insulin production to either prevent or treat T1D.

1.2 Pathogenesis of T1D

The etiology of human T1D is complex. Genetic predisposition and environmental factors play an important role in defining risk of disease development (5).

1.2.1 Genetic Factors

Family studies have indicated a strong genetic component in the etiology of T1D, with first-degree relatives of an affected individual having a higher risk of T1D development compared to the general population (6). Further evidence of genetic heritability of T1D is provided by twin studies, indicating a high concordance for disease development in monozygotic twins (7). Genetic association studies have identified over 50 susceptibility loci that influence T1D development (8),(9),(10),(11). Genes of the HLA class II (DQ, DR and DP) that are expressed on antigen presenting cells and involved in presenting peptide antigens to CD4+ T cells contribute towards more than half of the genetic susceptibility to T1D (12),(13). The second major susceptibility locus maps to polymorphisms in the 5' region of the insulin gene that controls thymic expression of insulin and influences central tolerance to insulin protein (14),(15). Several other genes such as *PTPN22, IL2* receptor, *CTLA-4 and IFIH1* that influence various immunological functions have been associated with increasing genetic risk of T1D development (9),(16). Certain HLA class I molecule encoding alleles, including HLA-A*24, HLA-B*18, and HLA-B*39, also contribute to susceptibility of T1D (17). Data from two recent studies suggest that the effect of HLA class II is limited to the early phase of the disease process characterized by seroconversion for islet autoantibodies. HLA class II alleles did not associate with progression rate from advanced autoimmunity to clinical diabetes (18); whereas HLA class I alleles were associated with a more rapid progression to clinical disease (19),(20). The finding of effect of HLA class II in the initiation of autoimmunity and HLA class I in the progression to beta cell damage and clinical diabetes is in accordance with the model where CD4 + T-helper

cells recognizing their antigens in context of HLA class II molecules are important in the afferent arm of autoimmunity and CD8+ cytotoxic T cells in efferent arm where they infiltrate pancreatic islets and recognize their cognate antigens presented by HLA class I molecules (21).

1.2.2 Environmental Factors.

Despite strong genetic influence on the risk of developing T1D, not all individuals carrying the high-risk alleles develop diabetes. The rapid increase in global incidence of T1D at an annual rate of ~ 3%, combined with the variability in incidence amongst populations that are genetically similar is suggestive of an environmental trigger (22). Moreover, monozygotic twins develop T1D with a concordance less than 100%, indicating a role for non-genetic determinants in disease development (1),(23),(24)and(25). Dietary factors that include, duration of breast-feeding introduction of cow milk proteins, early exposure to cereals and lack of vitamin D (26),(27),(28),(29) have been associated with T1D development. The role of viral infections as a trigger for T1D has been extensively examined (30),(31),(32),(33). Viral infections may lead to a misdirected immune response towards beta cells (34),(35). Type 1 interferon (IFN) signaling and anti-viral immune responses have been associated with the etiology of T1D (36),(37). Recently an IFN inducible and disease associated transcriptional gene signature was detected in children with genetic predisposition to T1D, prior to development of islet autoantibodies (38). These results further support the role of a viral trigger for T1D. Large-scale prospective longitudinal studies with an aim to identify early pathogenic mechanisms operating to cause islet autoimmunity are currently underway (39),(40).

1.2.3 Natural History of Type 1 Diabetes Development

A model of the natural history of T1D first described in the 1980's suggests stages of disease progression that commence with genetic predisposition followed by progressive autoimmunity that culminates in clinical disease (41), (42). Observations from numerous prospective and

longitudinal studies have shown that T1D represents a disease continuum with a variable asymptomatic phase (43). A recently proposed staging classification system suggests that progression to symptomatic T1D occurs through distinct identifiable stages, which include a pre-symptomatic stage, thus recognizing the earliest stages of human T1D (Figure 1-1) (44).

Stage I

Environmental factor(s) may trigger anti-islet immune responses as early as in utero and continue in early life thereby affecting the onset and progress of beta cell autoimmunity (43). Initiation of immune responses towards islet antigens is followed by an asymptomatic phase that can range from months to years, during which autoimmune destruction of beta cells is marked by the development of autoantibodies against one or more islet cell antigens. Detection of two or more autoantibodies in an individual is a reliable indicator of impending T1D, with 44% (5-year) and 70% (10-year) risk of developing clinical disease (45). Therefore, stage I represents individuals with pre-symptomatic diabetes who express two or more islet autoantibodies but are normoglycemic.

Stage II

In individuals with multiple islet autoantibodies, ongoing autoimmunity results in loss of functional beta cell mass. This leads to development of dysglycemia or impaired glucose

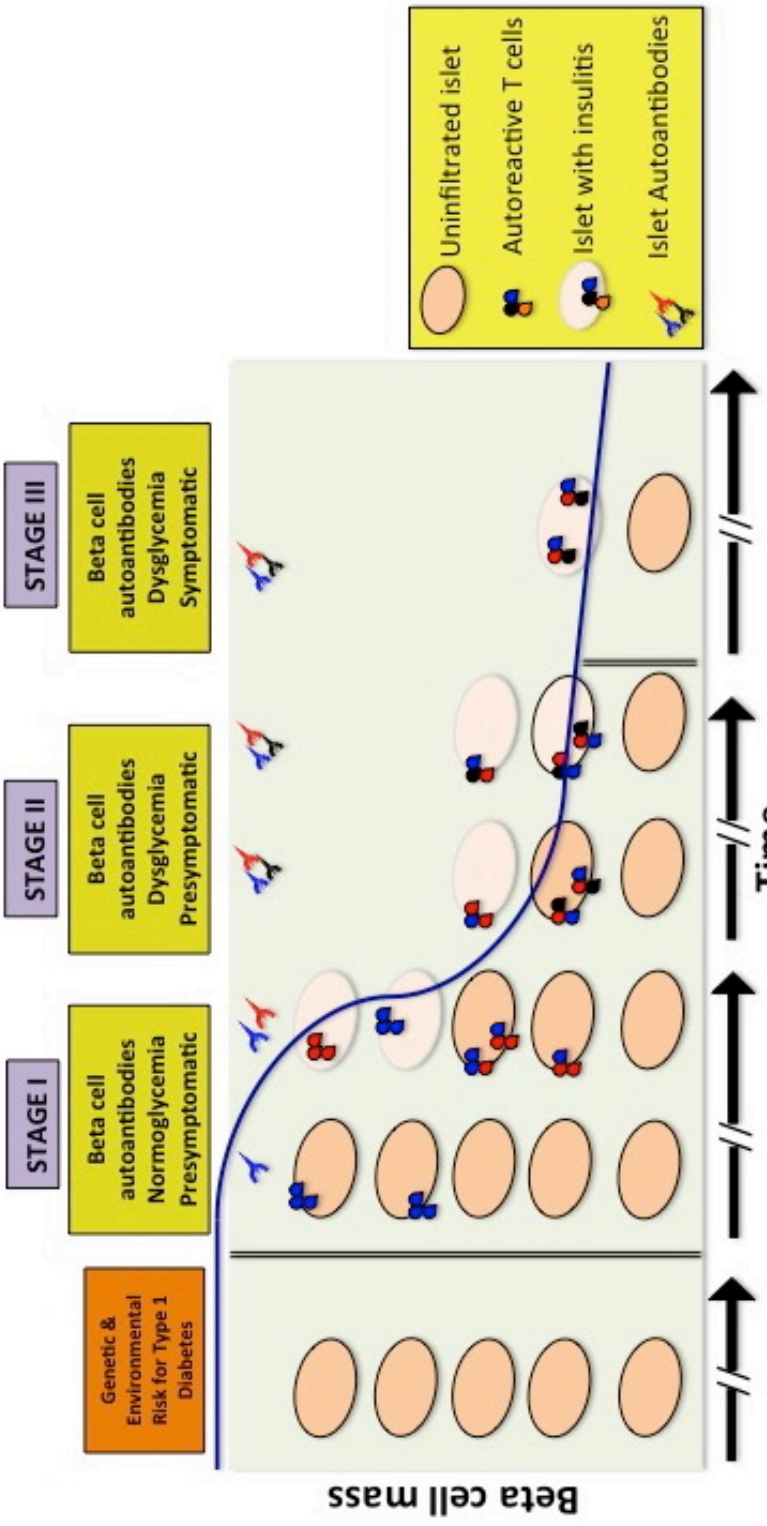

Figure 1-1: Stages of Type 1 diabetes

tolerance and gradual increase in hemoglobin A1C levels (46). The 5-year risk of developing symptomatic T1D at this stage is 75% with a lifetime risk approaching 100% (47). Individuals with beta cell autoimmunity marked by islet autoantibodies and dysglycemia can be classified as having progressed to pre-symptomatic stage II of T1D development.

Stage III

Stage III represents clinical onset of T1D marked by reduced insulin production hyperglycemia and decline in C-peptide levels and associated symptoms of diabetes that may include polyuria, polydipsia, fatigue, weight loss etc.

1.3 The NOD mouse model of autoimmune diabetes

The pathogenesis of T1D is complex and the study the pathogenesis of this human disease is difficult due to limited access to relevant pancreatic tissue. Invasive sampling for scientific studies cannot be routinely done since a vast majority of T1D patients are young children (48). The availability of animal models such as the non-obese diabetic (NOD) mouse, which spontaneously develop autoimmune diabetes, has vastly contributed to the current understanding of T cell mediated autoimmunity and pathogenesis of T1D.

The NOD mouse is a robust model for investigation of autoimmune diabetes. It mirrors the clinical features of human disease such as the presence of islet antigen specific autoantibodies and progressive lymphocytic infiltration of the pancreas. The NOD mouse also has similar polygenic determinants of the disease susceptibility to those in humans, such as specific major histocompatibility complex (MHC) alleles, and several potential disease modulating genes termed as *IDD* (Insulin dependent diabetes) loci. The major antigens targeted by T and B cells also overlap in human and NOD T1D (49), (50). In addition, the MHC class II (I-Ag7) of the NOD mouse and diabetes susceptible HLA-DQ2-DQ8 in humans share similarities at the amino acid

level (51). Moreover, the peptides that bound to MHC class II (I-Ag7) of the NOD mouse and those that bound to HLA-DQ8 in humans displayed identical sequence specificity (52).

Immunopathological mechanisms leading to autoimmune diabetes have been extensively studied in NOD mice. Recognition of islet-antigens presented on the APC by autoreactive T cells is the primary event in the pathogenesis of islet autoimmunity (53),(54). Mononuclear infiltrate termed “insulitis” comprising of various immune cell subsets is visible around the periphery of the islets in the pancreas of NOD mice around 3 weeks of age (55),(56),(57). Invading self-reactive T cells mediate progressive beta cell destruction by various effector mechanisms (58). Eventually, spontaneous diabetes ensues in 60-80% in female NOD mice and 20-30% in male mice (59).

1.3.1 Strengths and limitations of the NOD mouse model

For several decades studies in NOD mice have significantly contributed to the current understanding of autoimmune diabetes (60),(61). The use of the NOD mouse model has led to many important advances that have been informative about genetic susceptibility, environmental influences, mechanisms of immune tolerance and multiple autoantigens contributing to T1D development (62),(63),(64). Although NOD mice reflect crucial pathophysiological aspects of human T1D (65), shortfalls in translating various preventions and cures developed in NOD mice to clinical therapies for T1D have questioned the relevance of NOD mice to human disease (66),(67),(68). The concerns arise from several factors such as the differences in immune systems of NOD mice and humans (69),(70),(71), the highly inbred nature of NOD mice compared to outbred humans (72),(73), the strong gender bias for diabetes development in NOD mice(74),(75) and environmental influences on the penetrance of diabetes in various colonies of NOD mice (76). The pitfalls in accurately transposing outcomes from NOD mice to humans at risk of T1D, may be in part due to suboptimal design and execution of such preclinical studies (77).

Despite dissimilarities to the human disease, NOD mice continue to be an important animal model to address experimental questions and test therapies for clinical application. Rigorous design of studies can further enhance the value of NOD mice as a preclinical model (78),(79). The greatest strength of NOD mice is that they are tractable, as they facilitate powerful mechanistic studies by allowing manipulation of key genes and proteins, which would otherwise be impossible in human subjects.

1.4 Immunopathology of T1D Development

A series of immunological checkpoints operate to maintain tolerance to self-antigens in order to prevent autoimmunity, as well as modulate the amplitude of immune responses to infectious pathogens to prevent tissue damage (80),(81). Autoimmune destruction of pancreatic beta cells in T1D suggests that there are defects in such homeostatic checkpoints that allow the emergence and subsequent activation of autoreactive T cells.

1.4.1 Immune Tolerance to Self-Antigens

T cell development in the thymus is stringently regulated to maintain tolerance to self whilst allowing for a robust response against foreign pathogens. T-cell precursors arise from the hematopoietic stem cells in the bone marrow and migrate to the thymus and complete their maturation (82). During their maturation in the thymus T cells undergo a process of random recombination events to generate a wide variety of T cell receptors (TCRs) that are able to recognize both foreign and self-antigens. Generation of self-reactive T cells increases the risk of autoimmunity, and therefore a series of immune tolerance mechanisms have evolved to ensure that such self-reactive T cells are purged from the T-cell repertoire to maintain immune homeostasis (83).

1.4.1.1 Central tolerance

The mechanism of central tolerance is a crucial step during the maturation of T cells in the thymus, which prevents the development of potentially self-reactive T cells. T-cell progenitors express TCRs on their surface and interact with peptide-major histocompatibility complex (MHC) presented by various professional antigen-presenting cells (APCs)(84). The transcription factor autoimmune regulator (AIRE) influences central tolerance by driving the expression of an array of self-antigens in the medullary thymic epithelial cells (mTECs)(85). The affinity of a TCR for a self-peptide-MHC complex determines the fate of the thymocyte (86),(87). Thymocytes expressing TCRs that are unable to bind a self-peptide MHC complex are eliminated due to neglect. Naïve CD4- CD8- T cell precursors expressing TCRs with a low to intermediate affinity for a self-peptide presented by thymic APCs on MHC class II or MHC class I molecules further differentiate into CD4+CD8+ double positive and single positive CD4+ or CD8+ T cells, this process is termed positive-selection. Developing T cells that bind to self-peptide MHC complexes presented on mTECs and dendritic cells (DCs) with high affinity may cause autoimmunity. Therefore, these potentially self-reactive T cells are either eliminated by the process of clonal-deletion also termed negative selection or are converted to regulatory T cells (Tregs) that suppress immune responses to self through numerous mechanisms (88), (89),(90). Approximately 1-5% of thymocytes that have survived positive and negative selection processes emerge as functionally immature CD4+ and CD8+ T cells into the peripheral tissues where they transition to mature lymphocytes (91).

1.4.1.2 Peripheral tolerance

Even though central tolerance is a highly efficient process, some T cells with a self-reactive potential evade negative selection, as not all tissue-restricted self-antigens are expressed in the thymus. These self-reactive cells emigrate to peripheral tissues. Such T cells are kept in check

by mechanisms of peripheral tolerance (92). Peripheral tolerance is also necessary for control of T cells that encounter their cognate antigens only in the periphery, for example dietary and developmental antigens (83). T cells become activated in the periphery upon receiving a TCR signal along with a co-stimulatory signal provided by ligation of CD28 to its receptor on the APC. Peripheral tolerance mechanisms are induced when self-reactive T cells encounter the peptide MHC complex on APCs under conditions that do not elicit an adequate immune response. T cell activation in the absence of co-stimulation leads to a state of hyporesponsiveness and the T cells are rendered anergic (93),(94). Repeated stimulation of T cells can also lead to activation induced cell death via Fas and Fas-ligand mediated apoptosis (95). T regulatory cells (Tregs) also suppress the expansion of autoreactive T cells in the periphery by secreting immunosuppressive cytokines such as TGF-β and IL-10 (96). In addition, multiple peripheral cell types including Aire expressing lymph node stromal cells (LNSCs), lymph node endothelial cells and fibroblastic reticular cells also interact with autoreactive T cells and may play an important role in peripheral tolerance of the cells that have escaped central deletion (97),(98),(99),(100).

1.4.2 Impaired self-tolerance to islet antigens in T1D

Long-term studies in cohorts of individuals at risk of T1D have established that anti-islet autoimmunity can be triggered during childhood (45),(101) suggesting that loss of self-tolerance to islet antigens can occur early in life. The observation that self-reactive T cells specific for islet antigens are detectable not only in subjects with T1D but also often in healthy individuals suggests that thymic tolerance to islet antigens is incomplete (102),(103). Genetic mechanisms such as allelic variation, alternative splicing or epigenetic regulation (104),(105) in addition to antigen presentation by high-risk HLA can also influence the expression of self-antigens for establishment of thymic tolerance. In humans, expression of insulin in the thymic medulla is influenced by polymorphisms in regulatory elements of the insulin gene (14),(15). Alternative splicing of islet-antigens such as IA-2 and Islet specific glucose-6-phosphatase related protein

(IGRP) results in a mismatched expression pattern in thymus and pancreas (106),(107),(108). Such alterations in the display of islet-antigens lead to sub-optimal tolerance and allow for a T cell repertoire enriched for islet-reactive T cells to mature (109),(110).

1.5 The role of T cells in the pathogenesis of T1D

The role of T lymphocytes in autoimmune destruction of beta cells in NOD mice and humans has been demonstrated in a number of ways. Spleen cells or purified populations of CD4+ and CD8+ T cells adoptively transferred diabetes into athymic nude mice (111),(112) immunocompromised NOD.Scid mice (113) or NOD mice (114). Targeted depletion of CD4+ T cells (115),(116),(117),(118) and CD8+ T cells (119),(120) in NOD mice prevented diabetes onset suggesting that both CD4+ and CD8+ T cells are required for diabetes development.

Histological examination of pancreatic tissue from post-mortem specimens of patients with T1D has documented the presence of CD4+ and CD8+ T cells within the islets (121). Furthermore, both CD4+ and CD8+ T cells recognizing beta cell antigens have been detected in the peripheral blood of T1D patients. While these beta cell-specific T cells are also detected in healthy subjects at comparable frequencies, a key difference is that the autoreactive T cells in healthy subjects display a naïve phenotype whereas those found in T1D patients are memory T cells showing hallmarks of antigen exposure (102),(103),(122),(123). Using elegant approaches two recent studies have also demonstrated that CD4+ and CD8+ T cells found in the islets of T1D patients recognize islet antigens (124),(125). These findings implicate T cells in the pathogenesis of T1D and suggest that a breakdown in peripheral tolerance mechanisms leads to activation, differentiation and expansion of naïve circulating islet reactive T cells into highly pathogenic effector T cells ultimately resulting in beta cell destruction.

1.5.1 Priming of islet reactive T cells

Following their exit from the thymus naïve CD4+ and CD8+ T cells migrate to peripheral lymph nodes, where they encounter their cognate antigens. Activation of islet-specific T cells occurs in the draining pancreatic lymph node (PLN). T cells responding to islet antigens are detected in the PLN prior to onset of insulitis (126). Furthermore, removal of the PLN at 3 weeks of age in NOD mice prevented diabetes onset, whereas removal of the spleen had no impact on diabetes development (54).

1.5.2 Islet-resident APCs present beta cell antigens to T cells

Islet-resident APCs play a crucial role in presenting beta cell antigens to T cells in PLN (127). DCs loaded with islet antigens can be visualized within the PLN (128) and more recently it has been shown that beta cells transfer the contents of their secretory granules to islet-resident phagocytes for presentation to T cells (129). Typical islet-resident APCs capture extracellular antigens and process them for presentation on MHC class II to CD4+ T cells, however a subset of DCs (Cd11c+ CD8+ and CD11c+ CD11b+/- CD103+) can also acquire exogenous antigens and process them via MHC class I pathway and present the antigens to CD8+ T cells. This process termed “cross-presentation” is important in cross priming of CD8+ T cells for development of effector CD8+ T cell responses (130),(131),(132). Development of pathogenic CD8+ T cell response to islet antigen is also determined by provision of CD4+ T cell help and modification of the APC (133, 134). Complete protection from insulitis and diabetes in NOD mice lacking cross-presenting CD103+ Batf-3-dependent DCs, but having a fully competent repertoire of islet reactive T cells, further highlights the role of antigen presentation in the PLN by DCs in initiation of autoimmunity in NOD mice (135).

1.5.3 Islet infiltration by immune cells (Insulitis)

The term “insulitis” was coined by a Swiss pathologist von Meyenburg in 1940, and is defined as lymphocytic infiltration limited to the islets of Langerhans (136).

1.5.3.1 Insulitis in the NOD mouse

T cells that are activated in the PLN but not naïve T cells gain entry into the islets to initiate insulitis (137). Retention of T cells in the insulitic lesion is dictated by antigen-specificity, as it has been demonstrated that T cells that accumulate within the islet lesion are predominantly antigen specific (138),(139). The onset of insulitis amplifies the immune response by inducing expression of chemokines and adhesion molecules in the islet, making it more receptive to further infiltration of immune cells including bystander and naïve T cells (140),(141),(142).

Insulitis in NOD mice develops progressively with age. Immune infiltrate comprising of myleloid cells that include dendritic cells (DCs), macrophages and neutrophils is visible as early as 3 weeks of age (55),(57),(143). A small number of CD4+T cells are detected in early infiltrates that co localize with CD11c+ APCs in approximately 10% of the islets by 4 weeks of age (144),(145),(146). Between 8-12 weeks of age 50-60 % of islets in the pancreas show infiltration by all major inflammatory cell subsets such as CD4+, CD8+ T cells, CD11c+ DCs and B cells; however there is no significant reduction in the beta cell mass during this phase suggesting that the insulitis is predominantly non-destructive (147). The overall number of islet infiltrating immune cells increases with age and by 18 weeks of age all islets are affected by infiltrates. The total beta cell mass gradually decreases leading to onset of hyperglycemia, which is diagnosed by abnormally high urine and blood glucose measurements (148).

1.5.3.2 Insulitis in human islets

Histopathological analyses from approximately 150 pancreata obtained from diabetic patients have demonstrated that insulitis is a feature of T1D development in humans (149),(150),(151).

Immunophenotyping of the insulitic lesion in T1D patients has documented the presence of T cells, B cells and macrophages in the islet infiltrate (152). CD8+ T cells are the predominant population, whereas CD4+ T cells are less in number as compared to both CD8+ T cells and macrophages(121),(153),(154). Qualitatively, insulitis in humans is similar to NOD mice with a similar composition of immune cell types, however there are several major differences that are characterized by a mild nature of islet infiltration as compared to the florid insulitis observed in NOD mice. While almost all islets in an 18 weeks old female NOD mouse display invasive insulitis, histopathological examination of pancreata from autoantibody positive non-diabetic subjects revealed islet infiltration in less than 10 % of the islets screened (155). Moreover, a peculiar feature of T1D pathology in humans is the lobular nature of beta cell destruction, with insulitis detectable in some pancreatic lobes but not others (156). Detection of infiltrates in pancreata of long-standing diabetic subjects suggests that insulitis is an important feature of human T1D (157). Despite quantitative differences in the immune cells infiltrating the islets in humans and NOD mice, T cells dominate insulitis in both humans and NOD mice.

1.5.4 CD4+ T cells

CD4+ T cells play a crucial role in mediating adaptive immunity to pathogens, as well anti-tumor, allergic and autoimmune responses. Upon stimulation with their cognate antigen presented on MHC class II molecules on the surface of APCs naïve CD4+ T cells can differentiate into various specialized T helper cell (Th) subsets that include Th1, Th2, T regulatory (Treg) and Th17 cells. Each subset is characterized by production of distinct cytokines and effector functions (158),(159). Various CD4+ T helper cells orchestrate a wide range of immune responses by providing help to B cells to produce antibodies, enhancing the CD8+ T cell response and by regulating/suppressing undesirable autoimmunity (160),(161),(162).

1.5.4.1 CD4+ T effector cells in T1D

Strong genetic association of MHC class II region genes with T1D in humans implicates CD4+ T cells in pathology of the disease. Until recently it was unclear if CD4+ T cells interacted with beta cells to mediate beta cell destruction. Recent results from our group and others have shown that beta cells isolated from infiltrated islets express MHC class II, and exposure to IFN-γ increases MHC class II expression on beta cells, suggesting an important role for MHC class II molecules in beta cell interaction with CD4+ T cells (163),(164). Proinflammatory cytokines such as IFN-γ, TNF-α and interleukin-1 (IL-1) have been suggested as main mediators of beta cell destruction by CD4+ T cells (165). While cytokines are able to kill beta cells in vitro, studies indicate that cytokines are not directly cytotoxic to beta cells in vivo (166),(167).

T1D was considered to be a Th1 mediated disease as increased levels of IFN-γ and lower levels of IL-4 correlated with the disease both in NOD mice and humans (168),(169). However, the Th1 bias in T1D pathology was questioned because NOD mice lacking IFN-γ and its receptor developed diabetes similar to wild-type NOD mice (170),(171).

Treg cells produce the immunomodulatory cytokines IL-10 and TGF-β and play a vital role in maintaining immune homeostasis by suppressing aberrant autoimmunity in mice and humans(172),(173). Brusko et.al recently reported that T1D patients had similar frequency of Tregs as controls (174), however the suppressive capacity of Tregs in patients is impaired which may contribute to development of anti-islet autoimmunity (175),(176).

The emergence of interleukin-17 (IL-17) producing Th17 cells led to revision of Th1/Th2 paradigm and raised the possibility that Th17 cells may drive organ specific autoimmunity rather than Th1 cells (177),(178). Studies in humans point towards an increase in IL-17 production in T1D setting (179),(180) however, NOD mice lacking IL-17 were not protected from diabetes development (181) thus role of Th17 cells in T1D remains unclear at present. In summary,

recognition of self-antigens presented by MHC class II is a crucial step in the initiation of islet inflammation, which leads to a dynamic immune response by CD4+ T effector cells in T1D.

1.5.5 CD8+T cells

CD8+ T cells mediate adaptive immune responses directed against infectious agents and tumors. Naïve CD8+ T cells encounter their cognate antigenic peptide coupled with MHC class I along with appropriate co-stimulation delivered by the APCs. This priming event leads to clonal expansion and differentiation naïve CD8+ T cells into short-lived T effector cells and long-lived memory T cells (182). Cytotoxic effector cells release proinflammatory cytokines such as IFNγ, TNF-α and cytolytic granules perforins and granzymes to mediate rapid clearance of infected target cells (183),(184),(185). Memory T cells reside in peripheral tissues and mediate effector responses by rapid expansion upon antigen re-encounter (186),(187).

Incremental evidence suggests that autoreactive CD8+ T cells paly a pivotal role in destruction of beta cells, as they constitute a significant proportion of insulitic lesion in human pancreas (121),(153). Upregulation of MHC class I expression has been reported in the islets that have been infiltrated with immune cells, suggesting increased antigen presentation to CD8+ T cells (152),(188). Recently, using in situ tetramer staining, direct evidence for involvement of CD8+ T cells in beta cell killing was provided by detection of CD8+T cells specific for various islet antigens in pancreatic sections of T1D organ donors (124).

Studies in NOD mice have clearly demonstrated the absolute requirement for a direct interaction between MHC class I and CTL to mediate beta cell destruction. NOD mice deficient in β2-microglobulin (NODβ2mnull mice) fail to express MHC class I and lack CD8+T cells and are protected from diabetes (189),(190),(191). Restoration of MHC class I expression on beta cells (RIP-β2m mice) led to diabetes development upon transfer of diabetogenic T cells (192). Ablation of MHC class I specifically on beta cells in NOD mice resulted in insulitis and a reduced

incidence of diabetes, highlighting the requirement for CTL–beta cell interaction for beta cell cytotoxicity, but not for islet infiltration (193).

1.5.6 Effector mechanisms used by CTLs in beta cell destruction

Activated CTLs mainly utilize granule exocytosis pathway incorporating the release of perforin and granzymes for rapid killing of their targets (194),(195). The engagement of Fas death receptor (CD95) by its ligand FasL is another mechanism used by CTLs to induce apoptosis of their targets (196). However, blocking Fas signaling on beta cell does not protect them from CD8+ T cell mediated killing and only partially reduces spontaneous diabetes onset (197). Collectively these results suggest a minor role for Fas-FasL pathway in beta cell death. NOD mice lacking perforin have a delayed onset and significantly reduced diabetes incidence indicating that granule-mediated cytolysis is a dominant mechanism used by CTLs to cause beta cell destruction (198),(199),(200). Investigation of effector mechanisms utilized by CTLs to destroy human islets has also demonstrated a dominant role for perforin and a minor role for Fas in mediating beta cell death (201),(202).

1.5.7 Islet antigen specific CD4+ and CD8+ T cells

Several diabetogenic CD4+ and CD8+ T cell clones from the early insulitic lesion in NOD mice have been isolated and characterized (203). Since the majority of T-cell clones described from the early islet infiltrates were CD4+ T cells, they were thought to be the initiators of the disease (204). However, reports of CD8+ T cells that are pathogenic in absence of CD4+ T cells indicate that both CD4+ and CD8 + T cells have an important role in initiation and progression of T1D (205). Study of islet reactive T cell clones has led to identification of their cognate antigens and has provided new tools such as TCR transgenic mice to further dissect the role of T cells in T1D (206),(207),(208). Nomenclature, antigen-specificity and diabetogenic potential of the individual

CD4+ and CD8+ T cells characterized in NOD mice is listed in Table 1. Specific antigens recognized by T cells will be discussed below.

Table 1: Islet antigen specific CD4+ and CD8+ T cell clones in NOD mice

Autoantigen	Epitope	Clone	CD4+/CD8+	Diabetogenic?	Ref
Insulin	B:9-23	BDC12.4.1	CD4+	YES	(203)
	B:9-23	BDC12.4.4	CD4+	YES	(203)
	B:9-23	2H6	CD4+	NO	(209)
	B:12-25	2H6	CD4+	NO	(209)
	A:14-20	A14	CD8+	YES	(210)
	B:15-23	G9C8	CD8+	YES (with CD4+ T cell help)	(269),(271)
IGRP	206-214	NY8.3	CD8+	YES	(253)
Chromogranin A	359-372	BDC2.5	CD4+	YES	(249)
	359-372	BDC10.1	CD4+	YES	(249)
GAD65	286-300	B16.3	CD4+	NO	(211)
Hsp60	437-460	C9	CD4+	YES (if activated)	(212)
ZnT8	345-359	10.8	CD4+	YES (if recipient irradiated)	(213)

1.5.8 Role of B cells in T1D

In the pathogenesis of T1D, conventional view is that T cells mediate autoimmune destruction of the beta cells, whereas B cells secrete islet autoantibodies as markers of the ongoing disease (214). However, the observation that NOD mice lacking B cells, but with normal T cell numbers fail to develop diabetes (215), suggests that B cells also play an important role in T1D development. Furthermore, NOD mice with a majority of B cells expressing anti-insulin B cell receptors (BCR) develop accelerated diabetes, suggesting that autoreactive B cells likely propagate immune responses that cause beta cell loss (216). It is likely that B cells indirectly participate in beta cell destruction by presenting antigens to islet reactive CD4+ T cells as NOD mice with B cells lacking MHC class II (I-Ag7) had a reduced incidence of spontaneous diabetes (217),(218). Data from NOD mice suggest that islet autoantibodies produced by B cells are non-pathogenic and are unable to passively transfer disease on their own (219). Moreover, NOD mice with B cells engineered to be defective in antibody production developed insulitis and diabetes, indicating that the ability to produce autoantibodies is not required by B cells to mediate anti-islet autoimmunity (220). Roep and colleagues reported a case study of T1D development in a B cell deficient subject, thus challenging the role of B cells and autoantibodies in T1D (221). However, anti-B cell therapy in animal models reversed diabetes (222),(223) and B cell depletion using rituximab demonstrated beneficial outcomes, such as a delay in the decrease of C-peptide levels in patients with recent onset T1D (224). These data support the idea that B cells may play a role in T1D development.

1.5.9 Insulin Autoantibodies (IAA) predict onset of T1D.

Autoimmunity to beta cells during the asymptomatic phase prior to clinical disease is characterized by the emergence of autoantibodies against several islet antigens which include insulin (225), glutamic acid decarboxylase (GAD) (226), ICA-69 (227), IA-2 (Phogrin) (228), insulinoma antigen-2 (ICA512) (229), heat shock protein 60 (230), zinc-transporter 8 (ZnT8) (231) and tetraspanin-7 (232). Autoantibodies to insulin, GAD, ICA-512,(IA-2) and ZnT8 are well defined in human disease and are routinely measured in the clinic as they have a strong predictive value (233). Individuals that express 2 or more of these antibodies almost always progress to diabetes (45). The onset of anti-islet autoantibodies typically occurs between 9-24 months of age in children with genetic-risk of developing T1D. It has been reported that insulin autoantibodies (IAA) are usually the first to appear in young children, and the children who progress to diabetes also express antibodies to GAD either simultaneously or soon after the IAA response (6, 45, 234).

NOD mice develop IAA in a heterogenous manner with IAA being detectable as early as 4 weeks of age, reaching their peak by 8 weeks and declining with age. IAA expression at 8 weeks of age strongly correlates with early onset of diabetes by 16-18 weeks of age in NOD mice (235). Detection of autoantibodies to other antigens in NOD mice has not been successful as yet.

1.6 Autoantigens in T1D

Many antigenic targets in T1D patients have been identified by the presence of serum autoantibodies. Several other antigens have been identified by biochemical and molecular approaches and the list is ever expanding (50). The autoimmune response targeting insulin-producing beta cells is highly specific, as the other cell types producing glucagon, somatostatin or pancreatic polypeptide remain intact. Genetic approaches in NOD mice, that include gene-

knockouts of beta cell antigens, transgenic expression of an antigen to induce recessive tolerance and generation of transgenic or retrogenic mice with a majority of T cells specific for a single antigen, have been undertaken to dissect the relative importance of various islet antigens in the pathogenesis of T1D.

1.6.1 Insulin is a major autoantigen in the pathology of T1D

Insulin is synthesized as preproinsulin. After the cleavage of the signal peptide, proinsulin is packaged into secretory granules for export from the pancreas and is further cleaved into insulin and C-peptide (236). NOD mice have two insulin genes Ins1 and Ins2 that code for two distinct proteins (237). While Ins1 is predominantly expressed in the islets, Ins2 expression is detected in the thymus (238), and the thymic expression depends on the number of copies of the Ins2 gene (239). Evidence from studies involving manipulation of insulin gene expression underpins its importance in the NOD model of T1D.

1.6.1.1 Genetic ablation of insulin genes

The two insulin genes in mice were individually knocked out to generate Ins-1 (-/-) and Ins-2 (-/-) mice and these were backcrossed on to the NOD background. NOD mice lacking Ins-1 were completely protected from diabetes (240). The protection is most likely due to the removal of cognate insulin peptide in the target organ, thus rendering the beta cells unable to be recognized by T cells. In contrast Ins-2 (-/-) NOD mice showed accelerated progression to diabetes (241). This contrasting phenotype is attributed to a loss of central tolerance caused by the lack of thymic insulin expression. Further highlighting the importance of thymic insulin expression in mediating tolerance to autoreactive T cells, a recent report showed that thymus specific deletion of Ins 2 gene in AIRE expressing mTECs, led to accelerated diabetes development even on a non-autoimmune C57BL/6 genetic background (242).

1.6.1.2 Transgenic expression of insulin in APCs

In a complementary approach, transgenic expression of proinsulin 2 in APCs driven by a MHC class II promoter resulted in complete absence of insulitis and prevented diabetes development in the transgenic NOD mice (NOD-PI mice) (243). The protection from diabetes was hypothesized to be due to removal of pathogenic insulin-reactive T cells. Although T cells from NOD-PI mice were responsive to proinsulin peptide immunization, NOD-PI splenocytes were unable to adoptively transfer diabetes, indicating absence of pathogenic potential. Dominant tolerance due to antigen-specific Tregs was ruled out, as NOD-PI splenocytes failed to suppress diabetes transfer to an immunodeficient NOD recipient when co-injected with spleen cells from diabetic NOD mice. Another study using a similar approach of expressing proinsulin in APCs under the control of the invariant chain promoter observed mild insulitis and a marked reduction in the diabetes incidence in the transgenic mice (244). In contrast to the previous study by French et. al, T cells from the transgenic mice in this study did not respond to proinsulin peptide immunization, indicating a defect in T cell responsiveness.

1.6.2 Chromogranin is another pathogenic antigen in NOD mice

A series of CD4+ T cell clones called the BDC cells were identified from spleens and pancreatic lymph nodes of NOD mice (245). These cells responded to islets and cell extracts from beta cell adenomas, but not insulin (246). Chromogranin A (ChgA), a molecule present in the secretory granules of the beta cells was recently identified as the antigenic target of the highly pathogenic BDC2.5 CD4+T cell clone (247-249). CD4+ T cells specific for ChgA weakly respond to WE-14 a naturally occurring proteolytic cleavage product of ChgA. It was recently reported that post-translational modification of WE-14 peptide greatly enhanced its antigenicity (250). T cell responses to an antigenic epitope of ChgA have also been detected in T1D patients (251). NOD mice lacking ChgA are protected from diabetes development (252), indicating that ChgA is essential for diabetes development in NOD mice. The impact of ChgA expression in APCs to

induce T cell tolerance remains to be evaluated. The observation that NOD mice lacking Ins1 do not develop diabetes despite the presence of ChgA specific T cells, suggests that T cells reactive to ChgA might not be the primary mediators of the disease.

1.6.3 IGRP is a downstream pathogenic antigen in NOD mice

A substantial body of evidence suggests that IGRP is also an important antigen targeted in NOD mice and humans. Pathogenicity of IGRP specific T cells in NOD mice has been demonstrated by studies of a CD8+ T cell clone (NY8.3) specific for the $IGRP_{206-214}$ epitope (253),(206). These cells are present in the earliest islet infiltrates of NOD mice (254). NOD mice transgenic for the 8.3 TCR develop diabetes at an early age (between 40 -90 days of age) (206). Moreover using $IGRP_{206-214}$ peptide-MHC class I (H-2-Kd) tetramer complexes specific for 8.3 T cells it has been demonstrated that up to 1% of CD8+ T cells in the peripheral blood and 30% of islet-associated CD8+ T cells are IGRP specific. Quantification of IGRP specific T cells in peripheral blood is predictive of diabetes onset in NOD mice (255). Moreover, the number of IGRP reactive CD8+ T cells in the periphery of NOD mice increases with age and correlates with insulitis progression (256).

Overexpression of IGRP in APCs failed to protect NOD mice from diabetes development. Moreover, IGRP reactive T cells that are predictive of disease onset were not detected in NOD mice tolerant to proinsulin, indicating that the autoreactivity to IGRP was dependent on the immune response to insulin (257). Furthermore, NOD 8.3 mice showed dramatic reduction in diabetes development when crossed with proinsulin tolerant NOD-PI mice, indicating that immune responses to proinsulin are a pre-requisite for diabetes development in the majority of NOD8.3 mice with a pre-existing repertoire of IGRP specific T cells(258). Thus IGRP is likely to be a downstream antigen to insulin.

1.6.4 Other autoantigens

Detection of autoantibodies against antigens such as GAD, IA-2 and IA-2b in patients with T1D was followed by studies examining the role of these antigens in NOD mice. Although T cell reactivity to GAD has been reported in NOD mice (259),(260),(261), overexpression of GAD in APCs (262), or GAD deficiency (263) failed to alter diabetes incidence in NOD mice. Removal of IA-2 and IA-2b also did not prevent diabetes in NOD mice (264, 265). Therefore, autoreactivity to these antigens is not essential for diabetes development in NOD mice.

1.6.5 Insulin epitopes in T1D

Understanding how diabetogenic T cells recognize self-antigen epitopes is essential to gain insight into disease development. Despite a broad spectrum of autoreactivity reported in NOD mice, the response to the insulin $_{B:9-23}$ epitope is dominant and essential for diabetes initiation. Approximately, half of the several CD4+ T cell clones that have been established from pancreatic islets, lymph nodes and spleen of NOD mice react to insulin and in particular to the B:9-23 region of insulin (266-268). CD8+ T cells reactive to insulin B chain peptide B:15-23 have also been identified in the early islet infiltrates in NOD mice (269). TCR transgenic mice with CD4+ T cells specific for insulin $_{B:9-23}$ epitope develop spontaneous diabetes (270) whereas TCR transgenic mice with CD8+ T cells recognizing the insulin $_{B:15-23}$ epitope develop diabetes upon immunization and activation of the CD8+ T cells (271)

Genetic evidence that recognition of the insulin $_{B:9-23}$ epitope by autoreactive T cells is central to diabetes development in NOD mice comes from the study by Nakayama and colleagues in which NOD mice lacking both proinsulin 1 and 2, but expressing a hormonally active but immunologically inert insulin transgene with a mutation disabling the T cell epitope (Insulin $_{B:9-23}$), were free from diabetes and insulitis (272). Introduction of the native insulin $_{B:9-23}$ sequence

in insulin-knockout mice via peptide immunization or islet transplantation reversed the protection conferred by the mutated insulin molecule (273) suggesting that immune responses to insulin $_{B:9-23}$ epitope are crucial to pathogenesis of T1D in NOD mice.

Analysis of circulating T cells from T1D patients has identified CD4+ T cells specific for insulin $_{B:9-23}$ peptide (274). In addition CD4+ T cells specific for insulin $_{A:1-15}$ have been derived from pancreatic lymph nodes (275) and peripheral blood (276) of individuals with T1D. A pathogenic CD8+T clone recognizing a preproinsulin signal peptide (277), as well as other CD8+ T cells targeting insulin $_{B:10-18}$, insulin $_{B:18-27}$ and insulin $_{A:12-20}$ epitopes (278), (279) have been identified from human subjects. Moreover in a recent finding it was reported that more than 25% of CD4+T cells derived from the islets of an individual with T1D recognized 6 distinct but overlapping epitopes in the C-peptide of proinsulin (125). T cell responses to other autoantigens such as GAD, IGRP and IA-2 have also been described in T1D patients (280).

In summary, insulin autoreactivity is dominant in NOD diabetes and perhaps humans. In particular, the immunological recognition of insulin $_{B:9-23}$ epitope by CD4+ T cells is essential for diabetes initiation in NOD mice.

1.6.6 Presentation of insulin epitopes on MHC II I-Ag7

A major focus of research studies recently has been to unravel how the immuno-dominant insulin $_{B:9-23}$ epitope binds to the diabetes associated MHC class II I-Ag7 molecule and evades tolerance mechanisms leading to a pathogenic autoimmune response. Binding of a peptide to MHC class II is facilitated by the interaction of side-chains of “anchor” amino acids at positions P1, P4, P6 and P9 with four corresponding pockets in the MHC class II peptide-binding groove (281). Polymorphic amino acid residues lining the peptide-binding groove influence the

preference of each binding pocket for a particular peptide side chain and therefore dictate the position or “register” in which the peptide is bound and recognized by the corresponding T cells.

It was previously demonstrated that the insulin B chain peptide $_{B:9-23}$ filled the peptide-binding groove of MHC II in two adjacent registers with a core nonamer (9mer) of insulin $_{B:12-20}$ peptide binding in “register 1” and insulin $_{B:13-21}$ peptide binding in “register 2”. Both registers were recognized by a distinct set of insulin reactive T cells (282). A recent study showed that a diverse set of pathogenic insulin reactive CD4+ T cells were able to recognize an insulin $_{B\ 9-23}$ epitope, only when it was bound to the I-Ag7 molecule in an unfavourable register termed “register 3”. The minimal insulin $_{B:12-23}$ peptide bound to the I-Ag7 groove poorly, unless the peptide anchors were optimized for proper binding (283). It was concluded that the target of T cells previously thought to recognize insulin $_{B:12-23}$ in “register 1” or “register 2 “ was actually insulin $_{B:12-23}$ bound in “register 3”. A subsequent study by Crawford et.al showed that a series of insulin $_{B\ 9-23}$/IAg7 pMHC tetramers that displayed the optimized insulin $_{B:\ 9-23}$ epitope bound to I-Ag7 in “register 3” were able to stain a majority of insulin reactive CD4+ T cells, lending further support to the earlier findings (284).

These studies suggest that poor binding of the insulin $_{B\ 9-23}$ peptide to I-Ag7 may hinder optimal presentation of the antigenic epitopes for thymic deletion, allowing escape of insulin reactive T cells to the periphery. Polymorphisms that influence the MHC class II-peptide interaction in NOD mice are also present in human HLA-DQ8 alleles and therefore may play a similar role in human T1D susceptibility (285),(52).

1.6.7 Conventional and nonconventional CD4+ T cells reactive to insulin

The suggestion that poor presentation of insulin peptides bound to MHC II in an unfavourable register allows the escape of insulin reactive T cells from the thymus, raised the question as to how the T cells overcame this weak interaction in the periphery to become activated and cause disease in the target organ? A previous study analyzing T cell-specific tolerance to hen egg lysozyme (HEL) identified a set of T cells that responded to immunization with HEL peptide but not the protein in HEL transgenic mice (286). It is possible that some self-reactive T cells can become pathogenic in the periphery where increased concentration of the cognate antigen in the target organ or a differential processing of the antigen leads to presentation of antigenic peptides in an alternate register to that found in the thymus. Unanue and co-workers presented evidence supporting this possibility in NOD mice, where they identified two types of insulin reactive CD4+ T cells. “Type A” cells were able to respond to intact insulin protein and insulin $_{B:9-23}$ peptide, whereas “type B” cells only responded to insulin $_{B:9-23}$ peptide and formed the majority of pathogenic insulin reactive CD4+ T cells in NOD mice (282),(287). Subsequent reports suggest that “type A” CD4+ T cells represent the T cells that recognize the insulin B:13-21/I-Ag7 complexes and are deleted in the thymus, whereas “type B” CD4+ T cells bypass thymic selection and accumulate in the pancreatic islets. High concentration of insulin and insulin peptides in secretory granules of pancreatic islets facilitates the formation of abundant insulin B:12-20/I-Ag7 complexes that are non-conventionally loaded at the islet APC cell surface leading to activation of pathogenic type B CD4+ T cells(288, 289).

1.6.8 Generation of hybrid antigenic epitopes

Chromogranin A (ChgA) has been recently identified as an islet autoantigen. WE14, a naturally occurring peptide cleavage product of ChgA has been shown to be antigenic both in NOD mice and T1D patients (249),(251). Biochemical analyses of WE14 binding to I-Ag7 revealed that a

tissue specific modification resulting in amino terminal truncation and carboxy terminal extension was required for I-Ag7 binding and recognition of this ChgA epitope by BDC 2.5 T cells (290). Addition of a N-terminal amino acid extension to the WXRM(D/E) epitope of the truncated WE14 peptide greatly enhanced the T cell stimulation and activated a range of CD4+ T cells. It was suggested that post-translational modification of islet antigens could uniquely happen in the pancreas or draining lymph nodes via the process of trans-peptidation accounting for escape of T cells from thymic deletion (291). In an interesting finding, it has been recently shown that islet-infiltrating cells from NOD mice are able to recognize hybrid epitopes generated by covalent linking of pro-insulin derived peptides to ChgA derived peptides as well as other peptides present in the beta cell secretory granules. Importantly, pathogenic CD4+T cells that infiltrate human pancreatic islets were also able to recognize such hybrid proinsulin peptides suggesting an important role for hybrid epitopes in human T1D (292).

In summary, unconventional interaction of self-peptides with diabetes susceptible MHC II molecules bypasses central tolerance to promote generation of pathogenic CD4+ T cells leading to anti-islet autoimmunity.

1.7 Immunotherapies in Type 1 Diabetes

A major goal of preventive therapies is to re-establish immune tolerance in those at risk of developing T1D and to slow the progression to overt T1D, whereas interventional approaches in patients with established T1D are aimed at replacing, regenerating or preserving residual beta cell mass to maintain endogenous insulin secretion. Immunotherapeutic strategies to prevent or halt autoimmune diabetes have involved both antigen-specific and non-antigen specific approaches. Tables 2 and 3 summarize major T1D prevention and intervention trials.

1.8 Non-antigen-specific preventive trials

1.8.1 Primary prevention trials

Primary prevention trials aim to halt the development of autoimmunity in subjects carrying high-risk HLA genes before any evidence of immune activation directed against islet antigens. Safety of the therapy is a major criterion for any type of primary prevention as only a small fraction of those at-risk are expected to develop diabetes and therefore such trials have been limited to non-immune interventions such as dietary modifications. A multinational Trial to Reduce Incidence of Diabetes in Genetically at Risk (TRIGR study) tested the effect of prolonged breastfeeding, use of hydrolyzed casein and delayed introduction of solid food and reported no delay in rate of clinical disease onset, similar results were also reported from the BABYDIET study examining gluten free diet in the first year of life (293),(28). The Finnish Dietary Intervention Trial for Prevention of Type 1 Diabetes (FINDIA) examined the effect of insulin-free bovine formula and reported a delay in islet autoantibody development in the first three years of life (294). The Nutritional Intervention to Prevent Type 1 Diabetes (NIP) study assessed the anti-inflammatory effects of dietary omega-3 fatty acid supplementation by administration of

docosahexaenoic acid (DHA) to high-risk subjects, and reported no efficacy of the treatment (295).

Table 2: Primary and Secondary prevention trials in T1D

Therapy	Study	Intervention	Outcome	Reference
Non-antigen specific				
Dietary	**TRIGR**	Hydrolyzed casein	No prevention	(293)
	BABYDIET	Delayed gluten exposure	No prevention	(28)
	FINIDIA	Insulin-free formula	Delayed Auto antibody in 1st 3 yrs	(294)
	NIP	(DHA) Docosa-hexaenoic acid	No reduction in Inflammation	(295)
	ENDIT	Nicotinamide (vitamin B3)	No prevention	(300)
Immunomodulatory	**Teplizumab**	Teplizumab (anti-CD3)	Ongoing	
	Abatacept	Abatacept (CTLA4-Ig)	Ongoing	
Antigen specific				
Insulin	**DPT-1 (systemic)**	Parenteral insulin	No prevention	(359)
	DPT-1 (oral)	Oral insulin	Partial benefit in subgroup with high IAA	(350)(351)
	DIPP	Intranasal insulin	No prevention	(357)
	INIT-II	Intranasal insulin	Ongoing	
	Pre-POINT	Oral insulin	Safe therapy, induced regulatory responses	(355)
GAD	**DIAPREVIT**	Alum-GAD(Diamyd ®)	Ongoing	

Table 3: Intervention trials in T1D

Therapy	Intervention	Outcome	Reference
Non-antigen specific			
Immunosupression	**Cyclosporine**	Remission during Treatment, Adverse effects	(306-309)
	Mycophenolate mofetil + anti-CD25	No benefit	(296)
B- Cell depletion	**Anti-CD20 (Rituximab)**	Transient C-peptide preservation 3-6 months	(224)
T- cell depletion	**hOKT3**	Remission till 24 months	(323)
	Teplizumab	Remission till 24 months	(324)
	Otelixizumab	Ongoing	
	Anti-Thymocyte globulin (ATG)	No effect on C-peptide decline	(313),(314)
Co-Stimulation blockade	**CTLA4-Ig (Abatacept)**	Slower C-peptide loss over 24 months	(327),(328)
IL-1 antagonists	**Anakinra**	No benefit	(334)
	Canakinumab	No benefit	(335)
TNF-α blockade	**Etanercept**	No adverse effects, Lower HbA1C and insulin needs	(339)
Antigen-Specific			
Insulin	**Ins B chain in IFA**	No benefit	(297)
	NBI-6024 altered peptide ligand	No benefit	(361)
	BHT-3021 Proinsulin vaccine	Slower C-peptide loss, reduction in proinsulin reactive CD8+ T cells	(362)
GAD-65	**GAD-65 Alum**	No reduction in C-peptide loss	(298)

1.8.2 Secondary prevention trials

Secondary prevention trials aim to delay or stop the progression to clinical disease in individuals expressing humoral and metabolic markers indicating established islet autoimmunity. It is speculated that such therapies earlier in the disease process may be more successful given greater β-cell mass and a less aggressive disease process. The majority of secondary prevention studies have focused on antigen-specific therapies mainly utilizing various preparations of insulin and these are described in detail below. The first non-antigen specific secondary prevention trial conducted was the European Nicotinamide Diabetes Intervention Trial (ENDIT). Nicotinamide has been shown to reduce beta cell inflammation and T1D development in animal models (299). Individuals who had a first-degree relative with T1D and one or more islet cell antibodies were randomized to receive either nicotinamide or placebo for 5 years. No difference was observed in the rate of T1D development in both groups (300). The German Nicotinamide Diabetes Intervention Study (DENIS) (301) also reported similar results. Several other non-antigen specific therapies that include Ketotifen (histamine antagonist) and Bacille Calmette-Guerin (BCG) have been trialed without any success in preventing T1D development (302),(303).

1.8.3 Tertiary prevention (intervention) trials

Tertiary prevention or intervention trials focus on preservation of residual beta cell mass and C-peptide secretion early after the onset of clinical disease. Preservation of C-peptide both, short and long term leads to less hypoglycemia and fewer complications. With the recognition that a persistent and targeted autoimmune response towards islet-antigens is central to pathogenesis of T1D, interventional approaches have mainly focused on suppressing the immune response to stop the ongoing beta cell destruction (304). Non-antigen-specific interventional strategies trialed so far can be broadly classified into 3 groups as described below.

1.8.3.1 Immunosuppressive agents

Cyclosporine (305) was the first immunosuppressive agent trialed to treat T1D. Cyclosporine treatment resulted in remission of T1D; however chronic drug treatment was required for maintenance of remission and was associated with renal toxicity (306),(307),(308),(309). Therefore, risks associated with chronic immunosuppressive therapies make them unsuitable for treatment of T1D patients.

Based on promising pre-clinical studies in NOD mice (310), anti thymocyte globulin (ATG) a lymphocyte-targeting immunosuppressant was administered to newly diagnosed T1D patients. Beneficial effects observed in pilot studies were accompanied by adverse effects such as serum sickness and thrombocytopenia (311),(312). Recently completed efficacy trials reported no preservation of beta cell function upon ATG treatment in T1D patients. Mechanistic analysis suggested that ATG treatment led to preservation of T cells with a memory phenotype and a decline in Treg cells, which may have contributed to failure of ATG therapy (313),(314). Thus generalized depletion of T cells may be an ineffective treatment for T1D.

1.8.3.2 Immunomodulatory therapies

To counter the risks associated with long-term immunosuppressive treatments, biological agents such as monoclonal antibodies and engineered fusion proteins that induce depletion of specific immune cells such as T cells (anti-CD3 antibodies) (315) and B cells (anti-CD20) (316) or block critical signaling pathways leading to T cell activation (CTLA4-Ig) (317) have been developed. A short-term low dose treatment of newly diabetic NOD mice with anti-CD3 antibody led to a selective depletion of activated T cells, preservation of Tregs cells and induced durable remission of established T1D (318),(319),(320) Similarly, treatment of NOD mice with anti-CD20 antibody led to depletion of B cells and prevented or reversed diabetes (222), whereas blockade of co-stimulatory signals by CTLA4-Ig treatment prevented diabetes in NOD mice when administered early in the course of

autoimmunity (321). Interventional studies that have targeted multiple facets of the immune response in recent onset T1D patients either with humanized anti-CD3 antibodies (322),(323),(324),(325),(326), CTLA4-Ig (abatacept) (327),(328) or anti-CD20 (Rituximab) (224) demonstrated transient benefit with preservation of C-peptide and improved metabolic parameters in the first 12 months post therapy. The rate of beta cell loss slowed in the initial 12-24 months post therapy, and showed a trend parallel to the placebo group thereafter.

1.8.3.3 Anti-inflammatory therapies

Inflammatory processes also play an important role in the pathology of islet destruction in T1D (329),(330). Blockade of cytokines such as interleukin 1 (IL-1) and TNF-α has been effective in treatment of other autoimmune disorders such as rheumatoid arthritis (331),(332),(333) and has also been tested in T1D. Treatment of new onset T1D patients with anakinra, a recombinant form of human IL-1 receptor antagonist (334) or canakinumab, a human anti-IL-1 beta monoclonal antibody failed to impact disease progression (335), which in hindsight is perhaps not surprising because deficiency of IL-1 receptors did not dramatically alter diabetes development in NOD mice (336) or increase efficacy of anti-CD3 treatment in newly diagnosed NOD mice (79). TNF-α signaling via its receptor TNFR1 has been implicated in promoting local inflammation within pancreatic islets leading to beta cell destruction in humans and NOD mice (337),(338). Administration of etancercept a soluble TNF-α antagonist to children with new-onset T1D demonstrated preservation of beta cell function evidenced by decreased exogenous insulin usage (339). Larger trials are needed to evaluate the safety and efficacy of etanercept treatment for prevention or treatment of T1D.

1.8.4 Conclusions

Overall negative outcomes of non-antigen-specific preventive studies indicate the complex pathogenesis of T1D. Moreover, a major hurdle in conducting preventive studies is that it takes

several years before any conclusions can be drawn. In contrast recent onset T1D provides an ideal setting for testing and refinement of both immune-based and antigen-based interventions as potential candidates are readily identified and risk-benefit analysis can be done in a shorter duration. The transient benefit observed after immune-modulatory interventions suggests that underlying pathophysiology remained unaltered, and a multifactorial treatment approach may be required to achieve lasting remission from T1D.

1.9 Antigen-Specific Therapies in T1D

The overall aim of immunotherapy in autoimmune disease is to block destructive immune responses to self, without impacting an individual's capacity to mount protective immune responses against infectious pathogens. A therapy that specifically disables the T cells with a self-reactive potential is termed as antigen-specific therapy. The rationale behind antigen-specific therapy is to administer self-antigenic proteins so as to induce antigen-specific T regulatory cells (dominant tolerance) or anergize/delete pathogenic T cells (recessive tolerance). Induction of antigen-specific immune tolerance bypasses the risks associated with broad immunosuppression, and thus, promises to provide a safe and effective therapy for prevention of T1D. Identification of clinically relevant antigens and a safe approach to induce immune tolerance to those antigens are two critical components that form the basis of antigen-specific therapy.

1.10 Proinsulin-specific immunotherapies

Proinsulin is the initiating antigen in NOD mice and a major antigen involved in the pathogenesis of human T1D. Being the only beta cell restricted autoantigen, proinsulin is an attractive target for antigen-specific treatment of T1D. Immunotherapies based on whole insulin, insulin-derived peptides, altered peptide ligands or plasmids encoding proinsulin have been tested in both experimental and clinical settings.

1.10.1 Insulin-based therapies in NOD mice

Studies in NOD mice have explored the effects of immunotherapy with proinsulin, insulin or insulin-derived peptides. Delivery of insulin or insulin B-chain peptides orally (340),(341) or intranasally (342), (343) decreased spontaneous diabetes incidence. Protective effects of both oral and nasal delivery were associated with generation of either CD8+ $\gamma\delta$ T cells or CD4+ T cells that were shown to mediate suppression of diabetes development (344).

Parenteral administration of insulin (345) or insulin $_{B:9-23}$ peptide (343) as well as intermittent immunization with either insulin or insulin B chain in incomplete Freund's Adjuvant (IFA) prevented insulitis and diabetes development in NOD mice (346). Vaccination of NOD mice with an altered peptide ligand of insulin $_{B:9-23}$ peptide induced anti-inflammatory cytokine response and significantly delayed diabetes onset (347). Intramuscular injection of a plasmid encoding insulin $_{B:9-23}$ peptide (348) afforded protection from diabetes onset in NOD mice, whereas proinsulin II-encoding plasmid vaccination blunted diabetes progression in hyperglycemic NOD mice (349).

1.10.2 Insulin-based clinical trials

Promising results from studies evaluating insulin immunotherapy in NOD mice paved the way for clinical trials that examined the safety and efficacy of oral, nasal or systemic insulin delivery for prevention or treatment of T1D.

1.10.2.1 Oral insulin trials

The Diabetes Prevention Trial (DPT-1) was initiated to test if oral delivery of insulin could prevent development of diabetes in healthy islet-autoantibody positive relatives of T1D patients that were assessed to have a 25-50% risk of T1D development over 5 years. Primary analysis found no difference in the rate of diabetes development between the subjects receiving oral insulin or placebo, however a post-hoc analysis revealed that individuals with high IAA titres had a 5-year delay in disease onset (350). A long-term follow up of the same group of individuals suggested that the rate of progression of T1D was halted as long as oral insulin treatment was maintained (351). Other studies investigated oral delivery of insulin to treat recent-onset T1D patients, however none of these trials achieved success in reversing established disease (352),(353),(354).

Preclinical studies in NOD mice suggest that oral insulin reduces diabetes incidence when administered early in the course of disease (340). Preliminary data from the ongoing Pre-

POINT study, which is testing primary prevention by oral insulin treatment in islet autoantibody negative children with high genetic risk for T1D, indicate that this treatment is safe without any adverse effects. Furthermore, antigen-specific T cells from insulin treated subjects displayed a regulatory gene signature suggesting that this treatment may be more successful (355).

1.10.2.2 Intranasal insulin trials

To examine the effects of intranasal delivery of insulin, islet autoantibody positive individuals with high-risk HLA haplotypes were enrolled in the Intranasal Insulin Trial (INIT-1). Intranasal insulin treatment did not accelerate beta cell decline, moreover T cell responses to insulin were dampened consistent with the mucosal tolerance to insulin (356). In the T1D Prediction and Prevention Project (DIPP Study) children with high-genetic risk were enrolled and upon detection of two or more islet autoantibodies, participants were randomized to receive intranasal insulin or placebo. Intranasal insulin delivery failed to delay diabetes onset in this study (357). Adults with recent onset T1D not requiring insulin treatment initially, showed markedly suppressed antibody response to subcutaneously injected insulin when treated with intranasal insulin, however, progression to diabetes was not prevented (358). This study provided the first evidence of immune tolerance to a mucosally delivered autoantigen in T1D.

1.10.2.3 Systemic insulin trials

The DPT-1 study assessed the effects of parenteral insulin treatment in high-risk individuals; while systemic insulin delivery was well tolerated, it failed to demonstrate any benefit as T1D developed at a similar rate in both control and treated groups (359). Interventional studies using insulin peptides or altered peptide ligands reported no impact in recent onset T1D patients (297),(360),(361), whereas proinsulin DNA vaccination of T1D patients, resulted in preservation of C peptide levels, this was associated with reduction of proinsulin-specific CD8+ T cells, however the effect was lost upon withdrawal of the therapy (362). These

results indicate that proinsulin DNA vaccination is safe and specifically impacts insulin-specific T cells, but not other T cell specificities. Thus, proinsulin-based treatments may provide benefits in patients with advanced disease.

1.10.3 Factors impacting success of antigen-specific therapies in T1D

Despite the success of antigen specific therapies in treating various allergic conditions (363),(364),(365), and promising pre-clinical studies in NOD mice, major preventive clinical trials targeting proinsulin-specific T cells failed to prevent T1D in humans. A careful examination of studies that applied antigen-specific therapies in both humans and mice has identified key variables that may have contributed to the unsuccessful translation of NOD mouse studies into clinical trials (77).

Optimal Antigen Dose

Dosing of an antigen can drastically influence its efficacy. A 2.5 - 7.5 mg/day dose of oral insulin used in human clinical trials (350),(353),(352) is likely to have been too low to achieve any efficacy, as it is about 100 fold less based on body weight when compared to milligram quantity doses that were successful in NOD mice (340).

Route of antigen delivery

An oral route of antigen delivery may not be optimal, as the delivered protein may undergo degradation in the harsh gastric environment, leading to insufficient antigen availability to induce mucosal tolerance. Moreover, mucosal delivery of antigen can elicit either tolerogenic or inflammatory responses depending upon the nature of antigen and other local factors (366),(367).

Timing of antigen-specific intervention

Many studies using a variety of approaches have demonstrated that robust antigen-specific tolerance can be induced in naïve T cells (368),(369),(370). However, insulin-based therapies to prevent T1D have been administered at a time when patients have developed multiple islet autoantibodies, suggesting advanced inflammatory response and generation of

a diverse pool of antigen-experienced memory T cells. Current antigen based treatments in the clinic may not be effective in inducing tolerance to antigen-experienced memory T cells.

1.10.4 Aims

Effective antigen-specific therapies that prevent T1D in humans have not been developed yet, despite the knowledge of key antigens and mechanisms involved in its pathogenesis (371). Evidence from NOD mice suggests that antigen-specific treatments are most effective in preventing disease onset when started very early in life. Currently, prediction of T1D onset using immunological and biochemical markers in humans is most accurate at a stage when the anti-islet immune response is well established and interventions done at this stage have not been successful in preventing ongoing beta cell loss. Therefore, a major hurdle in T1D prevention studies is the identification of an optimal window for antigen-specific treatment to induce life-long immune tolerance. Based on the above observations we hypothesize that the perinatal period represents an ideal time for antigen-specific treatment to achieve lasting immune tolerance.

Aim 1: To determine the stage of life when antigen-specific therapy is most effective, the first aim of this study is to generate and validate transgenic NOD mice with tetracycline-regulated expression of the key islet autoantigens proinsulin and IGRP in the APCs, allowing the induction of tolerance to these antigens in a temporal and a cell-specific manner.

Aim 2: Parenteral or mucosal delivery of proinsulin, insulin or insulin B chain peptides prevented diabetes and insulitis in NOD mice when administered at a young age. Therefore a “window of opportunity” may be present during the perinatal period, a time of life when priming of the immune system critically influences the host susceptibility to allergic and autoimmune diseases later in life (372),(373).

To test the hypothesis that the perinatal period represents the optimal time for antigen-specific intervention to induce life-long immune tolerance, the second aim of this study is to induce expression of the islet antigens proinsulin or IGRP until weaning in NOD mice and

evaluate the frequency and phenotype of antigen-specific T cells, insulitis and development of autoimmune diabetes.

Aim 3: Mice have two insulin genes, namely proinsulin 1 and proinsulin 2. The two genes differ in terms of their expression with greater proinsulin 2 expression in the thymus, but similar levels of expression of both genes in the pancreatic islets. NOD mice lacking proinsulin 2 develop accelerated diabetes whereas deletion of proinsulin 1 confers protection from insulitis and diabetes in NOD mice. While overexpression of proinsulin 2 in APCs prevents diabetes in NOD mice, the effect of overexpression of proinsulin 1 in the APCs of NOD mice remains to be investigated.

The final aim of this study is to generate NOD mice with tetracycline regulated proinsulin 1 expression in the APCs and evaluate whether ectopic proinsulin 1 expression induces immune tolerance. Insulin and IGRP specific T cells will be tracked and the development of insulin autoantibodies, insulitis and autoimmune diabetes will be examined.

2 Generation and validation of TIP and TII mice

2.1 Summary

Immune responses to proinsulin initiate anti-islet autoimmunity in non-obese diabetic (NOD) mice and possibly in humans. Strategies that augment antigen-specific tolerance to proinsulin prevent diabetes in NOD mice, however therapeutic benefit in human type 1 diabetes (T1D) patients has not been achieved. To determine a stage of life when antigen-specific tolerance is most effective, NOD mice were engineered to express islet autoantigens proinsulin (TIP mice) and IGRP (TII mice) in the antigen presenting cells (APCs) in a tetracycline dependent manner. MHC class II IEα promoter in combination with tet-OFF transactivator induced robust, doxycycline dependent and APC specific expression of proinsulin and IGRP in TIP and TII mice respectively. TIP mice expressing proinsulin did not develop insulitis and were protected from cyclophosphamide-induced diabetes, suggesting that proinsulin expression in TIP mice was sufficient to induce functional antigen-specific tolerance. In summary, we have generated and validated a new transgenic mouse model that enables temporal expression of islet antigens in the APCs. Using this model we will study the impact of antigen-specific therapy on the development of autoreactive T cells and spontaneous diabetes in NOD mice.

2.2 Introduction

Autoimmune disorders such as T1D have a complex etiology. Elucidation of underlying molecular mechanisms that cause self-reactive immune responses is imperative to identify novel therapeutic targets. Clinical observations in patients are helpful in determining the progression of autoimmunity, however such data do not provide an insight into destructive mechanisms that cause the disease. Animal models that mirror the clinical features of the human disease are invaluable tools to study the detrimental cellular mechanisms contributing to autoimmunity (374),(203),(375).

Genetic manipulation in animal models has vastly contributed to dissection of mammalian gene function *in vivo*. Generation of transgenic animals facilitates overexpression of gene products in a tissue specific manner, whereas targeted gene modification in mouse embryonic stem cells allows production of mice with mutation in specific genes, or use of Cre-lox recombinases facilitates conditional deletion of genes in particular cell types (376),(377). Although much of our current knowledge about various cellular mechanisms has been obtained from studies employing genetically modified animal models; a major pitfall of classical transgenic and knock-out model systems is that they are irreversible. Consequently, ectopic expression or deletion of genes involved in key developmental processes may cause lethality during embryonic development (378). Moreover, continuous expression or lack of a gene product throughout the life of the animal is likely to elicit compensatory responses. This becomes a confounding factor especially when examining redundant systems such as intracellular signaling pathways (379). Importantly, model-systems with constitutive deletion or over-expression of a gene of interest do not permit the study of gene function at a precise time point relative to the disease process. This is a key drawback as the timing of the gene-function may be equally important as the site of its expression (380). Efforts to circumvent these limitations led to development of genetic “switches” that permit spatio-temporal regulation of gene expression.

Development of the tetracycline (tet)-regulated system for transcriptional regulation of gene-expression, offers the advantage of temporal and reversible control of transgene expression. Initially developed for transgene overexpression, it has been adapted to allow inducible genetic manipulation by Cre/Lox, shRNA or CRISPR-Cas technologies (381),(382),(383),(384),(385). The tet-regulated system comprises of two genetic components, a tet-response element (TRE) promoter that controls the mRNA expression and a tet-transactivator that activates the TRE-promoter. The tTA (tet-off) transactivator strongly binds and activates gene expression from TRE promoter, however this binding is inhibited by addition of tetracycline or its derivative doxycycline (Dox). Modification of tTA molecule led to design of rtTA (tet-ON) transactivator (386),(387), which is latent until activated by doxycycline. The tet-regulated system has been utilized to overexpress or knockout gene products in various transgenic models. The great advantage of the tet-regulated system is that the transgene expression can be modulated even in neonatal mice. Previous studies have demonstrated that doxycycline can cross the placental barrier in sufficient amounts to reach the foetus *in utero* and via breast milk in neonates to induce or suppress gene expression (388, 389). In addition to the temporal control of gene expression, the tet system can be further refined by expressing the transactivator from a tissue specific promoter thus, adding a spatial dimension to the design of a disease model (390).

The NOD mouse model has proven to be an indispensible tool for dissecting immune mechanisms that contribute to spontaneous autoimmune diabetes (50). Most importantly, genetic manipulation of NOD mice using both knock-out and transgenic approaches has firmly established Insulin as a key antigen in the pathogenesis of T1D (243),(272). Several insulin-based strategies have prevented or reversed T1D in NOD mice; however, the success of preclinical interventions has not been replicated in clinical trials (66). Although NOD mice do not perfectly reflect the pathogenesis of the human disease, outcomes from preclinical studies can still guide clinical approaches if variables such as dosage of the treatment and timing of intervention are carefully considered(64),(78)

Evidence from NOD mice suggests that antigen-based treatments prevent diabetes when administered very early in life; however, in the clinic such therapies are currently administered to individuals with established autoimmunity. An optimal window for antigen-specific intervention remains undefined. This chapter describes the generation and validation of transgenic NOD mice with Tet-regulated expression of two key islet autoantigens insulin and IGRP in APCs. This model will enable induction of tolerance to these antigens in a temporal and a cell-specific manner, in order to define a stage of life when antigen specific tolerance will be most effective.

2.3 Methods

2.3.1 Restriction Enzyme Digestion

Plasmid DNA vectors were digested using restriction enzymes to release desired DNA inserts for subsequent cloning or to clone DNA inserts into appropriate cloning sites on the desired vector backbone. Restriction digestion reactions were performed in 50 μl reaction volume containing 1x reaction buffer, 1x BSA and 1U/μg DNA restriction enzyme (New England Biolabs). Reactions were incubated at 37^{o} C for at least 3 hours or overnight.

2.3.2 DNA fragment purification from agarose gel

Digested plasmid DNA vectors and inserts were separated by agarose gel electrophoresis. Fragment sizes were determined using 1Kb DNA ladder or 100bp DNA ladder (New England Biolabs) size markers. Gel slices containing the DNA fragments of interest were excised and purified using QIAquick Gel Extraction kit (Qiagen), according to manufacturers protocol.

2.3.3 DNA ligation and transformation

Purified DNA inserts were ligated to appropriate vector backbones by DNA ligation. Ligation reactions (10μl) containing 1x T4 DNA ligase buffer, 1U T4 DNA ligase (New England Biolabs) and 3:1 insert: vector DNA ratio were incubated overnight at 4^{o}C. 5μl of each ligation reaction was used to transform 50μl of chemically competent TOP 10 *E.Coli* cells (Invitrogen) according to manufacturers protocol. 100 μl of each reaction was plated on prewarmed LB agar plates containing 100μg/μL ampicillin (Amresco), and incubated overnight at 37^{o}C.

2.3.4 Plasmid DNA isolation

Plasmid DNA was extracted from and purified bacterial cells using QIAprep Spin Mini /Midiprep kits (Qiagen), according to manufacturers instructions. Briefly, bacteria were lysed in alkaline conditions, followed by precipitation of genomic DNA upon neutralization and

removal by centrifugation. The supernatant containing plasmid DNA was applied to QIAprep mini or midiprep column. Plasmid DNA selectively binds to silica membrane under high-salt conditions. Plasmid DNA was then eluted from the column following a series of ethanol based washes. Concentration of plasmid DNA was determined by spectrophotometric quantification by measuring absorbance at 260nm using NanoDrop (ThermoScientific).

2.3.5 Purification of transgene insert for microinjection

Plasmid DNA purified using phenol free isolation methods such as the alkaline lysis and column purification method is suitable for preparation of inserts for microinjection. Approximately 15-20 ug of plasmid DNA was digested to remove plasmid backbone sequence flanking the transgene of interest. The digested DNA along with bromophenol blue (BPB) DNA loading dye was separated on a 1% low melting agarose gel (SeaPlaque, FMC) without ethidium bromide (EtBr) at 40 V in a cold room for 3-4 hours. Thin strips of gel were cut on either side of the well containing digested DNA and stained with EtBr to visualize the digested insert. A notch was cut on the gel to mark the location of the insert fragment on the gel strips. The gel was re-assembled on a clean surface and a slice of the gel corresponding to the insert was cut out. The slice was cut into 1 cm pieces and incubated overnight in 20 ml 1x Agarase buffer at 4^0C. Agarase buffer was changed next day and the agarose slices were further incubated for 4-6 hours. Agarose slices were placed in a pre-weighed 1.5 ml tube and the weight of each slice was determined and limited to 400 mg per tube. Agarose was spun to the bottom of the tube in a microcentrifuge for 5 seconds and the tube was placed on a heat block pre-warmed at 68^{o}C for 10 minutes. Tubes were immediately transferred to a water bath pre heated at 40^{o}C. Beta-Agarase (New England Biolabs, 1U/μl) enzyme was added (1U/100mg of agarose) to each tube, mixed and incubated further at 40^{o}C for 2 hours. Digested agarose solution from all the tubes was pooled and centrifuged at 13,000 rpm in a microfuge for 20 minutes at room temperature. Top two thirds of the agarose solution containing transgene DNA was transferred to a fresh tube.

2.3.6 Dialysis of transgene insert

The transgene insert purified from agarose gel as described above was subjected to dialysis for removal of residual impurities. Millipore filters (type VM, pore size 0.05μM) were pre-equilibrated over-night in 30 ml of microinjection buffer (10mM Tris/HCL pH 7.4, 0.1 mM EDTA) at room temperature by floating on surface with shiny side up in a petri dish. Equilibrated filters were transferred to a new petri dish with fresh microinjection buffer. 200μl of purified solution containing the transgene was pipetted on the dialysis filter and dialysed for 8 hours at room temperature. DNA was recovered in a fresh 1.5 ml tube and its concentration was ascertained by spectrophotometry. Dialysed transgene DNA was diluted at a concentration of 3-5 μg/ml for microinjection.

2.3.7 Generation of Transgenic Mice

2.3.7.1 NOD-IEα-rtTA mice

A 1.9kb fragment encoding the MHC class II IEα promoter was excised using the *NotI* and *SpeI* restriction sites from the previously described IE-IGRP construct (257) and subcloned into pBluescript SK^- vector to generate pBS-IEα plasmid. Subsequently, a 1.2 kb fragment comprising of reverse tetracycline transactivator (rtTA) and a polyA signal was excised from pTET-ON Advanced plasmid vector (Clontech) and was ligated into *EcoRI* and *HindIII* restriction sites of the pBS-IEα plasmid, thereby placing rtTA under the control of MHC class II IEα promoter (pBS-IE-rtTA). The ~3 Kb transgene cassette containing IE promoter-rtTA-sv40pA was excised from pBS-IE-rtTA plasmid using *NotI, HindIII and ScaI* restriction sites and purified. The purified transgene was injected into NOD ova using standard procedures. Founder mice for breeding and subsequent transgene positive offspring were identified by PCR analysis of tail biopsies using primers spanning the MHC class II IEα promoter (5'-GATGCATCCAGCAATAAG-3') and the rtTA gene (5'-CCTGTCCAGCATCTCGATTG3').

2.3.7.2 TetO-Ins2 mice

To generate the TetO-Ins2 construct, an 883 bp fragment spanning the coding region of murine proinsulin II (PI) was amplified by PCR from NOD genomic DNA and cloned into BamHI and HindIII sites of the pTRE2 plasmid (Clontech). The cloned PI gene was verified by sequencing to rule out any mutations. A 2868 bp transgene cassette comprising of the TetO-minimal CMV promoter, followed by the PI gene and a polyA signal was excised between XhoI and DrdI sites and purified for injection into fertilized NOD/Lt ova using standard techniques. Founders and transgene positive offspring were screened by PCR using primers spanning the PI gene (5'-GTTGCAGTAGTTCTCCA-3') and the tetO-CMV promoter (5'-GTGAACCGTCAGATCGCCTG -3').

2.3.7.3 TetO-IGRP mice

To generate the TetO-IGRP construct a 1718 bp fragment encompassing IGRP cDNA and polyA signal was excised between SpeI and Cla I restriction sites of the IE-IGRP construct previously described (257) and cloned into NheI (SpeI compatible ends) and ClaI sites of the pTRE2 plasmid (Clontech) under control of the TetO-CMV promoter. The 2126 bp transgene cassette consisting of the TetO-CMV promoter-IGRP cDNA and polyA signal was excised between Xho I and ClaI sites and purified for injection into fertilized NOD/Lt ova using standard techniques. Offspring were screened by PCR using primers spanning IGRP cDNA (5'-TCAAGATCCCGACTCACG -3') and TRE2 plasmid (5'-GAGATATCGTCGACAAGC-3'). The offspring carrying the TetO-IGRP transgene were crossed with NOD-IEα-tTA mice to generate dual transgenic Tet-inducible IGRP (TII) mice.

2.3.8 Other mice

NOD/Lt mice were bred and maintained at the Bioresources Centre, St. Vincent's hospital (Fitzroy, Victoria, Australia). The NOD-IEα-tTA mice that drive the expression of tetracycline transactivator (tTA) under the control of MHC class II IEα promoter have been previously described (389) and were obtained from Prof. C. Benoist and Prof. D. Mathis (Dept of

pathology, Harvard). NOD8.3 mice express the TCRαβ rearrangements of the H-2K^d-restricted, β cell-reactive CD8$^+$ T cell clone NY8.3 (206). TRE-GFP-Shluc is a reporter mouse strain where the expression of green fluorescent protein (GFP) is under the control of tetracycline responsive TRE promoter (391). The TRE-GFP-Shluc mice were obtained from Dr. R. Dickins (Walter and Eliza Hall Institute). All mice were bred, maintained and used under specific pathogen free conditions at St Vincent's Institute (Melbourne, Australia). All experimental procedures followed the guidelines approved by the institutional animal ethics committee.

2.3.9 Flow Cytometry

Spleen and thymus were dissected and treated with 0.02% Collagenase P (Roche Diagnostics, GmBH) to make single cell suspensions .For spleens, red blood cells were lysed with 0.747% ammonium chloride before further staining. Antibodies used were anti-CD3 (500 A2) V500 (all BD Biosciences), anti-CD11c (N418) conjugated to APC, anti-B220 (RA3-6B2) conjugated to PE, (all BioLegend) Data was collected on LSR Fortessa flow-cytometer (BD) and analyzed using FlowJo (Treestar) software.

2.3.10 Doxycycline treatment

Doxycycline was administered to TIP and TII mice via drinking water containing Doxycycline hyclate (Sigma Aldrich) powder (2mg/ml) for the desired duration. For long-term doxycycline treatment TIP and TII mice were fed with a diet containing 600mg doxycycline /kg food (Specialty Feeds, Glen Forrest, WA).

2.3.11 Proinsulin ELISA

Whole thymus and spleen were excised and snap frozen in liquid nitrogen. Frozen tissues were homogenized in ice-cold Phosphate Buffered Saline (PBS) supplemented with protease inhibitor cocktail (Sigma) and clarified by centrifugation. Proinsulin levels in tissue homogenates were determined using a mouse proinsulin ELISA kit (Mercodia, Sweden) according to manufacturer's instructions.

2.3.12 RT-PCR

Total RNA was extracted from freshly dispersed splenic or thymic tissue using Nucleospin RNA XS kits (Macherney-Nagel), and first strand cDNA was generated using High Capacity cDNA Reverse Transcription kits (Applied Biosystem) according to the manufacturers' instructions. Real-time PCR analysis was performed using Rotor-Gene-RG-3000 cycler (Corbett Research, Sydney, Australia). Taqman gene expression primers murine insulin 2 (Ins2; Mm00731595_gH) and murine β-actin (Actb; Mm00607939_s1) were purchased from Applied Biosystems. To determine relative expression, Ct values of Insulin gene were subtracted from Ct values of β-actin for each sample and difference was plotted to determine the abundance of gene of interest.

2.3.13 Western Blotting

Whole thymus was excised and snap frozen in liquid nitrogen. Frozen tissue was homogenized in ice-cold lysis buffer (50 mmol/LTris-HCl pH 8, 150 mmol/L NaCl, 0.5% Triton X-100) and protease inhibitor cocktail (Sigma) and clarified by centrifugation. Thymic homogenates were resolved by SDS-PAGE and transferred to nitrocellulose membrane using standard procedures. Monoclonal antibodies to insulin (L6B10, Cell Signaling) and beta-actin (Santa-Cruz) and horse-radish-peroxidase-conjugated anti-mouse Ig antibodies were used to detect proinsulin protein.

2.3.14 CFSE labeling and adoptive transfer

CFSE labeling of CD8+ T cells from NOD 8.3 mice was done as previously described (2). $5x10^6$ CFSE labeled cells were intravenously transferred into TII mice with induced IGRP expression. Hosts were sacrificed after 3 days and their inguinal and pancreatic lymph nodes were examined for CFSE+ cells.

2.3.15 Pancreas immunohistochemistry and Insulitis scoring

For insulitis scoring pancreata were snap frozen in OCT (Sakura Finetek, Torrance, CA). Five um sections were prepared from 3 levels (200um apart), and stained with anti-insulin, followed by anti-guinea pig- HRP (All from Dako Corp., Carpenteria, CA). Staining was developed with diaminobenzidine (Sigma Aldrich), and sections were counterstained with haemotoxylin. Insulitis was scored on three sections using the following scale 0= no infiltrate, 1= peri-islet-infiiltrate, 2= extensive (>50%) peri-islet infiltrate, 3= intraislet infiltrate and 4= extenstive intra-islet-infiltrate (>80%) or total beta cell loss. The percentage of islets with each score was calculated by addition of scores for the three sections.

2.3.16 Cyclophosphamide induced diabetes

To monitor the incidence of cyclophosphamide induced diabetes, 12-14 week old TIP mice (with or without doxycycline treatment from gestation onwards) or non-transgenic NOD mice, were injected (i.p) with 300mg/kg of Cyclophosphamide (Sigma-Aldrich). Diabetes onset was monitored by weekly measurement of urine glucose levels using Diastix (Bayer Diagnostics). Blood glucose levels were measured in mice with glycosuria using Advantage II Glucose strips (Roche). Animals displaying two consecutive blood glucose measurements of ≥ 15mM/L were considered diabetic.

2.4 Results

2.4.1 APC-specific reporter expression driven by IEα-rtTA (Tet-ON) promoter

Several transgene positive founder mice were obtained after injection of NOD-IEα-rtTA construct into NOD ova. Founders #80, #86, #91 & #96 were viable, fertile and transmitted the transgene to their offspring. To test the ability of the MHC class II IEα promoter to direct APC specific reporter expression in a doxycycline dependent manner, the driver IE-rtTA (tet-ON) founder lines were crossed to a strain of mice expressing a tetracycline inducible GFP reporter (tetO-eGFP) (392). The dual transgenic NOD-IEα-rtTA-GFP mice (hereafter referred to as rtTA-GFP mice) (Figure 2-1-A), mice were either given Doxycycline (2mg/ml in drinking water) for 7 days or were not treated. The induction of GFP reporter expression was analyzed by flow-cytometry in $CD11c^+$ dendritic cells (DCs), and $B220^+$ B cells harvested from spleen and thymus of rtTA-GFP mice. Out of the four transgenic lines obtained for the driver NOD-IEα-rtTA mice, line #80, induced robust GFP expression in B cells harvested from spleen and thymus (~33% and ~12% respectively) however, modest GFP expression was observed in DCs examined from thymus (~6%) and spleen (4%) of rtTA-GFP mice (Figure 2-1-B).

In comparison, line #86 induced low level of GFP reporter expression in B cells from spleen (~8%) and thymus (~2%), as well as DCs from spleen and thymus (~1% and 4% respectively), whereas founder lines #91 and #96 were unable to induce reporter expression upon dox treatment in rtTA-GFP mice (data not shown). Moreover, overall frequencies of APC subsets in rtTA-GFP (line #80 and #86) mice were comparable to that of their non-transgenic littermates indicating normal development of immune subsets in the transgenic mice (Data not shown). These data suggest that the IEα-rtTA promoter induced variable reporter gene expression that was predominantly restricted to B cells in the dual transgenic rtTA-GFP mice.

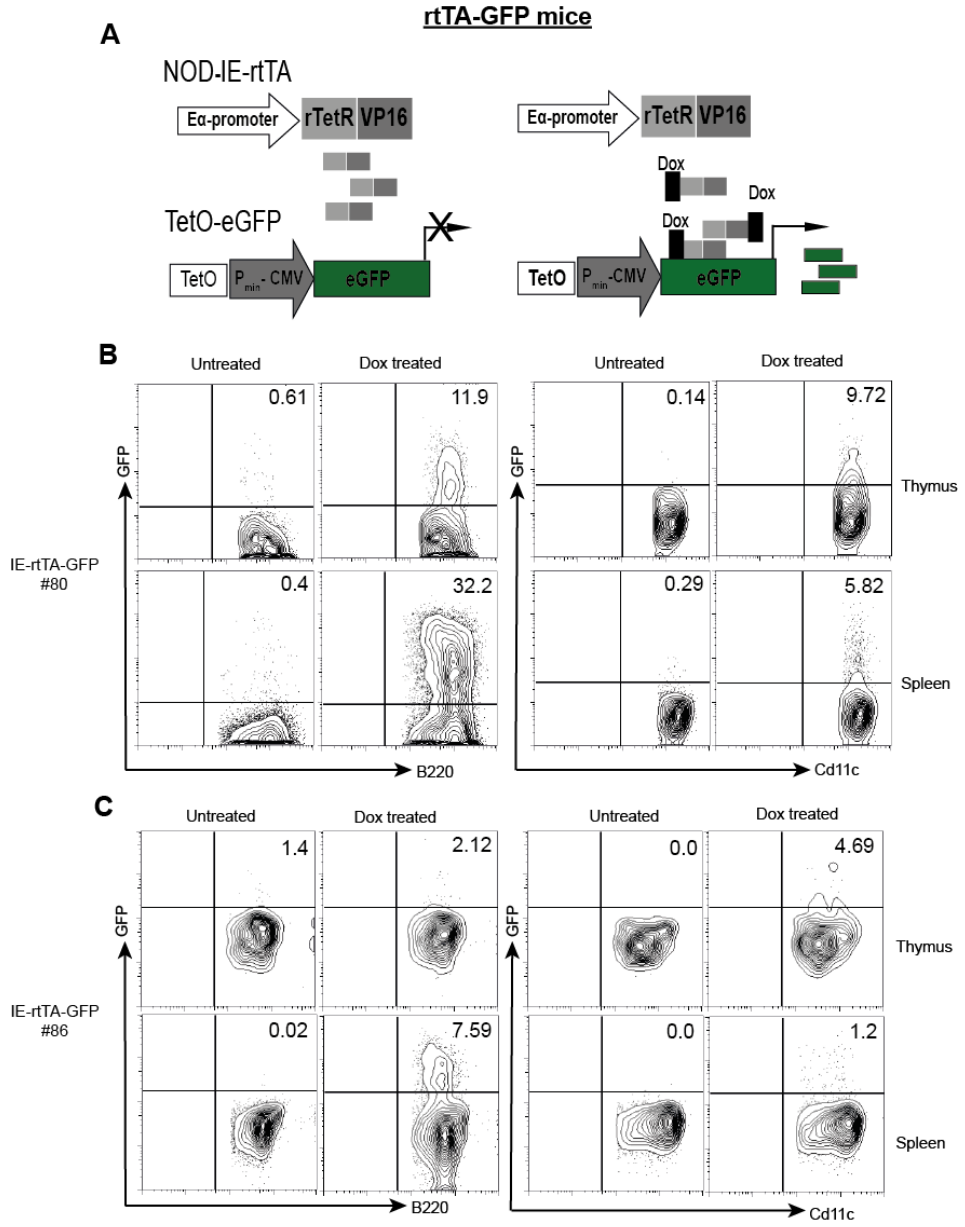

Figure 2-1 Reporter GFP expression in rtTA-GFP mice

(A) Scheme of generation of tetracycline regulated NOD.IEα-rtTA (IE-rtTA) and tetO-eGFP dual transgenic mice referred to herein as rtTA-GFP mice. Two independent lines (#80 & #86) of IE-rtTA mice were crossed with tetO-eGFP mice. Bi-transgenic animals were fed doxycycline (Dox) (2mg/ml) via drinking water to induce reporter expression. GFP expression in the APC subsets of was analyzed 1 week later. Representative FACS plots showing GFP expression in B220+ B cells (left) and CD11c+ dendritic cells (DCs) (right) isolated from thymus (top) and spleen (bottom) of IE-rtTA-GFP mice #80 **(B)** and IE-rtTA-GFP mice #86 **(C)** that were untreated or fed Dox. Numbers in top right quadrant of FACS plots indicate percentage of GFP+ cells. Data is representative of 2-3 independent experiments.

2.4.2 APC-specific reporter expression driven by IEα-tTA (Tet-OFF) promoter

NOD-IEα-tTA mice (referred to as TA-NOD mice, obtained from the laboratory of Prof. D.Mathis & C.Benoist) express the TetR-VP16 tetracycline transactivator protein (tTA) controlled by the IEα-MHC-II promoter, and constitutively drive the expression of the reporter construct under the control of Tetracycline response element (TRE). Upon doxycycline treatment, the tTA protein dissociates from the TRE thereby extinguishing the reporter expression. We bred TA-NOD mice to tetO-eGFP mice and examined the GFP expression in APCs by flow-cytometry. The dual transgenic TA-NOD-tetO-GFP mice (hereafter referred to as TA-GFP mice) (Figure 2-2-A) were untreated or given doxycycline to turn-off the reporter expression as indicated. The IEα-tTa promoter induced robust GFP expression in thymic DCs (~15 %) and B cells (~9%) as well as splenic DCs (~8%) and B cells (~13%) of TA-GFP mice (Figure 2-2-B). GFP expression was not detectable in APCs of TA-GFP mice after 1 week of doxycyline treatment, indicating a complete suppression of reporter expression. Thus, the IEα-tTA (tet-off) promoter offers the convenience of constitutive reporter gene expression in the APCs, with a rapid turn-off upon Dox treatment.

2.4.3 Conditional expression of IGRP in TII-ON and TII mice

2.4.3.1 TII-ON mice

$IGRP_{206-214}$-specific CD8+ T cells are the highest frequency antigen-specific T cells in the NOD mouse and expression of IGRP in APCs completely deletes $IGRP_{206-214}$-specific CD8+ T cells (257), and this deletion can be tracked in vivo after antigen-specific intervention to induce tolerance. We generated two transgenic lines of NOD mice expressing IGRP under the control of tetracycline-regulated tetO-CMV promoter (tetO-IGRP mice). To facilitate temporal and tet-regulated IGRP expression in APCs, tetO-IGRP founders lines #11 and #30 were bred with NOD-IEα-rtTA line #80 to generate bi-transgenic Tetracycline

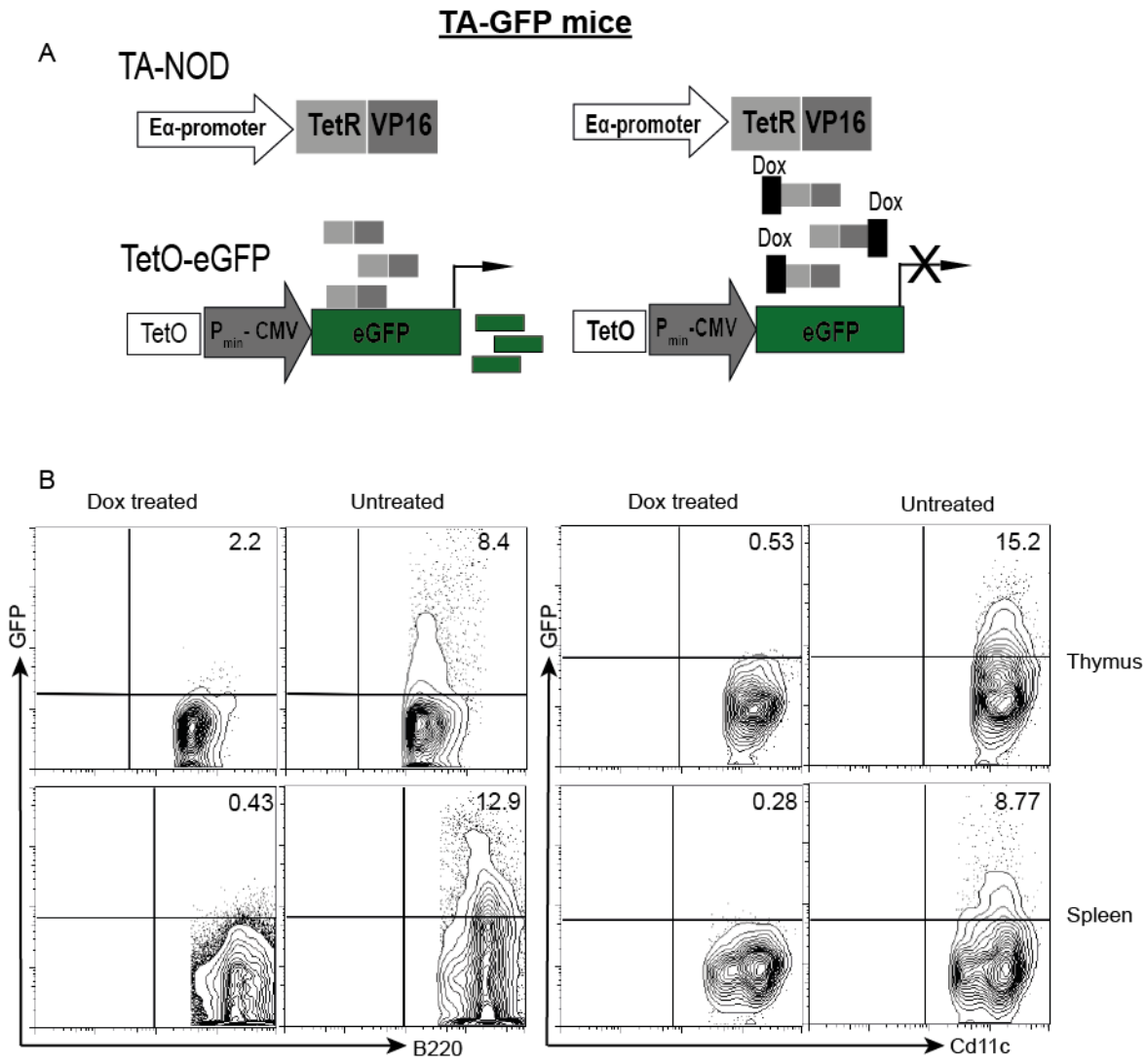

Figure 2-2: Reporter GFP expression in TA-GFP mice

(A) Scheme of generation of tetracycline regulated NOD.IEα-tTA (TA-NOD) and tetO-eGFP dual transgenic mice referred to herein as TA-GFP mice. TA-NOD mice were crossed with tetO-eGFP mice. Bi-transgenic animals constitutively express GFP in APCs and were fed doxycycline (Dox) (2mg/ml) via drinking water to suppress reporter expression. **(B)** Representative FACS plots showing GFP expression in B220+ B cells (left) and Cd11c+ dendritic cells (DCs) (right) isolated from thymus (top) and spleen (bottom) of TA-GFP mice that were untreated or fed Dox for 1 week. Numbers in top right quadrant of the FACS plots indicate percentage of GFP+ cells. Data is representative of 2 independent experiments.

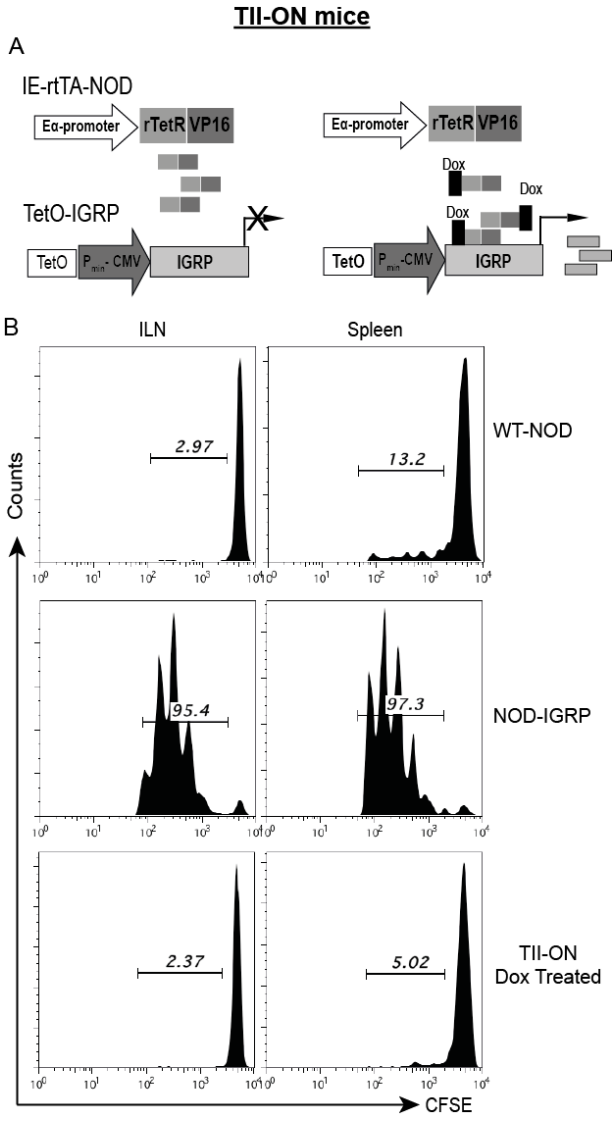

Figure 2-3: Lack of induced IGRP expression in TII-ON mice

(A) Scheme of generation of tetracycline regulated NOD.IEα-rtTA (IE-rtTA) and tetO-IGRP dual transgenic mice referred to herein as TII-ON mice. CFSE labelled CD8+ T cells from NOD 8.3 mice were transferred into non-transgenic NOD mice, NOD-IGRP mice and TII-ON mice that were fed doxycycline for 10 days to induce IGRP expression. Recipients were sacrificed 3 days later and inguinal lymph nodes (ILN) and spleen were analyzed for CFSE+ CD8+ T cells **(B)** Representative FACS profiles showing CFSE dilution in ILN (right) and spleen (left) of indicated strain of mice. Numbers in histograms indicate percentage of CFSE low cells. Data is representative of 2 independent experiments.

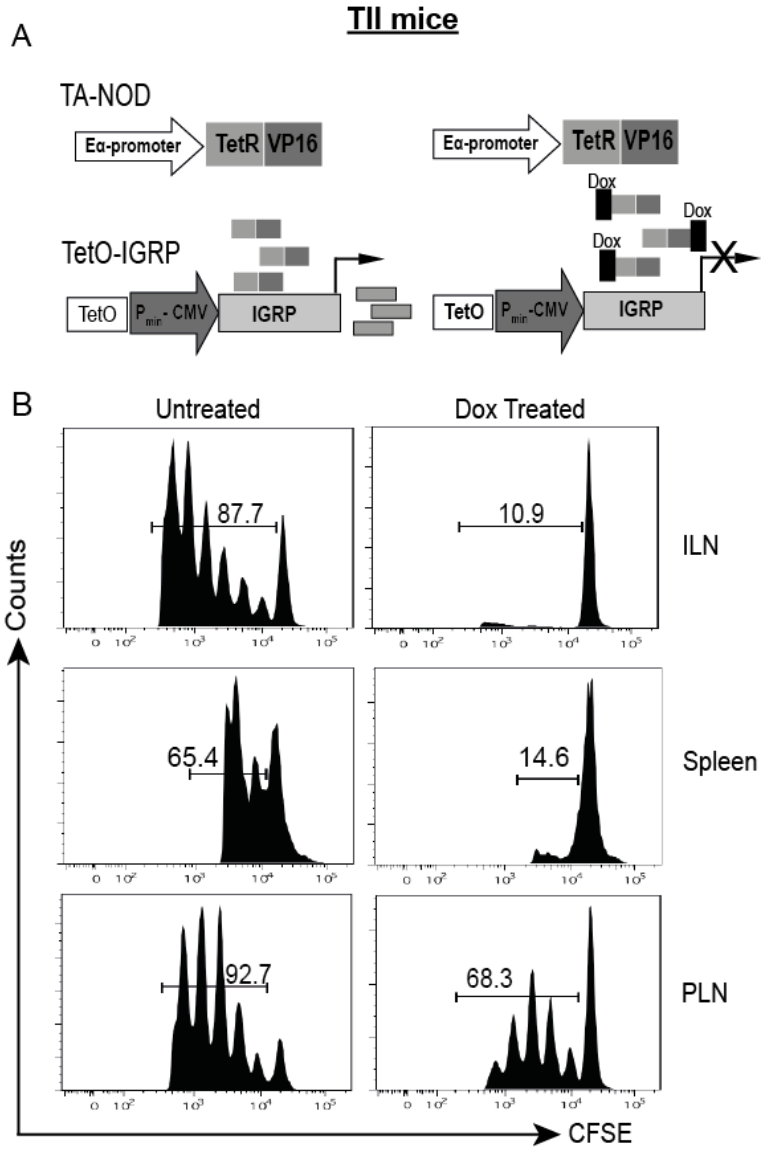

Figure 2-4: Conditional IGRP expression in TII mice

(A) Scheme of generation of tetracycline regulated NOD.IEα-tTA (TA-NOD) and tetO-IGRP dual transgenic mice referred to herein as TII mice. CFSE labelled CD8+ T cells from NOD 8.3 mice were transferred into 8 weeks old TII mice that were untreated or fed doxycycline for 10 days to turn-off IGRP expression. Recipients were sacrificed 3 days post transfer and inguinal lymph nodes (ILN), spleen and pancreatic lymph nodes (PLN) were analyzed for CFSE+ CD8+ T cells **(B)** Representative histograms showing CFSE dilution in ILN (top), spleen (middle) and PLN (bottom) of TII mice. Numbers in histograms indicate percentage of CFSE low cells. Data is representative of 3 independent experiments.

Inducible IGRP (TII-ON mice) (Figure 2-3-A). The conditional expression of IGRP in the APCs of TII-ON mice was validated by transfer of CFSE labeled IGRP specific CD8+ T cells from NOD 8.3 mice (206) into TII-ON mice that were treated with Dox to induce IGRP expression. Previously described NOD-IGRP mice (257) that constitutively express IGRP in the APCs were used as a positive control. Extensive proliferation of transferred IGRP specific T cells was observed in the spleen and ILN of NOD-IGRP mice as expected. However, transferred IGRP specific cells did not proliferate in the spleen and ILN of dox treated TII-ON mice (Data shown from tetO-IGRP line #30), indicating lack of induced IGRP expression in peripheral APCs (Fig 2-3-B).

2.4.3.2 TII mice

To examine the possibility that tetO-IGRP mice may not be expressing the IGRP transgene strongly, we crossed the tetO-IGRP lines to TA-NOD mice to generate TII mice (Figure 2-4-A) and evaluated if the IEα-tTA (tet-off) promoter was able to induce IGRP transgene. Extensive proliferation of transferred CFSE labeled IGRP specific CD8+ T cells was observed in ILN and PLN of TII mice that constitutively expressed IGRP whereas, IGRP specific T cells did not proliferate in peripheral lymph nodes of TII mice treated with Dox, indicating suppression of IGRP transgene (data shown for tetO-IGRP line #30) (Figure 2-4-B). The transferred IGRP specific T cells showed a basal level of proliferation in the PLN similar to that observed in WT NOD mice (257)which is most likely due to the presence of IGRP peptides in the draining lymph node as a result of ongoing insulitis in the host mice. The IGRP transgene was also expressed in the thymus of TII mice as determined by the proliferation of CFSE labeled 8.3 T cells when cultured in vitro for 3 days along with thymic APCs from TII mice (Data not shown). These results suggest lack of IGRP expression in TII-ON mice was most likely due to sub-optimal expression level of the IEα-rtTA (tet-ON) transgene, and hence for subsequent experiments TA-NOD mice were used to induce conditional expression of IGRP and proinsulin.

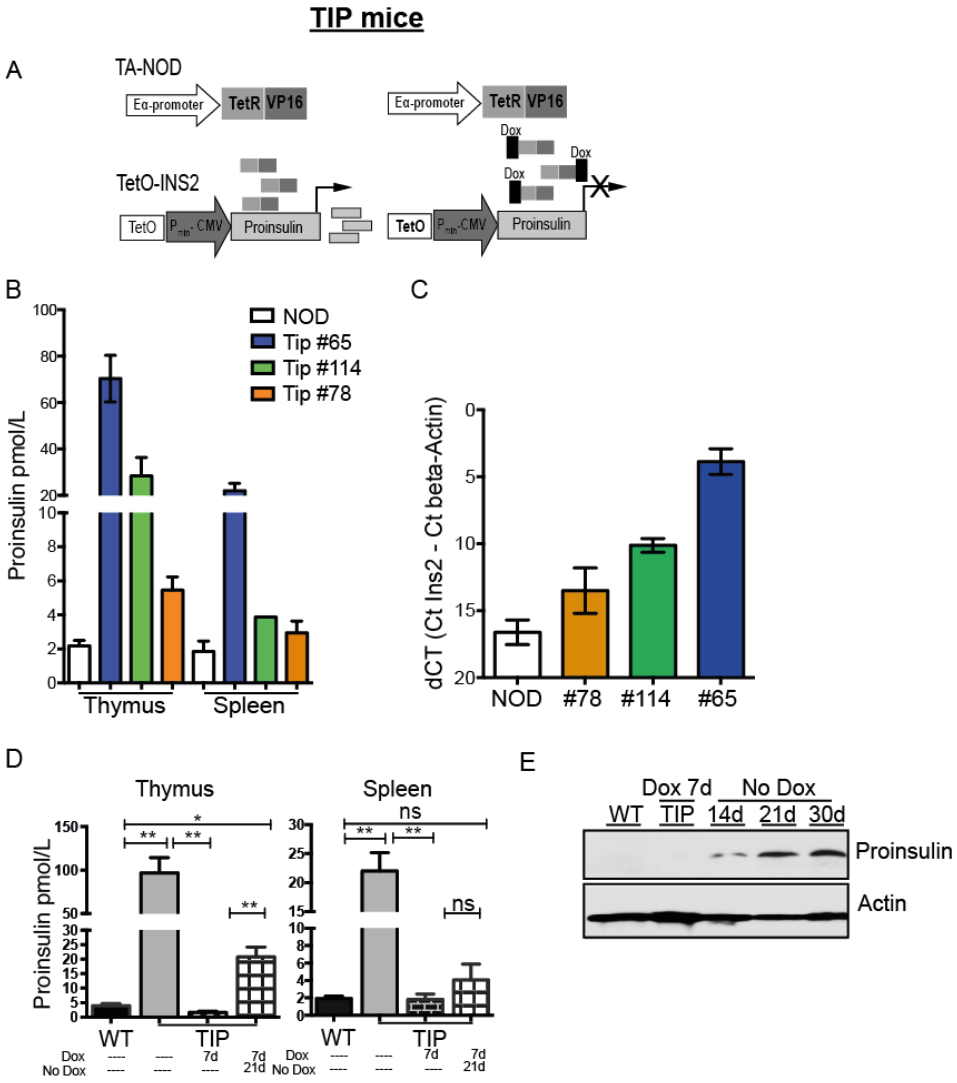

Figure 2-5: Temporal proinsulin expression in TIP mice

(A) Scheme of generating tetracycline regulated TA-NOD and TetO-Ins2 dual transgenic mice referred to as TIP mice. **(B)** ELISA assay showing level of proinsulin expression (Mean±SEM) in thymus and spleen of WT-NOD mice and three founder lines of TIP mice. **(C)** Quantitative RT-PCR for insulin and beta-actin in thymic lysates of WT-NOD mice and three founder lines of TIP mice. Data represent dCT values (Mean±SEM) from 2-3 independent experiments run in duplicate for each probe. **(D)** ELISA assay showing temporal proinsulin expression (Mean±SEM) in thymus and spleen of WT-NOD mice, untreated TIP mice and TIP mice (line #65) with indicated duration of Dox treatment. **(E)** Western-blot analysis of proinsulin expression in thymic lysates of WT-NOD mice and TIP mice (line #65) with indicated treatments (2-3 independent experiments with n≥2 per group). *P<0.05, **P<0.01. Values compared using One-way ANOVA with Tukey's multiple comparisons test **(D).**

2.4.4 Conditional expression of Proinsulin in TIP mice

Several founder lines of NOD mice expressing proinsulin (PI) under the control of the tetracycline-responsive promoter (tetO-Ins2 mice) were obtained after injection of the tetO-Ins2 construct into NOD ova. The tetO-Ins2 founder lines #65, #78 and #114 were bred with TA-NOD mice to generate bi-transgenic TIP (Tet Inducible PI) mice (Figure. 2-5-A). This enables conditional expression of PI in thymic and peripheral APCs in a doxycycline (Dox) dependent manner in the progeny carrying both transgenes. Analysis of PI expression in the individual founder lines of TIP mice by ELISA (Figure 2-5-B) and RT-PCR (Figure 2-5-C) revealed that PI transgene was robustly expressed in the thymus and spleen of line #65 followed by line #114 and line #78 respectively.

TIP mice from founder line #65 were treated with Dox to test the conditional expression of the PI transgene. After one week of Dox treatment, PI expression dropped to baseline levels in thymus and spleen as measured by ELISA (Figure 2-5-D), and was undetectable by Western blotting (Fig. 2-5-E). Re-expression of PI upon Dox withdrawal was much slower and recovery was observed after 3-4 weeks of Dox removal (Figure 2-5-D, E). Thus, PI expression in TIP mice was tightly regulated, inducible and reversible.

2.4.5 Doxycycline does not influence spontaneous diabetes development in NOD mice

In a typical NOD mouse colony ~70% of female mice develop autoimmune diabetes by 40 weeks of age (62). Cumulative incidence of hyperglycemia in the NOD mice is the highest in specific pathogen free animal facilities as compared to conventional facilities (76),(393),(394). Numerous environmental factors have been reported to modulate diabetes incidence in NOD mice, which include dietary factors (395),(396),(397), exposure to infectious organisms (398),(399),(400) and alteration in gut microbiota (401),(402). Antibiotic induced changes in gut microflora have been reported to alter immune responses and dampen diabetes incidence in rodent models (403),(404),(405),(406),(407).

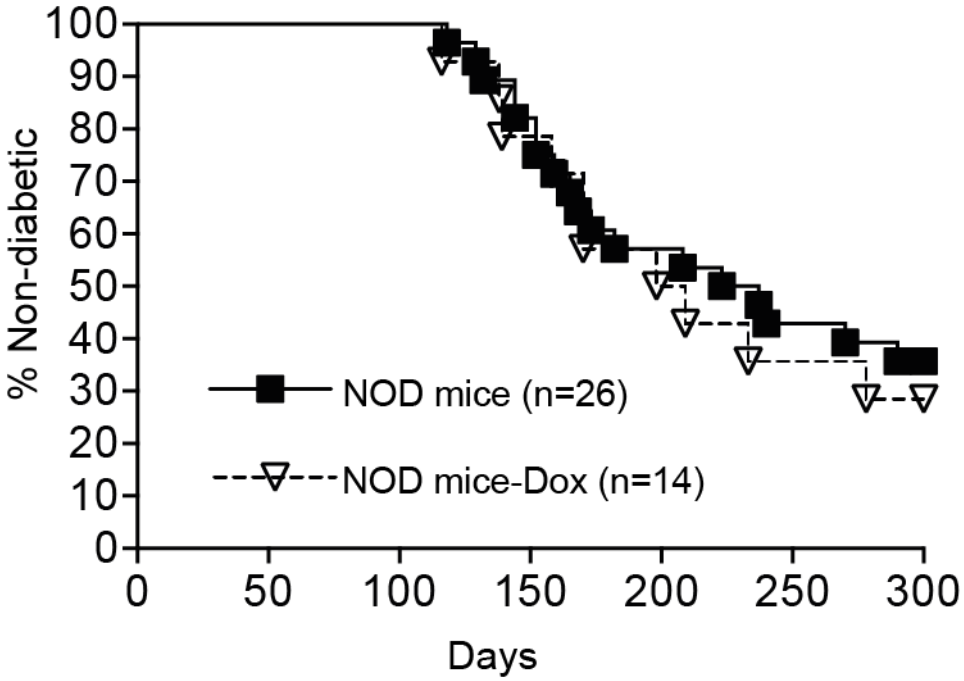

Figure 2-6: Doxycycline does not alter spontaneous diabetes incidence in NOD mice

Pregnant NOD breeders and their progeny were fed a diet containing doxycycline (600mg/kg) or standard chow diet. Spontaneous diabetes development was analyzed in female NOD mice of both groups until 300 days of age. Numbers in parentheses indicate the number of NOD mice analyzed. P= not significant. Survival curves were compared using log-rank test.

Prolonged administration of the antibiotic doxycycline would be required to turn-off antigen expression in the APC of TIP and TII mice described in this study. Therefore, we examined whether continuous administration of doxycycline had an influence on spontaneous diabetes development in non-transgenic NOD mice. NOD breeders and their offspring that were fed chow with doxycycline, developed diabetes at a similar frequency to NOD mice that were fed standard chow diet (Figure 2-6). Thus, doxycycline treatment did not alter diabetes incidence in our mouse colony.

2.4.6 Proinsulin expression in APCs of TIP mice protects from insulitis development

NOD mice develop progressive lymphocytic infiltrate (insulitis) in the pancreatic islets with age, which ultimately leads to destruction of beta cells, resulting in hyperglycemia. Constitutive expression of PI in APCs prevented insulitis in the previously described NOD-PI mice(243). We assessed whether the level of PI expression observed in APCs of TIP mice was sufficient to influence insulitis development. Analysis of pancreas histology from 12-14 week old TIP mice (lines #65, 78 and 114) continuously expressing PI revealed complete absence of cellular infiltrates in the islets as compared to age matched non-transgenic NOD mice where >70% islets had lymphocytic infiltrate. Moreover, protection from insulitis was dependent upon presence of both IEα-tTA and tetO-Ins2 transgenes, as mice transgenic for a single genetic component developed insulitis comparable to control NOD mice (Figure 2-7-A).

2.4.7 TIP mice are protected from cyclophosphamide-induced diabetes

Treatment of pre-diabetic NOD mice with cyclophosphamide leads to an accelerated and synchronous onset of spontaneous diabetes via a mechanism that involves ablation of CD4+ CD25+FoxP3+ regulatory T cells (408),(409),(410).

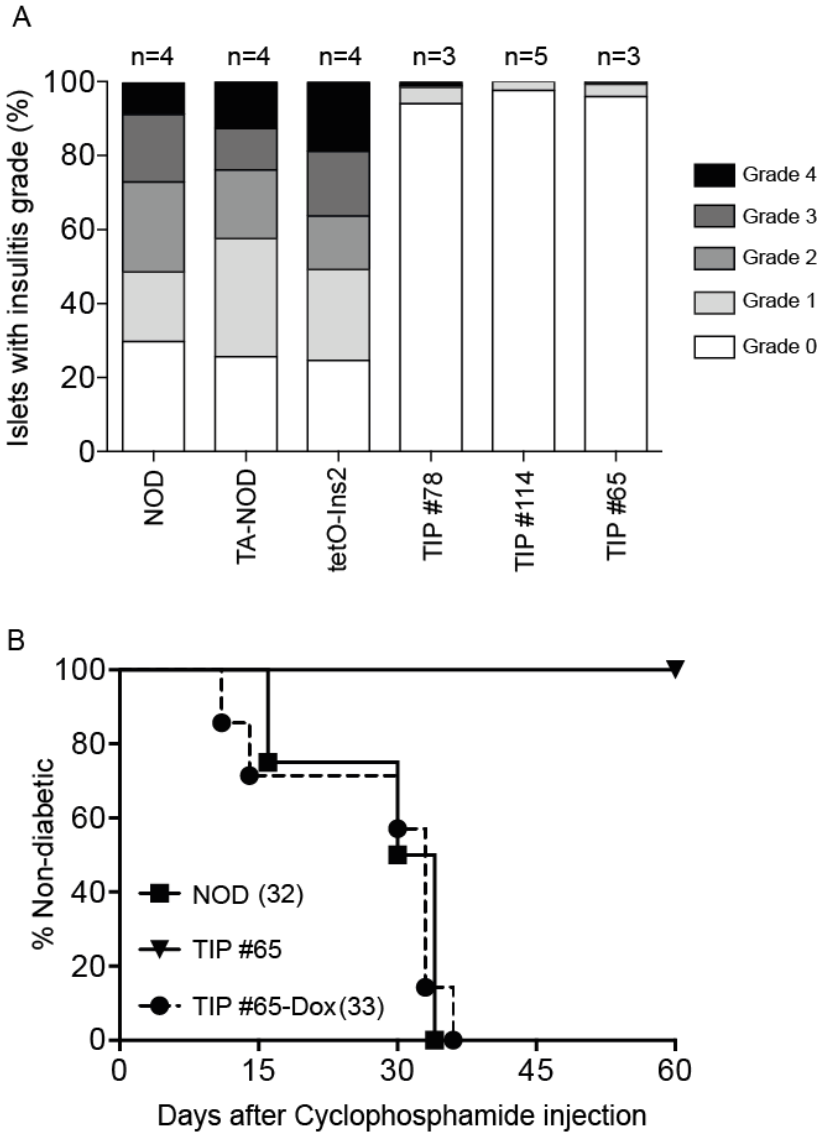

Figure 2-7:TIP mice are protected from insulitis and cyclophosphamide induced diabetes

(A) Histological grading of insulitis in 12-14 week old WT-NOD mice, mono transgenic TA-NOD mice, tetO-Ins2 mice or untreated TIP mice. Number on top of each column represents number of mice screened (≥ 90 islets scored per mouse). **(B)** Incidence of diabetes development in cyclophosphamide injected 12-14 week old WT-NOD mice (n=4), untreated TIP mice (n=5) or TIP mice treated with Dox continuously from gestation (n=7). Numbers in parantheses indicate median survival. P= 0.003 (Tip #65 vs Tip #65-Dox). Survival curves compared using log-rank test.

Cyclophosphamide treatment of TIP mice expressing PI did not result in accelerated diabetes, whereas dox treated TIP mice with no PI expression and non-transgenic NOD mice developed accelerated diabetes within 4 weeks of cyclophosphamide treatment (Figure 2-7-B). These results further confirm absence of insulitis in TIP mice that continuously express PI, resulting in protection from accelerated diabetes onset upon cyclophosphamide treatment.

2.5 Discussion

We have developed a new animal model that permits regulatable expression of islet antigens proinsulin and IGRP to study the impact of temporal antigen specific tolerance on autoimmune diabetes. NOD mice were engineered using the binary tetracycline regulated gene expression system to induce proinsulin and IGRP expression in the APCs under the dictates of MHC class II IEα promoter.

Although the MHC II IEα promoter driven rtTA (tet-ON) and tTA (tet-OFF) transactivators induced the expression of GFP in APCs of the tet-regulated rtTA-GFP and TA-GFP reporter mice; surprisingly, IGRP expression was not detected in APCs of bi-transgenic TII-ON mice upon induction with doxycycline. This discrepancy could be due to the site of tetO-IGRP transgene integration. While the tetO-GFP transgene in the reporter mice is targeted to the collagen type 1 alpha (Col1a1) locus, permitting tet-regulated expression of the the GFP reporter in a wide range of cell types (392),(411), the tetO-IGRP transgene may have integrated at a genomic location which may influence its accessibility by the tet-transactivator. Transgene silencing can occur when a transgene integrates into a genomic region that is transcriptionally inactive, or is influenced by DNA methylation or genomic imprinting (412),(413). Lack of IGRP expression in TII-ON mice could also be due to transgene-induced toxicity in the MHC class II expressing APCs. However, as the IEα-tTA (tet-OFF) transactivator robustly induced IGRP expression in the APCs of TII mice, the influence of genomic location on IGRP transgene as well as transgene toxicity can be ruled

out. A caveat around these experiments is that the expression of IGRP transgene in TII-ON and TII mice was evaluated using an indirect method rather than a direct readout such as qPCR or a western-blot.

The level of IEα-rtTA (tet-ON) transgene expression could be a possible reason for its inability to drive IGRP expression. In a recent study, sub-optimal transactivator protein expression resulted in heterogeneous expression of a fluorescent reporter protein in the hematopoietic system of various transgenic mouse strains (391). Tissue specific promoters or genes in transgenic models often do not reproduce endogenous expression levels as they lack critical enhancer or regulatory elements, and may be subject to positional effects such as epigenetic silencing (414). Inclusion of intronic sequences from rabbit beta-globin gene or simian virus 40 (SV40) intron in the design of the transgene construct can significantly enhance transgene expression (415),(416). The use of insulator sequences has been reported to decrease transgene silencing (417). The IEα-rtTA transgene used in this study did not incorporate any of the enhancer elements described above and this may have resulted in its sub-optimal expression.

The IEα-tTA transgenic mice have been previously reported to drive the expression of their tet-reponsive reporter exclusively in the thymus, with minimal induction of reporter expression in peripheral tissues (418),(389). We were able to detect both, IGRP and proinsulin expression in the peripheral tissues of TII and TIP mice (Figure 2-3 and 2-5-D); however, the level of proinsulin expression in all three lines of TIP mice was more in the thymus as compared to spleen (Figure 2-5-B). Whether this preferential expression influences antigen-specific tolerance in TIP and TII mice remains to be analyzed.

Two separate transgenic models expressing proinsulin in APC have been described with differing outcomes. The NOD-PI mice (243) were completely protected from insulitis and diabetes, whereas 20% of the transgenic NOD mice expressing proinsulin under the control of invariant chain promoter developed spontaneous diabetes (244). The tet-regulated TIP

mice described in this study are protected from insulitis and cyclophosphamide induced diabetes similar to the NOD-PI mice.

In conclusion, TII and TIP mice offer a novel means for regulating islet antigen expression in APC to induce tolerance to these key antigens in a temporal manner. Although proinsulin expression in APCs prevents diabetes in NOD mice, the change in immune function of antigen-specific cells targeted by such an approach remains to be analyzed. The impact of temporal proinsulin and IGRP expression on development of antigen-specific T cells, and spontaneous diabetes is described in chapter 3 of this thesis.

3 Perinatal tolerance to proinsulin is sufficient to prevent autoimmune diabetes

3.1 Summary

High-affinity self-reactive thymocytes are purged in the thymus, and residual self-reactive T cells, that are detectable in healthy subjects, are controlled by peripheral tolerance mechanisms. Breakdown in these mechanisms results in autoimmune disease, but antigen-specific therapy to augment natural mechanisms can prevent this. We aimed to determine when antigen-specific therapy is most effective. Islet autoantigens, Proinsulin (PI) and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) were expressed in the antigen presenting cells (APCs) of autoimmune diabetes-prone non-obese diabetic (NOD) mice in a temporally controlled manner. PI expression from gestation until weaning was sufficient to completely protect NOD mice from diabetes, insulitis and development of insulin autoantibodies. Insulin-specific T cells were significantly diminished, were naïve and did not express IFNγ when challenged. This long lasting effect of a brief period of treatment suggests autoreactive T cells are not produced subsequently. We tracked $IGRP_{206\text{-}214}$-specific $CD8^+$ T cells in NOD mice expressing IGRP in APCs. When IGRP was expressed only until weaning, $IGRP_{206\text{-}214}$-specific $CD8^+$ T cells were not detected later in life. Thus anti-islet autoimmunity is determined during early life and autoreactive T cells are not generated in later life. Bolstering tolerance to islet antigens in the perinatal period is sufficient to impart lasting protection from diabetes.

3.2 Introduction

The immune system normally functions to recognize and defend against foreign pathogens by utilizing a highly diverse repertoire of specific T and B cells. A large number of these cells recognize self-components, and must be eliminated or silenced by a process known as immune tolerance. Autoimmune disease results from failure of tolerance mechanisms. While it is normal to have detectable autoreactive T cells, clinical disease only affects a minority of the population (419). Restoration of the tolerant state is an important goal in the treatment of autoimmune diseases. Antigen-specific therapy is attractive to re-establish tolerance, but has not been successful thus far in autoimmune diseases (420).

One of the major hurdles for antigen-specific therapies is that it is often introduced when immunological markers of autoimmunity, such as autoantibodies, are detectable. At this time naive antigen-specific T cells have differentiated into antigen-experienced memory cells and the T cell responses have diversified beyond the initiating antigen (256). It is difficult to induce immune tolerance in these memory cells.

In contrast there may be a ‘window of opportunity’ during the neonatal period, a time that has several life-long consequences for the immune system. Epidemiological data in humans and mechanistic studies in animal models indicate that priming of the immune system during the neonatal period critically influences host susceptibility to allergic and autoimmune diseases in later life (372, 373). In humans and mice (421), the development of the immune system is said to be “layered” as hematopoiesis occurs in distinct waves. The initial wave of T cells that develops during early life adopts a tolerogenic fate upon encountering antigens (422). During this period, T cells have reduced capacity for acquisition of effector function due to immature antigen presentation (419). Consistent with this, it was recently shown that Aire, which controls expression of self-antigens in the thymus, was critical only until weaning but that it is dispensable beyond weaning age (389).

Autoreactivity to proinsulin (PI) is central to autoimmune diabetes development in non-obese diabetic (NOD) mice and in humans (257). Anti-islet autoimmunity in NOD mice is

preventable by tolerogenic expression of PI throughout life in the antigen presenting cells (APCs) (243, 244). However, insulin-based trials to prevent or reverse diabetes in humans have failed to demonstrate therapeutic benefit (423). While factors including delivery of insulin (instead of PI), which does not cover all the antigenic epitopes, uncertainty over the optimal dose, and the most efficacious route of administration may have contributed to lack of clinical efficacy, timing of intervention appears to be a more critical factor. Based on the evidence presented above, we hypothesize that the perinatal period represents the optimal time for intervention for life long tolerance. Here we studied the impact of induced ectopic islet autoantigen expression until weaning on the number and phenotype of antigen-specific T cells, pancreatic islet inflammation (insulitis) and autoimmune diabetes development in NOD mice.

3.3 Methods

3.3.1 Mice

NOD/Lt mice and C57BL/6 mice were bred and housed at the Bioresources Centre, St. Vincent's hospital (Fitzroy, Victoria, Australia). NOD-IEα-tTA mice were provided by Profs. C. Benoist & D. Mathis (Dept of Pathology, Harvard University). NOD mice expressing GFP under control of FoxP3 promoter (NOD.FoxP3.GFP mice), NOD mice lacking insulin 2 gene (NOD.Ins2.KO mice) and NOD.RAG1$^{-/-}$ mice were obtained from The Jackson Laboratory, (Maine USA). NOD8.3 mice express the TCRαβ rearrangements of the H-2K^{d}-restricted, β cell-reactive, CD8^{+} T cell clone NY8.3 (206). All mice were bred, maintained and used under specific pathogen free conditions at St Vincent's Institute (Melbourne, Australia). All experimental procedures followed the guidelines approved by the institutional animal ethics committee.

3.3.2 Doxycycline treatment

Doxycycline was administered to TIP and TII mice via drinking water containing Doxycycline hyclate (Sigma Aldrich) powder (2mg/ml) for the desired duration. For long-term doxycycline treatment TIP and TII mice were fed with a diet containing 600mg doxycycline /kg food (Specialty Feeds, Glen Forrest, WA).

3.3.3 Diabetes and Insulitis

Female mice were monitored for diabetes development for 300 days as described (424). Mice with two consecutive blood glucose measurements of ≥ 15mM/L were considered diabetic. Immunohistochemical staining of frozen pancreata for insulitis scoring was performed as previously described (273). For adoptive transfer of diabetes, 2x10^{7} splenocytes from TIP mice in cohorts 1-3 were transferred (i.v) into irradiated (9 Gy) 10-12week old NOD recipients. For co-transfer studies 2x10^{7} splenocytes from diabetic NOD mice were transferred along with 2x10^{7}splenocytes (1:1 ratio) or 2x10^{6} PLN cells (10:1 ratio)

pooled from PLN of 8 donor TIP mice or non-transgenic NOD mice into 8-9 week old NOD $Rag^{-/-}$ recipients and diabetes development was monitored as above.

3.3.4 Flow Cytometry

Antibodies used were anti-CD4 (RM4-5) conjugated to PerCpCy5.5, anti-CD3 (145-2C11) conjugated to FITC or anti-CD3 (500 A2) V500, anti-CD44 (1M7) conjugated to AlexaFlour700 (all BD Biosciences), anti-CD11c (N418), anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-F4/80 (BM8) conjugated to AlexaFlour450 (all BioLegend), anti-CD8a (5H10) conjugated to Pacific Orange (Invitrogen) or anti-CD8a (53-6.7) conjugated to PE-Cy7, anti-CD62L (MEL-14) conjugated to APC-Cy7, anti-IFN-gamma (XMG1.2) conjugated to FITC (all BD Biosciences) anti-CD73 (ebioTY11.8) conjugated to PE-Cy7, anti-FR4 (ebio12A5) conjugated to FITC, anti-FoxP3 (FJK-16S) conjugated to APC and anti-Helios (22F6) conjugated to FITC (All eBiosciences). Intracellular staining for IFN-gamma was performed using the Cytofix/Cytoperm kit (BD Biosciences), FoxP3 and Helios were stained intracellularly using FoxP3/Transcription Fixation/Permeabilization kit (eBiosciences). Data was collected on LSR Fortessa flow-cytometer (BD) and analyzed using FlowJo (Treestar) software.

3.3.5 Tetramer and magnetic-bead based enrichment

The tetramer and magnetic-bead based enrichment method was previously described (425). I-Ag7 tetramers were obtained from NIH tetramer core (USA), H-$2K^d$ tetramers were purchased from ImmunoID (Parkville, Victoria). To enrich insulin-specific $CD4^+$ T cells or insulin-specific $CD8^+$ T cells, single cell suspensions (10^7 cells) from peripheral lymphoid organs (PLO), (pooled spleen and non-draining lymph nodes), or pooled (3X) draining pancreatic lymph nodes (PLN) were stained with phycoerythrin (PE)-conjugated I-Ag7-INS_{B10-23} (HLVERLYLVCGGEG) tetramer for 1h at room temperature or PE-conjugated H-$2K^d$-INS_{B15-23} (LYLVCGGERL) for 1 hour on ice. Hen Egg Lysozyme I-Ag7-HEL (AMKRHGLDNYRGYSL) tetramer or H-$2K^d$-TUM (KYQAVYTTTL) were used as controls.

Cells were then washed and stained with anti-PE microbeads (Miltenyi Biotec) followed by magnetic separation using an AutoMACSpro (Miltenyi Biotec) according to manufacturer's instructions. The separated fractions were stained and analysed by flow cytometry. $IGRP_{206-214}$ specific $CD8^+$ T cells (H2-K^d, VYLKTNVFL) were stained and enriched as previously described (256). Gating strategy for tetramer enrichment was as follows: single cells were gated on forward and side scatter, and dead cells excluded using propidium iodide. From the live cell population, $CD11c^-CD11b^-B220^-F4/80^-CD3^+$ cells were gated as the T cell population for analysis. Further selection of $CD4^+$ T cells or $CD8^+$ T cells was followed by analysis of the insulin or IGRP tetramer positive population respectively.

3.3.6 Insulin autoantibody (IAA) assay

A non-competitive IAA assay was performed in a 96 well ELISA format as previously described (426). Briefly, an ELISA plate (Costar) was coated with or without human insulin (10ug/ml, Actrapid, Novo Nordisk) overnight at 4^0C. Wells were blocked with PBS containing 2% BSA for 2 hours and room-temperature and then probed with sera from 12-15 week old TIP mice, NOD or C57BL/6 mice (1:10 dilution) for 2 hours. Wells were washed 4 times and a biotinylated anti-mouse IgG1 (AbCam, 1:10000 dilution) antibody was added for 30 minutes. After washing, horse-radish-peroxidase conjugated streptavidin (BioLegend) was added for 15 minutes. The plate was washed 5 times, TMB substrate solution (BioLegend) was added and absorbance was measured at 450nm using a Polarstar (BMG labtech) microplate reader. Each sample was run in duplicate and absorbance (450nm) of test sample without plate bound insulin was subtracted from absorbance of test sample with plate bound insulin to calculate the actual absorbance value for each sample.

3.3.7 CFSE labeling and adoptive transfer

CFSE labeling of $CD8^+$ T cells from NOD 8.3 mice was done as previously described (2). $5x10^6$ CFSE labeled cells were intravenously transferred into TII mice with induced IGRP

expression. Hosts were sacrificed after 3 days and their inguinal and pancreatic lymph nodes were examined for $CFSE^+$ cells.

3.3.8 Immunization and cytokine staining

Insulin $_{B:9-23}$ peptide (100ug) was emulsified in complete Freund's adjuvant (CFA). TIP mice (cohorts 1-3) were immunized subcutaneously (s.c) with either CFA alone or CFA+insulin$_{B:9-23}$ peptide. Insulin tetramer binding $CD4^+$ T cells were enriched from peripheral lymphoid tissue of TIP mice 12-14 days after priming and stimulated in vitro in complete RPMI medium containing 50ng/ml PMA and 250ng/ml ionomycin. Brefeldin A was added 1 hr later and cells were further stimulated for 3 hours, followed by staining for surface markers and intracellular cytokine staining as per manufacturer's instructions (BD Biosciences)

3.3.9 Statistics

Groups were compared using One-way ANOVA with Tukey's multiple comparison test or 2-tailed unpaired t-test as indicated in figure legends. Diabetes incidence curves were compared using Log-Rank Analysis. Statistical significance was defined as P value of less than 0.05. Statistical analysis was performed using GraphPad Prism software (version 6.0).

3.4 Results

3.4.1 PI tolerance in early life is sufficient to prevent diabetes onset

To test if antigen-specific intervention limited to the perinatal period is sufficient to protect from autoimmune diabetes, we utilized TIP mice described in chapter 2 to enable temporal and cell-specific mouse PI expression in the APCs. To dissect the influence of timing of PI expression on tolerance, PI was expressed continuously from gestation until weaning and then turned off to test whether a 'tolerogenic vaccination' with PI for a short period in early life imparted lasting immune tolerance (Figure 3-1-A). When PI expression was induced until three weeks of life (cohort 3), the mice were completely protected from insulitis and diabetes, similar to mice that had PI expression induced though out life (cohort 2) and previously described NOD PI mice (243). Peri-islet infiltrate was observed in a fraction of islets screened from mice in cohort 3 at 12-15 weeks of age however; the insulitis did not progress further when examined at a later age around 30 weeks. In the control mice with no induction of PI expression (cohort 1), the severity of insulitis and diabetes was similar to WT NOD mice (Figure. 3-1-B, C and D). When PI expression was limited to gestation and turned off at birth in TIP mice, no protection from diabetes was observed (Figure 3-1-E), indicating that PI expression in APCs confined to gestation does not impact diabetes development.

3.4.2 T cells from proinsulin tolerant TIP mice lack pathogenic potential

Splenocytes from mice that had PI expression induced until weaning lacked diabetogenic potential as they were unable to transfer diabetes to irradiated NOD recipients, similar to splenocytes from mice that expressed PI throughout life. In contrast, splenocytes from mice with no induction of PI expression retained their diabetogenic potential (Figure. 3-2-A). Temporal tolerance to PI also impacted the response to the downstream antigen IGRP (257) as the number of $IGRP_{206\text{-}214}$-specific $CD8^+$ T cells was significantly reduced in mice that had PI expressed until three weeks of life or throughout life

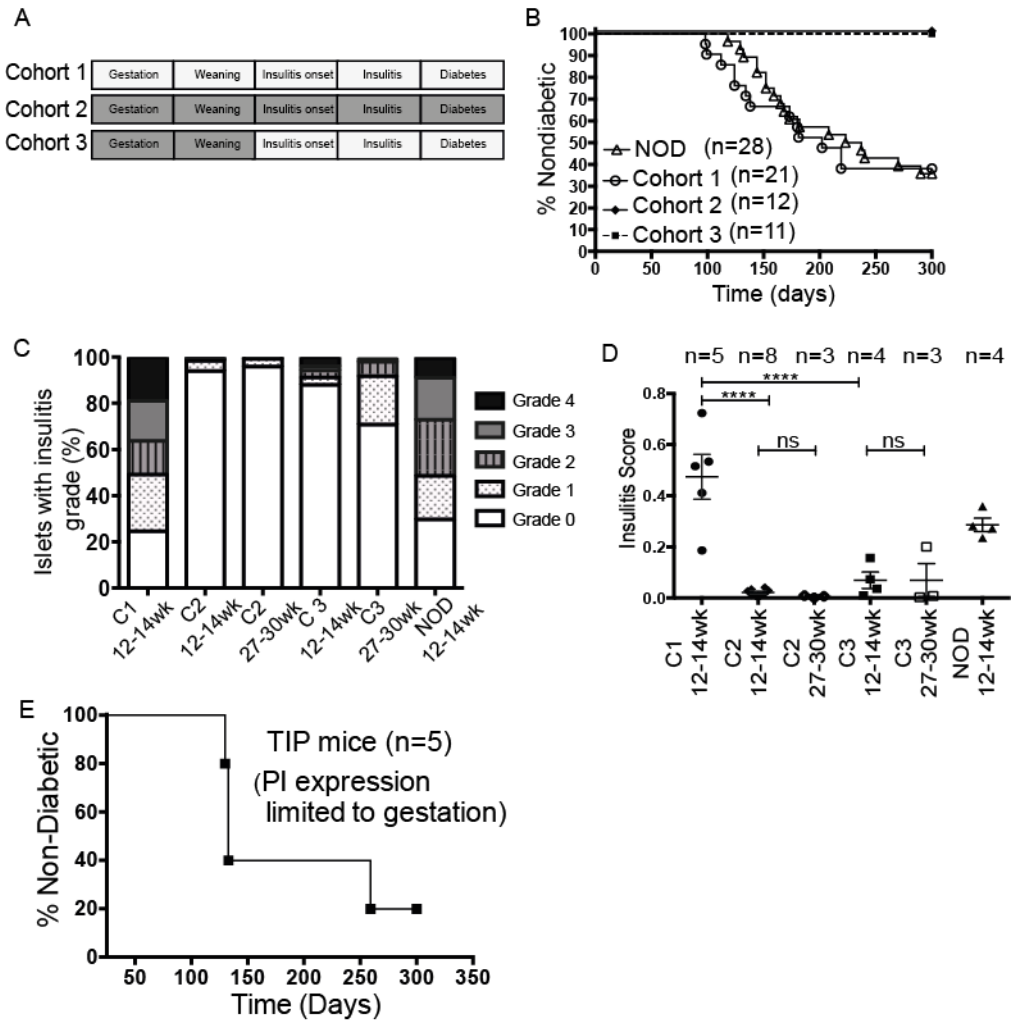

Figure 3-1: Temporal proinsulin expression and spontaneous diabetes development in TIP mice

(A) Study design depicting different cohorts of TIP mice with the duration of induced PI expression indicated by the filled area. **(B)** Incidence of diabetes in cohorts 1-3 of TIP and WT-NOD mice. P= 0.0006 NOD vs cohort 2, P= 0.0009 cohort 1 vs cohort 2 and P= 0.0015 cohort 3 vs cohort 1. **(C)** Histological grading of insulitis and **(D)** individual insulitis scores (Mean±SEM) for indicated cohorts of TIP mice, n≥3/group, >90 islets scored per mouse. Each symbol in scatter plot **(D)** represents data from an individual mouse. **(E)** Incidence of diabetes in TIP mice with PI expression limited to gestation. ****P<0.0001. Values compared using One-way ANOVA with Tukey's multiple comparisons test **(D)**. Survival curves **(B)** compared using Log-Rank analysis.

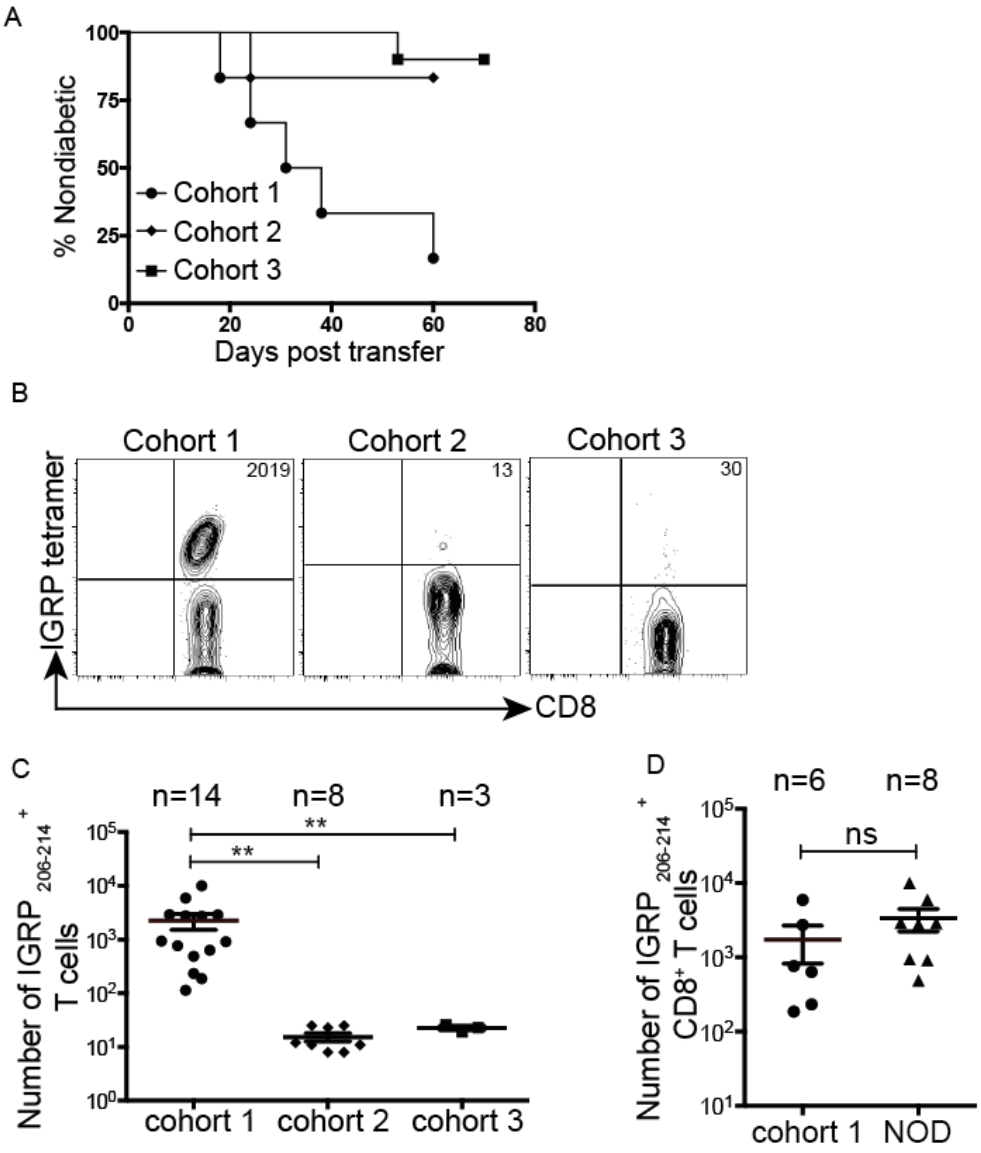

Figure 3-2: T cells from proinsulin tolerant TIP mice lack pathogenic potential

(A) Incidence of diabetes following transfer of splenocytes from 14-16 week old donor TIP mice (cohorts 1-3) into 10-12week old irradiated NOD recipients (n>5 each), P= 0.03 cohort 1 vs cohort 2, P= 0.0012 cohort 1 vs cohort 3. **(B)** IGRP $_{206-214}$ specific CD8+ T cells from pooled peripheral lymphoid organs (PLO) of 12-15 week old TIP and WT NOD mice were stained with tetramer and enriched using magnetic beads and enumerated by flow-cytometry. Representative FACS plots **(B)** and **(C)** enumeration of IGRP $_{206-214}$ tetramer+ CD8+ T cells from PLO of TIP mice in cohorts 1-3. **(D)** Enumeration of IGRP $_{206-214}$ tetramer+ CD8+ T cells from PLO of WT-NOD mice TIP mice (cohort 1). Each symbol in the scatter plots **(C)** and **(D)** (Mean ± SEM) represents data from an individual mouse. **P<0.01, ns = not significant. Data compared using 2-tailed unpaired t-test **(C & D)**. Survival curves **(A)** compared using log-rank analysis.

(Figure 3-2- B,C). Mice with no induction of PI showed expansion of IGRP $_{206-214}$-specific $CD8^+$ T cells comparable to wild-type NOD mice (Figure. 3-2-D).

3.4.3 TIP mice do not develop insulin autoantibodies

As proinsulin-specific $CD4^+$ T cell help is required for B cell humoral response (258), we measured insulin autoantibodies (IAA). IAA in 12-15 week old pre-diabetic mice that had PI expressed until three weeks or life or throughout life were similar to non-autoimmune prone C57BL/6 mice indicating lack of humoral autoimmunity towards insulin in these cohorts (Figure 3-3-A). In contrast, mice with no induction of PI expression had IAA comparable to wild-type NOD mice (Figure.3-3-B), indicating spontaneous anti-insulin autoimmunity. NOD mice express IAA in a heterogeneous manner with IAA being detectable as early as 4 weeks of age and reaching their peak by 8 weeks and declining with age (235). To rule out the possibility that lack of IAA in 12-15 week old TIP mice was due to their early appearance, we measured IAA in 5-6 week old NOD mice and TIP mice (cohort 2 and 3). Similar to TIP mice at 12-15 weeks of age, IAA were not detected at 5-6 weeks of age as compared to NOD mice (Figure 3-3-C), suggesting absence of immune responses to insulin in TIP mice. These results demonstrate that immune tolerance to PI only for a short period in early life is sufficient to completely prevent autoimmunity and diabetes development in NOD mice.

3.4.4 Insulin-specific CD4+ T cell tolerance to PI in TIP mice

In the previous studies showing protection from diabetes by transgenic expression of PI in the APCs, T cell tolerance to PI was not demonstrated directly (243, 244). Here, we demonstrate tolerance to PI in a number of ways. We enumerated the frequency of insulin reactive $CD4^+$ T cells using I-A (g^7) tetramer and an I-A (g^7) tetramer specific for a Hen Egg Lysozyme (HEL) epitope was used as a negative control (Figure 3-4-A).

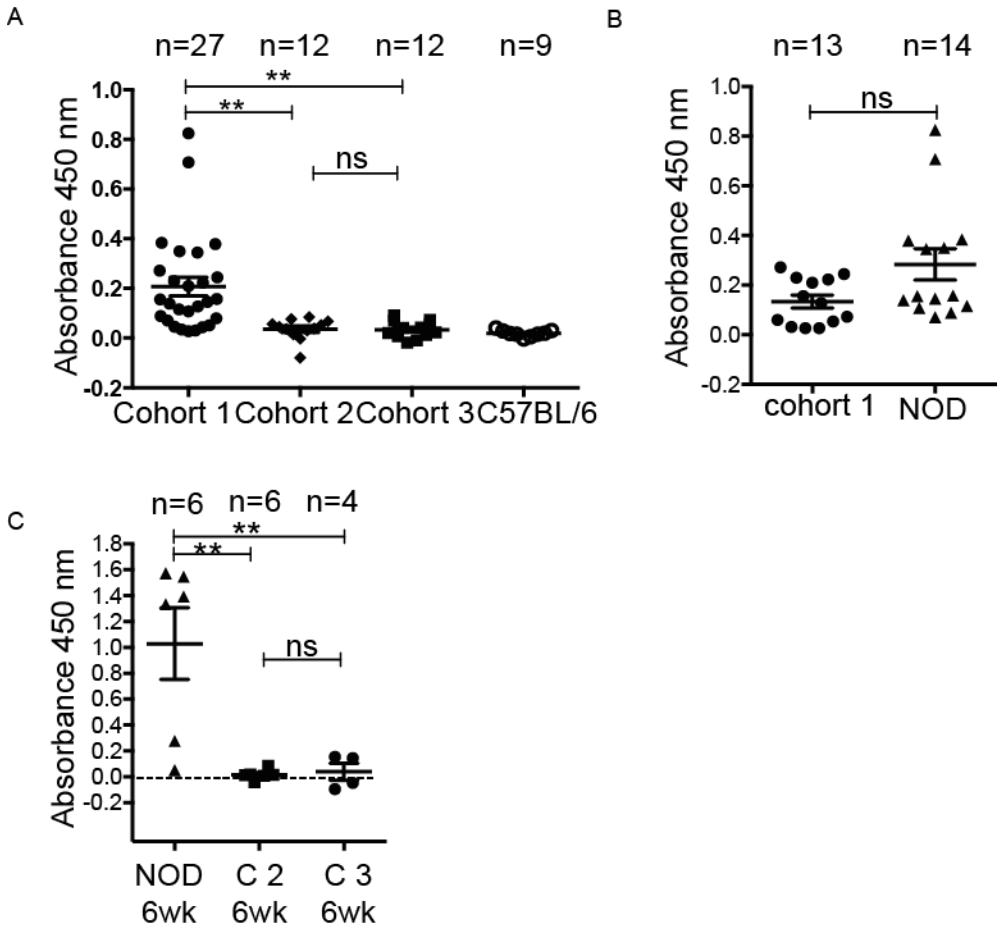

Figure 3-3: TIP mice do not develop insulin autoantibodies

(A) Sera from 12-15 weeks old TIP mice or control C57BL/6 mice, **(B)** 12-15 week old WT-NOD mice and TIP mice (cohort1) and **(C)** sera from 6 weeks old WT-NOD mice and TIP mice (cohorts 2 and 3) were tested for presence of insulin autoantibodies (IAA) by ELISA assay. Absorbance values at 450nm are plotted. Data plotted as Mean ± SEM. Each symbol in scatter plots **(A-C)** represents data from an individual mouse. **P<0.01, ns = not significant. Data compared using One-way ANOVA with Tukey's multiple comparisons test

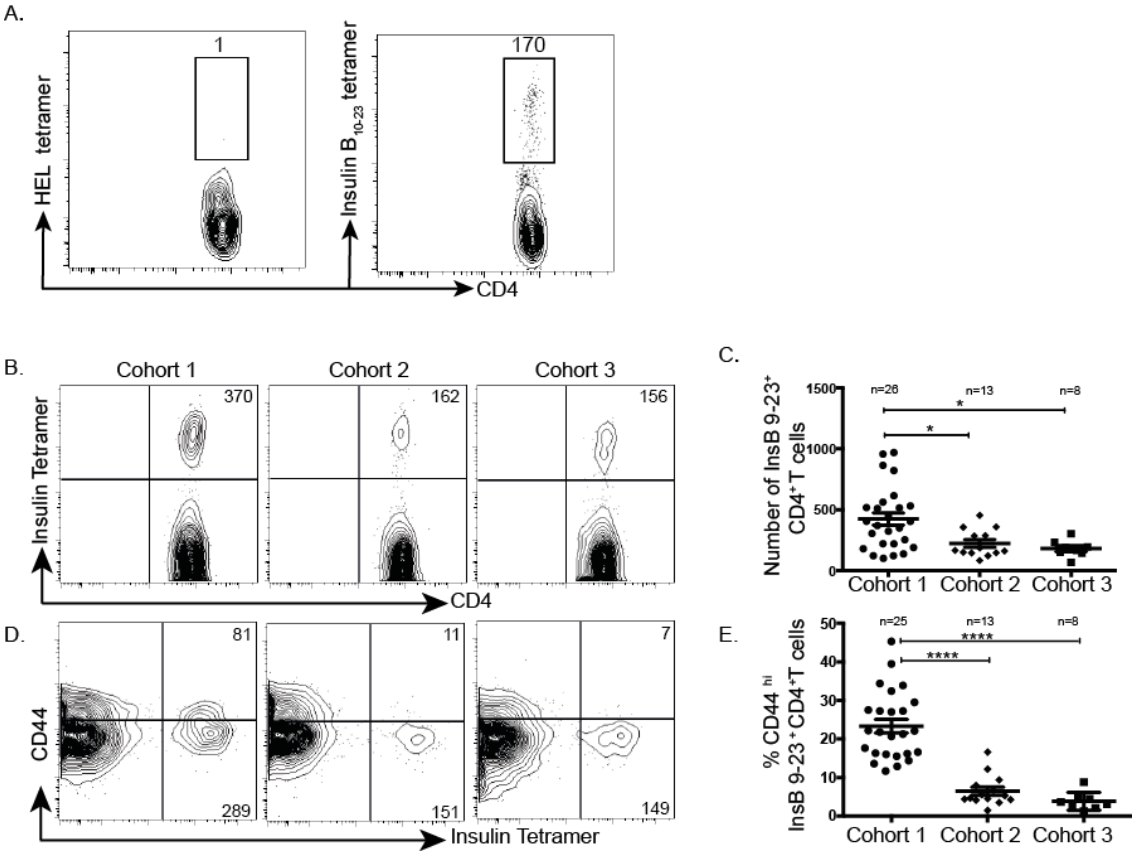

Figure 3-4: Immune tolerance to insulin-specific CD4+ T cells in TIP mice

T cells from pooled peripheral lymphoid organs (PLO) of 12-15 week old TIP mice (cohort 1-3) were stained with I-A (g^7)-Insulin B_{10-23} tetramer to enrich insulin-specific $CD4^+$ T cells using magnetic beads and enumerated by flow-cytometry. I-A (g^7)-Hen Egg Lysozyme (HEL) tetramer was used as a negative control. Representative FACS plots **(A)** showing specificity of I-A (g^7) Insulin tetramer (right) and lack of enrichment of CD4+ T cells due to lack of binding by HEL tetramer (left). Representative FACS plots **(B, D)** and enumeration **(C, E)** of insulin B_{10-23} $tetramer^+$ $CD4^+$ T cells and $CD44^{hi}$ insulin B_{10-23} $tetramer^+$ $CD4^+$ T cells in TIP mice. Values in the FACS plots show absolute number **(A, B and D)** in the indicated gate. Each symbol in the scatter plots (Mean±SEM) represents data from an individual mouse. *P<0.05, ****P<0.0001. One-way ANOVA with Tukey's multiple comparisons test was used to analyze the data.

Surprisingly, insulin specific $CD4^+$ T cells binding to insulin $_{B:10-23}$/ I-A (g^7) tetramer (284) were detected in all cohorts of mice. However, there was a significant reduction in the absolute number of insulin specific $CD4^+$ T cells (Figure. 3-4-B,C). The frequency of antigen-experienced $CD44^{hi}$ subset of insulin specific $CD4^+$ T cells was also significantly reduced in mice that had PI expressed until three weeks or life or throughout life as compared to mice with no induction of PI expression (Figure. 3-4-D,E).

3.4.5 Insulin-specific CD8+ T cell tolerance to PI in TIP mice

We also investigated the impact of transgenic PI expression in APCs by enumerating $CD8^+$ T cells recognizing the insulin B_{15-23} epitope (269) in TIP mice using the insulin H-$2K^d$ B_{15-23} tetramer and a negative control H-$2K^d$-TUM tetramer (Figure 3-5-A). Similar to insulin reactive $CD4^+$ T cells, both the absolute number (Figure 3-5-B,C) and the number of antigen experienced $CD44^{hi}$ (Figure 3-5-D,E) insulin H-$2K^d$ B_{15-23} tetramer binding $CD8^+$ T cells were significantly reduced in the periphery of TIP mice expressing proinsulin throughout life as compared to mice without any induced expression. This indicates that the remaining insulin B_{9-23}-specific $CD4^+$ T cells or insulin B_{15-23} reactive $CD8^+$ T cells in TIP mice could not become activated by antigen, despite it being expressed widely.

3.4.6 Dosage of antigen influences tolerance to proinsulin

The dosage of PI expression in APC had an impact on the frequency of $CD44^{hi}$ subset of insulin specific $CD4^+$ T cells (Figure.3-6-A,B), as we found the proportion of $CD44^{hi}$ $CD62L^{low}$ (Figure 3-6-C,D) subset of insulin specific $CD4^+$ T cells is least in NOD mice over-expressing PI in APC, intermediate in NOD mice and is most in NOD mice with Ins2 gene deleted (272). Collectively, these experiments demonstrate that, a few insulin specific $CD4^+$ and $CD8^+$ T cells (after negative selection of pathogenic T cells) are detectable in mice with PI expression for the first three weeks of life or throughout life.

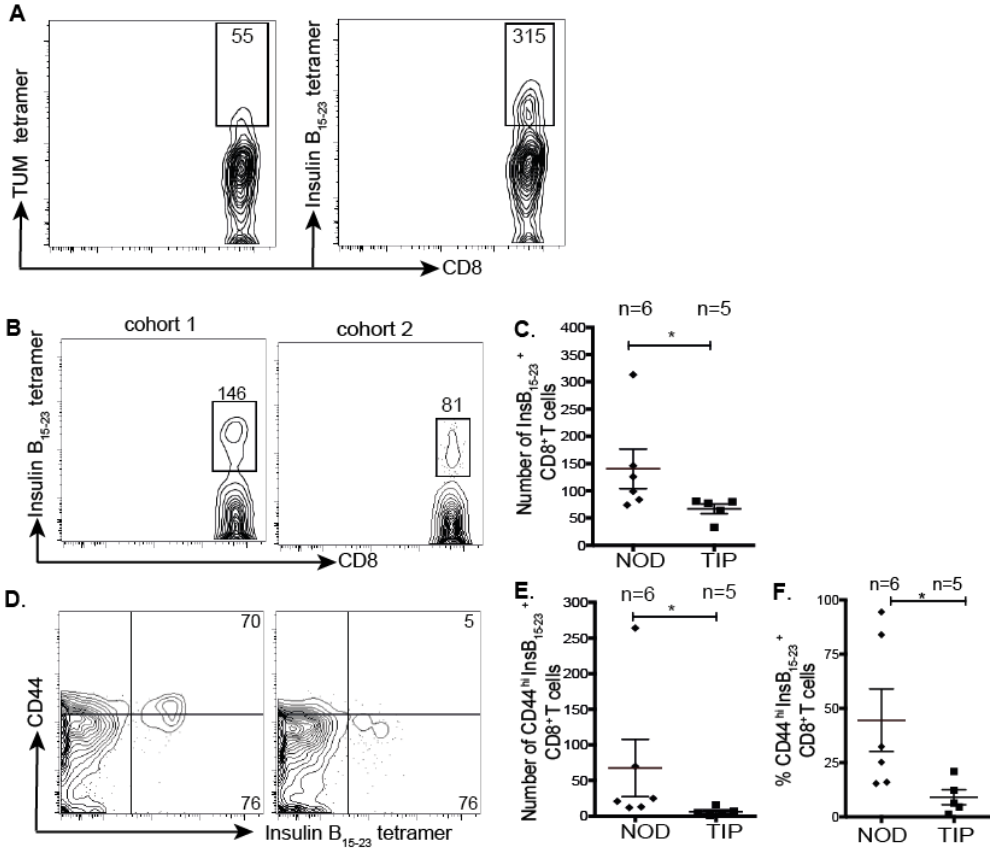

Figure 3-5: Immune tolerance to insulin-specific CD8+ T cells in TIP mice

T cells from pooled peripheral lymphoid organs (PLO) of 12-15 week old TIP mice were stained with H-2K^d-Insulin B $_{15\text{-}23}$ tetramer to enrich insulin-specific CD8$^+$ T cells using magnetic beads followed by flow-cytometry. H-2K^d-TUM tetramer was used as a negative control. **(A)** Representative FACS plot showing specificity of H-2K^d Insulin $B_{15\text{-}23}$ tetramer, (right) and no enrichment of CD8+ T cells due to lack of binding by TUM tetramer (left). Representative FACS plots **(B, D)** and enumeration **(C, E and F)** of Insulin $B_{15\text{-}23}$ tetramer$^+$ CD8$^+$ T and CD44^{hi} Insulin $B_{15\text{-}23}$ tetramer$^+$ CD8$^+$ T cells from PLO of TIP mice in cohorts 1 & 2.

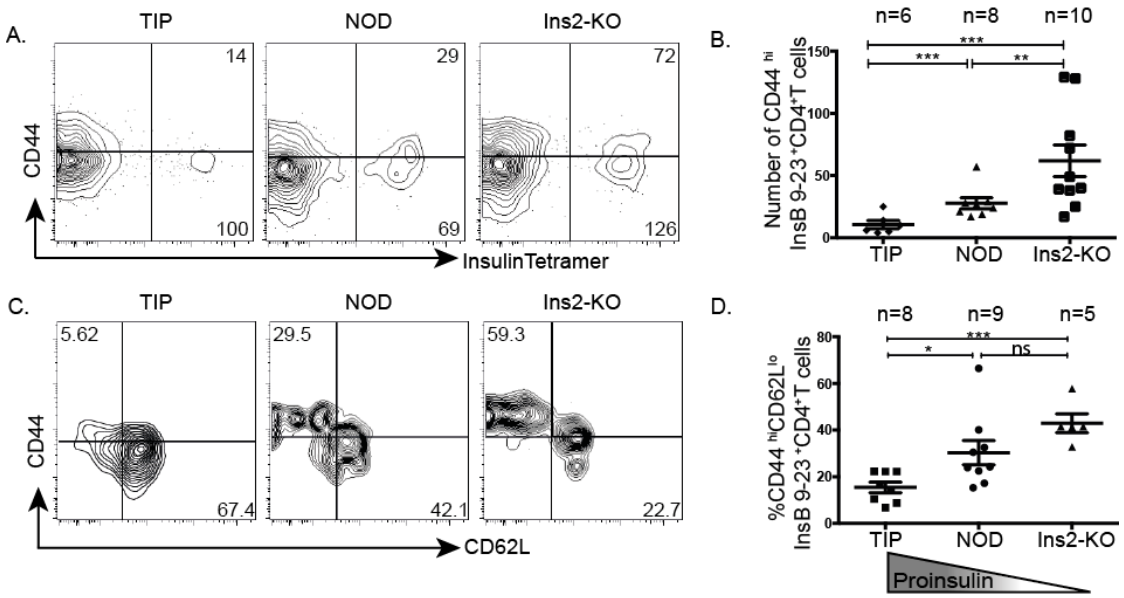

Figure 3-6: Dosage of antigen influences tolerance to proinsulin

Insulin specific $CD4^+$ T cells were stained with I-A (g^7) tetramer and enriched from pooled peripheral lymphoid organs (PLO) of 12-15 week old TIP mice (cohort 2), NOD mice or INS2-KO mice using magnetic beads and enumerated by flow-cytometry. Representative FACS plots **(A, C)** and enumeration **(B)** of insulin B_{10-23} tetramer+ CD44hi CD4+ T cells and percentage **(D)** of insulin B_{10-23} tetramer+ CD44hi CD62Llo CD4+ T cells in the TIP, NOD and Ins2.KO mice. Values in the FACS plots show absolute number **(A)** and percentage **(C)** in the indicated gate. Each symbol in the scatter plots (Mean±SEM) represents data from an individual mouse. *P<0.05, **P<0.01, ***P<0.001. One-way ANOVA with Tukey's multiple comparisons test was used to analyze the data

3.4.7 Residual insulin-specific T cells are not anergic

The inability of remaining insulin reactive T cells in the periphery of PI tolerant TIP mice to up-regulate activation markers may be due to induction of a state of unresponsiveness or anergy caused by prolonged antigen exposure. To investigate this possibility, we made use of CD73 and FR4 that have been recently described as markers of T cell anergy (427, 428) The frequency of insulin tetramer$^+$ CD4$^+$ T cells that expressed CD73 and FR4 was similar in TIP mice with and without induction of PI expression (Figure 3-7-A,B), indicating that induced PI expression did not enhance anergy induction in the residual insulin reactive CD4$^+$ T cells.

3.4.8 Residual insulin-specific T cells are functionally impaired

To further evaluate the functionality of the residual insulin specific CD4+ T cells, we examined their ability to produce IFNγ following stimulation, as it is a key cytokine implicated in pathogenesis of autoimmune diabetes (167). Following stimulation with PMA and ionomycin, very few insulin specific CD4$^+$ T cells from mice that had PI expressed until three weeks or life or throughout life produced IFNγ as compared with cells from mice with no induction of PI expression (Figure 3-7-C,D). These data suggest that residual insulin reactive CD4+ T cells in TIP mice are functionally impaired and their phenotype in mice expressing PI until three weeks is similar to mice with life long PI expression.

3.4.9 Frequency of regulatory T cells is not increased in TIP mice

Antigen expression in thymic APCs has been shown to induce regulatory T cells (Tregs) (429). Moreover, Tregs generated during neonatal period have been shown to be particularly important in protection from autoimmunity (430). To test this, TIP mice were crossed with NOD.FoxP3.GFP reporter mice. The frequency of Foxp3$^+$GFP$^+$ Insulin $B_{10\text{-}23}$ tetramer$^+$ CD4$^+$ T cells or non-tetramer binding Foxp3$^+$CD4$^+$ T cells was comparable in the periphery (Figure.3-8-A, B and C) and thymus (data not shown) of TIP.Foxp3.GFP and

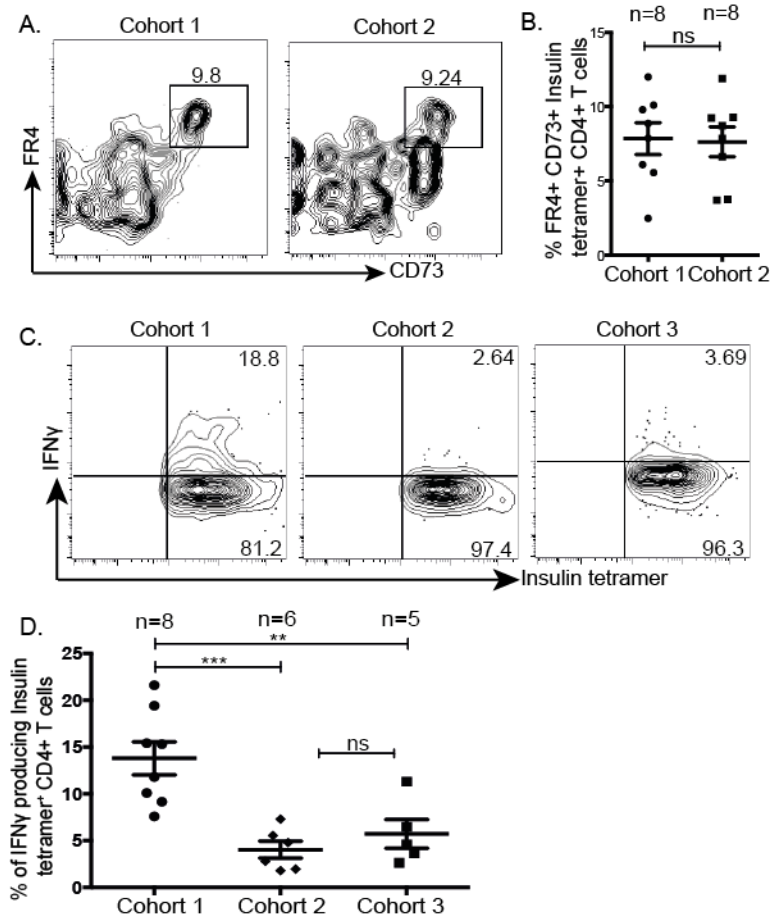

Figure 3-7: Phenotype of antigen-specific T cells in TIP mice

Insulin specific $CD4^+$ T cells were stained with insulin $B_{10\text{-}23}$-I-A(g^7) tetramer and enriched from pooled peripheral lymphoid organs (PLO) of 12-15 week old TIP mice in indicated cohorts using magnetic beads and enumerated by flow-cytometry. Representative FACS plots **(A)** and frequency **(B)** of $FR4^+$ $CD73^+$ Insulin $B_{10\text{-}23}$ $tetramer^+$ $CD4^+$ T cells in TIP mice. Representative FACS plots **(C)** and frequency **(D)** of intracellular IFNγ secreting insulin $tetramer^+$ $CD4^+$ T cells in indicated cohorts of TIP mice. Value in the FACS plots **(A, C)** shows percentage in the indicated gate. Each symbol in the scatter plots (Mean±SEM) represents data from an individual mouse. **P<0.01, ***P<0.001, ns= not significant. Values compared using 2-tailed unpaired *t*-test **(B)** and One-way ANOVA with Tukey's multiple comparisons test **(D).**

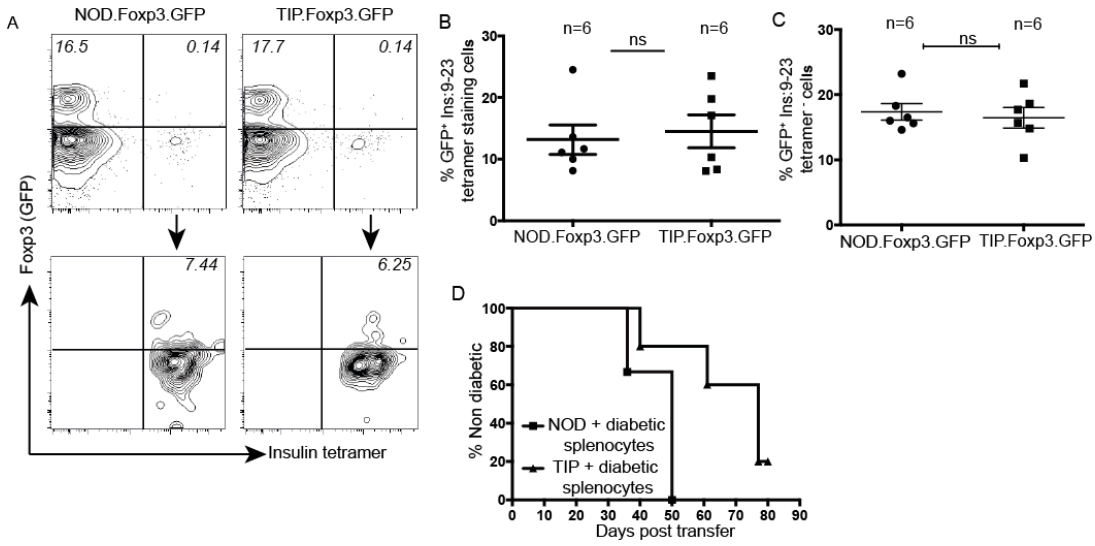

Figure 3-8: Frequency of regulatory T cells is not increased in TIP mice

TIP mice were bred with NOD.FoxP3.GFP reporter mice. Frequency of Foxp3 expressing insulin specific $CD4^+$ T cells was determined by enumerating GFP^+ insulin $tetramer^+$ $CD4^+$ T cells. **(A)** Representative FACS plots showing GFP reporter expression on insulin $tetramer^+$ and non-$tetramer^+$ $CD4^+$ T cells. Frequency of GFP^+ insulin $tetramer^+$ $CD4^+$ T cells **(B)** and GFP^+ insulin $tetramer^-$ CD4+ T cells **(C)** enriched from PLOs of indicated mice. Each symbol in the scatter plot (Mean±SEM) represents data from an individual mouse. **(D)** Incidence of diabetes in 8-9 week old NOD.Rag-/- recipient mice after co-injection of splenocytes from diabetic NOD mice along with splenocytes from either non-transgenic NOD mice (n=3 recipients) or TIP mice (n= 5 recipients) at an equal ratio ($2x10^7$ cells of each per recipient) (P= ns). Value in FACS plots **(A)** shown in italics indicates percentage. Data analysed using 2-tailed unpaired t-test, ns= not significant **(B, C).** Survival curves **(D)** compared using log-rank Test.

NOD.FoxP3.GFP reporter mice. Furthermore, splenocytes from TIP mice tolerant to PI, co-transferred with splenocytes from diabetic NOD mice (1:1 ratio) into NOD.Rag2$^{-/-}$ recipients were unable to delay or prevent diabetes onset in the recipients (Figure.3-8-D), indicating lack of dominant tolerance in TIP mice.

3.4.10 Protection from diabetes in TIP mice is not due to regulatory T cells.

The initial priming of autoreactive T cells in NOD mice occurs in the draining pancreatic lymph nodes (PLN) as early as 3 weeks of age (54). Therefore, induced PI expression in TIP mice during early life may lead to generation of antigen specific Tregs in the PLN. We addressed this possibility using two different approaches, firstly, we enumerated the frequency of insulin tetramer $^{+}$ Foxp3^{+} CD4^{+} T cells in PLN of TIP mice in cohort 1(No PI expression), cohort 2 (continuous PI expression) and cohort 3 (PI expression confined till weaning) (Figure 3-9-A,B). The absolute number of insulin tetramer binding Foxp3^{+} CD4^{+} T cells (Figure 3-9-C (top left panel)) was comparable in the PLN of TIP mice in all cohorts, however there was a reduction in the number of insulin tetramer $^{+}$, Foxp3^{-} CD4^{+} effector cells in the PLN of TIP mice in cohort 2 and 3 as compared to cohort 1 (Figure 3-9-C (bottom left panel)). The reduction in the number of insulin tetramer^{+} CD4^{+} effectors altered the ratio of insulin specific Treg: Teff cells in PLN of TIP mice (cohorts 2 and 3), evident by the increase in the proportion of insulin tetramer^{+} CD4+ FoxP3^{+} Tregs (Figure, 3-9-C (Top right panel), and a concomitant decrease in proportion of insulin specific CD4^{+} FoxP3^{-} effector T cells in PLN of TIP mice expressing PI continuously (cohort 2) or limited until weaning (cohort 3) as compared to those in cohort 1 without induced PI expression (Fig 3-9-C (bottom right panel). Also, the majority of insulin tetramer binding FoxP3+ CD4+ Treg cells detected in PLN were thymus-derived nTregs as determined by their expression of the transcription factor Helios (431) (Figure 3-9-D), suggesting that the Treg cells detected in the periphery were not induced T regulatory cells.

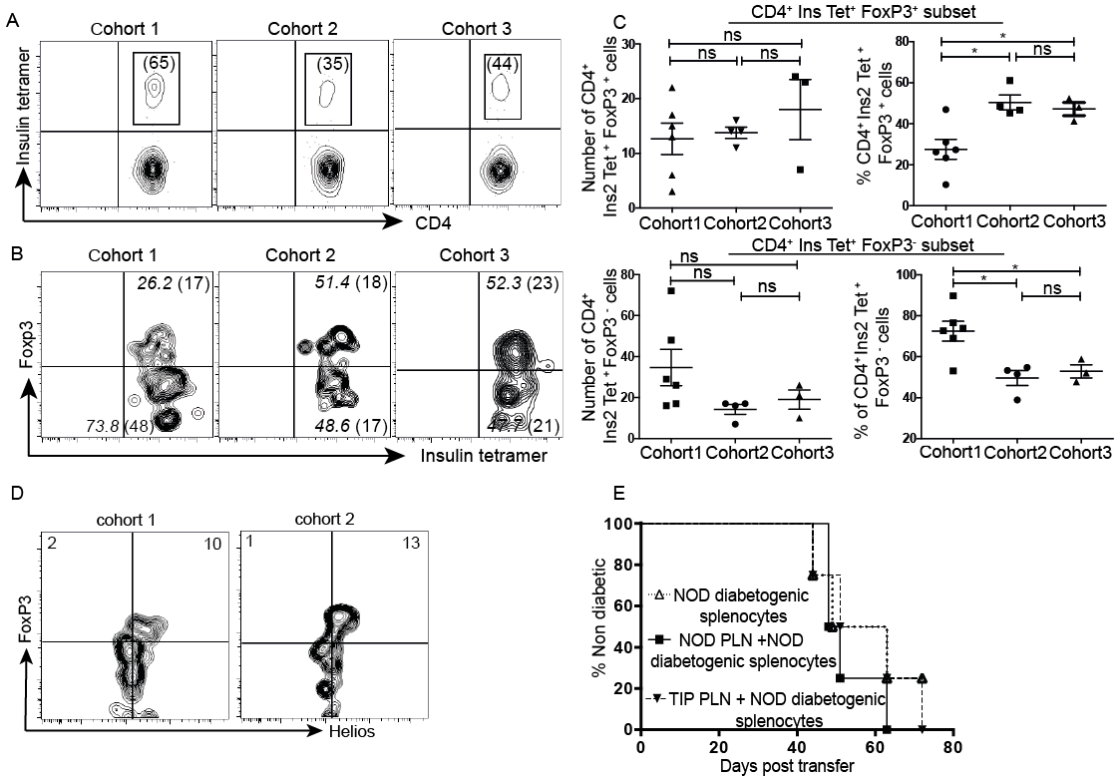

Figure 3-9: Protection from diabetes in TIP mice is not due to dominant tolerance

Insulin B_{10-23} tetramer$^+$ CD4$^+$ T cells were enriched from PLNs of TIP mice and intracellular FoxP3 expression was analysed by flow-cytometry. Representative FACS plots **(A,B)** showing FoxP3 expression on Insulin tetramer+ cells in cohorts 1-3 of TIP mice. Cumulative data from 3-4 independent experiments **(C)** showing number and proportion CD4+ tetramer+ Foxp3+ Treg subset (top) and CD4+ tetramer+ Foxp3- T effector subset (bottom). Each symbol in the scatter plots **(C)** represents data from pancreatic lymph nodes (PLNs) pooled from 3 mice (Mean±SEM). **(D)** Representative FACS plot showing expression of Helios and FoxP3 on Insulin B $_{10-23}$ tetramer$^+$ CD4$^+$ T cells enriched from pooled PLN of TIP mice. Value in FACS plots **(A, D)** indicates absolute numbers Value in FACS plots **(B)** shown in italics indicate percentage and values in parantheses indicate absolute numbers. Incidence of diabetes **(E)** in 8-9 week old NOD.Rag-/- recipient mice after co-injection of splenocytes from diabetic NOD mice alone or along with cells from PLN of 12-14 week old non-transgenic NOD mice or TIP mice (cohort 3) (10:1 ratio, $2x10^7$ splenocytes : $2x10^6$ PLN cells per recipient, n=4 recipients per group), P= ns. Data analysed using One-way ANOVA with Tukey's multiple comparisons test **(C)**. Survival curves **(E)** compared using log-rank Test. *P<0.05, ns= not significant

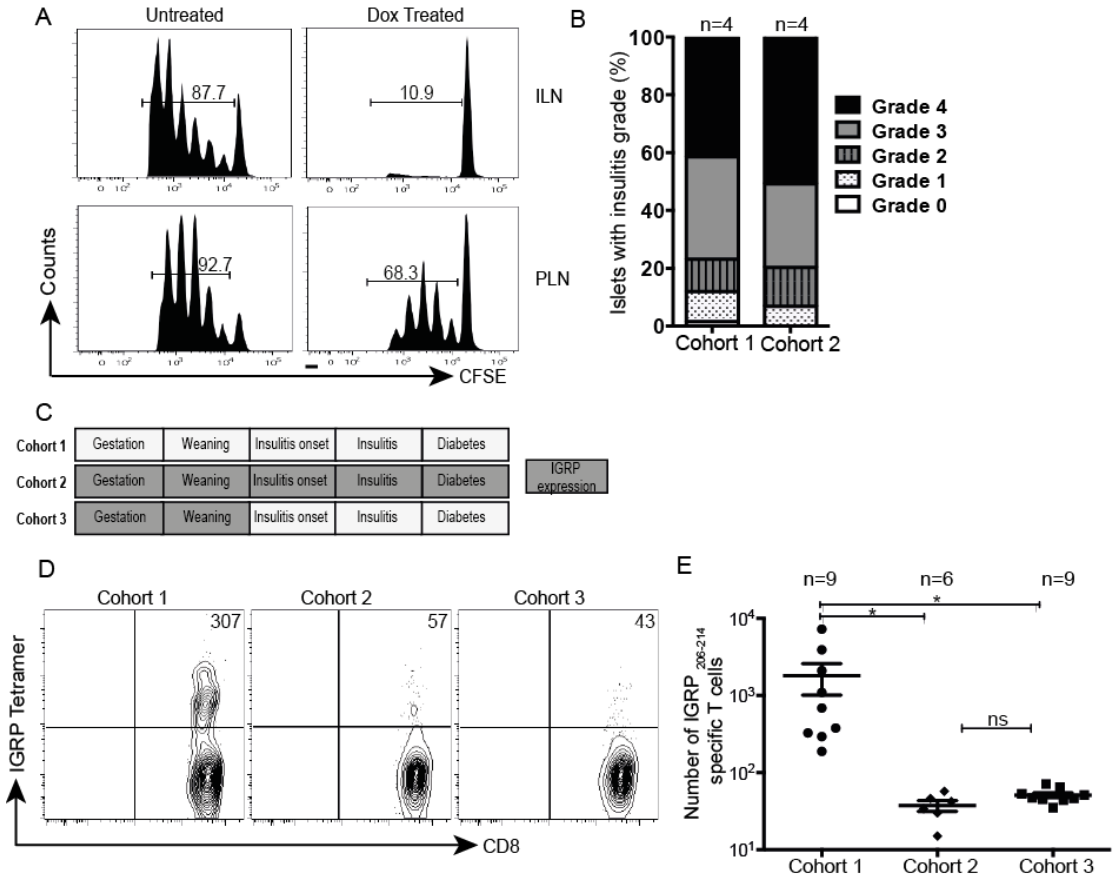

Figure 3-10: IGRP reactive T cells develop in early life

(A) Dox regulated and functional expression of IGRP in the APC of TII mice. CFSE labeled $CD8^+$ T cells from a NOD 8.3 mouse were transferred into 8-week old TII mice that were untreated, or fed Dox for 1 week to turn off IGRP transgene (n=2-3 per group). Recipients were sacrificed 3 days post-transfer and their inguinal (ILN) and pancreatic (PLN) lymph nodes were analyzed for $CFSE^+$ cells. Numbers within each histogram plot indicate percentage of CFSE low cells. Data is representative of 2 independent experiments. **(B)** Histological grading of insulitis development in pancreata of 15-18 week old TII mice (cohort 1 and 2) **(C)** Study design showing the different cohorts of TII mice with duration of IGRP expression indicated by the shaded area. **(D)** Representative FACS plots showing absolute number of tetramer+ IGRP206-214-specific CD8+ T cells and **(E)** enumeration of IGRP206-214-specific CD8+ T cells enriched from PLO of 12-14 week old TII mice (cohort 1-3). Values in the FACS plot show absolute number for the indicated gate. Each symbol in the scatter plot (Mean±SEM) represents data from an individual mouse. *P<0.05, ns= not significant. Values compared using One-way ANOVA with Tukey's multiple comparisons test **(E).**

Secondly, T cells from PLN of TIP mice with induced PI expression from gestation until weaning were co-transferred at a 1:10 ratio with splenocytes from diabetic WT- NOD mice into NOD.Rag2$^{-/-}$ recipients to examine their suppressive potential. Transfer of diabetes was similar in all groups tested (Figure 3-9 E). However, the data presented here need to be strengthened further by increasing the group sizes and replicates along with conclusive evidence using positive controls to demonstrate that Tregs transferred from PLN survive in the recipient mice. Taken together, these data along with the observation that induced insulin expression in APCs did not lead to an increase in the frequency of either insulin tetramer binding or non-tetramer binding FoxP3+ Tregs in other organs such as the thymus (data not shown) suggest that protection from diabetes observed in TIP mice expressing PI continuously or until weaning is not due to dominant tolerance exerted by insulin-specific FoxP3^{+} Tregs.

3.4.11 Autoreactive T cells predominantly exit the thymus during the early neonatal period.

IGRP$_{206-214}$-specific CD8^{+} T cells are the highest frequency antigen-specific T cells in the NOD mouse and expression of IGRP in APCs completely deletes IGRP$_{206-214}$-specific CD8^{+} T cells (257)**,** which makes them suitable to track *in vivo* after intervention to induce tolerance. To study the mechanism of robust life-long tolerance when autoantigen is expressed only until weaning, we generated Tetracycline Inducible IGRP (TII mice) for conditional expression of IGRP in APCs. The conditional expression of IGRP in the APCs of TII mice was validated as previously described (257) (Figure.3-10-A). As previously shown (257), transgenic expression of IGRP in TII mice did not impact insulitis progression (Figure 3-10-B). We divided these mice into three cohorts similar to TIP mice (Figure.3-10-C) and assessed the frequency of IGRP$_{206-214}$-specific CD8^{+} T cells in the PLO of 12-15 week old mice. As previously shown, we found very few cells when IGRP was expressed throughout

life and $IGRP_{206-214}$-specific $CD8^+$ T cells could be easily detected in TII mice with no induction of IGRP expression. Interestingly, we also found very few $IGRP_{206-214}$-specific $CD8^+$ T cells when IGRP was expressed until weaning (Figure3-10-D,E). This indicates that diabetogenic autoreactive T cells emerge from the thymus only before weaning and these cells undergo robust tolerance when antigen is expressed in APCs during this period.

3.5 Discussion

We show that early life, before the onset of islet autoimmunity in NOD mice, is a critical period for therapeutic intervention. In children who are genetically at-risk for developing T1D, onset of anti-islet autoantibodies peaks between 9-24 months of age, with anti-insulin antibody appearing first (45), underscoring the possibility that anti-insulin autoimmunity is established during the first few months of life in subjects with a genetic predisposition to T1D. It has been known for decades that early life is an important time for tolerance induction in immature T cells but this study suggests a different but possibly related finding – that autoreactive T cells are not generated throughout life as often thought but are uniquely produced in early life.

Our data both complement and contrast with previous studies from Mathis and Benoist (8, 18) that showed that neonatal Aire expression in AIRE-deficient mice, a model of human Autoimmune Polyglandular Syndrome type 1, is sufficient to prevent systemic autoimmunity and enhances the development of antigen-specific regulatory T cells. Systemic autoimmunity was prevented on the NOD background but autoimmune diabetes was restored. While regulatory T cells are an important tolerance mechanism, we did not find evidence that antigen-specific regulatory T cells play a significant role in augmentation of tolerance to proinsulin in our model. APS-1 is a complex and rare disorder involving multi-organ autoimmunity. We have studied the impact of perinatal expression of the single driver antigen proinsulin on development of type 1 diabetes, a common and prototypical organ-specific autoimmune disease of great clinical significance.

Our findings are consistent with the previous observation from a TCR transgenic mouse model of multiple sclerosis, which suggested that a window of maximum susceptibility to EAE existed at a younger age and that tolerance to myelin specific T cells correlated with levels of antigen expression in vivo (432). Moreover, a recent study using a thymus transplant approach demonstrated that thymic production of islet-reactive T cells in NOD mice was limited to a 10 day window after birth (433). Induction of PI expression in APCs in

TIP mice during embryonic stages up until the weaning age augments central tolerance to antigenic epitopes of PI presented by thymic APCs, by shaping the developing T cell repertoire to be devoid of high-affinity PI-reactive T cells.

Our observations raise the question of whether a tolerance-boosting approach in human T1D patients would mirror the mechanisms observed in NOD mice. The structural and biophysical properties of the MHC class II (I-Ag7) molecule of the NOD mouse and HLA-DQ2-DQ8 are highly conserved, and the MHC peptidome is also identical for both molecules (52). Furthermore, given that the insulin molecule is highly conserved between humans and NOD mice at the amino acid level, it can be envisaged that thymic selection of insulin-reactive T cells in response to peptide epitopes presented would follow similar principles in both NOD mice and humans.

To translate findings from the animal model presented here into an antigen-specific therapy in the clinic, PI tolerance-boosting therapies will need to be administered to very young children who are at risk of developing T1D. This demands that potential PI based therapies have a non-invasive mode of delivery. Oral administration of Lactococcus lactis (*L. lactis*) engineered to express PI and IL-10 in combination with anti-CD3 treatment reversed diabetes stably in NOD mice (434), another study administered *L. lactis* expressing HSP65 to 4-6 week old NOD mice and observed decreased incidence of diabetes (435), indicating that mucosal delivery of islet antigens via the gut presents an attractive non-invasive therapy for treatment of T1D. More recently, Mallone and colleagues using a clever approach fused proinsulin to Fc portion of an antibody, to enable the uptake of this fusion protein from mother to foetus via neonatal FcRn receptor. Treatment of pregnant NOD mice late in gestation with PPI.Fc fusion, reduced diabetes in the offspring (436). The FcRn receptor is also expressed in the gut in the neonates, thus making a case for ‘vaccine’ like antigen-specific treatment during perinatal period with a translational potential.

In conclusion, our finding that a brief exposure to PI confined to the perinatal period in NOD mice imparts long lasting protection from diabetes leads us to suggest that early life is a vulnerable period for the escape of insulin-specific T cells– a different conclusion to the more

accepted view that immature T cells are susceptible to tolerance induction. Ongoing tolerance therapy to insulin is not required in adult life because few, if any, new autoreactive cells develop then. Of course, some individuals develop T1D very late in life but this may be from T cells that originate in early life. Whether this is the case or not, therapeutic interventions targeting PI during early life in high-risk human subjects may prevent diabetes development.

4 Tolerance to proinsulin-1 partially protects NOD mice from autoimmune diabetes

4.1 Summary

Two highly homologous isoforms of proinsulin genes co-exist in mice. Immune tolerance to proinsulin-2 imparts robust protection from autoimmune diabetes in the non-obese diabetic (NOD) mice. Whether inducing tolerance to proinsulin-1 would influence diabetes development in NOD mice remains to be investigated. In this study we generated transgenic NOD mice that conditionally express proinsulin-1 in the antigen presenting cells (APCs) namely, **T**et-**I**nducible **P**roinsulin-1 (TIP-1) mice. TIP-1 mice displayed a significantly reduced incidence of spontaneous diabetes, which was associated with reduced severity of insulitis and insulin autoantibody development. Antigen experienced proinsulin specific T cells were significantly reduced in number in TIP-1 mice indicating immune tolerance. Proinsulin-1 expression in APCs impacted the immune response to the downstream antigen IGRP, however, was unable to prevent diabetes in NOD 8.3 mice with a pre-existing repertoire of IGRP reactive T cells. Thus, despite the high homology of proinsulin-1 and -2, tolerance to proinsulin-1 only partially prevents islet-autoimmunity in NOD mice, which suggests an ongoing residual immune response to proinsulin-2 epitopes in TIP-1 mice.

4.2 Introduction

Recognition of proinsulin by the immune system is a major determinant in the pathogenesis of autoimmune diabetes (437),(438). A polymorphic variable number of tandem repeats (VNTR) located in the promoter region of the insulin locus controls the transcription level of the *Ins* gene and is strongly associated with susceptibility to type 1 diabetes (T1D) in humans (14),(15),(439). Mice do not have a VNTR upstream of the insulin locus; however they express two proinsulin isoforms encoded by two distinct genes *Ins1* and *Ins2* (237). Both proinsulin-1 and 2 isoforms are expressed in the pancreatic islets, but differentially expressed in the thymus, with proinsulin-2 being the predominant isoform detected in the thymus (238),(239),(440). Proinsulin 1 and 2 proteins are highly homologous with identical A chains but differ by two amino acids in the B chain, three amino acids in the connecting peptide (C-peptide) and several differences also exist in the leader peptide of the respective preproinsulin molecules (Figure 4-1) (237).

Genetic and biochemical approaches have highlighted further differences between the two-proinsulin isoforms in terms of cellular and humoral immune responses as well as diabetes development in NOD mice. The two insulin genes were deleted to generate *Ins1 -/-* and *Ins2 -/-* mice in the 129 strain of mice (441). Loss of either isoform did not impair glucose tolerance or insulin secretion in the mutant mice. This was attributed to compensatory mechanisms as both genes are expressed in the pancreatic islets (442). To evaluate the consequences of proinsulin 1 or 2 deficiency on the development of autoimmune diabetes, *Ins2 -/-* and *Ins1 -/-* 129 mice were individually backcrossed onto the NOD genetic background. NOD *Ins2 -/-* mice developed accelerated diabetes, which related to loss of central tolerance to insulin peptides caused by the lack of

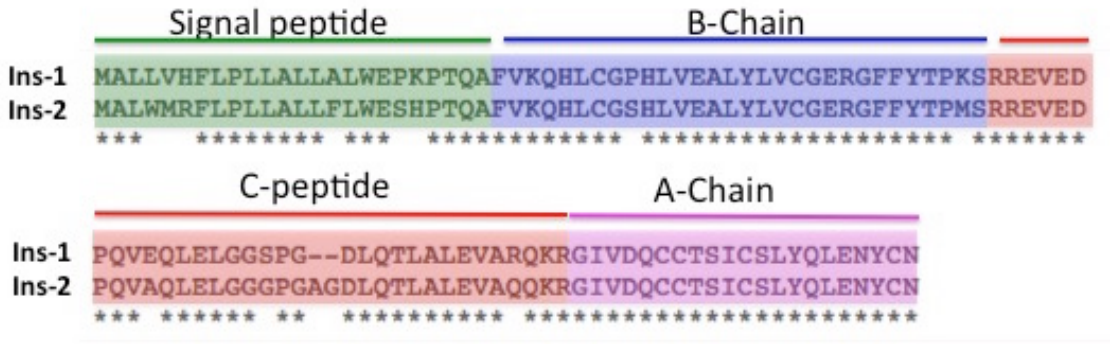

Figure 4-1: Mouse proinsulin proteins are highly conserved

Amino acid sequence alignment of mouse proinsulin-1 and 2 molecules shows a high degree of homology between the two proteins. Conserved residues in the various domains are marked with an asterisk (*), and the non-homologous residues are left blank.

thymic proinsulin 2 expression, moreover development of insulin autoantibodies (IAA) in *Ins2 -/-* mice suggested that proinsulin 1 was the autoantigen targeted by the self-reactive T cells (241). In contrast, loss of *Ins1* prevented insulitis and diabetes in NOD mice; however, IAA production in NOD *Ins1 -/-* mice was similar to unmanipulated mice. Protection from diabetes in NOD *Ins1 -/-* mice is likely due to absence of cognate antigen in the target tissue, suggesting that proinsulin 1 peptides are preferentially targeted by insulin reactive T cells. Detection of IAA may be related to continuing presence of the *Ins2* gene in *Ins1 -/-* mice (240).

A majority of islet infiltrating CD4+T cells isolated from prediabetic NOD mice react to insulin and in particular the B chain amino acids 9-23 (Ins $_{B:9-23}$) (266). Although the two insulin proteins differ by just two amino acids in the B chain (Figure 4-1) different outcomes occurred after immunization of NOD mice with the B:9-23 peptide from either insulin-1 or insulin-2 proteins. Immunization with insulin-2 $_{B:9-23}$ peptide resulted in protection from diabetes which was associated with regulatory responses, whereas insulin 1 $_{B:9-23}$ peptide immunization did not alter disease development and splenocytes from insulin 1 $_{B:9-23}$ immunized mice transferred diabetes with accelerated kinetics (343),(443). These data suggest that insulin 2 $_{B:9-23}$ peptide may induce cross-tolerance to proinsulin-1 reactive T cells, whereas insulin 1 $_{B:9-23}$ peptide may just tolerize proinsulin-1 specific T cells allowing proinsulin-2 specific immune responses to develop normally. A prime role for insulin $_{B:9-23}$ peptide in diabetes development was demonstrated as NOD mice lacking both insulin genes but expressing an insulin transgene with a single amino acid substitution in the B chain were protected from insulitis and diabetes (272). The divergent response to immunization with two insulin B chain peptides in addition to marked protection from diabetes in *Ins 1 -/-* NOD mice suggests that there may be peptide epitopes in the insulin-1 molecule that are diabetogenic.

A systematic study of preproinsulin epitope recognition in NOD mice identified immunogenic epitopes that localized to the leader sequence, C-peptide and B chain of preproinsulin-1 and

2 proteins; moreover, peptides spanning these epitopes were able to stimulate T cells from NOD islets (268). Furthermore, Levisetti and colleagues recently demonstrated that T cells recognizing proinsulin-1 47-64 (PI-1 47-64) epitope located in the C-peptide region could be isolated from prediabetic NOD mice, and that these cells were able to induce diabetes upon transfer into NOD.scid recipients (444). These reports suggest that although insulin-2 $_{B:9-23}$ peptide is the focus of spontaneous T cell reactivity in NOD mice, immune responses also extend to proinsulin-1 epitopes.

As described in chapter 3, expression of proinsulin-2 in the APCs of the NOD mouse induces antigen-specific tolerance and prevents diabetes. Whether inducing tolerance to proinsulin-1 would influence autoimmunity in NOD mice remains to be determined. In this study we generated NOD mice with tetracycline regulated proinsulin-1 expression (TIP-1 mice) in the APCs and examined the impact of ectopic proinsulin-1 expression on the development of antigen-specific T cells as well as insulin autoantibodies, insulitis and autoimmune diabetes.

4.3 Methods

4.3.1 Generation of Transgenic Mice

4.3.1.1 TetO-Ins1 mice

To generate the TetO-Ins1 construct, a 411 bp cDNA fragment spanning the coding region of murine proinsulin-1 (PI-1) was amplified by PCR using NOD pancreatic islet cDNA as a template, and cloned into HindIII and EcoRV sites of the pTRE-tight plasmid (Clontech). A 1100 bp transgene cassette comprising of the TetO-minimal CMV promoter, followed by the PI-1 gene and a polyA signal was excised between Xho I sites and purified for injection into fertilized NOD/Lt ova using standard techniques. Founders and transgene positive offspring were screened by PCR using primers spanning the PI-1 gene (5'-TTAAGATATCTTCATTCATTATAGAACTC -3') and the tetO-CMV promoter (5'-TCAGTGATAGAGAACGTATGTCG -3').

4.3.1.2 Other mice

NOD/Lt mice were bred and housed at the Bioresources centre St. Vincent's Hospital, Fitzroy. The NOD-IEα-tTA mice that drive the expression of tetracycline transactivator (tTA) under the control of MHC class II IEα promoter have been previously described (389) and were obtained from Prof. C. Benoist and Prof. D. Mathis (Dept of pathology, Harvard). NOD8.3 mice express the TCRαβ rearrangements of the H-2K^d-restricted, β cell-reactive, CD8+ T cell clone NY8.3 (206). All mice were bred, maintained and used under specific pathogen free conditions at St Vincent's Institute (Melbourne, Australia). All experimental procedures followed the guidelines approved by the institutional animal ethics committee.

4.3.2 RT-PCR

Total RNA was extracted from freshly dispersed splenic or thymic tissue using Nucleospin RNA XS kits (Macherney-Nagel), and first strand cDNA was generated using High Capacity cDNA Reverse Transcription kits (Applied Biosystem) according to the manufacturers' instructions. Real-time PCR analysis was performed using Rotor-Gene-RG-3000 cycler (Corbett Research, Sydney, Australia). Taqman gene expression primers murine insulin 1 (Ins1; Mm01950294_s1) and murine β-actin (Actb; Mm00607939_s1) were purchased from Applied Biosystems. To determine relative expression, Ct values of Insulin were subtracted from Ct values of β-actin for each sample and the difference was plotted to determine the abundance of the gene of interest.

4.3.3 Doxycycline treatment

Doxycycline was administered to TIP and TII mice via drinking water containing Doxycycline hyclate (Sigma Aldrich) powder (2mg/ml) for the desired duration. For long-term doxycycline treatment TIP and TII mice were fed with a diet containing 600mg doxycycline/kg food (Specialty Feeds, Glen Forrest, WA).

4.3.4 Pancreas immunohistochemistry and Insulitis scoring

For insulitis scoring pancreata were snap frozen in OCT (Sakura Finetek, Torrance, CA). Five µm sections were prepared from 3 levels (200µm apart), and stained with anti-insulin, followed by anti-guinea pig- HRP (All from Dako Corp., Carpenteria, CA). Staining was developed with diaminobenzidine (Sigma Aldrich), and sections were counterstained with haemotoxylin. Insulitis was scored on three sections/mouse using the following scale 0= no infiltrate, 1= peri-islet-infiiltrate, 2= extensive (>50%) peri-islet infiltrate, 3= intraislet infiltrate and 4= extenstive intra-islet-infiltrate (>80%) or total beta cell loss. The percentage of islets with each score was calculated by addition of scores for the three sections.

4.3.5 Flow Cytometry

Antibodies used were anti-CD4 (RM4-5) conjugated to PerCpCy5.5, anti-CD3 (145-2C11) conjugated to FITC or anti-CD3 (500 A2) V500, anti-CD44 (1M7) conjugated to AlexaFlour700 (all BD Biosciences), anti-CD11c (N418), anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-F4/80 (BM8) conjugated to AlexaFlour450 (all BioLegend), anti-CD8a (5H10) conjugated to Pacific Orange (Invitrogen) or anti-CD8a (53-6.7) conjugated to PE-Cy7, anti-CD62L (MEL-14) conjugated to APC-Cy7 (BD Biosciences). Data was collected on an LSR Fortessa flow-cytometer (BD) and analyzed using FlowJo (Treestar) software.

4.3.6 Tetramer and magnetic-bead based enrichment

The tetramer and magnetic-bead based enrichment method was previously described (425). I-Ag7 tetramers were obtained from the NIH tetramer core (USA), H-2K^d tetramers were purchased from ImmunoID (Parkville, Victoria). To enrich insulin-specific CD4+ T cells and IGRP-specific CD8+ T cells, single cell suspensions (10^7 cells) from peripheral lymphoid organs (PLO), (pooled spleen and non-draining lymph nodes), were stained with phycoerythrin (PE)-conjugated I-Ag7-INSB$_{10\text{-}23}$ (HLVERLYLVCGGEG) tetramer for 1 hour at room temperature or PE-conjugated H-2K^d- IGRP$_{206\text{-}214}$ (VYLKTNVFL) tetramer for 1 hour on ice. Hen Egg Lysozyme I-Ag7-HEL (AMKRHGLDNYRGYSL) tetramer or H-2K^d-TUM (KYQAVYTTTL) were used as controls. Cells were then washed and stained with anti-PE microbeads (Miltenyi Biotec) followed by magnetic separation using an AutoMACSpro (Miltenyi Biotec) according to manufacturer's instructions. The separated fractions were stained and analysed by flow cytometry. Gating strategy for tetramer enrichment was as follows: single cells were gated on forward and side scatter, and dead cells excluded using propidium iodide. From the live cell population, CD11c-CD11b-B220-F4/80-CD3+ cells were gated as the T cell population for analysis. Further selection of CD4+ T cells or CD8+ T cells was followed by analysis of the insulin or IGRP tetramer positive population respectively.

4.3.7 Insulin autoantibody (IAA) assay

A non-competitive IAA assay was performed in a 96 well ELISA format as previously described (426). Briefly, an ELISA plate (Costar) was coated with or without human insulin (10ug/ml, Actrapid, Novo Nordisk) overnight at 4^0C. Wells were blocked with PBS containing 2% BSA for 2 hours and room-temperature and then probed with sera from 12-15 week old TIP mice, NOD or C57BL/6 mice (1:10 dilution) for 2 hours. Wells were washed 4 times and a biotinylated anti-mouse IgG1 (AbCam, 1:10000 dilution) antibody was added for 30 minutes. After washing, horse-radish-peroxidase conjugated streptavidin (BioLegend) was added for 15 minutes. The plate was washed 5 times, TMB substrate solution (BioLegend) was added and absorbance was measured at 450nm using a Polarstar (BMG labtech) microplate reader. Each sample was run in duplicate and absorbance (450nm) of test sample without plate bound insulin was subtracted from absorbance of test sample with plate bound insulin to calculate the actual absorbance value for each sample.

4.3.8 Incidence of spontaneous diabetes

Diabetes onset was monitored by weekly measurement of urine glucose levels using Diastix (Bayer Diagnostics). Blood glucose levels were measured in mice with glycosuria using Advantage II Glucose strips (Roche). Animals displaying two consecutive blood glucose measurements of ≥ 15mmol/L were considered diabetic.

4.4 Results

4.4.1 Conditional expression of Proinsulin-1 in TIP-1 mice

NOD mice expressing proinsulin-1 (PI-1) under the control of the tetracycline-responsive promoter (tetO-Ins1 mice) were obtained after injection of the tetO-Ins1 construct into NOD ova. Of the two tetO-Ins1 founder lines (#12 and #45), line #45 was viable and fertile and was crossed with TA-NOD mice to generate bi-transgenic TIP-1 (Tet Inducible PI-1) mice (Figure. 4-2A). This enables conditional expression of PI-1 in thymic and peripheral APCs in a doxycycline (Dox) dependent manner in the progeny carrying both transgenes. Analysis of PI-1 expression in TIP-1 mice revealed that PI-1 transgene was expressed in the thymus and after one week of Dox treatment, PI-1 expression dropped to baseline levels in thymus of TIP-1 mice as measured by RT-PCR (Figure 4-2B). Thus, PI-1 expression in TIP mice was inducible and Dox regulated.

4.4.2 Proinsulin-1 expression in APCs of TIP-1 mice partially protects from insulitis development

Progressive lymphocytic infiltration (insulitis) in the pancreatic islets is the hallmark of disease progression in NOD mice. As described in chapter 3, constitutive or temporal expression of proinsulin-2 in the APCs limited to the perinatal period prevented insulitis in TIP mice. We assessed whether the continuous expression of PI-1 in the APCs of TIP-1 mice was sufficient to influence insulitis development. Analysis of pancreas histology from 10-12 week old TIP-1 mice continuously expressing PI-1 revealed an absence of cellular infiltrates in 80% of the islets examined as compared to age matched TIP-1 mice fed dox to turn-off PI-1 expression or non-transgenic NOD mice where >70% islets had lymphocytic infiltrate. Moreover, protection from insulitis was dependent upon the presence of both IEα-tTA and tetO-Ins2 transgenes, as mice transgenic for a single genetic component developed insulitis comparable to control NOD mice (Figure 4-3A). We examined if the protection from insulitis observed in TIP-1 mice was durable, by assessing islet infiltration at a later age.

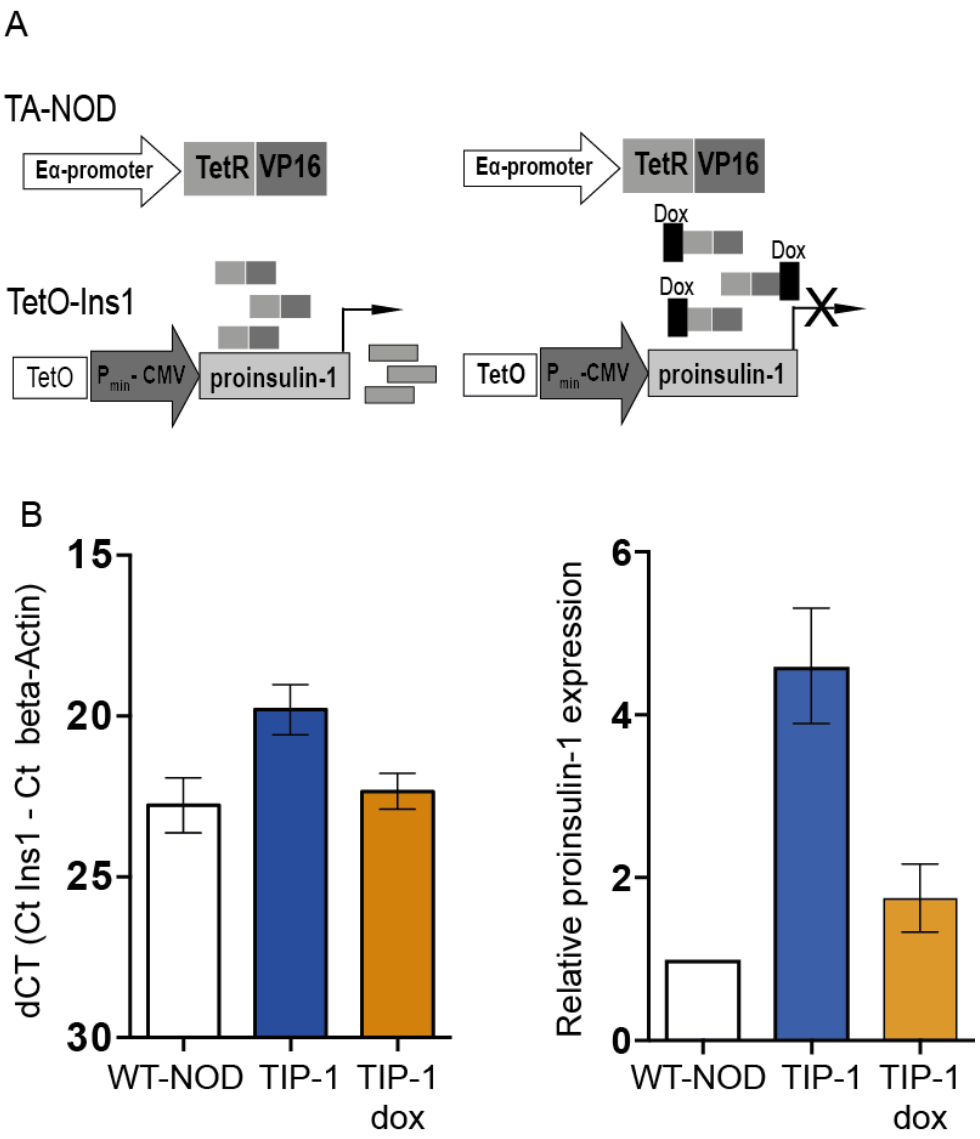

Figure 4-2: Conditional proinsulin-1 expression in TIP-1 mice

(A) Scheme of generation of tetracycline regulated NOD.IEα-tTA (TA-NOD) and tetO-Ins1 dual transgenic mice referred to herein as TIP-1 mice. TA-NOD mice were crossed with tetO-Ins1 mice. Bi-transgenic animals constitutively express proinsulin-1 in APCs and were fed doxycycline (Dox) (2mg/ml) via drinking water to suppress transgene expression. **(B)** Quantitative RT-PCR was performed using Taqman probe for insulin-1 and beta-actin in thymic lysates of WT-NOD mice and three founder lines of TIP mice. dCt values (right) were calculated by normalizing the test gene (insulin-1) to housekeeping gene (beta-actin). Proinsulin-1 expression relative to NOD mice (right) was calculated using ddCT method Data represent values (Mean±SEM) from 2-3 independent experiments run in duplicate for each probe.

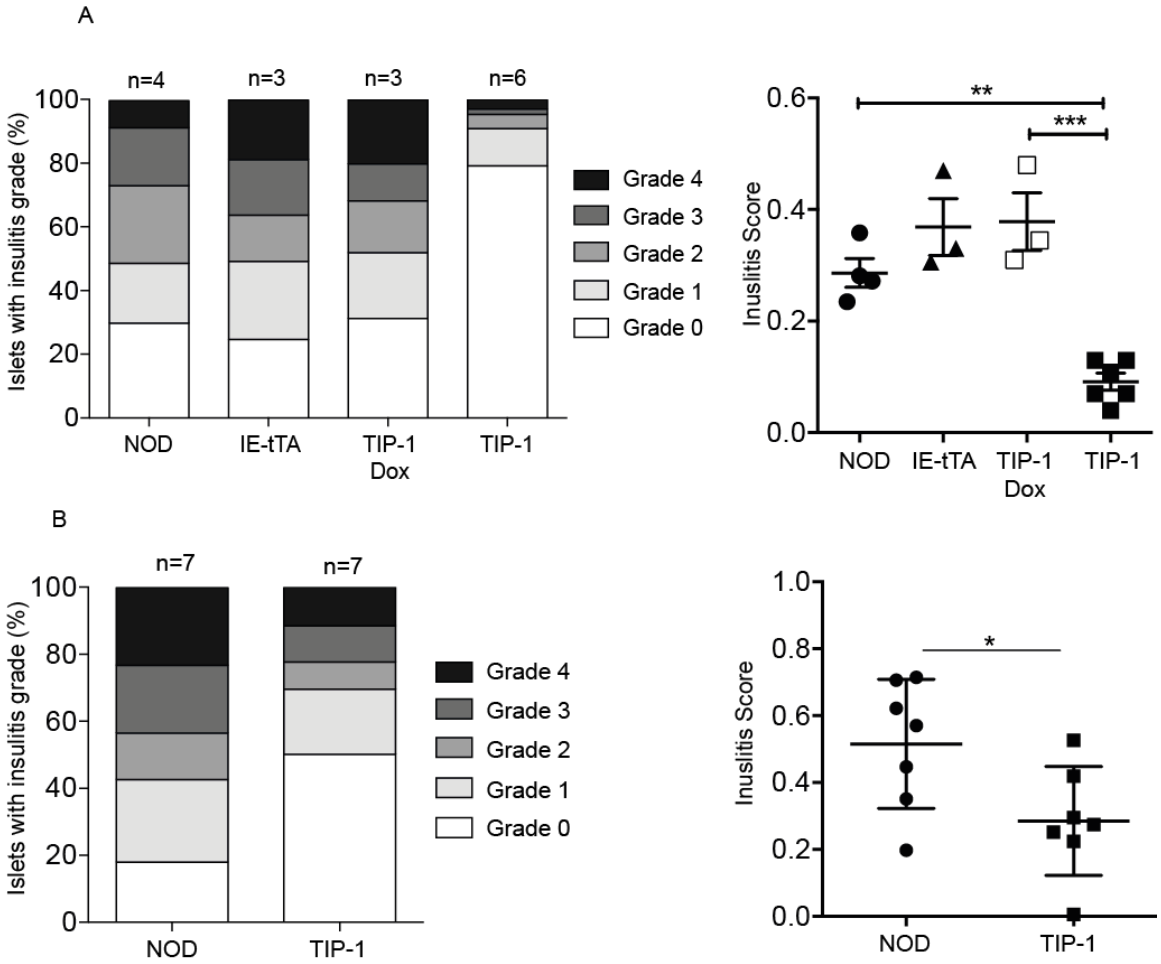

Figure 4-3: Insulitis in TIP-1 mice

(A) Histological grading of insulitis (left) and individual insulitis scores (right) in 10-12 week old WT-NOD mice, mono transgenic TA-NOD mice and untreated TIP-1 mice. Number on top of each column represents number of mice screened (≥ 90 islets scored per mouse) **(B)** Histological grading of insulitis (left) and individual insulitis scores (right) in 18-20 week old WT-NOD mice, and TIP-1 mice. Number on top of each column represents number of mice screened (≥ 90 islets scored per mouse). *P<0.05, **P<0.01, ***P<0.001. Data compared using One-way ANOVA with Tukey's multiple comparisons test **(A)** and 2-tailed unpaired t-test **(B)**.

Analysis of pancreas histology from 18-20 week old TIP-1 mice continuously expressing PI-1 revealed infiltration in approximately 50% of the islets, whereas non-transgenic littermates had >80% of islets destroyed (Figure 4-3 B and C). These data suggest that proinsulin-1 expression in the APCs provides partial protection from insulitis.

4.4.3 TIP- I mice develop reduced insulin autoantibodies (IAA)

Development of IAA is predictive of impending diabetes onset in both humans and NOD mice (235),(45). Moreover, NOD mice tolerant to proinsulin-2 (NOD-PI mice), do not develop IAA suggesting that proinsulin specific CD4+ T cell help is required for the humoral response by B cells (243),(258). We examined whether tolerance to PI-1 had an influence on B cell mediated humoral responses by measuring IAA in TIP-1 mice. IAA were significantly reduced in 12-15 week old TIP-1 mice as compared to age matched non-transgenic NOD mice. Although a few TIP-1 mice displayed IAA level above the baseline, overall they were not significantly higher than proinsulin-2 tolerant TIP-2 mice or non-autoimmune prone C57BL/6 mice (Figure 4-4) indicating that tolerance to PI-1 had a moderate influence on B cell mediated IAA production.

4.4.4 TIP- I mice have a reduced incidence of spontaneous diabetes

We next tested whether proinsulin-1 expression in the APCs influenced spontaneous diabetes development. TIP-1 mice developed diabetes but at a reduced incidence compared to non-transgenic control NOD mice. By 300 days of age 40% of TIP-1 mice and 65% of the control mice developed diabetes (Figure 4-5). This result suggests that immune tolerance to proinsulin-1 is able to partially prevent diabetes development in NOD mice and is in agreement with the partial protection from insulitis and IAA development observed in TIP-1 mice.

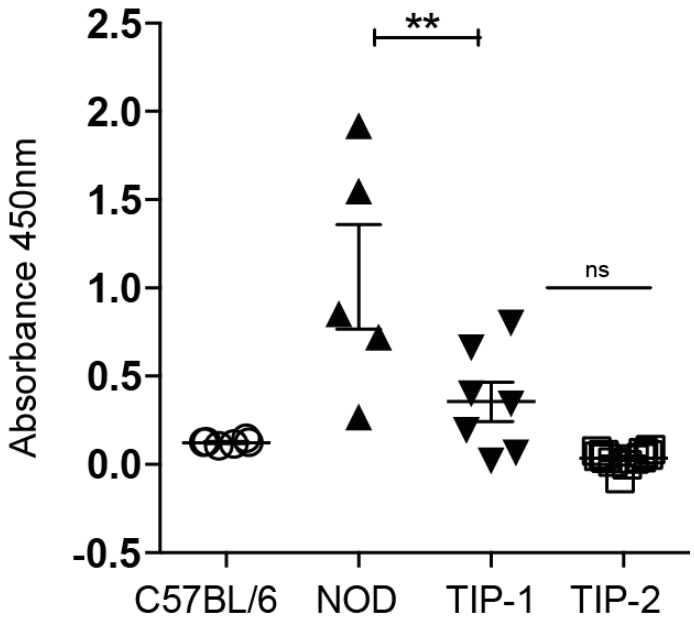

Figure 4-4: Insulin autoantibody (IAA) development in TIP-1 mice

Sera from 12-15 week old C57BL/6 mice, WT-NOD mice, TIP-1 and TIP-2 mice were tested for presence of insulin autoantibodies (IAA) by ELISA assay. Absorbance values at 450nm are plotted. Data plotted as Mean ± SEM **P<0.01, ns = not significant. Data compared using One-way ANOVA with Tukey's multiple comparisons test.

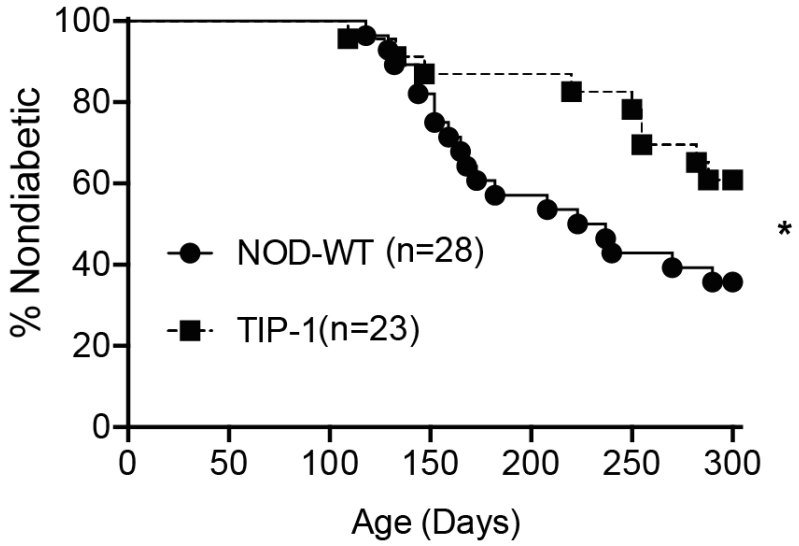

Figure 4-5: Spontaneous diabetes incidence in TIP-1 mice

Spontaneous diabetes development was analyzed in female TIP-1 mice and non-transgenic littermates until 300 days of age. Numbers in parentheses indicate the number of mice analyzed. *P<0.05. Survival curves were compared using log-rank test.

4.4.5 Proinsulin specific tolerance in TIP-1 mice

The partial protection from insulitis and diabetes in TIP-1 mice expressing proinsulin-1 in the APCs could be due to immune tolerance to proinsulin-1. Murine proinsulin-1 differs from proinsulin-2 by 1 amino acid at position 9 in the immunodominant insulin B chain epitope Ins $_{B:9-23}$ (240),(272). To directly demonstrate tolerance to PI-1, we enumerated the frequency of CD4+ T cells reactive to the insulin in the periphery of TIP-1 mice. Similar to TIP mice described in chapter 3, we detected insulin specific CD4+ T cells binding to the insulin $_{B:10-23}$/I-A (g^7) tetramer in TIP-1 mice. As the peptide Ins B:10-23 is identical between both PI-1 and 2, it is possible that the insulin $_{B:10-23}$/I-A (g^7) tetramer may detect CD4+ T cells specific for either molecule.

The absolute number of insulin tetramer binding cells in the control NOD mice (Figure 4-6 A, B) was much lower as compared to control mice (cohort 1) used in chapter 3 (figure 3-4), this is most likely due to batch to batch variation in tetramer binding efficiency. There was a trend towards reduction in absolute number of insulin specific CD4+ T cells (Figure 4-6 A, B), whereas the antigen-experienced $CD44^{hi}$ subset of insulin specific CD4+ T cells was significantly reduced in TIP-1 mice (Figure 4-6 B,C). These data suggest that residual insulin specific CD4+ T cells in the periphery of TIP-1 mice fail to become activated in response to ectopic proinsulin-1 expression. Insulin reactive CD4+ T cells in TIP mice expressing proinsulin-2 also exhibited a similar phenotype (Chapter 3). Given that the immunodominant epitope in both proinsulin-1 and 2 is highly conserved, it is likely that tolerance to proinsulin-1 may primarily impact proinsulin-1 reactive T cells, as well as a cross-reactive subset of proinsulin-2 specific CD4+ T cells. The residual proinsulin-2 specific T cells possibly mediate the reduced level of diabetes observed in TIP-1 mice.

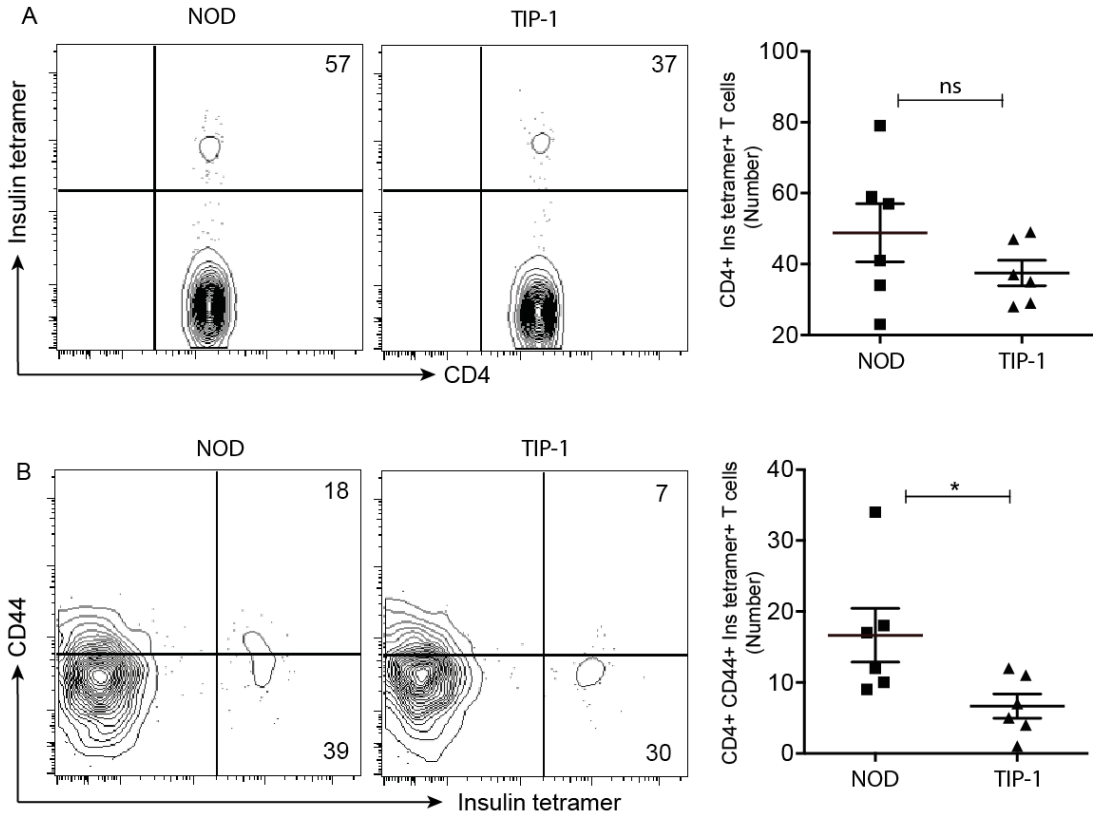

Figure 4-6: Immune tolerance to insulin-specific T cells in TIP-1 mice

Insulin B:10-23-specific CD4+ T cells were stained with I-A (g7) tetramer and enriched from pooled peripheral lymphoid organs (PLO) of 12-15 week old TIP-1 mice and NOD mice using magnetic beads and enumerated by flow-cytometry. (A) Representative FACS plots and enumeration of insulin B:10-23 tetramer+ CD4+ T cells and (B) $CD44^{hi}$ insulin B:10-23 tetramer+ CD4+ T cells in TIP-1 mice. Values in the FACS plots indicate absolute number of tetramer binding cells in the respective gate (A, B). Each symbol in the scatter plots (Mean±SEM) represents data from an individual mouse. *P<0.05, ns= not significant. Data compared using 2-tailed unpaired t-test

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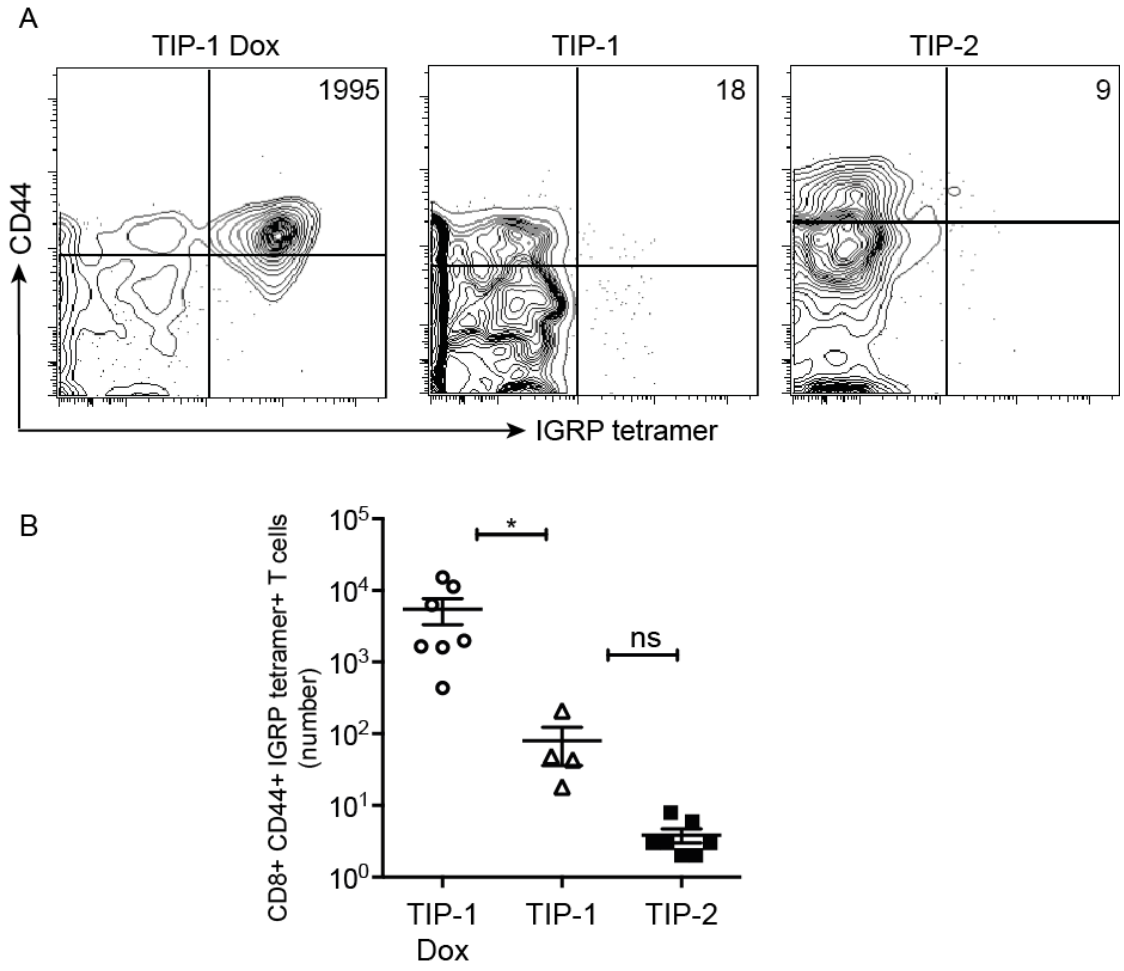

Figure 4-7: Enumeration of IGRP-specific CD8+ T cells in TIP-1 mice

(A) Representative FACS plots showing absolute number of tetramer+ IGRP $_{206\text{-}214}$-specific CD8+ T cells and **(B)** enumeration of IGRP $_{206\text{-}214}$-specific CD8+ T cells enriched from PLO of 12-14 week old TIP-1 mice treated with Dox from gestation,TIP-1 mice and TIP-2 mice. Values in the FACS plot show absolute number for the indicated gate. Each symbol in the scatter plot (Mean±SEM) represents data from an individual mouse. *P<0.05, ns= not significant. Values compared using One-way ANOVA with Fisher's LSD test.

4.4.6 Downstream responses to IGRP are dampened in TIP-1 mice

Previous work from our group has demonstrated that IGRP is a downstream antigen, and autoreactive responses to IGRP are dependent upon immune response to proinsulin-2 (257). We examined the frequency of pathogenic IGRP $_{206\text{-}214}$ reactive CD8+ T cells to evaluate if tolerance to proinsulin-1 influenced the immune response to IGRP in TIP-1 mice. The number of IGRP $_{206\text{-}214}$ specific CD8+ T cells in TIP-1 mice expressing proinsulin-1 was significantly reduced as compared to TIP-1 mice with no induction of proinsulin-1 expression indicating that tolerance to proinsulin-1 affected the response to downstream antigen IGRP. Compared to proinsulin-2 tolerant TIP mice, the number of IGRP specific T cells in TIP-1 mice was more; although not statistically significant (Figure 4-7 A,B).

4.4.7 Immune response to proinsulin-1 is not required for diabetes in NOD 8.3 mice

TCR transgenic NOD 8.3 mice have >90% of their CD8+ T cells specific for IGRP 206-214 and develop accelerated diabetes (206). Krishnamurthy et al have shown that autoreactivity to proinsulin II is required for diabetes development in the NOD 8.3 mice that have a pre-existing repertoire of IGRP specific T cells (258). Since we observed reduced frequency of IGRP reactive CD8+ T cells in TIP-1 mice, we wished to know if immune responses to proinsulin-1 were necessary for diabetes development in NOD 8.3 mice. TIP-1 mice were crossed with NOD 8.3 mice to generate offspring that were TIP-1/NOD8.3 double transgenic or NOD 8.3 transgenic alone. As previously reported, 100% NOD8.3 mice developed diabetes (median survival 70 days), whereas the TIP-1/8.3 mice developed diabetes with delayed kinetics (median survival 97 days) (Figure 4-8). Therefore, tolerance to proinsulin-1 significantly delays but does not prevent diabetes development in NOD 8.3 mice.

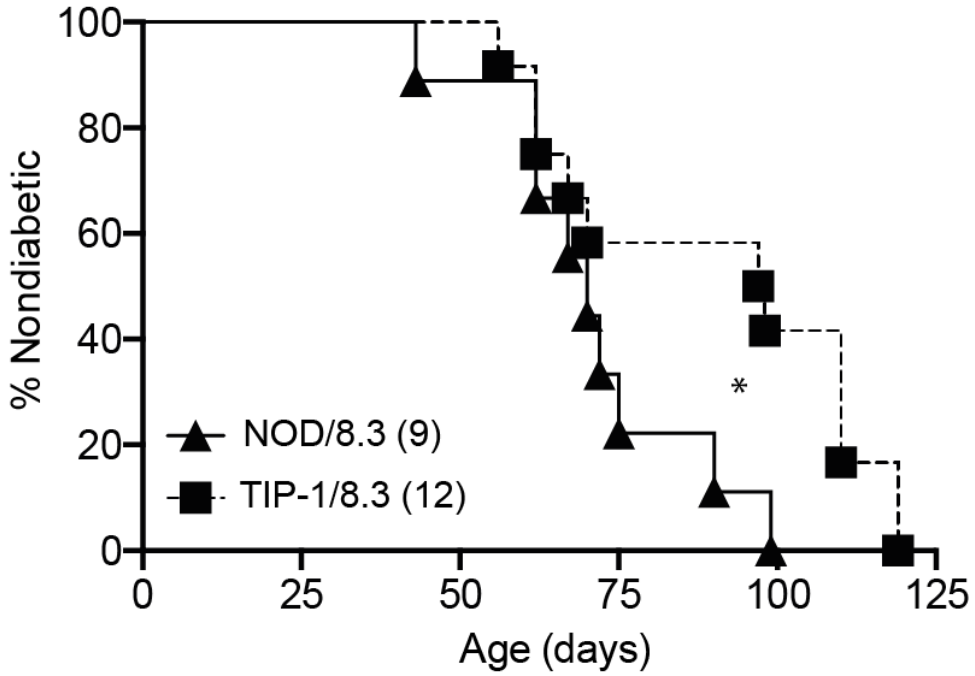

Figure 4-8:Spontaneous diabetes incidence in TIP-1/8.3 mice

Spontaneous diabetes development was analyzed in female TIP-1/8.3 mice and NOD 8.3 littermates. Numbers in parentheses indicate the number of mice analyzed. *P<0.05. Survival curves were compared using log-rank test.

4.5 Discussion

Two isoforms of proinsulin encoded by distinct genes co-exist in the NOD mouse. While antigen-specific tolerance to proinsulin-2 prevents diabetes development (243),(244) the impact of immune tolerance to proinsulin-1 on diabetes development in NOD mice has not been assessed. In this study we generated transgenic NOD mice to induce proinsulin-1 expression in the APCs and examined the impact of antigen specific tolerance on autoimmune diabetes. The main findings of this study are 1) TIP-1 mice expressing proinsulin-1 in the APCs show significantly reduced incidence of diabetes, which is associated with reduced insulitis and insulin autoantibody (IAA) expression. 2) Proinsulin specific T cells are detectable in TIP-1 mice but are not activated. 3) Immune responses to downstream antigen IGRP are reduced but not absent, and tolerance to proinsulin-1 delays but does not prevent diabetes in NOD 8.3 mice with a pre-existing repertoire of IGRP specific T cells.

Given the high degree of homology between proinsulin 1 and 2 proteins, especially in the immunodominant insulin B chain epitope $Ins_{B:9-23}$ we expected to achieve robust protection from diabetes onset in TIP-1 mice, similar to TIP mice described in chapter 3 , and the previously described NOD-PI mice; however partial protection from insulitis and diabetes observed in TIP-1 mice points to the existence of distinct pathogenic peptide epitopes in the proinsulin-2 protein that can precipitate autoimmunity in NOD mice.

It is likely that a single amino acid difference in the Ins-1 $_{B:9-23}$ peptide as compared to Ins-2 $_{B:9-23}$ epitope (443) accounts for the partial protection observed in TIP-1 mice. One possibility is that the difference at position 9 of the immunodominant insulin B chain peptide B:9-23 may lead to a change in the stability or binding of the INS B:9-23 peptide within MHC-class II I-Ag7 peptide binding groove and this may give rise to distinct immunogenic epitopes. However it has been shown previously that the core nonamer (9mer) of the insulin B chain that promiscuously binds to MHC class II I-Ag7 encompasses amino acids at

positions 12-23 (282),(283). Therefore, a single amino acid difference at position 9 in the insulin B chain between PI-1 and PI-2 is unlikely to influence the binding of INS B:9-23 peptide to MHC-class II I-Ag7 and account for reduced diabetes incidence in TIP-1 mice.

Another explanation for robust protection from diabetes observed in TIP mice in comparison to TIP-1 mice could be that proinsulin-2 expression in APC induces tolerance to most proinsulin-1 reactive T cells in addition to proinsulin-2 reactive T cells, whereas proinsulin-1 expression in APCs primarily targets proinsulin-1 specific T cells, and some cross-reactive proinsulin-2 specific T cells, allowing the residual proinsulin-2 reactive cells to induce diabetes in TIP-1 mice.

A drawback of our study is that we have analyzed a single transgenic founder line expressing proinsulin-1 in the APCs. It is therefore possible that the lack of robust protection from diabetes observed in TIP-1 mice may be due to insufficient antigen expression in the APCs. Chentoufi and Polychronakos previously reported that *Ins2* is expressed at more than 3 fold higher level than *Ins1* in the thymus of NOD mice (239). In TIP-1 mice analyzed here, induction of proinsulin-1 results in approximately 5 fold higher expression as compared to non-transgenic NOD mice or uninduced TIP-1 mice. Moreover, protection from insulitis in TIP-1 mice is associated with the expression of proinsulin-1 transgene, as TIP-1 mice fed doxycycline to suppress proinsulin-1 expression develop islet infiltration comparable to non-transgenic controls indicating that ectopic proinsulin-1 expression in APCs influences anti-islet immunity.

Our data complement the previous observations that reported detection of proinsulin-1 reactive T cells (268),(444) in NOD mice. While previous studies did not directly demonstrate the role of proinsulin -1 reactive T cells in spontaneous disease, the significant reduction in diabetes incidence in TIP-1 mice suggests that proinsulin-1 specific T cells participate in autoimmune destruction of beta cells. On the other hand, development of IAA and diabetes in TIP-1 mice may be related to ongoing immune responses to proinsulin-2 peptides.

Does the reduction in the incidence of spontaneous diabetes in TIP-1 mice correlate with deletion of proinsulin-1 specific T cells? Ins $_{B:10-23}$ specific tetramer used in our study is likely to detect both proinsulin-1 and 2 reactive CD4+T cells, due to the invariant nature of the peptide between the two isoforms. While the absolute number of Ins $_{B:10-23}$ binding CD4+ T cells was similar in WT and TIP-1 mice, the reduction in the antigen-experienced subset of tetramer binding CD4+ T cells is suggestive of antigen-specific tolerance. Ins $_{B:9-23}$ represents just one of the multiple proinsulin-1 epitopes, thus, it is possible that T cells specific for other proinsulin-1 epitopes may have been affected by antigen expression.

Autoimmunity to insulin determines immune responses to other downstream antigens such as IGRP (257). IGRP reactive T cells were reduced in TIP-1 mice; but tolerance to proinsulin-1 did not prevent diabetes onset in TIP-1/8.3 mice. The precursor frequency of IGRP reactive CD8+ T cells is low in NOD mice (445), and the residual immune response to proinsulin-2 in TIP-1 mice may not be sufficient to induce robust expansion of IGRP specific T cells as observed in non-transgenic NOD mice. However, the residual immune response to proinsulin-2 in TIP-1/8.3 mice with a pre-existing repertoire of IGRP specific T cells may be sufficient to help IGRP specific CD8 + T cells to mediate beta-cell destruction.

In summary, we find that immune tolerance to proinsulin-1, whilst partly protective, is not sufficient to prevent spontaneous diabetes in NOD mice. This study clarifies the role of proinsulin-1 in the pathogenesis of autoimmune diabetes in NOD mice. Whether proinsulin-1 specific immune responses are important in initiation or maintenance of anti-islet autoimmunity in NOD mice remains to be determined.

5 General Discussion

5.1 Summary of results

Loss of immune tolerance to islet antigens such as proinsulin results in autoimmune destruction of insulin-secreting beta cells leading to type 1 diabetes (T1D). Antigen-specific therapy to boost immune tolerance is highly desirable but has not been successful so far in treatment of T1D. In this thesis I generated transgenic NOD mice expressing islet antigens proinsulin and IGRP in the antigen presenting cells (APCs) during defined periods to augment antigen-specific T cell tolerance (TIP and TII mice). My findings suggest that a brief exposure to proinsulin confined to the perinatal period in TIP mice prior to weaning imparts durable protection from diabetes onset. By tracking IGRP-reactive T cells in TII mice that temporally express IGRP in the APCs I demonstrate that IGRP reactive T cells are uniquely produced in early life. Major conclusions from this study are: 1) early life is a vulnerable period of escape of islet-reactive T cells and 2) ongoing tolerance therapy to islet antigens is not required after weaning as few if any new self-reactive T cells develop in adult life. Thus, results from this study will likely inform the design of future antigen-specific interventions for prevention of T1D.

5.2 Antigen-based primary prevention in individuals at risk for T1D

The increasing incidence and prevalence of T1D globally (22) points to an urgent need for safe and effective clinical approaches to prevent the progression and onset of T1D. Interventions around the time of disease onset have been partially effective, as the autoimmune processes that mediate beta cell destruction are irreversibly established (423). A current paradigm suggests that an approach aimed at primary prevention or prevention of islet autoimmunity is most likely to be successful in prevention of clinical onset of T1D (Figure 5-1) (446).

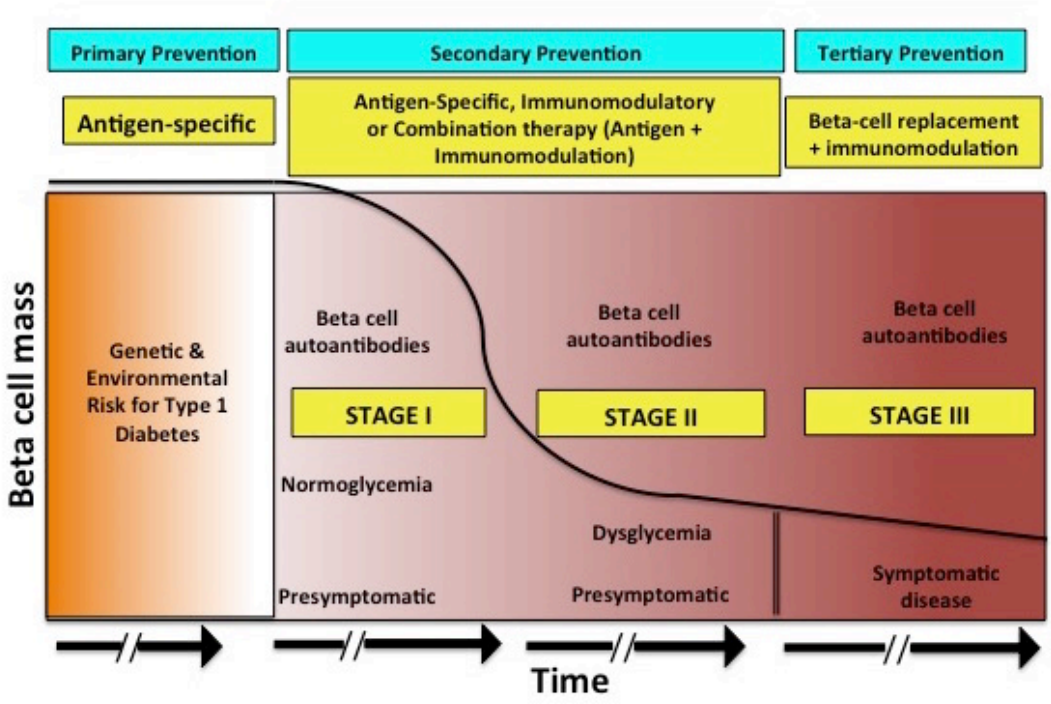

Figure 5-1: Clinical strategies to preserve beta cell mass for prevention of type 1 diabetes

Based on recently defined stages in the development of type 1 diabetes primary prevention strategies that are antigen-specific can be applied prior to stage 1 of the disease in individuals with high genetic risk for developing T1D, secondary prevention using either immunomodulatory therapies alone or in combination with antigens can be applied during stage 1 and 2 to halt the decline of beta cell mass. Approaches such as beta cell replacement combined with immunosuppressive treatment can be used as a tertiary prevention or new-onset intervention during stage 3 when functional beta cell mass is insufficient.

Primary prevention of T1D would require: (1) Early identification of individuals who are at risk of developing T1D and (2) antigen-specific therapy that is safe because the vast majority of the target population would be young children.

In many children who go on to develop T1D, islet autoantibodies reach peak levels between 9 months and 2.5 years of age (45). Therefore, to achieve efficacy, primary prevention needs to commence during early life prior to development of autoimmunity. This is supported by preclinical studies including the data presented in chapter 3 of this thesis, which suggest that antigen-specific therapies may be most effective when administered during early-life, a period when natural mechanisms of immune tolerance are fully active (436),(447). Relevant to antigen-based primary prevention of T1D is the recently reported success in the field of peanut allergy where infants exposed to peanut proteins in early life had a dramatic reduction in the prevalence of peanut allergy (448).

Translation of the above experimental studies would require tolerogenic vaccination with self-antigen, preferably in the thymus either during gestation or during neonatal life, to augment central tolerance. Previous studies have reported that it is possible to boost central tolerance by delivering antigens to the thymus by intrathymic transplantation of pancreatic islets (449) or injection of lentiviral vectors (450). More recently, a clever experimental approach with a potential for therapeutic application has been described, wherein, proinsulin was fused to Fc portion of an antibody, to enable the uptake of this fusion protein from mother to foetus via neonatal FcRn receptor. Treatment of pregnant NOD mice late in gestation with PPI.Fc fusion, reduced diabetes in the offspring (436). Clinical translation of such an approach would require demonstration of safety and efficacy as well as a non-invasive mode of antigen delivery either *in utero* or to newborns.

The recently completed Pre-POINT trial was the first pilot study to administer an autoantigen to children aged 2-7 years with family history and high-genetic risk for T1D development. The findings of Pre-POINT trial demonstrated that oral administration of insulin at escalating doses was safe and resulted in a measureable immune response that was tolerogenic (355).

Based on the foundation for antigen-based primary prevention laid by the Pre-POINT study, the Global Platform for the Prevention of Autoimmune Diabetes (GPPAD) has been established in Europe. GPPAD aims to develop the first randomized controlled phase II/III trial using antigen-based therapy for primary prevention of earliest events in newborns that lead to islet autoimmunity (451). Neonates who are first-degree relatives of individuals with T1D and carry diabetes susceptible HLA genes will be recruited initially. Antigen therapy will commence around 4-6 months of age just prior to the 9-12 month age window during which an increase in the beta-cell autoantibody seroconversion is observed (452). The primary outcome that will be assessed in this trial is the development of multiple beta-cell autoantibodies or T1D development at 4 years of age. Such a platform will also provide an opportunity for implementing secondary prevention strategies if the children develop islet-autoantibodies. These studies pave the way for development of effective primary prevention strategies for T1D.

5.3 Population-based screening for risk of T1D in early life

Current risk screening approaches mainly target first-degree relatives of T1D patients as this population has a 20 fold increased risk of disease development (453). However, since more than 80% of individuals who develop T1D do not have a family history (454) such an approach to risk screening has limited potential to capture the great majority of susceptible patients. Early diagnosis of pre-clinical T1D in the general population is possible by detection of islet autoantibodies as nearly all of the children with multiple beta-cell antibodies go on to develop symptomatic diabetes regardless of their family history (45). Emphasizing the importance of population based screening in early life is the observation that around 80% of children who go on to develop T1D have multiple islet autoantibodies by the age of 5 years (45, 452).

Efficient and cost-effective methods of screening the general population such as the measurement of islet autoantibodies from dried blood spots on filter paper have been described (455) which bypass the need for invasive venipuncture especially in young

children. Furthermore, a high-throughput ELISA assay for detection of multiple diabetes-associated autoantibodies has also been developed to facilitate large-scale population screening for children at risk of developing T1D (456).

The Fr1da study currently underway in Bravaria, Germany, is examining the feasibility of early staging of T1D at a population-based level in children aged 2-5 years. Islet autoantibodies will be measured in capillary blood samples from 100,000 children without pre-selection of the target population. Preliminary results from this study suggest that early diagnosis of pre-clinical T1D within public health setting is feasible (457). The overall aim of this study is to assess (1) whether such an approach prevents severe complications observed at clinical diagnosis and (2) if preventive education and care reduces psychological distress.

Early screening of general population for risk of T1D if implemented as a component of public health can offer several potential benefits. Early diagnosis of pre-symptomatic T1D can decrease morbidity and mortality associated with diabetic ketoacidosis (DKA). Other advantages of early screening include earlier insulin administration, which may preserve residual beta-cell function and reduce hypoglycemia and other complications. Early diagnosis of risk for T1D also provides an opportunity for educating and preparing children for later interventional studies.

5.4 Novel strategies for antigen-based vaccination in established autoimmunity

The prophylactic potential of antigen-specific treatments has been extensively demonstrated in preclinical studies (340),(346),(343),(342), but restoration of immune tolerance in the setting of ongoing inflammation has not been achieved in the clinic (350),(352),(357),(362). A possible reason for the lack of efficacy of antigen-specific treatments in the clinic is that at the time of intervention, naïve autoreactive T cells have differentiated into antigen-experienced memory T cells that are refractory to tolerance inducing strategies (458),(64).

Tackling antigen-specific memory T cells is also crucial for therapies attempting to replace beta cell mass in T1D patients. Pancreas or islet transplantation can elicit a memory response against islet antigens and recurrence of autoimmunity, ultimately resulting in the failure of the islet graft (459),(460).

Although antigen-specific therapies have failed to eliminate memory T cells, reassuringly, introduction of antigens in an already primed environment did not aggravate T1D indicating that such interventions are safe (356). Immunomodulatory therapies that target memory cells or co-stimulatory signals showed transient benefit but chronic administration may be associated with the risk of compromising systemic immunity (461),(328). While eliminating memory T cells may be difficult, it may possible to modulate memory T cells to alter the disease progression or outcome. Combining antigen-specific treatment with a short-term immunomodulatory therapy may provide synergistic benefit by restoring immune tolerance and avoiding long-term side effects. For example, anti-CD3 Ab treatment combined with mucosal delivery of antigens such as insulin or glutamic acid decarboxylase (GAD), induced remission from established disease in NOD mice (462),(434),(463). Recently it has been reported that transfer of bone marrow encoding cognate antigen targeted to dendritic cells was able to inactivate established memory CD8+ T cells and generate a durable tolerogenic environment, thus making a case for hematopoietic stem cell mediated therapy to disable memory T cell responses (464).

Several novel strategies for efficient antigen delivery have been tested in NOD mice for prevention or treatment of autoimmune diabetes. *Lactococcus lactis* were engineered to express proinsulin or GAD65 and the Th2 cytokine IL-10. Oral administration of these recombinant bacteria combined with a low dose of anti-CD3 Ab treatment induced long-term remission in newly diabetic NOD mice by boosting antigen-specific regulatory T cells in the gut mucosa (434),(465).

Other approaches have used methods to chemically couple islet antigens to the surface of apoptotic cells and demonstrated antigen-specific tolerance in experimental settings (466),(467). A recent study reported a decrease in myelin specific T cell responses in

patients with multiple sclerosis who received myelin peptides coupled with autologous peripheral blood mononuclear cells, thus demonstrating the feasibility and safety of such a therapy (468). Blocking insulin specific T cells by using a monoclonal antibody targeting MHC class II IA(g7)-insulin B:10-23 peptide complex modulated disease kinetics in NOD mice (469), demonstrating the potential of peptide-MHC-specific antibodies as a treatment for prevention of T1D.

Santamaria and colleagues recently reported a novel therapeutic strategy using nanoparticles coated with MHC class II /peptide complexes for treatment of established autoimmune disease. Stimulation of CD4+ T cells with cognate peptide-MHC class II coated nanoparticles not only reversed autoimmune diabetes in NOD mice, but also blunted inflammatory responses in collagen induced arthritis and experimental autoimmune encephalomyelitis models by converting Th1 memory T cells into Tr1 regulatory cells that produce IL-10 and TGF-beta (470). Notably, the therapy was efficacious even when subdominant epitopes were targeted. In the clinical setting such a therapy precludes the need for identification of a dominant or primary antigen, thereby removing a major hurdle in the epitope-based immunotherapies. The only drawback of this strategy is the need for ongoing treatment, as withdrawal of antigen led to recurrence of diabetes in NOD mice. Although these novel approaches for antigen delivery have been validated in a preclinical setting, the mechanistic data is encouraging and holds promise for clinical translation for prevention or treatment of T1D.

The success of preclinical antigen-specific interventions in the setting of established autoimmunity as described above was mainly due to peripheral tolerance mechanisms such as generation of Tregs or induction of anergy in the autoreactive T cells. This is in contrast to the results described in chapter 3 of this study where thymic deletion of insulin-specific T cells is the likely mechanism imparting protection from T1D in TIP mice. Whether the thymic deletion of insulin reactive T cells is mediated by resident thymic dendritic cells or migratory dendritic cells remains to be determined. Moreover, whether peripheral tolerance mechanisms contribute to the protection from T1D in TIP mice is still unclear.

Future studies using the TIP and TII mice that I developed in this thesis aim to induce expression of proinsulin or IGRP in APCs after the onset of islet inflammation to study the impact of peripheral tolerance and explore the combination of immunomodulatory treatments with induced antigen expression. In these studies antigen-experienced memory T cells will be modulated and thus potentially alter the disease course or outcome.

5.5 Developing biomarkers for early identification of anti-islet autoimmunity

The earliest environmental or endogenous events that determine progression from genetic susceptibility to development of clinical onset of T1D remain elusive. These events may be separated by months or years from the detection of autoantibodies, which mark the onset of autoimmunity. Development of diagnostic tools or biological measures that can predict the earliest stages of autoimmunity will greatly aid in earlier prediction of risk for development of T1D. Large-scale efforts using metabolomics (471),(472), transcriptomics (473),(474) and analysis of gut microbiome (475),(476) are underway to identify biomarkers preceding autoimmunity. Recently, a type I interferon signature has been detected in children with genetic risk for T1D, prior to autoantibody development (38). Furthermore, decreased levels of phospholipids in cord-blood have been detected in at-risk children who progress to clinical T1D early in life (477). Identification of such diagnostic signatures for early risk detection may further define a new stage prior to the recently defined stage 1 in the natural history of T1D (44).

5.6 Developing biomarkers to assess the efficacy of immunotherapies

A current obstacle in the design of prevention trials is the lack of biomarkers that can accurately predict the course of disease progression. Given the variable length of the latent phase prior to symptomatic disease, individuals may be at different stages of disease progression with varying beta cell reserves when a therapy is initiated (423). This heterogeneity may contribute to a dilution of any therapeutic effect. For example, in the study examining the effect of anti-CD3 treatment in new-onset T1D patients, a greater benefit was observed in those with higher residual beta cell function thus, directly correlating beta cell mass with the outcome (326).

5.6.1 Biomarkers to assess beta cell mass

Several assays with the potential of determining beta cell loss at the level of DNA, RNA and protein have been developed in recent years (478). A promising approach in biomarker development for measuring beta-cell death is the detection of differentially methylated DNA fragments. Insulin expression inversely correlates with CpG DNA methylation. Therefore appearance of circulating cell-free hypomethylated insulin DNA fragments might reflect an increase in beta cell death (479). Herold and colleagues have shown that individuals at-risk who later progressed to T1D had a significantly higher ratio of unmethylated/methylated insulin DNA as compared to controls, and preservation of C-peptide in T1D patients treated with anti-CD3 antibody was associated with decreased unmethylated insulin DNA (480),(481).

MicroRNAs (miRNAs) post-transcriptionally regulate gene expression, and function in a cell-autonomous fashion. Emerging evidence suggests that miRNAs can serve as biomarkers of T1D progression or disease complications (482),(483). Upregulation of several miRNAs has been reported in NOD mice as well as T1D patients (484),(485),(486). While a miRNA signature can potentially serve as biomarker of beta-cell death in T1D subjects, more

comprehensive studies need to be done in high-risk populations. A hallmark of beta cell dysfunction is the accumulation and secretion of inadequately processed proinsulin molecules. Therefore beta cell dysfunction can be detected in a non-invasive manner by measuring the ratio of circulating proinsulin relative to C-peptide (PI/C ratio), and alterations in this ratio could identify individuals at risk of T1D prior to acute beta-cell loss. Elevated PI/C ratios were found to be predictive of T1D onset in autoantibody positive individuals who went on to develop clinical disease (487). Other studies also reported increased PI/C ratios in recent onset T1D patients (488), and reduction in this ratio was associated with remission from T1D (489),(490). Thus PI/C ratios are an important biomarker in T1D prediction algorithms. The inability to monitor the initiation and progress of lymphocytic infiltration in the pancreatic islets is a major impediment in understanding the pathogenesis of T1D. A non-invasive method based on the uptake of magnetic nanoparticles by the macrophages infiltrating the islets followed by their visualization by magnetic resonance imaging (MRI) has been previously utilized to follow the progress of pancreatic inflammation in the NOD mouse (491) and the feasibility of this approach has been examined in humans (492). In a recent proof-of-principle study, T1D patients showed a clear difference in nanoparticle accumulation in the pancreas as compared to healthy controls (493). Thus, uptake of nanoparticles by macrophages infiltrating the pancreas could be a non-invasive biomarker of islet inflammation in T1D and may be applied to monitor the efficacy of immunomodulatory therapies in T1D patients.

5.6.2 T cell biomarkers

T cells play a central role in the pathogenesis of T1D therefore it is equally important to have an immunological readout to predict the therapeutic outcome of an intervention. The clinical course of T1D onset is heterogeneous and greatly varies between individuals with similar genetic risk; this heterogeneity is also reflected in the response of the patients to an intervention (494). Measurement of T cell populations in patients enrolled in clinical trials has revealed differences between responders and non-responders before and after the

treatment. For example an increase in the frequency of CD8+ central memory T cells with decreased activation markers and increased regulatory gene expression was associated with clinical responses to teplizumab (anti-CD3 Ab) in recent onset T1D patients (495). In another study treatment of T1D patients with alefacept (CD2 blockade) led to a reduction in the frequency of effector T cells and this led to an increased Treg/Teff ratio (496).

Emerging evidence suggests that enumeration of T cells combined with in-depth functional and phenotypic analyses may provide gene signatures with prognostic value in autoimmune disease. A recent study compared gene expression using single cell analysis in CD8+ T cells form patients with chronic infection and autoimmune disease and reported a gene expression profile indicative of T cell exhaustion to be associated with a beneficial clinical outcome in autoimmune disease. A single surrogate marker KAT2B, which is an anti-apoptotic transcription factor, correlated with progression to clinical T1D onset in children with genetic risk (497). Advances in understanding the molecular basis of disease heterogeneity in T1D will aid in better design of future clinical approaches by facilitating studies with homogenous patient cohorts and set the stage for personalized therapies.

5.7 Concluding remarks

In this study I have demonstrated that a brief exposure to proinsulin in early life is sufficient to impart durable protection from autoimmune diabetes in NOD mice. I provide mechanistic evidence that the protection from islet autoimmunity is due to reduction in the insulin reactive T cells and not due to increased T regulatory cells. In addition, my results show that islet-reactive T cells emerge only during early life, which provides a different but a related conclusion to the widely accepted view that immature T cells are susceptible to tolerance induction. This report did not study the impact of antigen-specific tolerance after the onset of autoimmunity, but future work using the transgenic mice described here will address this question. Our preclinical study will aid the design of future antigen-specific therapies to prevent T1D.

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