PREDICTION OF AUTOIMMUNE DIABETES IN THE NON-OBESE DIABETIC MOUSE BY *EX VIVO* ANALYSIS OF AUTOREACTIVE CYTOTOXIC T LYMPHOCYTES

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Title of Thesis/Project/Extended Essay

Prediction of autoimmune diabetes in the non-obese diabetic mouse by ex vivo analysis of autoreactive cytotoxic T lymphocytes

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Abstract

Type 1 diabetes affects approximately 20 million people worldwide, with 120 new cases diagnosed each year in British Columbia. Although individuals with type 1 diabetes are kept alive with daily injections of insulin, this treatment is not a cure, and does not protect individuals from early mortality as a result of disease-associated complications. As potential therapy for treatment of individuals with type 1 diabetes, promising strategies include islet transplantation in patients with established disease, or the treatment of newly-diagnosed patients with drugs that interfere with the immune response. Unfortunately, at the time of diagnosis, most individuals with type 1 diabetes have lost the majority of pancreatic β-cells as a result of a prolonged immune attack by the body's "defense" system. The ability to predict those individuals that will develop diabetes prior to extensive β-cell loss would provide a larger window of opportunity for therapeutic intervention.

In this thesis, work is presented that focuses on a population of T cells that is highly pathogenic to β-cells in the pancreas of non-obese diabetic mice, a model of type 1 diabetes. Using a novel reagent, the dynamics of this T cell population were studied in order to determine the role of these autoimmune cells in disease pathogenesis. Quantification of this T cell population in the peripheral blood during the pre-diabetic period permitted identification of those mice that would develop diabetes. These T cells were examined also following islet transplantation in order to determine whether their presence in the blood would be an effective means of monitoring islet graft health.

As interventions for type 1 diabetes become more effective, identification of individuals prior to development of disease will be critical for implementing treatment to preserve beta cell mass. The principles and practices for monitoring pathogenic T cells outlined in this thesis suggest that upon identification of important autoantigens in human diabetes, prediction may be possible using these methods.

Acknowledgements

There are many individuals that have contributed immensely to my personal growth and success during the tenure of my Ph.D. With the leadership, example, and support of each of the individuals below, I have managed to learn, laugh, and grow through what will surely be some of the best years of my life.

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Chapter 1:

Introduction

Diabetes Mellitus

Diabetes mellitus is a disease in which the body is unable to regulate blood sugar. The term diabetes mellitus comes from the Greek words "flow" and "honey". Ancient physicians characterized their patients as being very thirsty and having abnormally large amounts of urine. It was also noted that the urine produced a sweet-smelling, honey-like odour, and by tasting the urine, the physician could make a diagnosis; sweet urine indicated that the patient had diabetes mellitus (1, 2). There are two forms of diabetes mellitus: type 1, also known as "juvenile-onset" or "insulin-dependent" diabetes mellitus, and type 2, also known as "late-onset" or "non-insulin-dependent" diabetes mellitus. The American Diabetes Association standardized the nomenclature for the two forms of diabetes as type 1 and type 2 diabetes mellitus in 1997 (3). In both forms of diabetes, the β-cells in the pancreas (Figure 1) are unable to secrete sufficient insulin to maintain normal glucose levels in the blood. Type 1 diabetes is the focus of this thesis and will be discussed in detail. Briefly, type 2 diabetes is a disease that is thought to typically affect obese adults, however it affects people of differing body masses and is becoming more commonly seen in younger adults and children (4). It is a disease of insulin resistance,

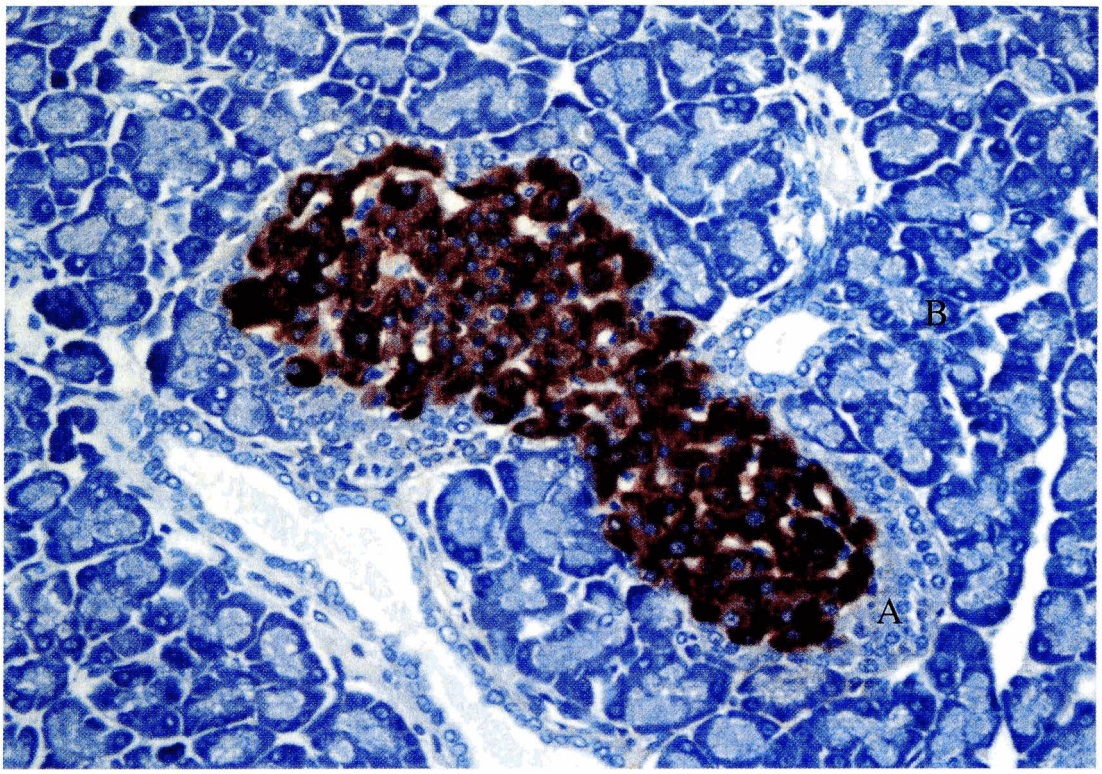

Figure 1. *Insulin is secreted from β-cells in the pancreatic islets of Langerhans.* Shown is a section of pancreas from a healthy rodent that has been stained with an antibody to insulin (brown). The islet also contains other endocrine hormone-secreting cells (surrounding the β-cells) (A), and is surrounded by pancreatic exocrine tissue (B).

whereby the β-cells in the pancreas cannot produce enough insulin to regulate blood sugar in a body where the main users of insulin, the muscle and fat tissue, are resistant to the actions of insulin. In contrast, individuals that develop Type 1 diabetes lose essentially all of their pancreatic β-cells, and as a result are unable to produce any insulin. The complete loss of β-cells is a result of an autoimmune attack that specifically targets the β-cells of the pancreas.

Type 1 diabetes mellitus (T1D)

T1D affects approximately 20 million people worldwide, and depending on geographical region, can affect from 0.7 – 40.2 per 100 000 births (5, 6). Canada has the third-highest worldwide incidence of T1D (7) and each year, 120 new children in British Columbia are affected (D. Metzger, B.C.'s Children's Hospital, personal communication). The incidence of T1D worldwide is increasing at a rate of approximately 3 - 4% annually (5, 6), and extrapolation of these trends suggest that the incidence of T1D will be 40% higher in the year 2010 than it was in 1998 (5). T1D is usually diagnosed when the concentration of sugars in the blood rise (hyperglycemia) to very high levels, and the individual's body is digesting proteins for fuel, thus producing a state of ketoacidosis. These patients are said to be "starving in the midst of plenty", as they are unable to use the glucose in their bloodstream for fuel. Diagnosis is preceded by a prolonged (months to years), asymptomatic period during which the pancreatic β-cells are slowly destroyed by the body's immune system.

Genetics of T1D

The initiation of β-cell killing by the cells of the immune system is dependent upon both environmental and genetic factors. Half of monozygotic twins whose parents have T1D progress to diabetes, but the concordance rates vary from 60% if the first twin developed T1D before 5 years of age, to 5% if the first age of onset is after 25 years (8), suggesting that there is also a strong environmental component to the development of the disease. Many genes are known to provide both susceptibility, and protection from T1D. One set of critical genes that confer susceptibility or protection from disease are those found within the major histocompatibility (MHC) locus. The MHC class II genes on chromosome 6p21 can account for approximately 50% of genetic susceptibility (formerly called IDDM1), with the protective genes also found at this locus (9, 10). The expression of protective alleles is dominant over susceptibility alleles and thus protects from disease. T1D is a polygenic disorder with over 15 identified "susceptibility loci" that are located outside the MHC locus (11, 12). The identity and function of the individual genes at the susceptibility loci are largely unknown, and their contribution to the development of T1D in many cases is poorly understood (13).

Proposed environmental factors

The contribution of the environment to the development of T1D is not disputed, but the nature of the environmental trigger(s) is unknown, and contentious (13). The environmental triggers that are most actively investigated are viral infections, including coxsackie virus, congenital rubella and cytomegalovirus (14-16); dietary intake, particularly of the young infant (17); childhood vaccinations (16, 18); and exposure to toxins such as N-nitroso derivatives (19). Large population-based studies aimed at

identifying the roles of such factors have not yielded consistently positive results, and underscore the difficulties associated with identifying simple inciting factors during a time in life when multiple initial environmental exposures are occurring (13).

Complications of T1D

Insulin injections are able to sustain life in individuals with T1D but cannot prevent the devastating complications that are associated with the disease. Prolonged elevated blood glucose levels cause damage to blood vessels and nerves, resulting in irreversible organ damage. Damage to the small blood vessels in the retina results in decreased vision and in its advanced form (proliferative retinopathy), blindness can occur. Almost everyone develops some visual problems after living with T1D for 20 years, while 20 – 30% develop the advanced form. High glucose levels also cause damage to large blood vessels leading to cardiovascular disease, which is the leading cause of death in individuals with T1D. Another common complication of disease is diabetic nephropathy, which can eventually result in kidney failure. In addition, damage to nerves causes neuropathy and affects approximately 60% of individuals with T1D (The Juvenile Diabetes Research Foundation, http://www.jdrf.org/).

The non-obese diabetic (NOD) mouse as a model for human disease

T1D is diagnosed very late in the β-cell destruction process. In fact, it is estimated that by the time individuals become hyperglycemic and are diagnosed with T1D, approximately 80% of their β-cell mass has been destroyed by the immune system (20-23). For these reasons, animal models have been developed and extensively studied as a means of understanding the disease process that precedes and results in death of β-cells.

Animal models of spontaneous T1D include the BioBreeding (BB) rat, the Tokushima rat, and the non-obese diabetic (NOD) mouse (8). The development of advanced techniques in immunology and genetics for the study of murine models has facilitated study of the NOD mouse as compared to the two rat models. In fact, the NOD mouse is the best-studied model of any spontaneous organ-specific autoimmune disease, and has provided significant insights into the development, regulation, and modulation of T1D (24, 25).

The NOD mouse was described first in Japan in the late 1970s (26). It shares many of the features of human T1D, including the polygenic control exhibited by the inheritance of particular MHC alleles and other non-MHC susceptibility genes. Importantly, the MHC class II molecule carried by NOD mice, I-A^{g7}, is similar in structure to the MHC class II DQ8 allele that confers diabetes susceptibility in humans. Specifically, the P9 peptide-binding pocket in both I-A^{g7} and DQ8 are lacking a negatively charged residue at β57, resulting in a preference for negatively charged amino acids at this position, a characteristic not seen in other mouse strains. Both DQ8 and I-A^{g7} similarly bind peptides that are different from other DQ and I-A molecules, including the dominant epitopes from putative MHC class II antigens (8, 27). The NOD mouse model has other commonalities to the human disease such as an early, progressive invasion of the pancreatic islets by cells of the immune system (insulitis), the development of β-cell autoantibodies, the transmission of disease by the transfer of bone marrow-derived stem cells, and the dependence on autoreactive T cells for the development of disease (25).

There are differences that exist between NOD mouse diabetes and human T1D. NOD mice do not develop ketoacidosis in response to hyperglycemia, they lack the complement protein C5, and a murine homolog for human HLA-DR on antigen presenting cells, and they are deaf (27). In addition, it is only in strict pathogen-free conditions that full disease penetrance is observed (25, 27).

Despite some differences, the NOD mouse model has proven invaluable as a tool for gaining understanding of the pathogenesis of T1D in humans. Undoubtedly, T1D in humans is a more complex disease and many investigators view the inbred NOD mouse merely as a single human case study (27). Nonetheless, discoveries in NOD mice have engendered many human clinical trials, and its study is essential to basic T1D research. The studies presented in this thesis may be applicable not only to human T1D, but also to other autoimmune diseases, and likely would not have been possible without this particular animal model.

Progression of diabetes in the NOD mouse

The initiation and progression of diabetes is a clinically silent process heralded by the presence of islet inflammation, or insulitis. Insulitis can be detected first at 4 – 5 weeks of age, beginning with the infiltration of macrophages (Mϕ) and dendritic cells (DC), and followed quickly by $CD4^+$ and $CD8^+$ T cells and B cells (25, 28, 29). Hyperglycemia does not develop until 4 – 6 months of age. During this intervening period, the insulitis becomes more pervasive, until a time when immune cells have completely invaded all of the islets, and the majority of β-cells are destroyed (Figure 2). While some degree of insulitis can be seen in all NOD mice, not all mice develop fully

invasive insulitis and diabetes. Seventy – 90% of females develop disease, while only 20 – 50% of males develop disease- a gender bias that is not seen in humans (25). The variation in disease penetrance is partly dependent on the mouse colony, in that an increasing number of pathogens introduced into the environment decreases the incidence of disease (27, 30). The incidence of diabetes in our mouse colony is shown in Figure 3.

Immunology of T1D

T1D develops as a consequence of a spontaneous autoimmune process that culminates in the destruction of the insulin-producing pancreatic β-cells. Many of the immunological events that conspire to destroy β-cells are understood largely as a result of studying the NOD mouse. What is currently understood about the immunology of T1D will therefore be discussed in the context of the NOD mouse with examples from human studies where available.

Initiation of the autoimmune process- What triggers T1D?

The event(s) that trigger the β-cell-directed autoimmune attack are not known. A conventional view is that a single event triggers the autoimmune process in genetically susceptible individuals, and that in the absence of this trigger, these individuals would remain disease-free. Recent evidence obtained from human and animal studies suggest a more complex model. In this model, life-long exposure to environmental agents such as viruses, dietary factors, etc. (see section 1.2.2) continually act upon 'genetically susceptible' individuals that have inherent defects in the immune system and the target organ (13). For example, data suggest that the frequency and timing of exposure to viral infections can differentially affect the development of T1D. Children that experience

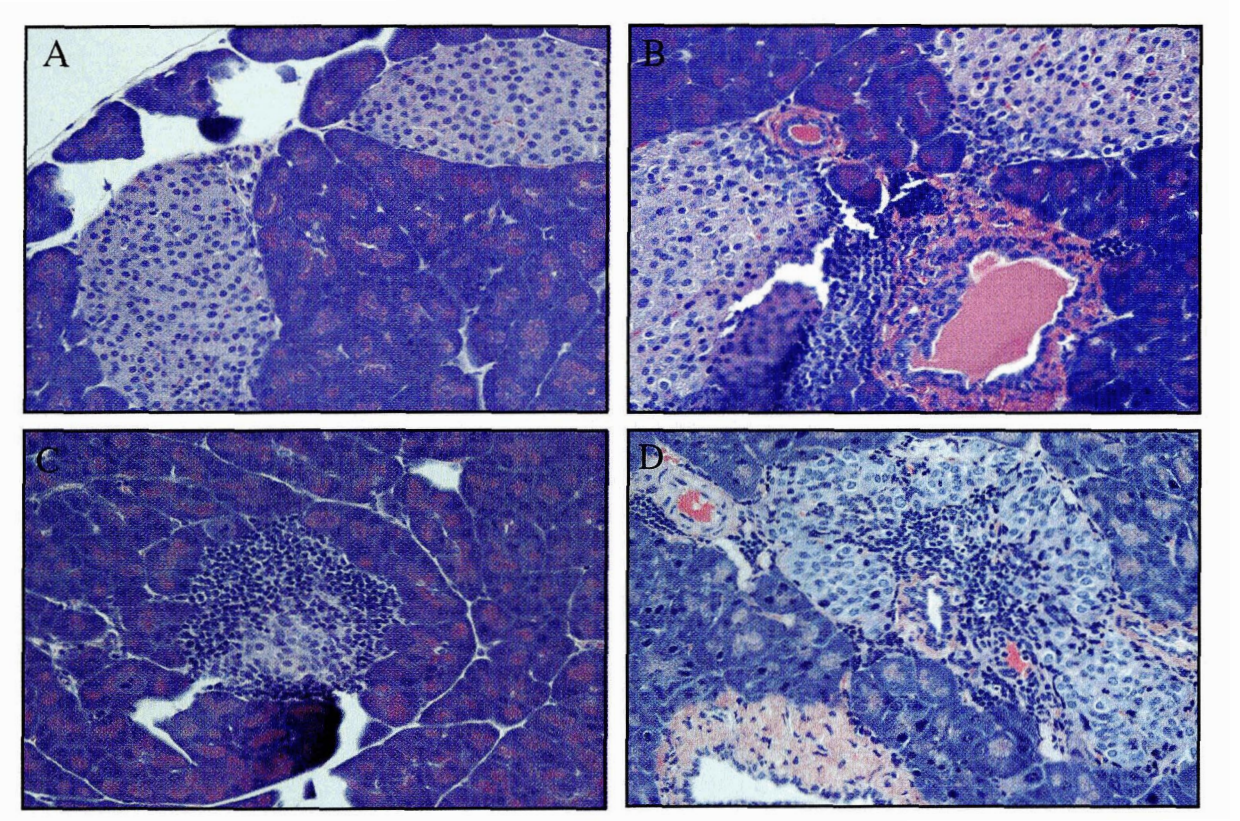

Figure 2. *Progression of insulitis in the pancreatic islets of pre-diabetic NOD mice.* Pancreases were removed at 3 (A), 9 (B), 15 (C), and 18 (D) weeks of age, fixed in paraformaldehyde, and paraffin sections were stained with hematoxylin. As mice aged and approached the onset of hyperglycemia, the percentage of islet area that was infiltrated by immune cells increased dramatically. The mouse depicted above in D, developed diabetes at 18 weeks, and the islet shown was one of very few islets remaining in the tissue.

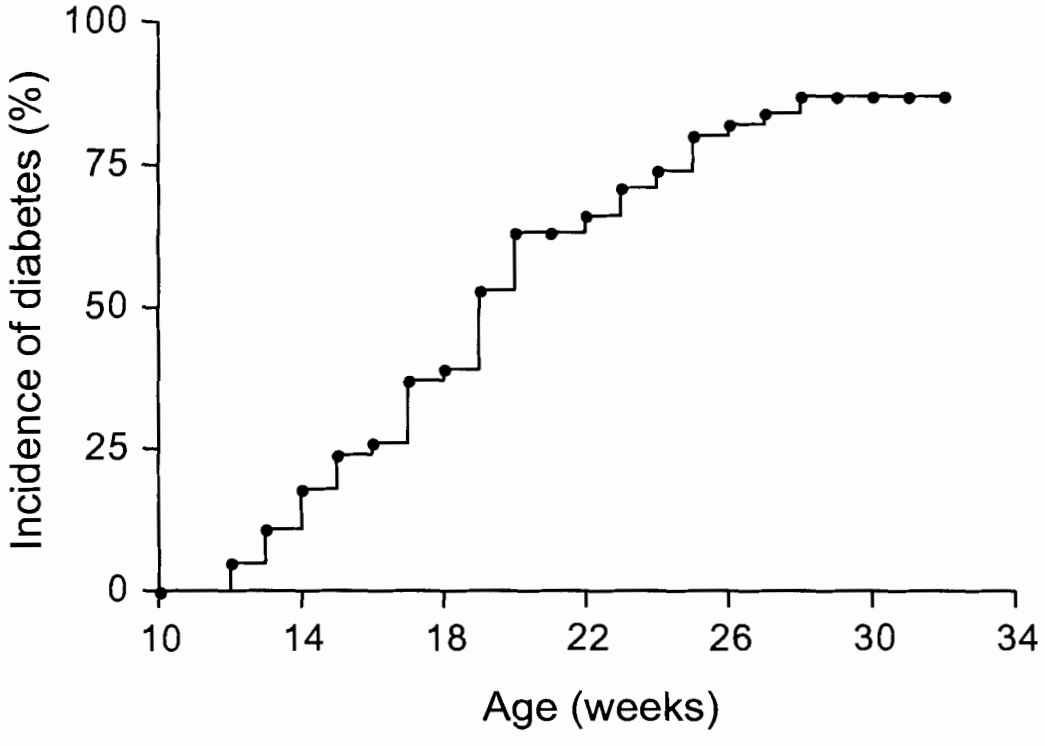

Figure 3. *Incidence of autoimmune diabetes in non-obese diabetic (NOD) mice.* Blood glucose was monitored weekly, and mice having a blood glucose >15mM were measured again the following day. Mice were considered diabetic if they had two consecutive blood glucose measurements >15mM.

multiple viral infections during the first few years of life, or those who are enrolled in preschool, have a decreased risk of developing T1D, whereas viral infections during the perinatal period are associated with increased risk (16, 31, 32). Although not well understood, the triggering event(s), particularly for humans, are undoubtedly many and varied.

Independent of what triggers the autoimmune process, β-cell apoptosis is a unifying event that influences the impending immune response. As a part of normal development and maintenance of tissues, cells continually undergo apoptosis. In the disease-free adult rodent pancreas, β-cells replicate at a rate of approximately 3%/day (33, 34). In the absence of any cell death, this rate of replication would result in a doubling of the β-cell mass within one month (35). These data demonstrate that the β-cell mass is dynamic- with the growth in mass due to replication and proliferation, balanced by a loss of β-cells as a result of apoptosis. Moreover, as a part of normal neonatal development, there is a period of increased apoptosis that peaks ~10 – 14 days after birth, where up to 10% of β-cells die each day (36). There is an analogous perinatal wave in humans that peaks at birth (37). Rodent studies comparing the rate of apoptosis during the neonatal period in diabetes prone and diabetes resistant strains have shown that the number of β-cells that are undergoing apoptosis are not statistically different between the two strains (36). However, the number of apoptotic β-cells that can be seen histologically is increased in pre-diabetic animals, suggesting that the pre-diabetic animals have a deficiency in the ability to clear dead cells (36). Apoptotic cells are normally cleared very efficiently from the body by macrophages (Mϕ), thereby preventing an immune response (Figure 4). Not only are apoptotic cells taken up very quickly, Mϕ also secrete cytokines

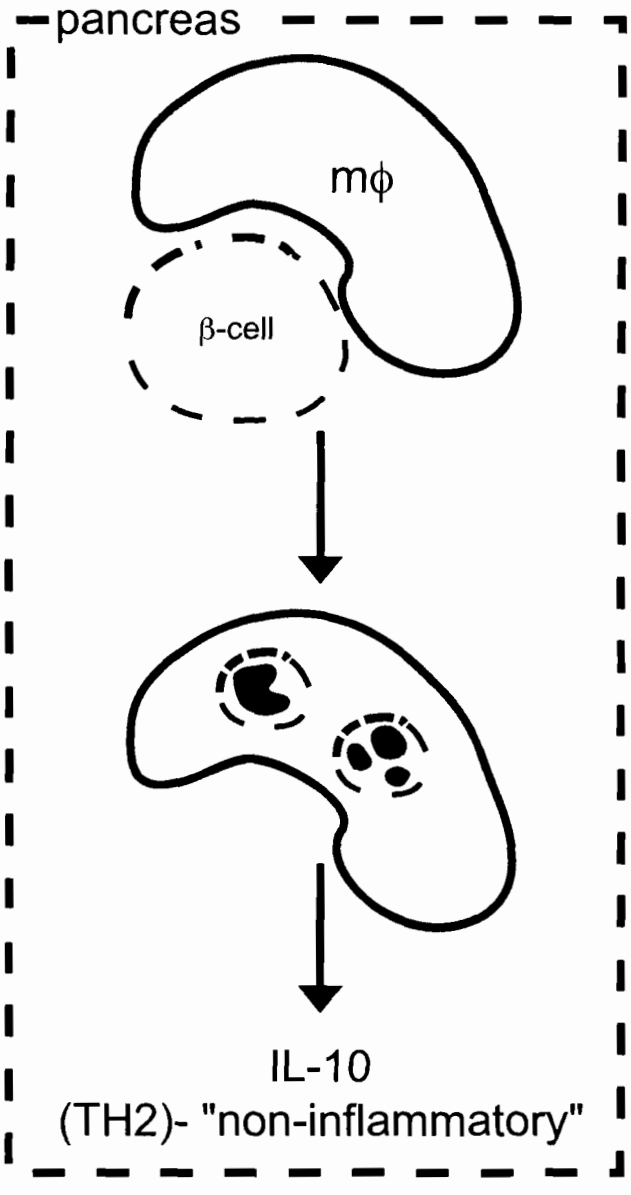

Figure 4. *Non-inflammatory clearance of apoptotic β-cells in the normal pancreas.* As cells in the body continually undergo apoptosis as a part of normal tissue turnover, they are efficiently cleared by macrophages (and other cells), thereby preventing apoptotic debris (and cellular antigen) from remaining within tissue. A simultaneous release of "non-inflammatory" T_H2-type cytokines such as IL-10 are released, further preventing any inflammatory response.

that are aimed at preventing an immune response. However, as apoptotic cells remain uncleared in the tissue, they undergo further cellular degradation, releasing cellular antigens that have the potential to initiate an immune response (38). Some investigators have hypothesized that it is an increased load of apoptotic cells, combined with an inability to clear the cellular debris, that initiates the autoimmune process in diabetes-prone strains (36, 39, 40). Other autoimmune diseases such as systemic lupus erythamatosus (SLE) have been shown to be caused, at least in part, by an inability to efficiently clear apoptotic cells (41). The neonatal period when the rate of β-cell apoptosis is increased, as well as other proposed environmental triggers such as viral infections, results in an increased load of apoptotic β-cells that may be sufficient to initiate the immune attack against β-cells.

A working model- How autoimmunity is initiated

Apoptotic β-cells have the potential to alert the immune system to impending 'danger'. As apoptotic cells remain in the tissue without being cleared, they are further broken down, releasing their cellular contents (38). These cellular contents, in the form of β-cell protein antigens, can be taken up by antigen presenting cells (APCs) in the pancreas, and shuttled to the pancreatic draining lymph nodes (PLN), where they can then be seen by the immune system. Several critical events must work in concert for such an event to produce an immune response.

First, the β-cell protein antigens must be taken to the PLN where they can be seen by T lymphocytes (the nature of these autoantigen(s) will be discussed in a later section). It is controversial as to where the initial activation of T cells takes place, but it is likely

that the PLN are involved, at least initially, because T cells that have not previously been activated are excluded from peripheral tissues like the pancreas, but move freely between the lymphoid organs and the blood (40, 42). That activation of β-cell-specific T cells occurs in the PLN is supported by adoptive transfer studies in the NOD mouse, where initial expansion of transferred β-cell-cell specific T cells occurs in the PLN, but not in the pancreatic islets (8). In addition, surgical removal of the PLNs prevents priming of naïve β-cell-specific autoreactive T cells and diabetes development in NOD mice (43).

A second requirement is that the APC, usually in the form of a dendritic cell (DC), after acquiring β-cell antigen, is activated in such a way that it is capable of initiating an immune response. DCs are normally quiescent and their activation is required to activate T cells (44, 45). DC activation is thought to occur in response to a "danger signal". These danger signals can arise from self; such as cellular mutations that lead to stress or inappropriate cell death, or insufficient scavenging of cells undergoing physiological cell death, or they can arise from non-self; such as virally infected cells that are forced to die, or pathogens and environmental toxins (44-46).

The third requirement is that T cells exist that are specific for the β-cell antigen, and that they come into contact, and are activated by the DC presenting the β-cell antigen. T cells that recognize autologous tissues usually do not cause problems within the body because of two mechanisms: central and peripheral tolerance. In the thymus, as T cells develop, they are introduced to virtually all of the proteins in the body in the context of self-MHC molecules. T cells develop with T cell receptors (TCR) that result from random recombination of 3 gene segments. It is estimated that upon recombination,

a possible 10^9 - 10^{11}different TCRs can arise (47). Any T cell with a TCR that recognizes protein antigens from the body with a high affinity (and therefore likely to cause death of autologous cells) are programmed to undergo apoptosis- a process termed negative selection. Those T cells that do not have any affinity for peptide plus MHC (pMHC) are also programmed to die- a process termed death by neglect. It is only the T cells that recognize self-pMHC with a low to moderate affinity that survive. These T cells recognize pMHC with sufficient affinity to be useful in recognizing foreign pMHC to ward off foreign pathogens, but do not recognize self-pMHC with enough affinity to cause damage (autoimmunity). These cells are selected to exit the thymus and constitute the body's T cell repertoire- a process termed positive selection (48). At one time it was thought that development of autoimmune disease was a failure of central tolerance, resulting in self-reactive T cells in the periphery. Central tolerance cannot explain why the body does not reject tissues that are introduced later in life, such as a fetus. It is now apparent that potentially self-reactive T cells exist in all individuals (49-51), but do not, under normal circumstances, cause autoimmune disease because of peripheral tolerance mechanisms.

It is thought that autoreactive T cells are not normally activated in the periphery because of their low avidity for pMHC and/or lack of co-stimulation from unactivated APCs, i.e. they "ignore self tissue". Under these circumstances, autoreactive T cells can undergo further peripheral tolerance mechanisms- they can be rendered anergic upon encounter with self antigen, they can be deleted, or dominantly controlled by regulatory T cells (52, 53). The danger hypothesis suggests that it is only in the presence of danger signals that autoreactive T cells can be activated to proliferate and destroy tissues.

Successful occurrence of the aforementioned events permits autoreactive T cells to achieve contact with pMHC on activated APC and undergo clonal proliferation, thus producing identical progeny that recognize the same antigen. There are two general types of T cells that can be activated by such a process; they are termed $CD4^+$, or helper T lymphocytes (T_H cells), and $CD8^+$, or cytotoxic T lymphocytes (CTL). These activated β-cell-specific T cells are then able to traffic to the pancreas where together they begin to destroy β-cells. This begins the positive feedback process of insulitis: as T cells destroy β-cells, more antigen is taken up by APCs and presented to T cells (either in the PLN or the pancreas itself), which leads to activation of more β-cell-specific T cells and more β-cell killing. A working schematic of this process is shown in Figures 5a and 5b. The final destruction of β-cells is accomplished by T cells, and the role of $CD8^+$ T cells in particular, will be the focus of this thesis.

Activation of T cells by pMHC complexes

A central feature of the initiation and propagation of β-cell death is the activation of T cells by pMHC molecules on the surface of APCs. It is easy to understand then, why the MHC haplotype of an individual is vital in determining ones susceptibility to, or protection from disease. Once apoptotic cells or cellular proteins are acquired by APCs, they are processed into small peptide fragments and presented on their cell surface in the context of MHC molecules. In this way, the immune system is able to "survey" the contents of all cells and is alerted by abnormal cells and foreign substances. In the presence of a danger signal, the APC becomes activated in such a way that it is able to activate T cells. It is the combination of peptide plus MHC (pMHC) that is able to specifically activate only certain TCRs. $CD8^+$ CTL are activated by 8 – 10 amino acid

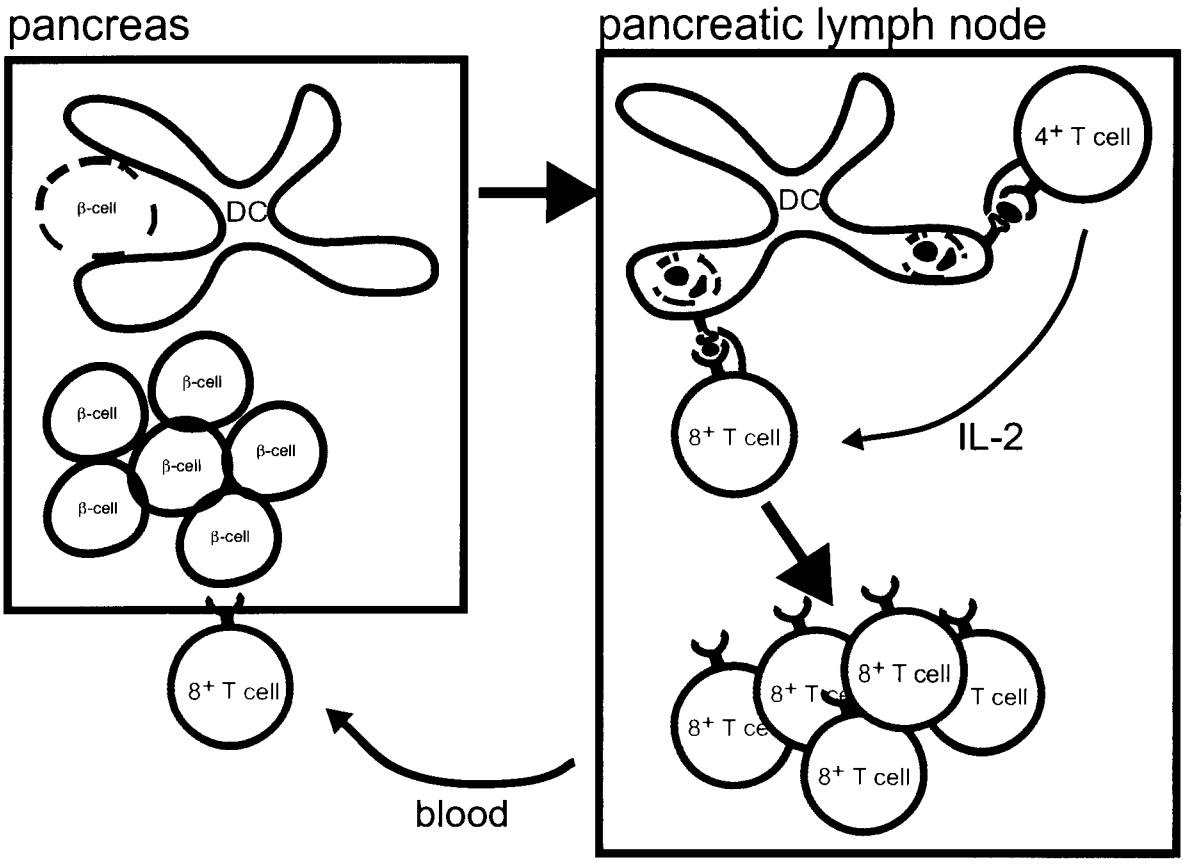

Figure 5a. *Bone marrow-derived dendritic cells acquire β-cell antigen in the pancreatic islets.* β-cells that undergo apoptosis in the presence of a "danger signal" can be taken up and processed by local dendritic cells (DCs). In the presence of a danger signal, DCs move to the local draining lymph nodes and become ideal cells for activating T cells. As DC are able to process β-cell antigen and present them on both MHC class I and class II, they are able to activate both $CD4^+$ and $CD8^+$ β-cell-specific T cells.

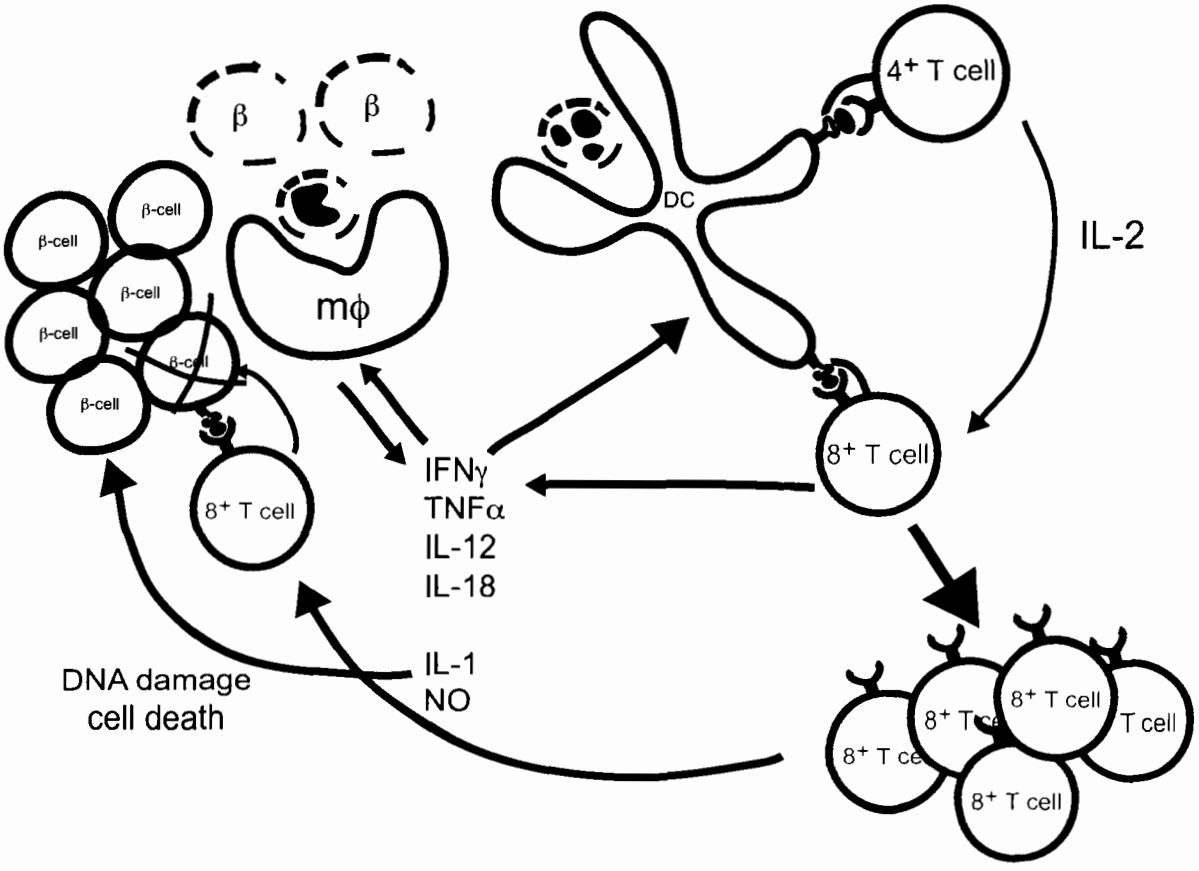

Figure 5b. *Autoreactive β-cell-specific T cells are activated by antigen presenting cells in the pancreatic lymph nodes and recirculate to the pancreas to target β-cells.* This schematic demonstrates a simple model whereby activated β-cell-specific $CD4^+$ and $CD8^+$ T cells proliferate and travel back to the pancreatic islets where they destroy β-cells, resulting in further release of β-cell antigen. Activation of both $CD4^+$ and $CD8^+$ T cells results in the release of cytokines that function to aid in T cell growth and function (IL-2 released from $CD4^+$ T cells), and also that are involved in pathogenicity (IFN-γ from $CD8^+$ T cells). In addition, the release of oxygen free radicals such as IL-1 and nitric oxide (NO) from macrophages is thought to be highly toxic to β-cells.

peptide fragments in the context of MHC class I, while $CD4^+$ T_H cells are activated by longer (10 – 30 or more amino acids) peptide fragments in the context of MHC class II molecules.

The importance of T cells in the pathogenesis of T1D

Studies of both humans (23) and NOD mice (25, 27) have implicated T lymphocytes as important mediators of β-cell damage. For example, induction of diabetes in immunodeficient NOD mice by the adoptive transfer of spleen cells from a diabetic NOD mouse requires both $CD4^+$ and $CD8^+$ T cells (54-58). In addition, disease can be prevented by neonatal thymectomy, by immunosuppressive drugs that target T cells, and by monoclonal antibodies that target either $CD4^+$ or $CD8^+$ T cells (25, 27). While numerous studies have indicated that $CD8^+$ CTL do not function optimally in the absence of $CD4^+$ T cells (59-62), many have also clearly demonstrated that $CD8^+$ CTL are required for diabetes, and mediate β-cell damage *in vivo* (29, 55-58, 63, 64). For instance, neither $CD8^+$ T cell-depleted NOD mice nor β2-microglobulin (β2m) deficient NOD mice that lack $CD8^+$ T cells develop insulitis or diabetes (64-67).

While both subtypes of T cells are necessary, and conspire to cause T1D, β-cells, which express only MHC class I, are eventually destroyed by the action of β-cell-specific CTL. Studies of $CD8^+$ T cell deficient mice have suggested that the initial β-cell insult is due to the action of β-cell-specific CTL, whose actions are dependent upon the presence of $CD4^+$ T cells (29, 68). Autopsy studies of pancreases from individuals with newly diagnosed T1D confirmed that the predominate cell type within the infiltrated islets were CTL, and that the remaining β-cells had very high levels of MHC class I expression (69,

70). When CTL make sufficiently strong contact with pMHC in the PLN, they are activated to divide, and by virtue of a chemokine gradient, will travel through the blood to the pancreas where they will kill β-cells by inducing apoptosis (40). The apoptotic process is caused both by the release of perforin/granzyme B and through Fas-ligand triggered signaling of Fas-expressing cells (71). Thus, while numerous susceptibility factors are necessary to produce T1D, the loss of β-cells is likely mediated primarily by CTL recognition of β-cells. The overall aim of this thesis is to identify and characterize a population of β-cell-specific CTL in the many weeks that precede the onset of hyperglycemia. To date, the inability to identify and study the natural history and evolution of autoreactive T cells has hampered the understanding, prediction, and prevention of T1D.

Detection and characterization of antigen-specific T cells- MHC class I tetramers

Immunologists have long questioned the magnitude of the adaptive immune response to foreign pathogens. Until recently, methods for quantifying the number of antigen-specific T cells that had proliferated in response to a virus, for example, were extremely laborious, and not very accurate, often underestimating the magnitude of antigen-specific T cell responses (72). In 1996, the description of MHC class I tetramers was a major advance in the field of cellular immunology as it allowed scientists to specifically examine antigen-specific T cell responses (73). MHC tetramer technology exploits the specific interaction between the TCR and pMHC complex. They are soluble, fluorescent, multimeric complexes of MHC bound to a peptide antigen, and thus permit the sensitive and specific detection of antigen-specific T cells. Created by combining four

identical biotinylated pMHC complexes (monomers) to a central fluorescent avidin molecule, MHC tetramers are able to detect very specifically a very small percentage of T cells within a mixed cell population (Figure 6). Selection of an appropriate 9 amino acid sequence permits the reagent to bind to T cells with TCRs that recognize the chosen peptide. The inclusion of four TCR binding sites increases the avidity of the reagent to a level that permits detection. A single pMHC monomer bound to a fluorochrome can bind to a TCR, but does not do so with sufficient affinity to allow detection of the T cell (74-76).

Detection of antigen-specific $CD8^+$ T cells using MHC class I tetramers has permitted careful characterization of T cell responses to many viral and bacterial infections (77, 78). The expansion of activated, pathogen-specific CTL is much greater than was previously appreciated. For example, following lymphocytic choriomeningitis virus (LCMV) infection of mice, as many as 55% of all $CD8^+$ spleen cells are virus specific (following both primary and secondary infection) (79-81). In humans acutely infected with Epstein-Barr virus (EBV), as many as 44% of all $CD8^+$ T cells within the peripheral blood are EBV-specific (82). In addition, the number of CTL that comprise the virus-specific memory T cell pool is dependent on the magnitude of this initial response (79). Studies using MHC class I tetramers have also modernized the field of HIV research: they have been used to show that the proportion of HIV-specific T cells is inversely related to viral load (83), that the degree of tetramer staining within 1 –2 years of seroconversion strongly predicted the length of AIDS-free survival (84), and to test the efficacy of various vaccine strategies (85, 86) and antiretroviral regimens (83, 87).

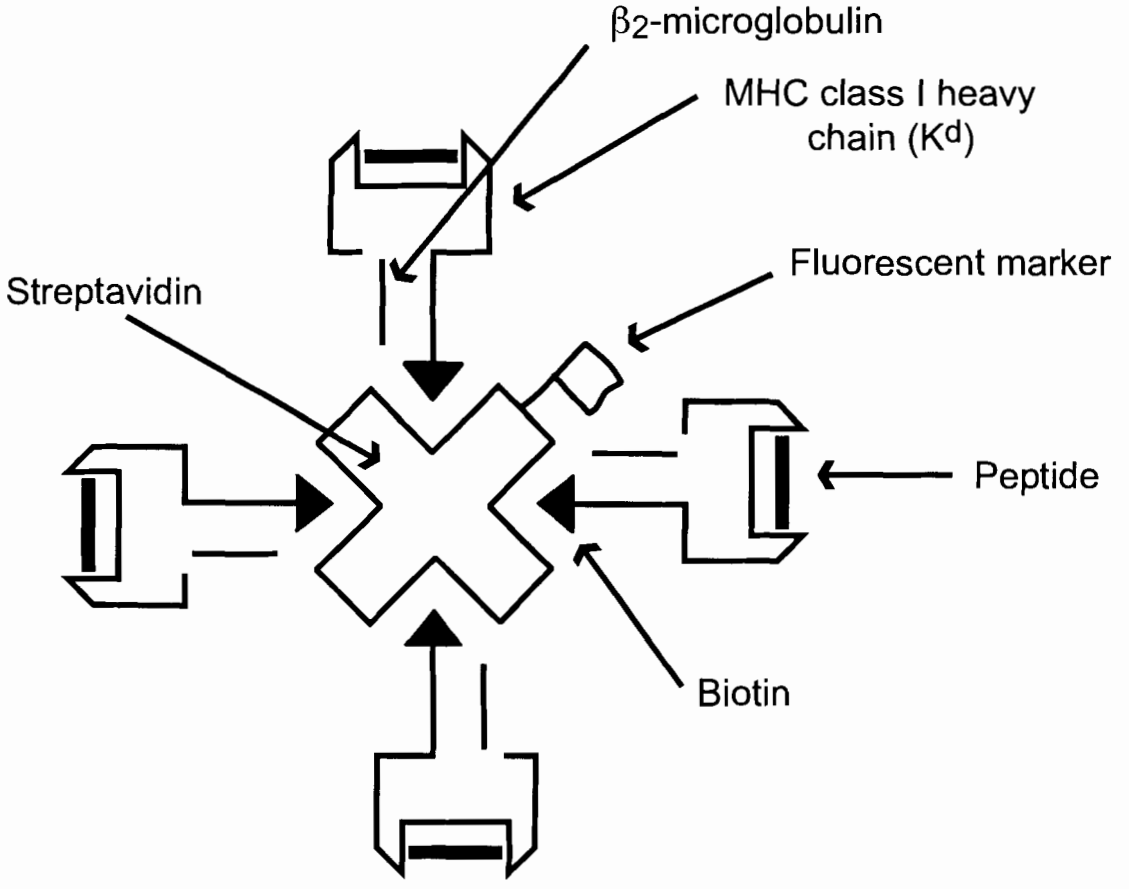

Figure 6. *Schematic of MHC class I tetramer.* MHC class I proteins (K^d and β_2-microglobulin) are made in and purified from *E. coli*, and subsequently refolded with a chosen 9 amino acid peptide. The MHC class I heavy chain is biotinylated permitting binding of 4 identical peptide-MHC complexes to a central streptavidin molecule, which has 4 biotin-binding sites. The streptavidin molecule is conjugated to a fluorochrome to allow detection of the reagent.

In contrast to viral and bacterial infection, the natural history and mechanistic role of antigen-specific self-reactive T cells in autoimmune diseases is less clear. Several reasons likely account for the difficulty in tracking autoreactive T cells with tetramers, including the lack of identified immunodominant (self) epitopes in T1D (and other autoimmune disorders); the low frequencies of autoreactive T cells in autoimmunity (in comparison to acute viral infection); and the low avidity that autoimmune T cells have for self-antigen (in comparison to T cells that recognize viral or bacterial antigens) (88, 89). This latter phenomenon is likely a consequence of autoimmunity in general; in which self-reactive T cells are derived from a subset of mature T cells whose low-avidity TCR's have escaped negative selection in the thymus (88). In addition, attempts to detect self-reactive lymphocytes after clinical presentation of diabetes is hampered by the fact that the relative lack of antigen (β-cells) post-disease onset limits the prevalence of autoreactive T cells.

β-cell autoantigens

T1D is an antigen-driven disease; β-cell autoantigens must be present to initiate and maintain a β-cell directed immune response, at least in the NOD mouse. For example, spleen cells from NOD mice in which all of the β-cells have been removed, cannot transfer disease into irradiated hosts, and diabetogenic T cells that are "parked" in these animals for 2-4 weeks lose their ability to transfer disease (90, 91). Identification of β-cell-specific T cells and their target antigens in human individuals that will eventually develop T1D will be an important step in the development of immune therapy that targets only β-cell pathogenic T cells. However, the difficulties outlined in the previous section

have prevented definitive identification of the β-cell antigen(s) that cause disease. Of the putative β-cell epitopes that have been identified, in the subsequent study of humans with T1D, or those identified as at risk of developing the disease, none has consistently stood out as being exceptionally important in the disease process.

Several candidate $CD4^+$ T cell antigens have been identified and are the focus of many T1D studies worldwide. These include: a 65kDa isoform of glutamic acid decarboxylase (GAD65), a protein tyrosine phosphatase-like molecule (IA-2), insulin, and heat shock protein 60 (hsp60). Despite the fact that these antigens are available in purified form, the proliferation assays for measuring $CD4^+$ T cell responses to these β-cell antigens have been inconsistent and contradictory, particularly in human studies (92, 93). For this reason, many of the studies that have been done investigating T cell responses to particular β-cell antigens have used the NOD mouse model. Although often considered a single human case study, the NOD mouse shares with humans the same putative T cell epitopes, and it also develops autoantibodies to the same β-cell proteins.

There are many lines of evidence suggesting that the above autoantigens play a role in T1D. Treatment of new-onset T1D patients with a $CD4^+$ T cell epitope from hsp60 (DiaPep277) resulted in increased plasma C-peptide concentrations, and a decreased insulin requirement, both implicating that the injected peptide preserved β-cell function (presumably by slowing further destruction of β-cells) (94). In the NOD mouse, induction of neonatal tolerance to GAD65 eliminated subsequent insulitis and diabetes development (95-97). Treatment with insulin or insulin B chain (98-101), proinsulin

(102), or hsp60 (103, 104) also prevented diabetes and responses to the other autoantigens.

Given that diabetes development has long been known to be strongly associated with the MHC class II complex (and therefore presumably the $CD4^+$ T cell response), the majority of human and mouse studies investigating T cell responses to β-cell antigens have focused primarily on $CD4^+$ T cells. Only in very recent years has an association to MHC class I, and therefore the $CD8^+$ T cell response, been established (105-108). The MHC class I alleles expressed in NOD mice ($H\text{-}2K^d$ and $H\text{-}2D^b$) are also required for disease development (109, 110). This association, in combination with convincing evidence of the critical role of $CD8^+$ T cells in the β-cell destructive process (discussed above), has prompted investigations into the specificity and function of β-cell-specific CTL. Only three $CD8^+$ T cell β-cell epitopes have been identified and studied (primarily in the NOD mouse): 1) amino acids 15-23 from the insulin B chain ($INS_{15\text{-}23}$) (111), 2) amino acids 206-214 of the β-cell protein islet-specific glucose-6-phosphatase catalytic subunit-related protein ($IGRP_{206\text{-}214}$) (112) and the NRP family of peptide mimotopes (113, 114), and 3) amino acids 5-13 of the leader sequence of the β-cell protein prepro-islet amyloid polypeptide (IAPP) (115). The work in this thesis focuses almost entirely on the $CD8^+$ T cell response to the peptide mimotope NRP, although some T cell responses to $INS_{15\text{-}23}$ will be presented and discussed.

$IGRP_{206\text{-}214}$ and the NRP family of peptide mimotopes

Two independent studies, both done in the mid-1990s, established that the majority of the $CD8^+$ T cells isolated from the islets of pre-diabetic and acutely diabetic

NOD mice had highly homologous TCRα chains (29, 91). Despite the fact that NOD mice express both H-2K^d and H-2D^b MHC class I molecules, all of the CD8$^+$ T cells characterized from the insulitic lesions of the pre-diabetic mice were restricted by H-2K^d (29, 91). Although the CD8$^+$ T cells isolated used a relatively diverse set of TCR Vβ and Vα gene families, these β-cell autoreactive CD8$^+$ T cells were characterized by a TCRα chain repertoire that was restricted in several important ways. In particular, many of the CD8$^+$ T cell lines contained a TCRα chain that used a Vα17-Jα42 gene segment- a significant finding because the Vα17-Jα42 gene segment is found very infrequently on the naïve CD8$^+$ T cells found in spleens of NOD mice (91). In addition, the portion of the TCRα chain responsible for antigen contact (complementarity-determining region 3 or CDR3 loops) (116-120) was identical in approximately half of all CD8$^+$ cells, suggesting that this particular CD8$^+$ T cell population was proliferating in response to a particular antigen (29, 91, 121). Although the TCRβ chains were variable, other studies have shown that recognition of identical pMHC complexes by many different TCRs often involves T cells with very limited TCRα chains, but variable β chains (122, 123).

Evidence for the biological importance of T cells with Vα17-Jα42 containing TCRα chains, comes from the development of a transgenic NOD mouse bearing exclusively one of the corresponding TCRβ chains (taken from one of the Vα17-Jα42-containing β-cell-reactive CD8$^+$ T cell clones) (121). Strikingly, these mice have restricted usage of the original Vα17-Jα42 gene segments (matching that of the original clone) by CD8$^+$ T cells in the pancreatic islets, but not in the periphery, and develop earlier onset diabetes (121). In addition, transgenic NOD mice were generated that

contained $CD8^+$ T cells bearing both the α and β TCR chains from one of the original clones (8.3 clone) isolated from an acutely diabetic NOD mouse. In this case, approximately 90% of the $CD8^+$ T cells are generated under the influence of the transgene and therefore express the 8.3 TCR (8.3-TCR NOD) (124). In this case, animals had vastly accelerated diabetes onset, with ~70% of female mice diabetic by 6 – 7 weeks of age (124). These 8.3-TCR-bearing CTL are also capable of driving β-cell damage and diabetes onset in the absence of other T cells ($CD4^+$ or $CD8^+$), as 8.3-TCR NOD mice on a recombination activating gene (RAG) knockout background also develop diabetes (124).

These studies established not only that $CD8^+$ T cells were required for disease development, but also that the repertoire of $CD8^+$ T cells in the islets was very skewed, potentially by the presence of a restricted set of antigenic determinants on β-cells. At this time however, no $CD8^+$ T cell-restricted antigens had been identified. Using a random combinatorial peptide library approach, a 9 amino acid peptide mimotope (KYNKANWFL), capable of stimulating 8.3-TCR T cells was identified, and named NRP (113). NRP was able to elicit the proliferation, cytokine secretion, differentiation, and cytotoxicity of naïve $CD8^+$ T cells from 8.3-TCR NOD mouse T cells, and was also recognized by, and stimulated $CD8^+$ T cells within islets from pre-diabetic NOD mice (113). Further studies established that modification of the TCR contact residue at position 7 increased the affinity of the TCR-pMHC interaction and therefore induced better proliferation, cytokine secretion and cytotoxicity of 8.3-TCR T cells (113, 114). Specifically, substitution of an alanine (NRP-A7; KYNKANAFL) or a valine (NRP-V7;

KYNKANVFL) for the tryptophan (W) at position 7 resulted in progressively better T cell responses (NRP < NRP-A7 < NRP-V7).

Recently, the endogenous β-cell epitope targeted by 8.3-TCR-like T cells was identified as amino acids 206-214 of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (112). $IGRP_{206-214}$ was shown to exhibit moderately strong H-$2K^d$ binding, and was capable of stimulating not only the 8.3-TCR clone, but also a previously isolated islet-specific $CD8^+$ T cell clone sharing the Vα17-Jα42 TCRα chain (AI12.B1.3) (29). In addition, a T cell clone that expressed a Vα17-Jα5 TCRα chain and a similar TRCβ chain to the 8.3-TCR clone (AI15.F5) also responded to $IGRP_{206-214}$ and to NRP-V7, but did not respond to NRP-A7.

Insulin 15 – 23

Prior to the identification of any β-cell-specific $CD8^+$ T cell epitopes, several $CD8^+$ T cell clones had been isolated from the islets of pre-diabetic and acutely diabetic NOD mice. These clones had varying capacities to kill β-cells both *in vitro* and *in vivo*, secrete cytokines and proliferate in response to β-cell antigens (125-127). One such clone, G9C8, was isolated from the islets of 7-week-old NOD mice, showed H-$2K^d$-restricted responses, and was able to transfer diabetes to H-$2K^d$-expressing mouse strains within 4 – 7 days in the absence of $CD4^+$ T cells (125). The autoantigen that stimulated the G9C8 clone was subsequently shown to be a 9 amino acid peptide from the insulin B chain (INS_{15-23}) (111). The INS_{15-23} peptide was shown to exhibit poor binding to the H-$2K^d$ molecule and it was suggested that this resulted in insufficient peptide presentation to result in negative T cell selection in the thymus (128). The inaugural paper describing the

islet-derived CTL response to INS_{15-23} stated that 87% of $CD3^+$ cells within the islets of 4-week-old NOD mice were specific for INS_{15-23}, and this percentage decreased steadily to 12% by 9 weeks of age (111).

Objectives and Approach

The ability to identify and characterize the T cell response to β-cell antigens during the course of diabetes development will likely lead to a better understanding of the disease process, and possibly the development of immune therapies that specifically target β-cell-specific T cells. Understanding the temporal relationship between the appearance of β-cell-specific T cells and β-cell destruction will hopefully result in the ability to predict those individuals that are in the process of developing T1D. This thesis attempts to take an initial step toward the goal of understanding the dynamics of CTL both in the pre- and post-diabetic period, and in response to islet transplantation using the NOD mouse model. Using MHC class I tetramers, CTL reactive to the NRP family of peptides were analyzed, and their contribution to the disease process examined. This work has revealed that in the NOD mouse model of disease, this particular population of CTL is pivotal to the disease process, and that T cell responses to IGRP may be largely responsible for disease pathogenesis. These data provide evidence for predictive and therapeutic strategies aimed at IGRP-reactive CTL.

Chapter 2:

Materials and Methods

Mice

NOD/ltj, NOD*scid*, Balb/c, C57Bl/6, and NOR/ltj mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and maintained in a specific-pathogen free animal facility at The British Columbia Research Institute for Children's & Women's Health. 8.3-TCR transgenic (Tg) NOD mice were obtained from Dr. Pere Santamaria (University of Calgary). To monitor diabetes development in NOD mice, glucose levels were monitored weekly beginning at 12 weeks of age by urine analysis (Uristix, Bayer, Canada). As animals developed glucosuria, blood glucose was monitored bi-weekly using a One Touch Ultra glucose monitor and strips (Lifescan, Johnson & Johnson). Diabetes was defined as 2 consecutive readings ≥ 15mM. Diabetic mice were maintained on 0.5 – 1.0 U/day of Humulin NPH (Eli Lilly) or with LinBit insulin implants (LinShin Canada). Animals were considered "non-diabetic" if they maintained normal glucose levels until 32 weeks of age. All animal experiments were performed in accordance with the rules of the Animal Care Committee, University of British Columbia.

Animal Procedures

All surgeries were done under general anesthetic using 2% isofluorane with 2 litres/minute oxygen. When necessary, anesthetized animals were sacrificed by cervical dislocation.

Islet Isolation

Islets were obtained by the Islet Isolation Core (Department of Pathology and Laboratory Medicine, University of British Columbia) in the laboratory of Dr. Bruce Verchere. Briefly, mice were anesthetized and the common bile duct was perfused with 2.5 ml of ice cold collagenase (Type XI, Sigma). Following engorgement of the pancreas by the injected collagenase, the pancreas was removed and placed on ice. Pancreases were digested for 6 – 12 minutes (depending on the age of the mouse and the batch of collagenase) in a shaking water bath at 37°C. Islets were then separated from other pancreatic tissue by isolation on a dextran gradient. The appropriate layers were removed from the gradient and the islets were hand picked using a pipette to obtain a clean islet preparation. For analysis by flow cytometry or for use in ELISpot assays, islets were further digested into single cell preparations by pipetting up and down for 10 minutes in 200µl Cell Dissociation Buffer (Gibco-BRL). The purpose of dispersing the islets into single cells was to permit analysis at the single cell level, and also to release the islet infiltrating lymphocytes. Whole islets isolated for transplantation experiments were incubated in Ham's F10 media overnight.

MHC class I tetramer generation

MHC class I tetramers were generated according to the protocol of Altman *et al.* (73). Briefly, the generation of tetramers involved: the production of the MHC class I proteins, refolding these proteins with the appropriate 9 amino acid peptide to form a peptide-MHC class I complex (pMHC), biotinylation of the pMHC complex, and then isolation of the correctly folded, biotinylated pMHC using gel filtration and anion exchange. The properly folded, biotinylated pMHC are then combined with an avidin-bound fluorochrome at a 4:1 molecular ratio. All of these steps were carried out at 4°C over 4 – 5 days and are detailed below.

Generation of MHC class I proteins and peptide synthsis

MHC class I proteins were generated by transfection of BL21(DE3)pLysS competent cells (Stratagene) with plasmids containing the constructs for H-2K^d and β_2m (gift of Andrew McMichael, University of Oxford, England). The H-2K^d construct contained a BirA biotinylation signal sequence. Bacteria were grown and then spun at high speed. Proteins were liberated from inclusion bodies by sonication and homogenation. Proteins were solubilized in 10M urea. Stable transfection and production of proteins was confirmed using gel electrophoresis (Figure 7). The peptides NRP (KYNKANWFL), NRP-A7 (KYNKANAFL), NRP-V7 (KYNKANVFL), INS (LYLVCGERL), and TUM (KYQAVTTTL) were prepared by FMOC chemistry and purified by reverse phase high-performance liquid chromatography (> 90% purity) at the University of British Columbia. The INS peptide was modified (Gly9Leu) from its endogenous counterpart in order to increase MHC Class I stability, without affecting CTL binding ability.

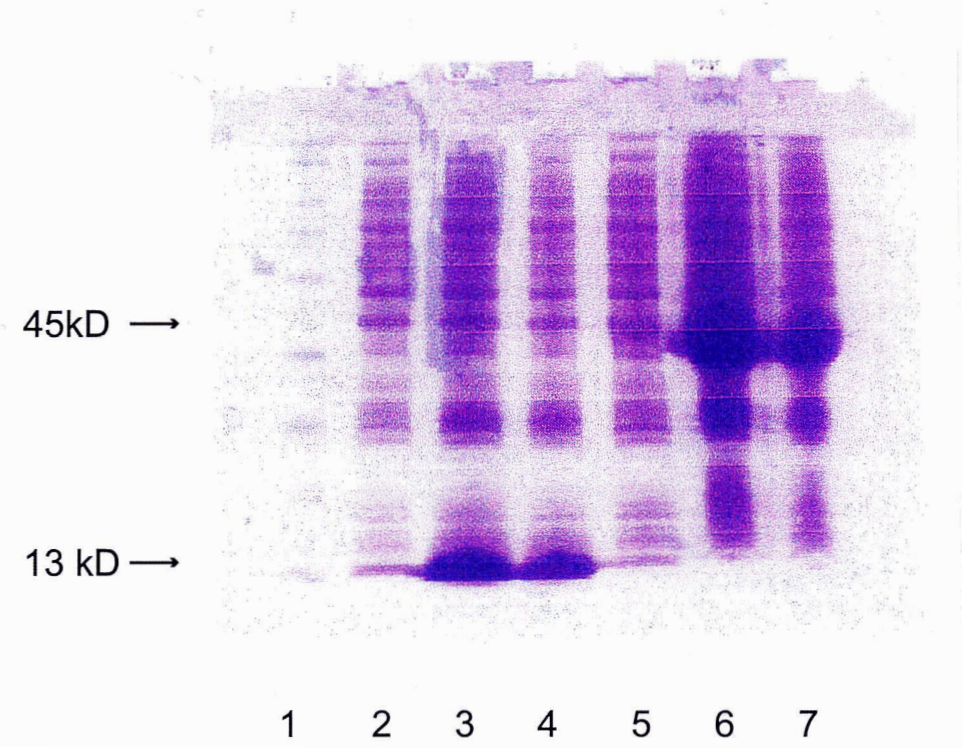

Figure 7. *Transfection and expression of MHC class I proteins shown by gel electrophoresis.* Lane 1: pre-stained broad range protein standard; Lane 2: *E. coli* transfected with β_2-microglobulin prior to induction with IPTG; Lane 3, 4: β_2-microglobulin-transfected *E. coli* following induction; Lane 5: *E. coli* transfected with the MHC class I molecule K^d prior to induction with IPTG; Lane 6, 7: K^d-transfected *E. coli* following induction.

Refolding of MHC class I proteins with peptide to generate pMHC complexes

Fifty mg H-2K^d and 15 mg β_2m were refolded with 10 mg peptide (dissolved in 200µl DMSO) over 48 hours in 1 litre of Tris-based buffer (100mM Tris pH 8.0, 400mM L-arginine, 2mM EDTA, 5mM reduced glutathione, 0.5mM oxidized glutathione, 0.1mM PMSF) at 4°C. The MHC class I heavy chain (H-2K^d) was slowly added in 4 aliquots of heavy chain dissolving buffer (6M guanidine, 50mM Tris, pH 8.0, 100mM NaCl, 10mM EDTA, 10mM DTT) over 48 hours to prevent the protein from precipitating out of solution, and to maximize the amount of correctly refolded pMHC.

Concentration, biotinylation and fractionation of pMHC complexes

After 48 hours of incubation, the refolded product (1 litre) was concentrated to 2 ml. Initially, the solution was concentrated to 100ml using a tangential flow device with a 10kDa membrane (Ultrasette, Filtron). It was then concentrated to 10ml using a centrifuged based concentrator with a 10kDa membrane (Centriprep, Amicon Bioseparations). Next, the buffer was exchanged to one that would facilitate biotin binding using a G25 gel filtration column (Amersham Pharmacia Biotech). The product was further concentrated to 2ml and incubated overnight (at room temperature) with 50µl of 100mM d-biotin, 200µl of 100mM ATP, 3µl of 3 mg/ml biotin protein ligase (BirA enzyme, Avidity, LCC) and 2µl each of the protease inhibitors PMSF, pepsin, and leupeptin (1 mg/ml). The following morning, using a G75 Superdex column (Amersham Pharmacia Biotech), the properly folded pMHC complexes were separated from the solution. The eluents from the column included: aggregated and misfolded MHC complexes (> 60kDa; a), correctly folded pMHC (58kDa; b), H-2K^d heavy chain only (45kDa; c), β_2m only (13kDa; d) and small proteins such as peptide and biotin (< 13kDa;

e) (Figure 8). Peak 'b' was collected, and then using an anion exchange column (Mono Q HR 5/5, Amersham Pharmacia Biotech), the fraction of peak 'b' that was biotinylated (Fig 9; a) was separated from the non-biotinylated fractions (Fig 9; b). The biotinylated pMHC eluent was collected and tested using a colourimetric assay to confirm the presence of biotin (Figure 10). Only those fractions that were strongly reactive were concentrated to 1 – 2 mg/ml and then used to make the tetramer. The concentration of the pMHC monomeric solution was tested using the Bradford colourimetric assay (Biorad). The pMHC monomers were then combined with a fluorochrome-bound avidin molecule at a 4:1 molar ratio. All of the MHC class I tetramers for this thesis work were generated with streptavidin-phycoerythrin (SA-PE) (Rockland Immunochemicals).

Testing of MHC class I tetramer efficacy

The function of the NRP family of tetramers was verified using spleen cells from 8.3-TCR Tg NOD mice. Cells were harvested and stained (as described below) with each batch of tetramer to determine if the tetramer was functional and to determine the optimal concentration for cell staining (Figure 11a). The function of the INS-L tetramer was confirmed using a $CD8^+$ T cell line that is specific for the $INS_{15\text{-}23}$ peptide (G9C8 $CD8^+$ T cell clone, gift of F.Susan Wong, University of Bristol).

In order to visualize tetramer staining and also confirm their function, spleen cells from 8.3-TCR tg NOD mice were stained and spun onto microscope slides and viewed under fluorescence microscopy. Following staining (as described below) cells were fixed in 4% paraformaldehyde (PFA) and spun at 1000 rpm for 5 minutes onto microscope

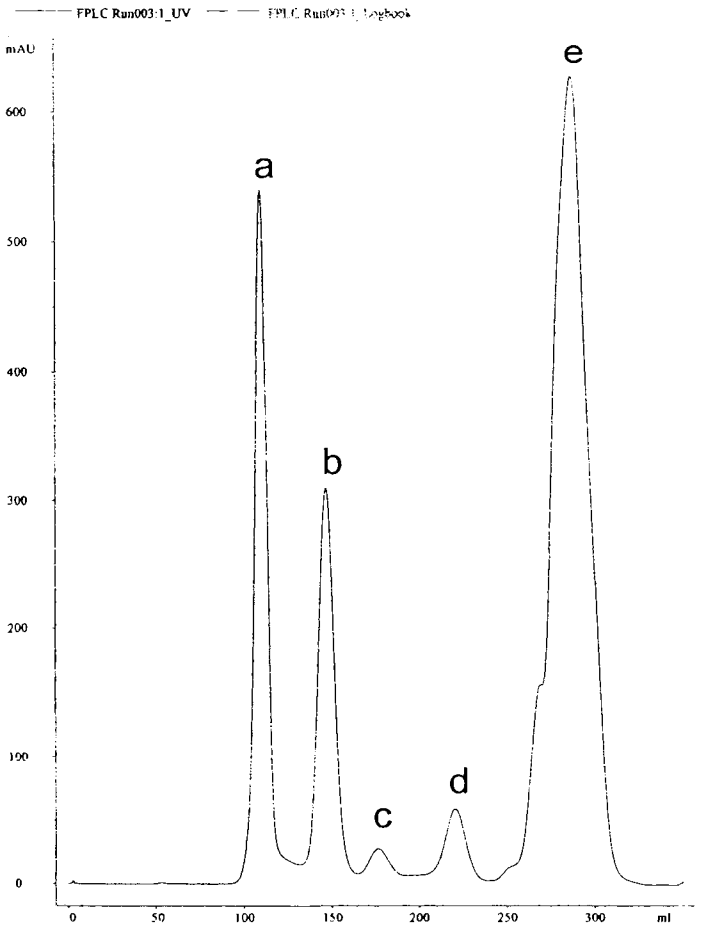

Figure 8. *Separation of peptide-MHC monomers by gel filtration.* Following a 48 hour period when K^d, β_2-microglobulin, and peptide were incubated together to form pMHC, the correctly formed monomers were separated from the other proteins based on size, by gel filtration on a G75 Superdex column using FPLC. Properly formed pMHC is 45kD (fraction b) and is excluded from the column at approximately 150 ml (4 ml/min flow). The other fractions correspond to mis-folded and aggregated proteins (a), K^d (c), β_2-microglobulin (d), and other small proteins such as biotin, peptide and ATP (e).

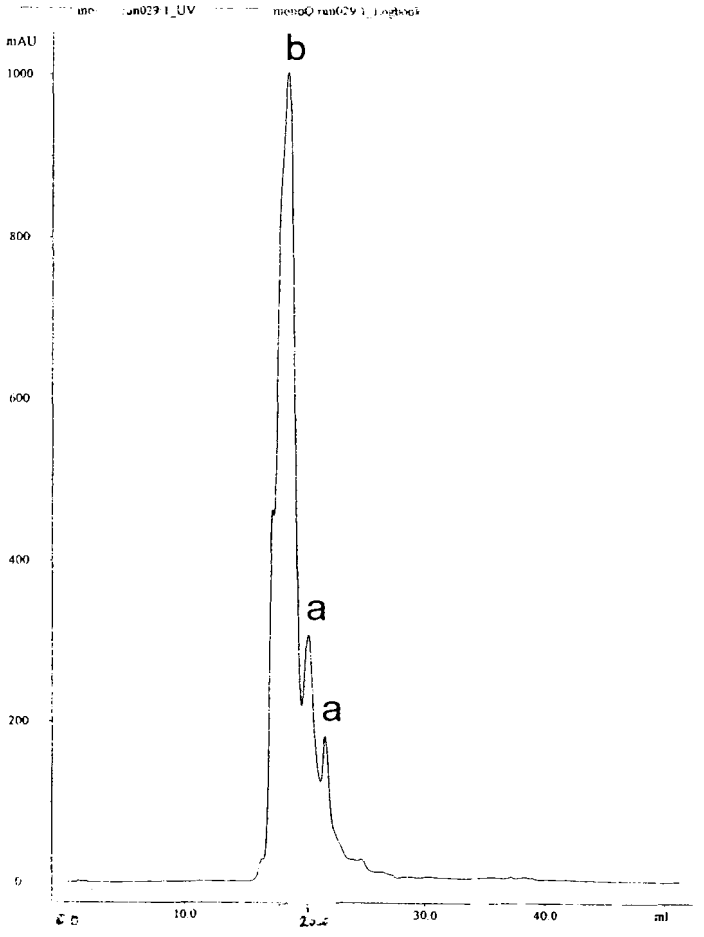

Figure 9. *Separation of biotinylated peptide-MHC monomers by anion exchange.* Fraction "b" extracted from the gel filtration procedure is run through an anion exchange column (MonoQ HR 5/5) by FPLC to separate the biotinylated from the unbiotinylated pMHC monomers. The eluent is collected in 1 ml fractions, which are subsequently tested in a biotinylation colour-change assay. Fractions labeled "a" are typically biotinylated, while those fractions that come off of the column earlier (b) are generally unbiotinylated.

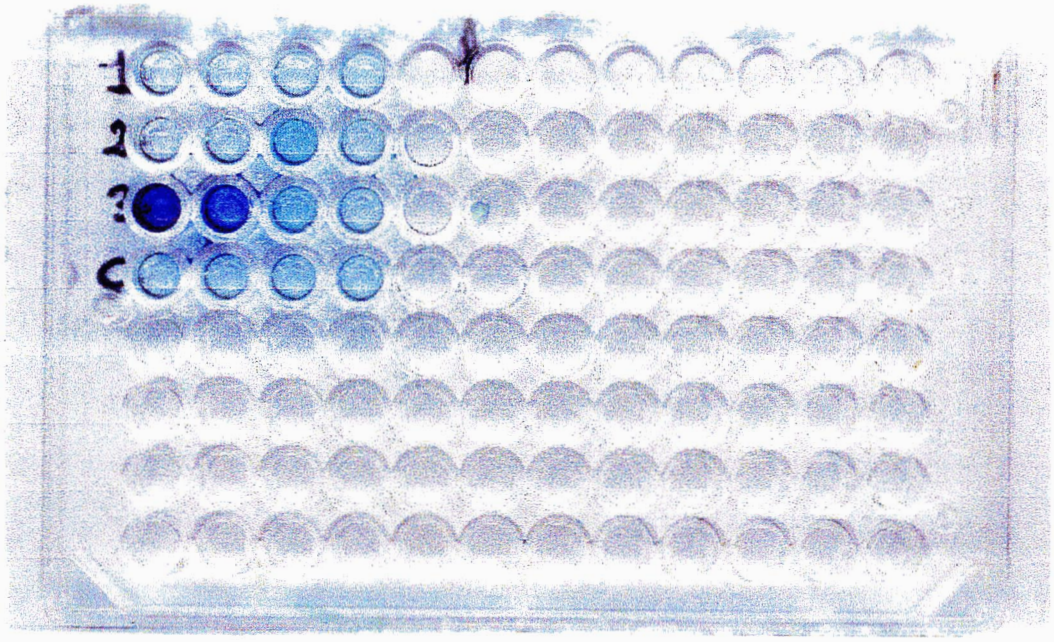

Figure 10. *Colourimetric assay to measure biotinylation.* A small sample (1 μl) of each 1 ml fraction obtained from the anion exchange column was plated in a 96-well flat-bottomed plate and incubated for 1 hour at 37°C or overnight at 4°C. The plates were washed, and incubated with extravidin peroxidase for 15 minutes. They were again washed and subsequently incubated with the substrate TMB (3,3',5,5'-tetramethylbenzidine). Samples that contained biotin (and hence bound extravidin) resulted in a blue colour change. This figure shows the results of the validation experiment that was done to confirm the presence of biotin in sample 3, but not in sample 1 or 2. The control lane (C) was used in all experiments and was a sample of unbiotinylated, purified peptide-MHC class I complex.

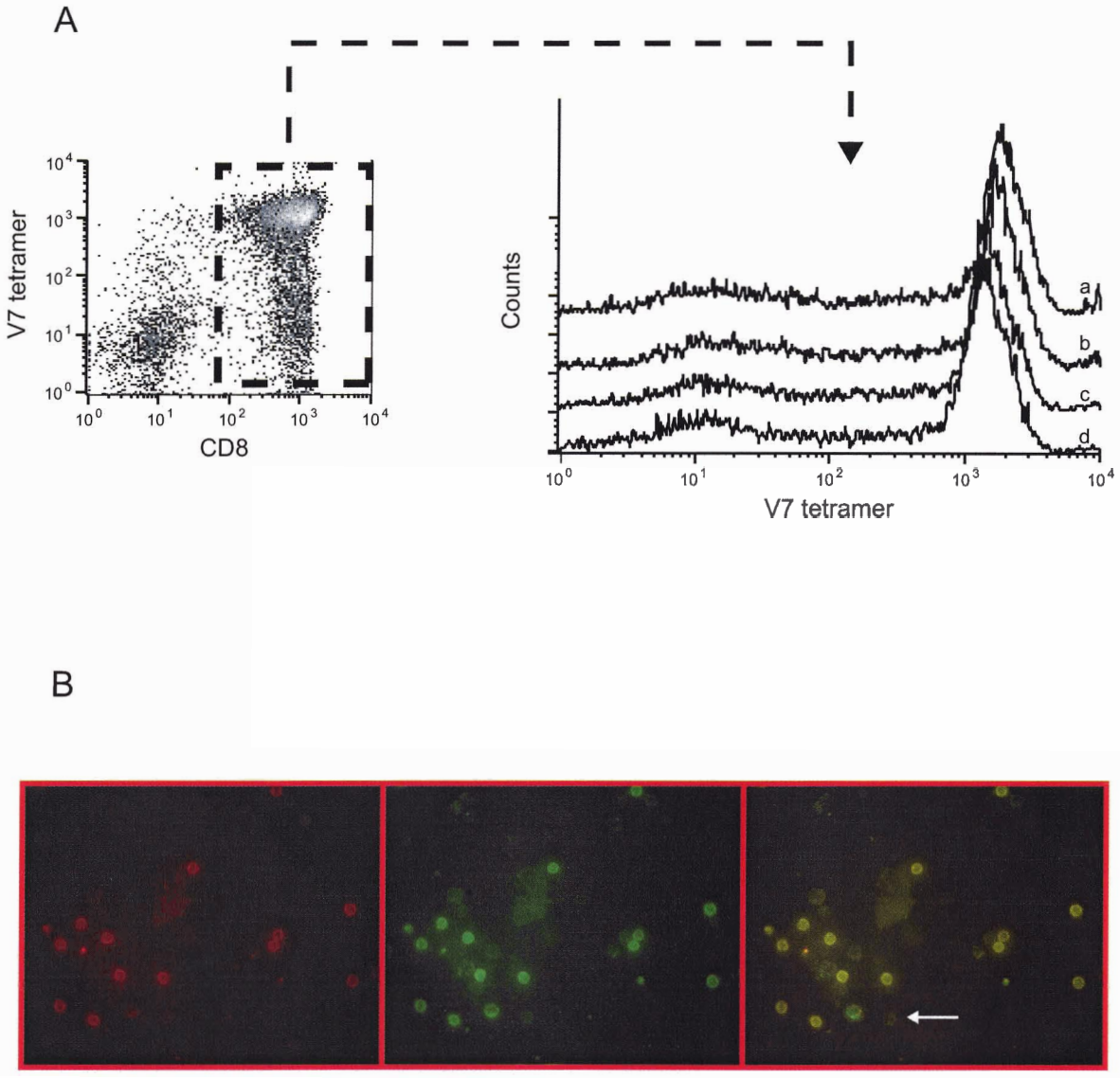

Figure 11. *Validation of MHC class I tetramers.* A. Spleen cells from 8.3-TCR tg NOD mice were stained with NRP-V7 tetramer at various dilutions (a: 1:25; b: 1:50; c: 1:100; d: 1:200) to determine the optimal concentration of tetramer for FACS analysis (tetramer batches ranged in concentration from 1-2 mg/ml). After gating on the CD8+ cells (left, hatched box), the fluorescence intensity of the tetramer positive and tetramer negative cells were analyzed. (The histograms are separated from one another vertically for clarity.) A concentration of tetramer was chosen based on optimal brightness and minimal background. B. Spleen cells from 8.3-TCR tg NOD mice were stained with NRP-V7 tetramer-PE alone (left), CD8 FITC alone (middle), or double stained with both. Using a fluorescence microscope, the proportion of CD8+ cells that stain also with tetramer can be clearly seen (right, orange). With the odd exception (arrow), the majority of transgenic cells, as expected, stained with the NRP-V7 tetramer.

slides using a Cytospin 3 (Shandon). It was evident that the tetramer was binding to the majority of $CD8^+$ cells in the sample (Figure 11b).

Flow cytometry

Tissue sample preparation

In order to stain cells from peripheral blood, spleen, lymph nodes, and islet cells for analysis by flow cytometry, single cell suspensions were prepared. Peripheral blood was collected from the femoral vein using heparinized capillary tubes (Fisher Scientific, Nepean, Canada) and placed in 1 ml red blood cell lysis buffer (0.15M NH_4Cl, 1.0mM $KHCO_3$, 0.1mM Na_2EDTA) to remove red blood cells prior to staining. For each experiment, 130µl of blood was collected. According to the Canadian Council on Animal Care, up to 135µl (7.5% of total blood volume) of blood can be safely taken from a 25 gram mouse each week. To prepare single cell suspensions from spleen and lymph nodes, the tissues were collected and mechanically disrupted by rubbing them between two sterile glass microscope slides. Red blood cells were removed from spleen samples by incubation with 1 ml red blood cell lysis buffer. Single cell suspensions of islet cells were obtained as outlined in section 2.2. In all cases, samples were washed with FACS staining buffer (1x Hank's Balanced Salt Solution, 0.1% sodium azide, 1mM EDTA, 2% fetal calf serum) prior to staining.

Staining of single cell suspensions

Cells were stained with MHC class I tetramer for 3 hours, and with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (clone YTS 169.4, Cedar Lane Laboratories) and PerCP-conjugated anti-B220 (clone RA3-6B2, Pharmingen) for the last 30 minutes,

all on ice. On occasion biotin-conjugated CD44 (clone IM7, Pharmingen) or CD69 (clone H1.2F3, Pharmingen) followed by streptavidin-allophycocyanin (SA-APC, Pharmingen) was used to stain cells prior to tetramer staining. Cells were washed 3 times with FACS staining buffer prior to analysis. If biotinylated antibodies such as CD44 were used (rather than antibodies that were conjugated directly to a fluorochrome) they were incubated with the cells for 30 minutes, and then washed prior to incubation with tetramer. The time and temperature of staining was determined in early experiments that demonstrated that 3 hours on ice was the condition that yielded the highest percentage of specific staining (data not shown).

Data collection and analysis

Single cell suspensions stained with tetramer and antibody were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, SanDiego, California). For peripheral blood samples, 100 000 events within a lymphocyte gate were analyzed. For spleen and lymph node samples, 250 000 events within a lymphocyte gate were analyzed. For samples of islet cells, as many events as possible within a lymphocyte gate were captured (often in between 10 000 and 50 000 events). Throughout this thesis, the cell population of interest is the percentage of $CD8^+$ cells that recognize or stain with the MHC class I tetramer. Cells that are both $CD8^+$ and $tetramer^+$ appear in the top right corner of flow cytometry 'dot plots'- they are the cells that have stained with both the $CD8^+$ antibody (+ in the green FL1 channel) and the MHC class I tetramer (+ in the red FL2 channel). B220 PerCP was added to all samples that were stained with tetramer to serve as an exclusion or 'dump' gate. Because the percentage of $CD8^+$ and $tetramer^+$ cells was often small, the dump gate was used to decrease the total number of cells included in the analysis. B220 is

an antigen found on B cells and non-MHC restricted lytic cells, but not on MHC class I restricted $CD8^+$ T cells (129, 130). Therefore, any cell that bound B220 (positive in the FL3 channel) was excluded from the analysis. Such a situation is analogous to looking for a needle ($CD8^+$ $tetramer^+$ cells) in a haystack (total population of spleen cells). The addition of B220, and exclusion of any $B220^+$ cells, permitted reduction of the size of the haystack, thereby allowing better detection of the needle. Therefore, the tetramer positivity throughout was determined using a lymphocyte gate and exclusion of $B220^+$ cells. Where mean data are shown, tetramer positivity is expressed as % of $CD8^+$ $B220^-$ cells, minus the percentage of TUM $tetramer^+$ ($CD8^+$ $B220^-$ cells). The background level of staining, determined with the control (TUM) tetramer was < 0.05%. Data were analyzed using FCSpress software (FCSpress, Cambridge, UK) or CellQuest software (Becton Dickinson).

Enzyme-linked immunospot (ELISpot) assays

Polyvinylidene fluoride 96-well ELISpot plates (MAIP N45, Millipore) were pre-coated with 75µl/well of IFN-γ antibody (10 µg/ml) (clone R4-6A2, Pharmingen) overnight at 4°C, and blocked with complete medium containing fetal calf serum for 1 hour at room temperature. Dispersed islet cells were co-incubated with 5 x 10^5 P815 cells (ATCC) and NRP-V7, INS, or TUM peptides (1 µg/ml) for 36 hours at 37°C. P815 cells are a mastocytoma cell line that expresses the MHC class I molecule H-$2K^d$. The addition of large amounts of β-cell peptides resulted in the displacement of the P815 antigens on the cell surface, thereby allowing the cells to act as antigen presenting cells to previously activated β-cell-specific $CD8^+$ T cells. Following 36 hours of incubation, IFN-γ secretion

was detected with a second, biotinylated IFN-γ antibody (5 μg/ml, 75 μl/well for 3 hours at room temperature) (clone XMG1.2, Pharmingen). Spots were developed using alkaline phosphatase conjugate substrate buffer (Biorad, Hercules, CA), magnified and counted, and expressed as a proportion of the total number of islet cells.

Islet Transplantation

Islets were isolated from NOD*scid*, Balb/c or C57B6 mice and transplanted into diabetic NOD mice that had been maintained on insulin for 4 – 6 weeks. Insulin treatment ended the day prior to transplantation either by cessation of insulin injections or removal of the insulin implant. The blood glucose of the transplant recipient was measured prior to surgery to confirm hyperglycemia. The animal was placed under anesthetic and its back shaved to expose the area of the left kidney. Using a micromanipulator and 12 inches of 0.58mm i.d. tubing, 550 islets were taken into the tubing. The tubing was clamped and spun for 30 seconds to obtain an islet pellet at the end of the tubing. The animal was opened to expose the left kidney. Using a 32g needle a small hole was made in the kidney capsule and a small glass pipette inserted into this hole along the long axis of the kidney and moved side to side to create a 'pocket'. The end of the tubing is cut and placed into the pocket. Using the micromanipulator, the islets were inserted into the pocket, the tubing removed, and the small hole cauterized. The animal was then sutured, and removed from the anesthetic. Normally, within 24 – 48 hours the animal regained normal glucose levels. Blood glucose was monitored daily.

Analysis of islet grafts by flow cytometry

To examine the immune cell content of the islet grafts the animals were sacrificed and their kidneys removed. The graft was cut away from the kidney as cleanly as possible and placed in sterile PBS. The graft was mechanically dissolved between two sterile glass microscope slides, washed with PBS, and spun into a pellet. To obtain a single cell suspension, the pellet was incubated with 2 ml of Cell Dissociation Buffer (Gibco-BRL) for 8 minutes at 37°C. Every 2 – 3 minutes the sample was removed from the incubator and agitated vigorously. A homogeneous single cell suspension was verified under the microscope, cells were strained through a 40μm nylon mesh and then washed in FACS staining buffer, and stained with CD8-FITC, tetramer- PE and B220-PerCP (as above). Five hundred thousand events were analyzed.

Statistical analysis

Differences between means were analyzed with Student's *t*, simple analysis of variance, or a repeated measures design, where appropriate. Post hoc analysis was done using Tukey's test. Sensitivity and specificity were defined as the proportion of true positives and true negatives that were corrected identified by the test, respectively (131). Positive predictive value was defined as the proportion of animals with positive results that were correctly identified, while the negative predictive value was defined as the proportion of animals with negative results that were correctly identified (132).

Chapter 3:

Prediction of spontaneous diabetes in NOD mice by quantification of autoreactive CTL

Introduction

The introduction of MHC tetramers to immunologists in the late 1990s immediately advanced the study of adaptive immune responses to foreign pathogens. Increasingly sophisticated applications of this technology are now being employed to study the evolution of the immune response, and its response to therapeutics and vaccination strategies. Comparatively little is known about the immune response to self-tissue(s) during the evolution of an autoimmune disease. Difficulties associated with identification of autoantigens, in combination with the low avidity, and what is commonly thought to be low frequency of autoreactive T cell populations has resulted in slower progress. Many autoimmune diseases, such as T1D, are driven by the action of T cells, and it is therefore critical to understand their role in disease pathogenesis in order to implement effective therapeutic strategies. The ability to detect autoreactive T cells in the peripheral blood of patients or individuals at risk for disease may permit prediction of disease, and would likely aid in monitoring of potential therapies.

Detection and characterization of autoreactive T cells in T1D and other autoimmune diseases

Using MHC tetramers, investigators have attempted to characterize the role of autoreactive CTL in several autoimmune diseases including diabetes (51, 63, 111, 133-137). In almost every case, detection of autoreactive T cells directly *ex vivo*, particularly from the peripheral blood, has proved difficult or impossible. In diabetes, CTL specific for a peptide derived from the insulin B chain (INS_{15-23}) have been reported to comprise a large fraction of the T cells within the islets of young NOD mice, but were undetectable in the peripheral blood or secondary lymphoid organs (111). Previous attempts to examine the proportion of islet-derived NRP-reactive CTL were unsuccessful without prior expansion of T cells *in vitro* (63). Attempts to detect glutamic acid decarboxylase (GAD)-specific CTL in blood or islets from NOD mice have also proved unsuccessful without first expanding T cell numbers by short-term *in vitro* cell culture (135). Similarly, attempts to detect GAD-specific $CD4^+$ T cells in the peripheral blood of humans at risk for developing T1D required *in vitro* T cell expansion (51).

A significant drawback to experiments that require expansion of T cells is that the *in vitro* manipulation disrupts the true proportion of antigen-specific cells present, and the use of exogenous soluble antigens and/or cytokines in the short or long-term cell culture almost certainly changes the effector state of the T cell population of interest. Akin to limiting dilution assays, the assay is no longer testing for the frequency of antigen-specific cells in the body, but rather the number of cells that survive *in vitro*. For example, many lymphocyte expansion protocols continue to rely on the addition of the lymphocyte growth factor, interleukin (IL)-2. Recent studies have shown that, contrary to

its positive effects on naïve or effector CTL, IL-2 induces *apoptosis* of memory T cells (138, 139), which instead require the growth factor(s) IL-15 (138, 140-142) and/or IL-7 (141-145). Therefore, IL-2 expansion may be successful during a period when effector cells abound, but not during a period where memory T cells prevail (such as after onset of disease) and accordingly, these experimental manipulations affect the data obtained. Nonetheless, taken together, these previous reports indicate that autoreactive T cells are present in pre-diabetic and diabetic NOD mice and humans, albeit at frequencies and avidities that are too low to be detected *ex vivo* with conventional MHC-tetramers.

Current methods for prediction of T1D

At present, the prediction of T1D is based on the presence of circulating autoantibodies targeted to the β-cell proteins insulin, glutamic acid decarboxylase (GAD), and ICA512/IA-2. In humans, the presence of all three autoantibodies predicts the development of diabetes within 5-10 years with a sensitivity ranging from 60-100% (146, 147). In mice, the presence and first appearance of serum autoantibodies has been shown to vary considerably between both diabetic and non-diabetic mice, and shown by many to be correlated only modestly with development of disease (148-150). A recent workshop validated the presence of anti-insulin autoantibodies (IAA) in pre-diabetic NOD mice, but questioned the role of both ICA512/IA-2 and GAD antibodies (149). Consistent with these findings is a recent prospective study showing that the presence of IAA in NOD mice is strongly associated with development of diabetes, and that the age of IAA appearance correlates with the age of disease onset (151). Despite the relative success of autoantibodies for prediction of diabetes occurrence, a method for monitoring the CTL that are directly responsible for β-cell damage would be highly desirable (152,

153) not only for its potential to be an earlier, more accurate and reliable means of assessing the severity of pancreatic islet inflammation, but particularly as a means of directly monitoring the β-cell-specific T cell response to therapies. The appearance of IgG antibodies would be predicted to follow the cellular response, as CTL responses can be detected during acute infections (hours to days post infection) whereas the appearance of IgG requires days or weeks (47). Moreover, the detection of high affinity IgG requires affinity maturation in lymph nodes, a process that may delay antibody detection further (47). In addition to the delay in initial detection, autoantibodies exist in the body over a lifetime, and their presence may be difficult to interpret following therapy such as islet transplantation. Because tetramer-based assays directly measure the number of autoreactive CTL present, it may be a more sensitive test for the degree of ongoing β-cell damage.

Results

Detection of autoreactive CTL with high avidity pMHC class I tetramers

To overcome the limitations of detecting low avidity autoreactive T cells *ex vivo*, high affinity altered peptide ligands (APLs) were used in conjunction with MHC tetramers to increase the avidity of the pMHC for autoreactive TCRs. The increased avidity of the pMHC-TCR interaction would allow the MHC-tetramer to bind more easily and with a longer half-life to antigen-specific T cells and in turn, allow for the detection of a larger subset of cells, that includes both lower and higher avidity T cells (as opposed to just higher avidity cells). A similar strategy has been used by others to increase the avidity of interaction between tumor-specific T cells and tumor cells (154). The NRP

mimotope (KYNKANWFL) was modified by substitution of an alanine or a valine residue for the tryptophan at position 7 (NRP-A7 and NRP-V7, respectively). NRP-A7 and NRP-V7 are heteroclitic analogs of NRP that possess superior agonistic activity (63, 114). NRP, NRP-A7, NRP-V7 and the control peptide TUM were used to create MHC class I tetramers, and used to stain islet-derived T cells. The TUM peptide (KYQAVTTTL) is an H-2K^d-binding peptide derived from P198.3 tumour cells (155). It binds to the MHC class I molecule H-2K^d, however no TUM-specific T cells are expected to exist in NOD mice, thereby serving as a good negative control for tetramer binding studies.

In contrast to previous findings (63), a significant fraction of islet-derived CD8$^+$ cells were NRP-reactive and detectable *ex vivo* (Figure 12). Moreover, a significantly higher percentage of CD8$^+$ cells stained with the NRP-V7 tetramer (20.0% of all CD8$^+$ cells) as compared to the less avid NRP-A7 tetramer (8.2% of all CD8$^+$ cells) and the even less avid NRP tetramer (4.1% of all CD8$^+$ cells) (Figure 12). As MHC class I tetramers complexed to NRP-V7 stained islet-associated T cells more intensely and bound to a significantly greater number of T cells than tetramers bearing the peptides NRP or NRP-A7, the NRP-V7 tetramer was chosen for the majority of further analyses.

Detection of islet-derived autoreactive CTL populations *ex vivo*

It has been reported previously that a large majority of the CD8$^+$ T cells in the pancreatic islets of pre-diabetic NOD mice, particularly in very young mice, are specific for the $INS_{15\text{-}23}$ peptide, derived from the insulin B chain (111). In attempt to confirm this finding, as well as to determine the proportion of CD8$^+$ T cells within freshly isolated

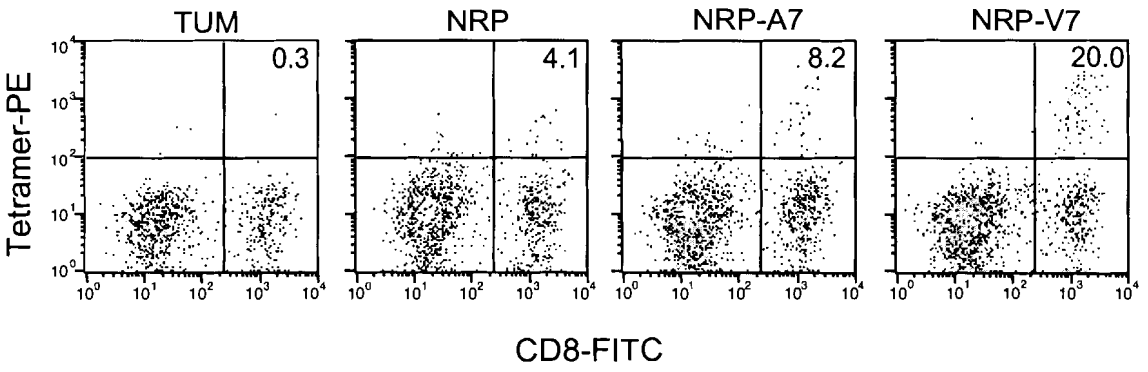

Figure 12. *High avidity peptide/MHC class I tetramers detect a higher frequency of autoreactive T cells from freshly isolated islets.* Pancreatic islets derived from 8-week-old female NOD mice were stained with tetramers associated with the peptides TUM, NRP, NRP-A7 or NRP-V7. Numbers (top right quadrant) indicate the percentage of $CD8^+$ $B220^-$ $tetramer^+$ cells. The data is representative of six independent experiments from mice 6 – 15 weeks of age. A similar result was observed with peripheral blood (not shown; % of tetramer-positive cells ~10-fold lower).

islets from NOD mice that were NRP-reactive, islets were examined at various stages of pre-diabetes. Islets were obtained from NOD mice beginning at 4 – 5 weeks of age (the earliest signs of insulitis), at 7 – 10 and 11 –14 weeks (progressively more intense periods of insulitis), and at 15 –18 weeks of age (just prior to the onset of hyperglycemia). Islets were isolated and then dispersed into single cell suspensions in order to liberate the intra-islet lymphocytes, and to permit single cell staining. For animals younger than 9 weeks of age, it was necessary to pool the islets from several animals in order to obtain a sufficient number of cells for analysis. Cells from freshly isolated islets were stained with tetramers bearing the control peptide TUM, the NRP-V7 peptide as well as the index peptide NRP, and the INS_{15-23} peptide (Figure 13 a and b). In freshly isolated islets from 4 – 5 week old mice, the number of INS_{15-23}-reactive $CD8^+$ T cells ranged from 0.60 – 10.0% (Table 3-1), a value significantly less than that reported previously (111). The proportion of INS_{15-23}-reactive $CD8^+$ cells in the islets declined to less than 3 percent by 7 – 10 weeks of age, and declined further thereafter. In contrast, the NRP-reactive population of $CD8^+$ T cells first appeared significantly at 7 weeks of age, and peaked at 11 – 14 weeks of age, when the proportion of $CD8^+$ cells within islets that stained with the NRP-V7 tetramer ranged from 1.61 – 36.8%. Although the age of peak NRP-V7 staining roughly paralleled that of NRP, use of the high avidity NRP-V7 analog permitted detection of a much higher number of autoreactive T cells as compared to the index NRP mimotope (Figure 13b and Table 3-1). Islets from non-diabetic mice at 32 weeks of age (the end of the study) were examined to determine the proportion of islet-infiltrating lymphocytes that were NRP-reactive. NOD mice that remain non-diabetic have insulitis, albeit a much less invasive

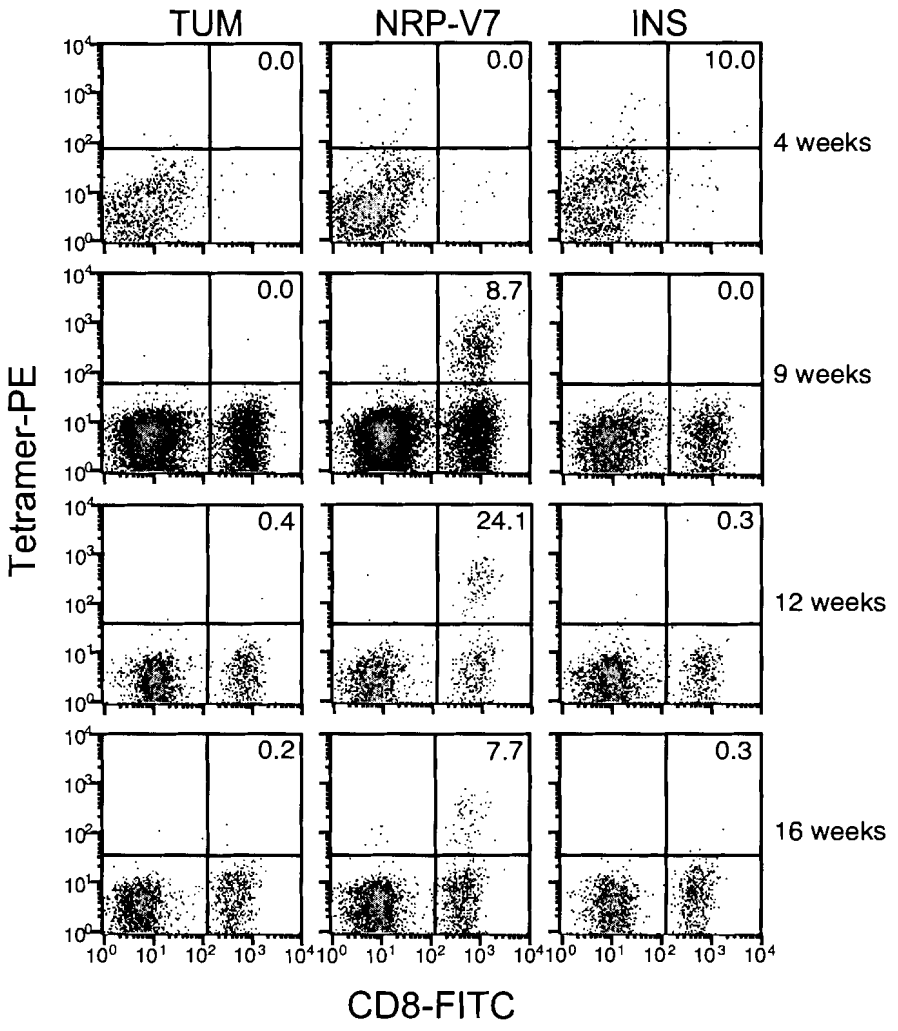

Figure 13a. *A significant fraction of CD8+ cells from the pancreatic islets stain with MHC class I tetramers ex vivo.* Shown is a representative example of TUM, NRP-V7, or INS tetramer staining of islets at 4, 9, 12, and 16 weeks of age. Numbers (top right quadrant) indicate the percentage of CD8+ B220- tetramer+ cells.

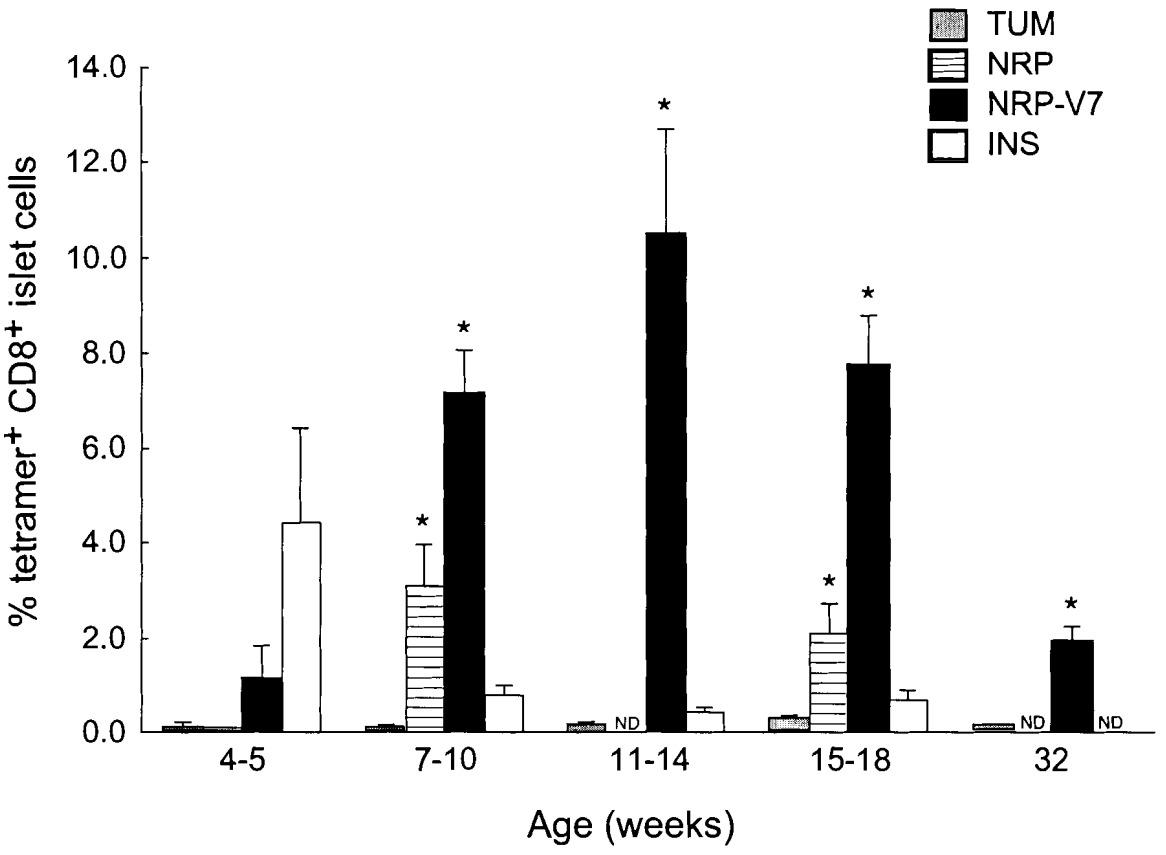

Figure 13b. *Islet-associated CD8+ cells are strongly NRP-reactive beginning at 7 weeks of age.* Mean percentage (± SEM) of tetramer positive cells from mice at 4 – 5 (n = 4), 7 – 10 (n = 14), 11 – 14 (n = 17), and 15 – 18 (n = 14) weeks of age. The percentage of NRP+ cells was not determined in all experiments: at 4 – 5 weeks, n = 3; 7 – 10 weeks, n = 7; 15 – 18 weeks, n = 10. For animals younger than 8 weeks of age, islets were pooled from several animals to obtain sufficient cells for analysis. Data shown also for non-diabetic mice at 32 weeks of age (n = 5), ND = not determined. * Indicates a significant difference from TUM at each age, $P < 0.001$.

Table 3-1. Frequency of autoreactive $CD8^+$ T cells in freshly isolated pancreatic islets. (The range for each age group is indicated.)

Age (weeks)	NRP-V7$^+$ $CD8^+$ cells (%)	NRP$^+$ $CD8^+$ cells (%)	INS$^+$ $CD8^+$ cells (%)
4 – 5	0.0 – 2.68	0.0	0.60 – 10.0
7 – 10	0.32 – 14.1	1.01 – 7.10	0.0 – 2.48
11 – 14	1.61 – 36.8	Not determined	0.0 – 1.37
15 – 18	1.79 – 18.7	0.25 – 7.13	0.0 – 1.52
32	1.18 – 3.01	Not determined	Not determined

form. Nonetheless, a small but significant percentage of the $CD8^+$ T cells within islets from 32-week-old animals stained with the NRP-V7 tetramer (Figure 13b).

Analysis of the number of antigen-specific cells with MHC tetramers provides valuable quantitative information. Although many studies have confirmed that during acute infection the number of tetramer positive cells correlated with the number of functional cells as measured by ELISpot assay (79, 156), the tetramer-based analysis does not confirm the phenotype or function of the tetramer positive cells. ELISpot (enzyme-linked immunospot) assays are ELISA-based assays whereby specific antibodies are used to detect cytokines secreted after a memory (or antigen experienced) T cell comes into contact with its specific antigen. With respect to experiments outlined in this thesis, islet-derived T cells are co-incubated with peptide-loaded P815 cells ($H\text{-}2K^d$) that are coated with NRP-V7, $INS_{15\text{-}23}$, or TUM peptides. Antibodies are used to detect the IFN-γ secreted when an antigen-specific $CD8^+$ T cell comes into contact with the peptide-coated P815 cell. As a negative and positive control, naïve spleen cells and islet-derived T cells, respectively, from 8.3-TCR NOD mice were used in the assay. The use of ELISpot assays was necessary and effective for provision of functional data for islet-based assays because the number of effector cells was very low (too small to conduct standard chromium release assays as a measure of antigen-specific lysis by CTL). IFN-γ ELISpot assays however, are very sensitive and have been shown in previous experiments to correlate with the lytic activity shown in chromium release assays (80).

To confirm that the islet-derived NRP-V7-reactive cells measured by tetramer analysis were functional, the number of cells that produced IFN-γ in response to NRP-

V7, INS_{15-23}, or TUM peptides was counted (Figure 14). Approximately 0.2% of total islet cells secreted IFN-γ in response to NRP-V7 – a number that was consistent with the proportion of NRP-V7-reactive T cells contained within the total islet cell population as determined by NRP-V7 tetramer staining, and a value significantly greater than that obtained using either INS_{15-23} or TUM.

Detection of peripheral blood autoreactive T cells

Having established that autoreactive $CD8^+$ T cells could be detected from islets *ex vivo*, it was necessary next to determine whether they were detectable in peripheral blood, and if their presence in peripheral blood could act as a surrogate marker for infiltration of pancreatic islets. Previous attempts by others to detect autoreactive β-cell-specific T cells in the peripheral blood prior to the onset of an autoimmune disease using MHC class II tetramers in both humans (51) and mice (135) was not possible without first expanding cells *in vitro*. Previous attempts to stain both NRP-reactive (63) and INS_{15-23}-reactive (111) $CD8^+$ T cells from the spleen and pancreatic lymph nodes *ex vivo* were unsuccessful, and the work in this thesis suggests that the peripheral blood staining mirrors that of the spleen (see below). Of the studies that attempted to characterize autoreactive T cells in the peripheral blood of patients with ongoing autoimmune disease, one of three has been successful. Two of the studies used MHC class II tetramers: one to examine Borrelia-reactive $CD4^+$ T cells in patients with lyme disease (136) and the other collagen-reactive $CD4^+$ T cells in patients with relapsing polychondritis (134). In both instances, the frequency of autoreactive T cells was too low to be detectable with the MHC tetramers. On the other hand, using an MHC class I tetramer to detect melanocyte-specific CTL in the peripheral blood of patients with autoimmune vitiligo, Ogg *et al.*

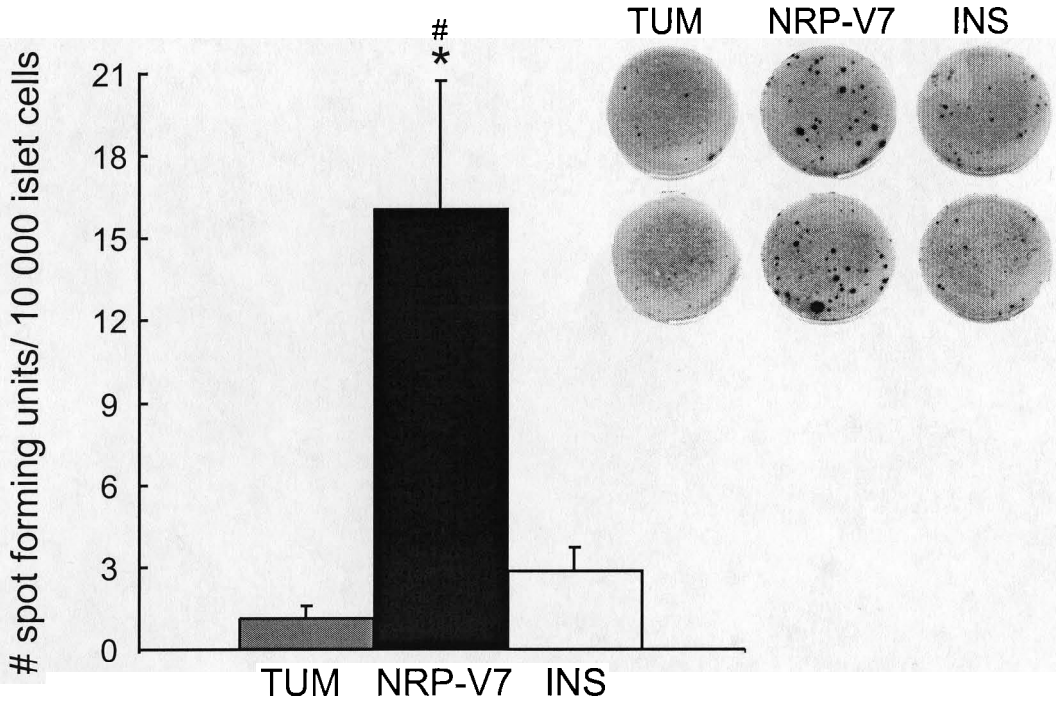

Figure 14. *Islet-associated NRP-reactive CD8⁺ T cells from pre-diabetic NOD mice secrete IFN-γ.* Islet cells pooled from 4 mice (13 – 16 weeks of age) were tested for their ability to secrete IFNγ in response to the peptides TUM, NRP-V7, or INS in ELISpot assays (n = 4). Naïve splenic T cells and islet-associated T cells from an 8.3-TCR NOD mouse were used as negative and positive controls, respectively. * Indicates a significant difference from TUM, $P < 0.001$, # Indicates a significant difference from INS, $P < 0.05$. Inset: Sample ELISpot assay showing duplicate wells containing 20 000 islet cells from four - 14 week old NOD mice.

were able to show that the frequency of tetramer-positive cells correlated with the severity of disease (133).

Although a small number of INS_{15-23}-reactive $CD8^+$ T cells were detectable in the islets of 4 – 5 week old mice (Figure 13a and 13b), at all time points examined (weekly intervals from 4 – 18 weeks of age), INS-reactive T cells could not be detected in the peripheral blood (data not shown). The NRP-V7 tetramer was chosen to analyze the peripheral blood of NOD mice, because in a similar fashion to the pancreatic islets (Figure 12), NRP-V7 tetramers detected a higher proportion of NRP-reactive cells in the peripheral blood than the NRP-A7 or NRP tetramers. Mice from three age-specific groups representing early (9-10 weeks), middle (11-14 weeks), and late (15-17 weeks) stages of disease pathogenesis were sacrificed, and blood, islets, and lymphoid organs from each mouse were stained directly *ex vivo* with the NRP-V7 and TUM tetramers. Autoreactive T cells were clearly detectable in peripheral blood from each group of mice, and their presence in circulation was associated with the presence of autoreactive T cell infiltration of both pancreatic islets and lymphoid organs (Figure 15a). In the vast majority of the animals examined (27/30), the presence of NRP-V7-reactive T cells in peripheral blood correlated with the presence of a similar population of T cells in pancreatic islets; however the exact percentage of NRP-$V7^+$ $CD8^+$ cells in the peripheral blood did not correlate with the percentage present in the islet infiltrate ($r = 0.23$, for all ages). There were also a few instances (3/30), where NRP-V7-reactive T cells were present in islets, but could not be detected in peripheral blood.

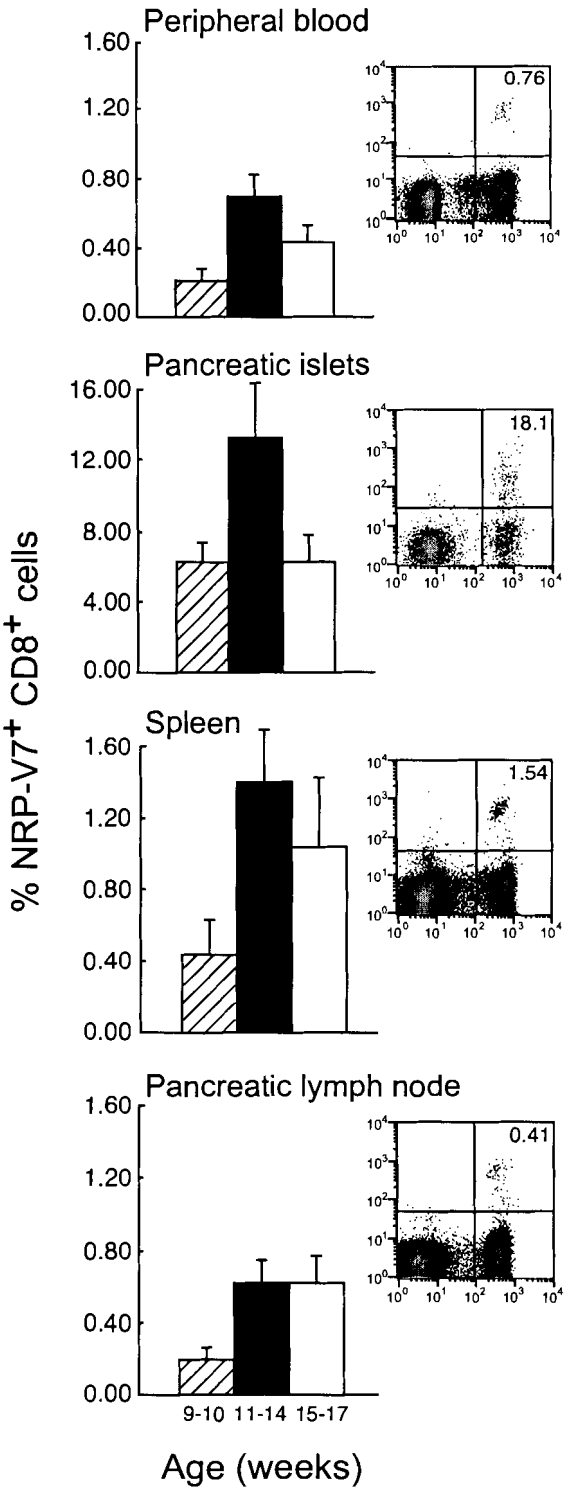

Figure 15a. *NRP-V7-reactive T cells in peripheral blood are a surrogate marker for their presence in pancreatic islets.* The mean (± SEM) percentage of NRP-V7-tetramer positive CD8+ B220- cells (minus percent TUM-tetramer positive CD8+ cells) within peripheral blood, pancreatic islets, spleen, and pancreatic draining lymph nodes are shown for individual NOD mice of 9 – 10 (hatched bars; n = 7), 11 – 14 (filled bars; n = 13), and 15 – 17 (unfilled bars; n = 10) weeks of age. The inset shows a representative staining of each tissue from a single 12-week-old mouse. Numbers indicate the percentage of CD8+ B220- tetramer+ (minus control) cells.

The peripheral blood of 11 week old NOR/Ltj, Balb/c, and C57Bl/6 mice was examined to assess the percentage of cells in other H2-K^d-expressing and non-H2-K^d-expressing mice that would bind the NRP-V7 tetramer (Figure 15b). NOR/Ltj mice are genetically similar to NOD mice; they express H2-K^d and develop some insulitis, but not diabetes (157). The percentage of NRP-V7 tetramer$^+$ $CD8^+$ cells (- TUM control) in the peripheral blood ranged from 0.01 – 0.09 (mean 0.04%; n = 5). In Balb/c mice, a non-diabetogenic strain that expresses H2-K^d, the range was 0.0 – 0.17 (mean 0.10%; n = 5), and in C57Bl/6 mice, a non-diabetogenic strain that does not express H2-K^d, the range was 0.0 – 0.6 (mean 0.01%; n = 8). There was a small, but convincing population of NRP-V7-reactive cells that was seen in 4/5 Balb/c mice (Figure 15b).

Dynamics of peripheral blood autoreactive T cells during diabetes development

Detection of antigen-specific T cells in pancreatic islets offers a static picture of the various T cell specificities present at any given time during progression of disease and thus cannot establish when T cells first appeared in individual animals, how the autoreactive T cell population evolved with time, and how the accumulation of autoreactive T cells related to development of diabetes. Since the presence of autoreactive T cells in peripheral blood reflected their accumulation in pancreatic islets, it was reasoned that screening the peripheral blood of individual animals over time might allow prediction of which animals would develop diabetes. Blood was obtained weekly from 6 to 32 weeks of age, or until the onset of diabetes, and autoreactive $CD8^+$ T cells were tracked using the NRP-V7 and TUM tetramers. Mice that became diabetic were maintained with exogenous insulin and monitored for four additional weeks. Several initial observations were made regarding the dynamics of circulating autoreactive T cells

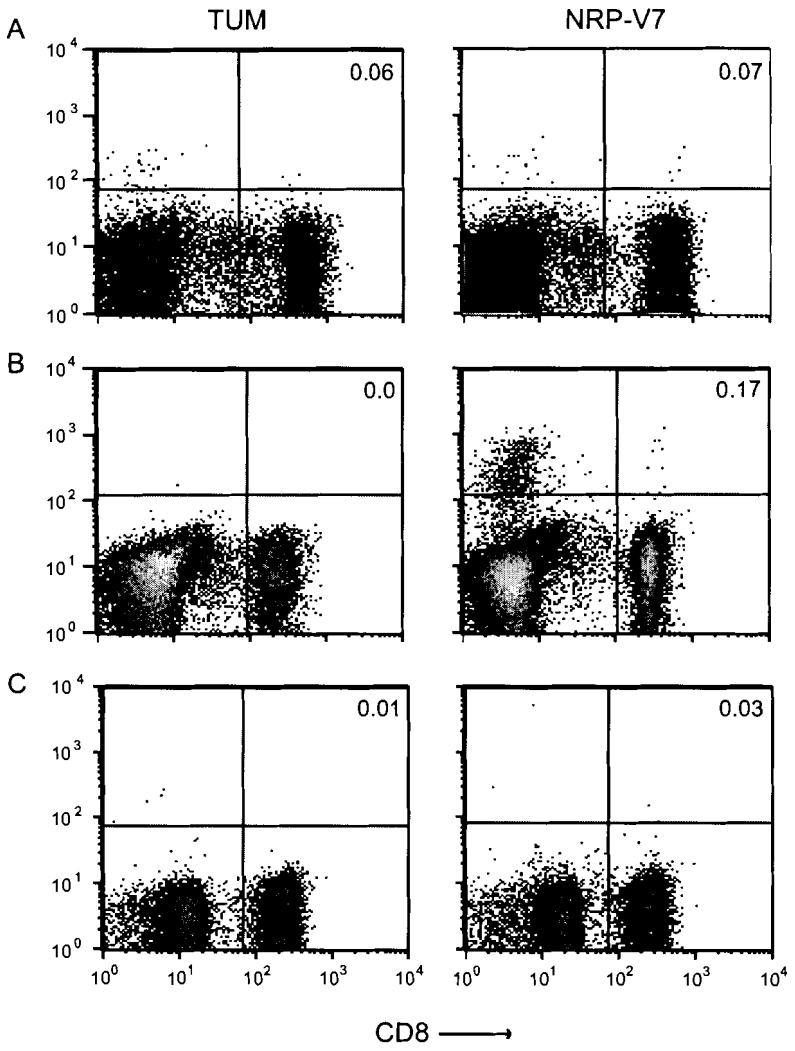

Figure 15b. *The peripheral blood from Balb/c mice contains a small fraction of NRP-V7-reactive cells.* Peripheral blood from NOR/Ltj (n=5) (A), Balb/c (n=5) (B), and C57Bl/6 (n=8) (C) mice was examined for the presence of NRP-V7 tetramer binding cells. Numbers (top right quadrant) are the percentage of tetramer$^+$ CD8$^+$ B220$^-$ cells.

prior to diabetes onset (Figure 16). The earliest detection of NRP-V7 specific T cells in the peripheral blood occurred at 9 weeks of age, suggesting that before this time, there was insufficient priming and/or proliferation of this autoreactive T cell population to allow visualization in blood. Since insulitis begins at approximately 3 – 4 weeks of age (39), these data also imply that approximately six weeks is required for sufficient expansion of primed T cells in pancreatic lymph nodes for them to become visible in peripheral blood. Importantly, mice destined to develop diabetes had significantly larger populations of NRP-V7-reactive T cells in the peripheral blood prior to diabetes onset (i.e. between 9 and 16 weeks of age). Interestingly, these populations appeared in distinct cycles prior to the onset of hyperglycemia: in animals that were destined to develop diabetes, hyperglycemia was preceded by at least one distinct peak of NRP-V7-specific $CD8^+$ cells, but more often by 2-3 peaks.

To examine the relationship between the appearance of tetramer-positive peaks in the blood and diabetes development, the onset of hyperglycemia was normalized to time 0 and the tetramer positivity in the weeks preceding diabetes was analyzed (Figure 17). Tetramer positivity first appeared on average 7 weeks prior to hyperglycemia, although the timing and magnitude of autoreactive T cell appearance during the pre-diabetic period varied between animals (as seen by the large standard error bars). Importantly, following the onset of hyperglycemia, the number of tetramer-positive cells declined rapidly, presumably due to lack of antigen as a result of β-cell destruction.

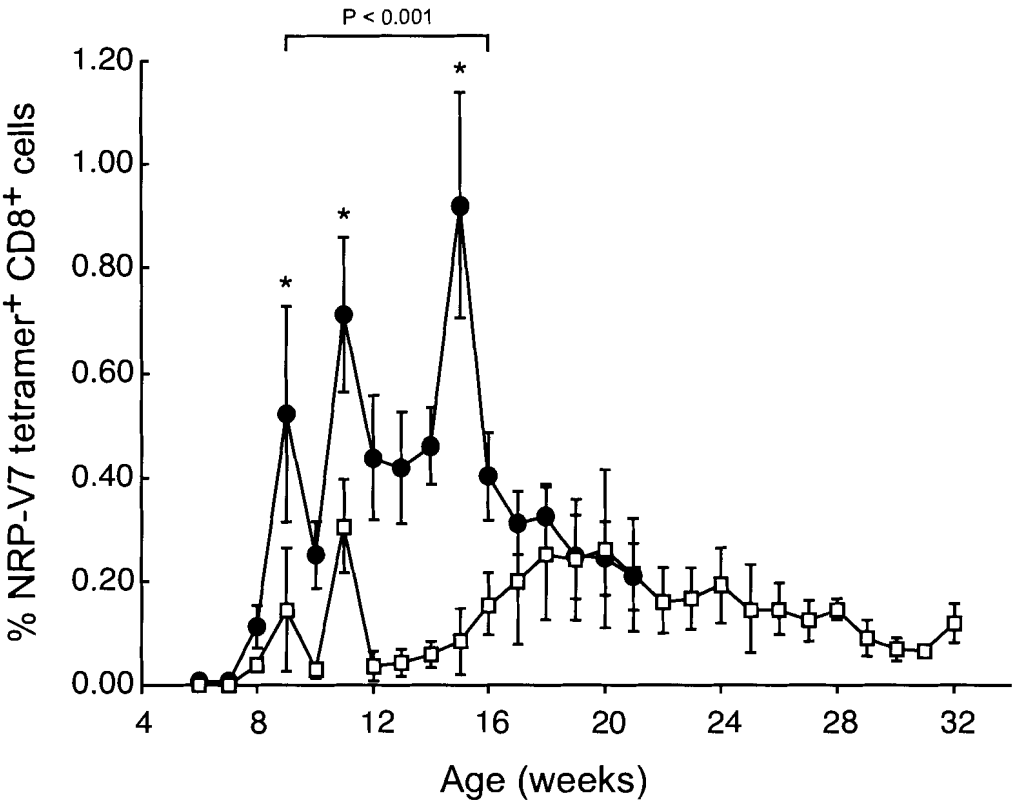

Figure 16. *NOD mice that are destined to develop diabetes have a higher proportion of NRP-reactive CD8+ T cells in the peripheral blood.* Mean (± SEM) proportion of NRP-V7-tetramer positive CD8+ B220- cells (minus percent TUM-tetramer postive CD8+ cells) in the peripheral blood of female mice that went on to develop diabetes (●; n = 13) versus mice that failed to develop diabetes by 32 weeks of age (□; n = 5). Mice from several different litters were analyzed in two separate groups beginning at 6 (n = 6), or 9 weeks of age (n = 12). Most diabetic mice were removed from the study after 21 weeks of age. The proportion of NRP-V7-tetramer positive cells was significantly different between diabetic and non-diabetic mice at each time point from 9 weeks to 16 weeks of age (bar; $P < 0.001$). For diabetic animals, there was a significant difference between NRP-V7-tetramer positive peaks and the time points immediately prior to, and following the indicated peak (*; $P < 0.001$).

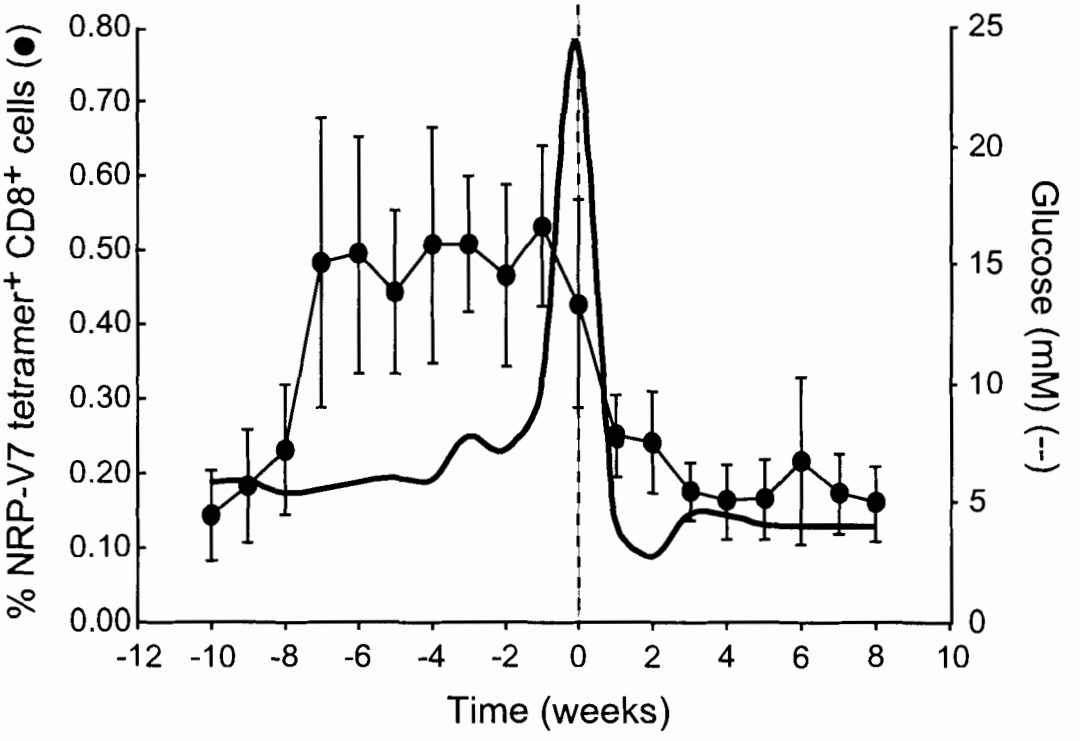

Figure 17. *Autoreactive CD8+ T cells are detectable in the peripheral blood prior to the onset of hyperglycemia.* The onset of hyperglycemia was normalized to time 0 (solid line), and the proportion (mean ± SEM) of NRP-V7-tetramer positive CD8+ B220- cells (minus percent TUM-tetramer postive CD8+ cells) was determined for the weeks prior to and following development of hyperglycemia (●). Decline in blood glucose levels following the onset of hyperglycemia (after time 0) was due to insulin treatment.

Prediction of autoimmune diabetes in pre-diabetic female NOD mice

To determine whether the presence of β-cell-specific T cells in peripheral blood could be used to predict diabetes development, the cumulative percentage of NRP-V7-specific T cells during the pre-diabetic period was calculated (Figure 18a). Mice that eventually developed diabetes accumulated a significantly larger proportion of NRP-V7-reactive T cells over time, evident several weeks prior to the average age of diabetes onset (18.5 weeks). A threshold was chosen post-hoc from this analysis that could serve as a predictor of diabetes development. For example, all mice (13/13) that accumulated 0.75% NRP-V7 tetramer positive cells by 15 weeks of age developed diabetes, whereas all of those that did *not* (5/5), remained diabetes free for the entire 32 week study period (Table 3-2). Further analysis of the individual mouse data revealed that in the context of weekly monitoring, a single value of greater than 0.50% NRP-V7-reactive T cells in the peripheral blood is a strong predictor of diabetes. For example, of the 13 mice that went on to become diabetic, 11 had at least one blood sample showing greater than 0.50% NRP-V7-reactive $CD8^+$ T cells (Figure 18b). In contrast, none of the 5 mice that failed to progress to diabetes by 32 weeks of age ever exhibited NRP-V7 staining greater than 0.50%. The data show also that if only a single measurement of NRP-reactive cells were to be made, the criterion of greater than 0.50% NRP-$V7^+$ $CD8^+$ cells would not have had predictive value since the majority of samplings contained less than 0.50%, even for animals that went on to develop diabetes (Figure 18b).

Autoreactive CTL dynamics in the peripheral blood of male NOD mice

Male NOD mice develop diabetes less frequently than female NOD mice (on average, disease incidence in males is 20 – 40% as compared to 70 – 90% incidence in

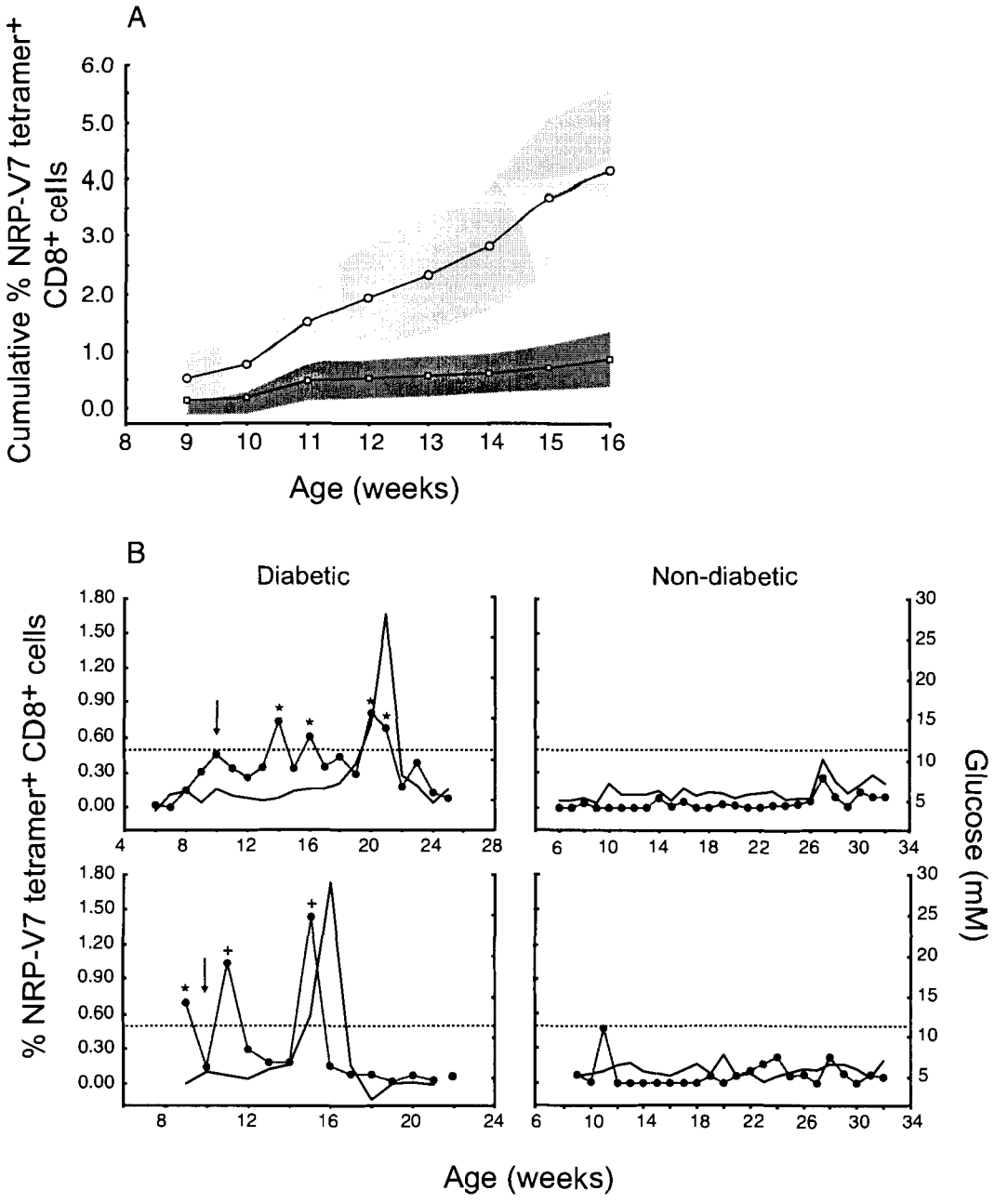

Figure 18. *NRP-V7-specific T cells detected in the peripheral blood can be used to predict diabetes development.* A. Accumulation of NRP-V7-tetramer positive cells analyzed from peripheral blood for diabetic (○; n = 13) and non-diabetic (□; n = 5) mice from 9 – 16 weeks of age. For each animal, the percentage of NRP-V7-tetramer positive cells at each time point (minus control) was summed with the cumulative total of NRP-V7-tetramer positive cells from all previous weeks, and expressed as a mean for diabetic vs. non-diabetic animals. The shaded area indicates the 95% confidence interval of the mean. B. Representative data from individual mice showing the proportion of NRP-V7-tetramer positive cells present in peripheral blood (●) as it relates to blood glucose (solid line, no symbols) for the entire period of study. Decline in blood glucose following onset of hyperglycemia was a result of insulin treatment. Arrows indicate the time at which mice had accumulated 0.75% NRP-V7-tetramer positive cells, predictive of diabetes development. * Indicates NRP-V7-tetramer positivity >0.50% for a single time point (0.50% indicated by dashed line), + indicates NRP-V7-tetramer positivity >1.00% for a single time point.

Table 3-2. Accuracy of cumulative peripheral blood NRP-V7 tetramer measurements for the prediction of diabetes in female NOD mice. (Average age of diabetes onset = 18.5 weeks.)

Age (weeks)	11	13	15
Sensitivity (%)	62	85	100
Specificity (%)	80	80	60
Positive Predictive Value (%)	89	92	87
Negative Predictive Value (%)	44	67	100

females) (25, 27), and therefore may be a more rigorous model for determining whether mice that eventually develop diabetes have a higher proportion of NRP-V7-reactive cells in the peripheral blood prior to the onset of hyperglycemia, and whether their presence can predict disease occurrence. Peripheral blood from male NOD mice was examined weekly, beginning at 6 weeks of age, with NRP-V7 and TUM tetramers. The methods used for the analysis of peripheral blood, and the subsequent treatment of diabetic animals was identical to that used for the female mouse experiments. Male mice developed diabetes later on average than did female mice (19.7 versus 18.5 weeks of age), and diabetic mice were therefore kept in the study until 26 weeks of age. As was seen with the female NOD mice, male NOD mice that went on to develop diabetes had a significantly higher proportion of NRP-V7-reactive $CD8^+$ T cells in the peripheral blood than did animals that remained non-diabetic for 32 weeks of age (Figure 19). Similar to female mice, the first appearance of NRP-V7-reactive cells in the peripheral blood occurred at 10 weeks of age, presumably many weeks following the onset of insulitis. The appearance of NRP-V7-reactive cells in the peripheral blood of male animals however, was less regular than the pattern seen with female mice. The appearance of NRP-V7-reactive cells in the female mice occurred in distinct cycles prior to the onset of disease (Figure 16), while in males the pattern was not cyclic, synchronous, nor did it appear to occur exclusively prior to disease onset. Normalization of the data to reflect the onset of hyperglycemia as time 0 permitted analysis of the average appearance of NRP-V7-reactive cells prior to, and following onset of hyperglycemia (Figure 20). The vast majority of NRP-V7-reactive cells were present in the peripheral blood prior to the onset of hyperglycemia. Although there was a decrease in the proportion of autoreactive cells

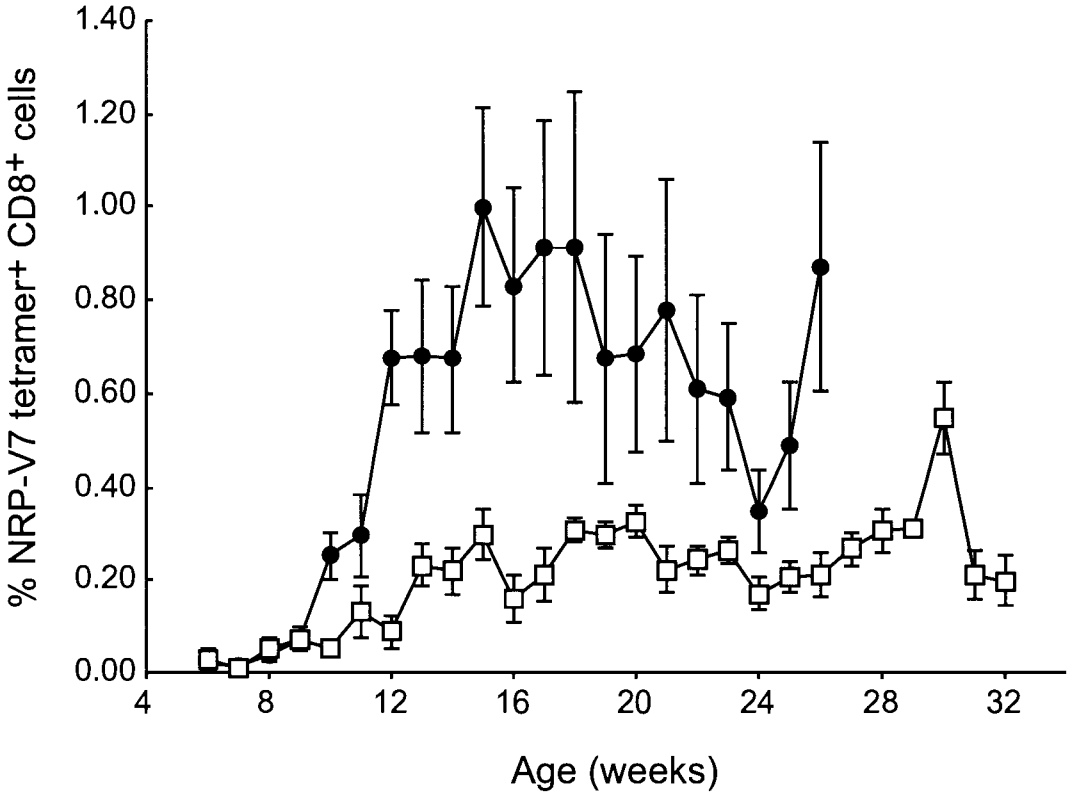

Figure 19. *The proportion of NRP-V7-tetramer positive cells is higher in the peripheral blood of male NOD mice that go on to develop diabetes.* Mean (± SEM) proportion of NRP-V7-tetramer positive CD8+ B220- cells (minus percent TUM-tetramer postive CD8+ cells) in the peripheral blood of male mice that went on to develop diabetes (● ; n = 10) versus mice that failed to develop diabetes by 32 weeks of age (□ ; n = 5). Mice from two different litters were analyzed in two separate groups beginning at 6 weeks of age. Diabetic mice were removed from the study by 26 weeks of age. The proportion of NRP-V7-tetramer positive cells was significantly different between diabetic and non-diabetic mice, $P < 0.001$.

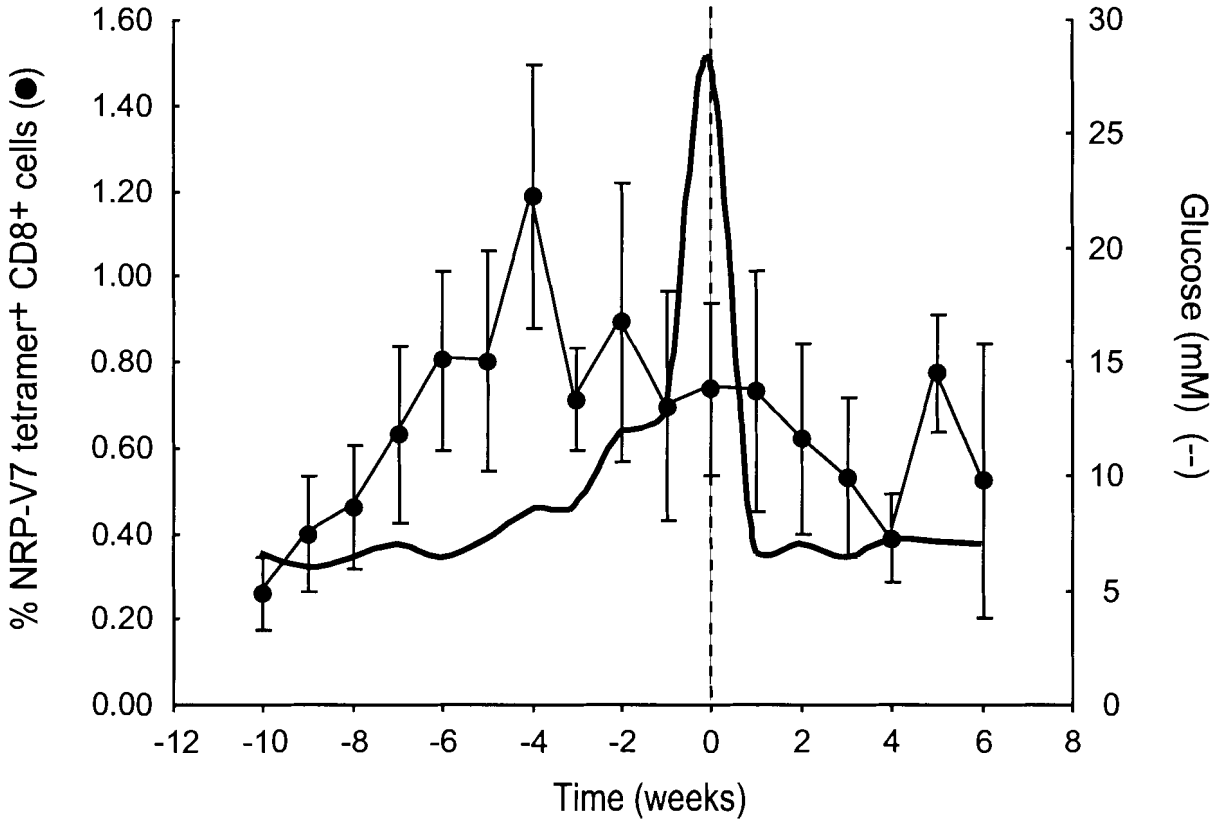

Figure 20. *Autoreactive CD8+ T cells are detectable in the peripheral blood of male NOD mice prior to the onset of diabetes.* The onset of hyperglycemia was normalized to time 0 (solid line), and the proportion (mean ± SEM) of NRP-V7-tetramer positive CD8+ B220- cells (minus percent TUM-tetramer postive CD8+ cells) was determined for the weeks prior to and following development of hyperglycemia ●). Decline in blood glucose levels following the onset of hyperglycemia (after time 0) was due to insulin treatment.

detected following the onset of hyperglycemia, the results were more variable than those demonstrated by the female mice- indicating that in many of the male mice, the proportion of NRP-V7-reactive cells did *not* decrease dramatically following hyperglycemia, nor was the decrease as pronounced as that seen for the female cohort (the average number of NRP-V7-reactive cells present following hyperglycemia was higher in male mice).

Prediction of autoimmune diabetes in pre-diabetic male NOD mice

To determine whether the presence of β-cell-specific T cells in peripheral blood could in fact be used to predict diabetes outcome for male NOD mice, the cumulative sum of NRP-V7-reactive, $CD8^+$ cells in the period preceding diabetes onset was calculated (Figure 21a). The calculated cumulative percentage of NRP-V7 tetramer$^+$ $CD8^+$ cells in male mice between the ages of 9 and 16 weeks was very similar to the results seen for female mice (compare Figures 18a and 21a). However, a greater calculated cumulative percentage was required to predict diabetes for the male cohort of mice. The threshold chosen to predict diabetes for the male mice was a calculated cumulative percent of 2.00 NRP-V7 tetramer$^+$ $CD8^+$ cells. By 15 weeks of age, 100% of mice that had a cumulative percentage value of 2.00% NRP-V7$^+$ $CD8^+$ cells went on to develop diabetes (average age of diabetes onset was 19.7 weeks), while all of the mice that remained non-diabetic did not measure beyond this threshold (Table 3-3). In addition, a single value of greater than 0.50% NRP-V7-reactive T cells in peripheral blood was also a strong predictor of diabetes for male mice (Figure 21b). All (10/10) of the male mice that went on to develop diabetes had at least one weekly sample that contained >0.50% NRP-V7$^+$ $CD8^+$ cells. In contrast, each weekly sampling for all (0/5) of

the mice that remained diabetes free remained below this threshold. The probability of successfully identifying a pre-diabetic animal simply by analysis of a single blood sample containing >0.50% NRP-V7$^+$ CD8$^+$ cells was higher for the male mice, as the frequency of single samples that exceeded this value was much greater in the male, as compared to the female population (Figure 21b and Table 3-4).

Tetramer analysis of peripheral blood as a predictor for diabetes outcome

As shown in Tables 3-2 and 3-3, the sensitivity of using a calculated cumulative threshold for identification of pre-diabetic mice increases with age. This is only a useful predictor for disease outcome if the calculated threshold is achieved several weeks prior to disease onset. The number of weeks spanning when the calculated cumulative threshold was reached (0.75 and 2.00% NRP-V7-tetramer$^+$ CD8$^+$ cells for females and males, respectively) and the age at which the animal developed diabetes is directly proportional (Figure 22). This indicates that there is no relationship between when the pre-diabetic animal accumulated a sufficient number of autoreactive cells to predict diabetes, and the onset of disease. In fact, the age at which the pre-diabetic animal reached the predictive threshold was independent of age of disease onset (Figure 23). Interestingly, all animals, both male and female attained the calculated cumulative threshold in between 9 and 15 weeks of age (Figure 23), with an average age of 11.2 and 13.5 weeks for females and males, respectively.

Sexual differences in the proportion of autoreactive T cells in NOD mice

Several observations suggest that pre-diabetic male mice have an increased number of NRP-V7$^+$ CD8$^+$ cells in their peripheral blood as compared to pre-diabetic

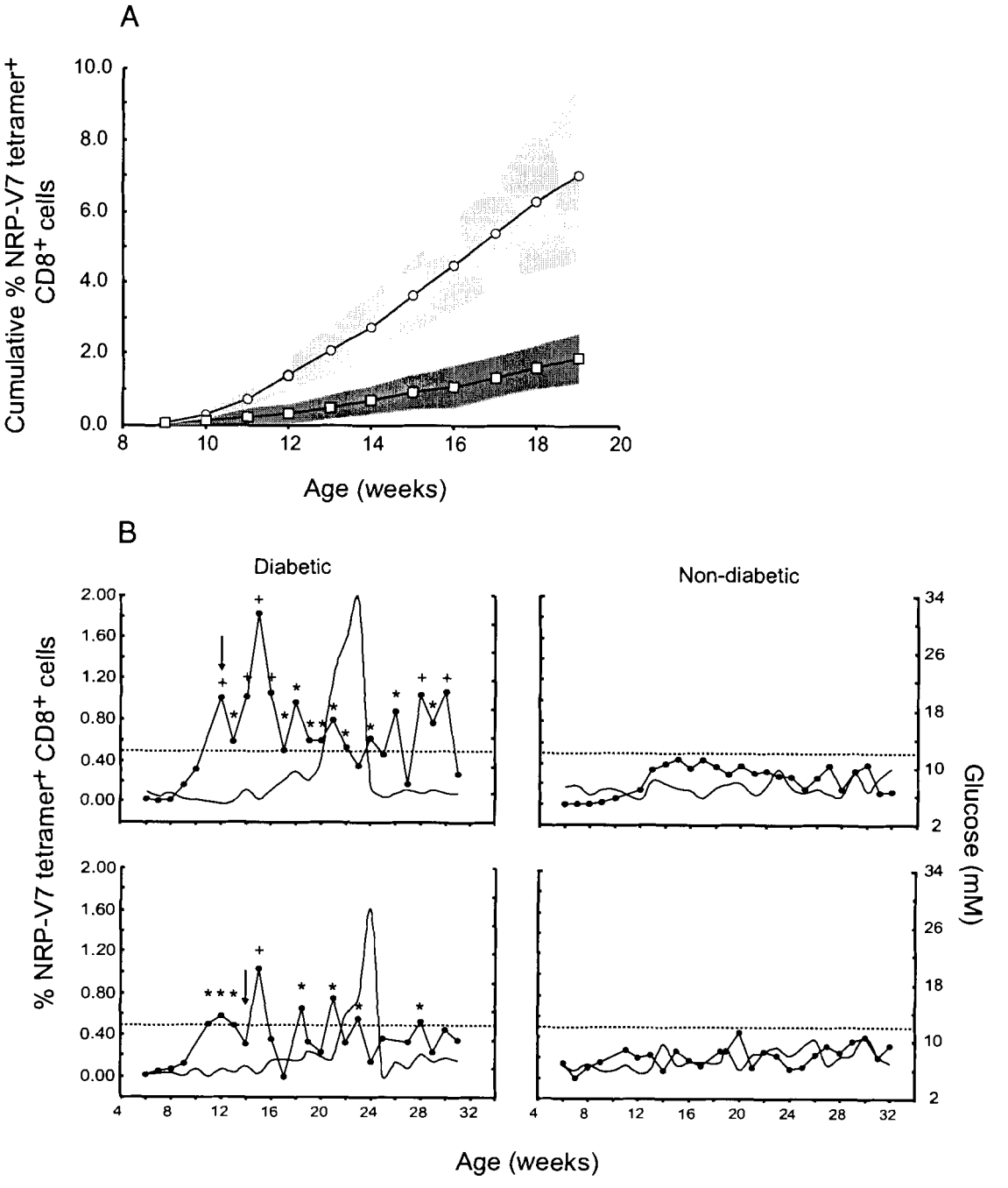

Figure 21. *NRP-V7-specific T cells detected in the peripheral blood of male mice is also predictive of diabetes development.* A. Accumulation of NRP-V7-tetramer positive cells analyzed from the peripheral blood of male diabetic (○; n = 10) and non-diabetic (□; n = 5) mice from 9 – 19 weeks of age. For each animal, the percentage of NRP-V7-tetramer positive cells at each time point (minus control) was summed with the cumulative total of NRP-V7-tetramer positive cells from all previous weeks, and expressed as a mean for diabetic vs. non-diabetic animals. The shaded area indicates the 95% confidence interval of the mean. B. Representative data from individual male mice showing the proportion of NRP-V7-tetramer positive cells present in peripheral blood (●) as it relates to blood glucose (solid line, no symbols) for the entire period of study. Decline in blood glucose following onset of hyperglycemia was a result of insulin treatment. Arrows indicate the time at which mice had accumulated 2.00% NRP-V7-tetramer positive cells, predictive of diabetes development. * Indicates NRP-V7-tetramer positivity >0.50% for a single time point (0.50% indicated by dashed line), + indicates NRP-V7-tetramer positivity >1.00% for a single time point.

Table 3-3. Accuracy of cumulative peripheral blood NRP-V7 tetramer measurements for the prediction of diabetes in male NOD mice. (Average age of diabetes onset = 19.7 weeks.)

Age (weeks)	13	15	17
Sensitivity (%)	50	100	100
Specificity (%)	100	100	80
Positive Predictive Value (%)	100	100	91
Negative Predictive Value (%)	50	83	100

Table 3-4. Frequency of peripheral blood samples that exceeded the 0.50% threshold of NRP-V7$^+$ CD8$^+$ cells in a single sample prior to diabetes onset.

	Number of weekly samplings containing >0.50% NRP-V7+ CD8$^+$ cells
female	0
female	1
female	4
female	9
female	4
female	3
female	1
female	5
female	0
female	2
female	3
female	5
female	6
male	1
male	9
male	2
male	6
male	10
male	9
male	2
male	5
male	9
male	7

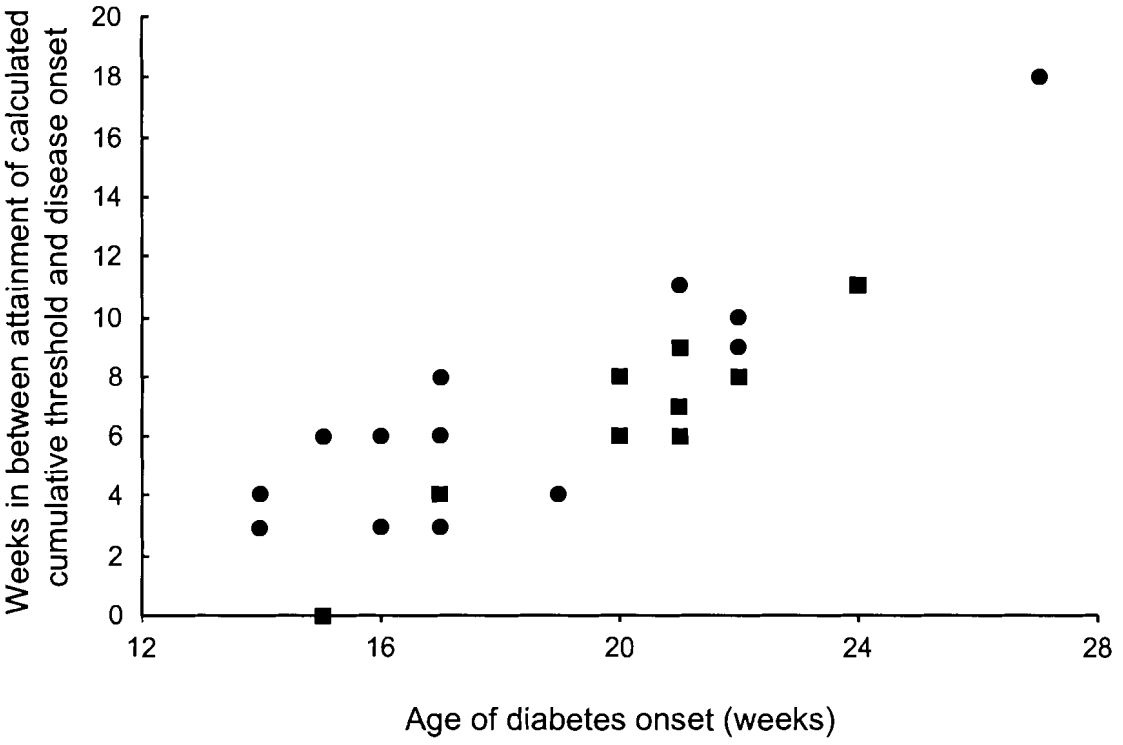

Figure 22. *In individual animals, the timing between individual animals reaching the cumulative predictive threshold and the onset of hyperglycemia is not constant.* For each animal that goes on to develop diabetes, the number of weeks in between attaininment of the calculated cumulative threshold for disease prediction (0.75 and 2.00% NRP-V7$^+$ CD8$^+$ cells for females and males, respectively) and the onset of hyperglycemia was calculated and is displayed as a function of age of diabetes onset. Male mice are represented as ■ (n = 10), and females as ● (n = 13).

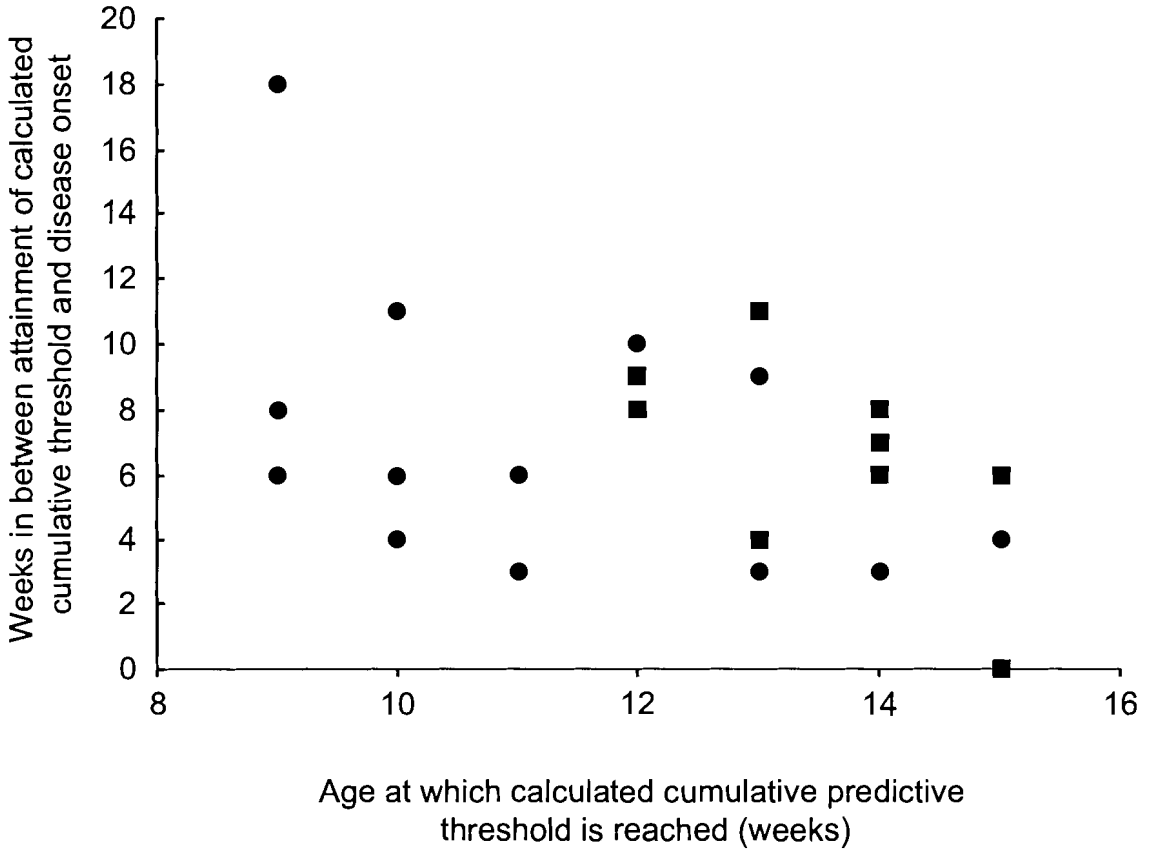

Figure 23. *The age of diabetes onset is independent of the age at which the threshold accumulation of NRP-V7$^+$ CD8$^+$ cells is reached.* For each animal that goes on to develop diabetes, the age at which they reach the calculated cumulative threshold for disease prediction (0.75 and 2.00% NRP-V7$^+$ CD8$^+$ cells for females and males, respectively) is plotted relative to the number of weeks in between attainment of that threshold and the onset of diabetes. Male mice are represented as ■ (n = 10), and females as ● (n = 13).

female mice. Firstly, prediction of diabetes in male mice necessitates a higher calculated cumulative threshold than for female mice. This increased threshold (likely in addition to a later average age of disease onset for male mice) results in a longer time on average, for the male mice to reach the cumulative threshold for prediction of disease (Figure 23). Secondly, male mice have a greater number of single weekly blood samples with >0.50% NRP-V7$^+$ CD8$^+$ cells (Table 3-4). Indeed, when the calculated cumulative percentage of NRP-V7$^+$ CD8$^+$ cells is compared between female and male, diabetic and non-diabetic mice, it is clear that male mice (both pre-diabetic and non-diabetic) have a greater proportion of NRP-V7-reactive cells in their peripheral blood (Figure 24). It also suggests that a greater number of autoreactive cells were required to cause disease in male mice as compared to female mice. Interestingly, by 32 weeks of age, both male and female mice that remained non-diabetic had reached the calculated cumulative threshold of circulating autoreactive cells that was predictive of diabetes development.

Discussion

These studies have shown that, with the use of a high affinity pMHC class I tetramer, it is possible to identify autoreactive CD8$^+$ T cells in the pancreatic islets, secondary lymphoid organs, and peripheral blood of pre-diabetic NOD mice *ex vivo*. This was accomplished with the use of an altered peptide ligand (APL) of a known β-cell mimotope complexed to an MHC class I tetramer. The presence of NRP-V7-reactive CTL in the peripheral blood was indicative of their presence also in the pancreatic islets, although the proportion of cells in each of these locations was not correlated. In addition, weekly monitoring of the peripheral blood of pre-diabetic mice permitted identification of

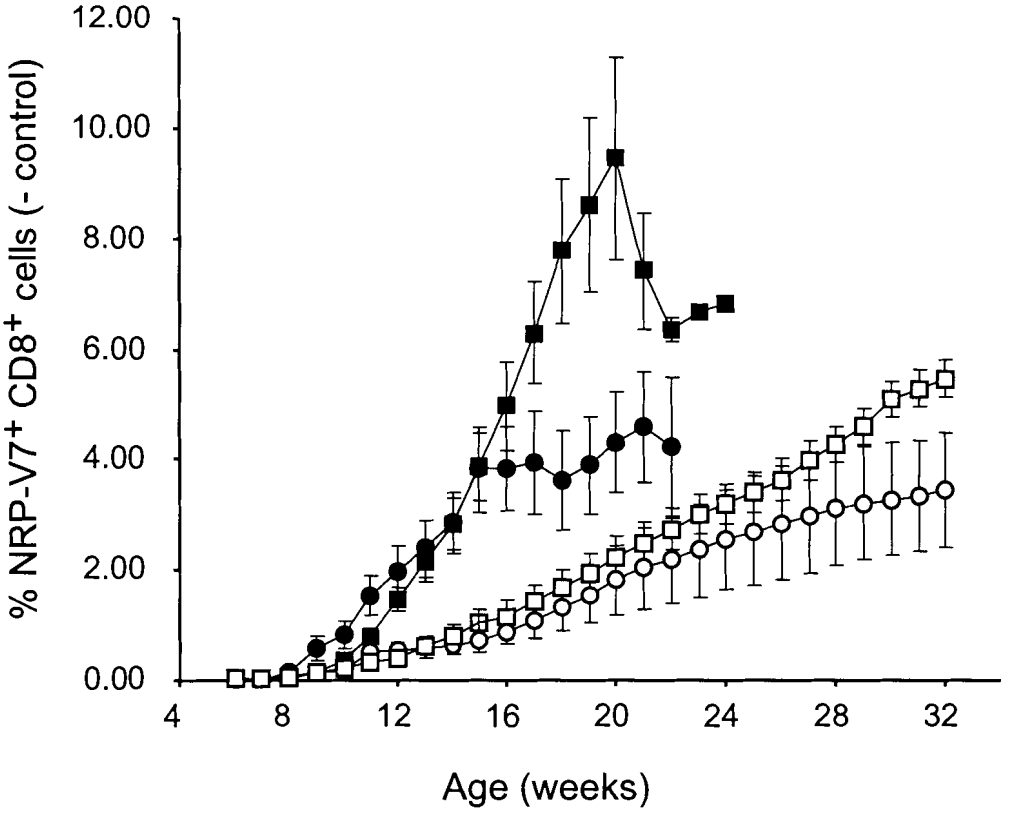

Figure 24. *Cumulative sum of NRP-V7-reactive cells in the peripheral blood of female (circles) and male (squares) NOD mice prior to diabetes onset.* For each animal, the percentage of NRP-V7 tetramer and CD8 positive cells at each time point was summed with the cumulative total of NRP-V7 tetramer and CD8 positive cells from all previous weeks, and expressed as a mean (±SEM) for diabetic (closed symbols; female n=13, male n=10) vs. non-diabetic (open symbols; female n=5, male n=5) animals. Mean age of diabetes onset was 18.5 and 19.7 weeks for females and males, respectively. For animals that eventually developed diabetes, data were included for each week up to and including the week that hyperglycemia developed. The mice that remained non-diabetic were studied for 32 weeks. As mice became hyperglycemic, they were removed from the analysis, and consequently, the number of animals remaining in the later weeks was smaller and the error margins larger.

the mice that would eventually develop disease. Notably, there were many interesting sexual differences in the appearance of NRP-V7-reactive cells, and the results indicated that there are unique requirements for diabetes development in the male and female NOD mice.

The ability to visualize a significant number of autoreactive $CD8^+$ T cells *ex vivo* resulted from the application of an MHC class I tetramer complexed with a high avidity peptide analog of a β-cell epitope. Development of high avidity mimics of low avidity autoepitopes thus introduces a novel general principle for the design and use of tetramers aimed at detecting autoreactive T cells. In light of these results, it is reasonable to speculate that previous attempts to detect autoreactive T cells in peripheral blood were hampered by the low avidity, in combination with the low frequency, of target T cells. It has also been shown using a mouse model, that following infection with *Listeria*, antigen-specific $CD8^+$ and $CD4^+$ T cells have intrinsically different capacities for proliferation (158). While it appears that $CD4^+$ T cells have a very limited proliferative capacity that is independent of infectious dose, $CD8^+$ T cells undergo extensive proliferation. It is unknown whether autoreactive $CD4^+$ T cells exhibit a similarly restricted proliferation pattern, but this may be an additional reason for the difficulties associated with the *ex vivo* identification of autoreactive $CD4^+$ T cells.

Indeed, previous attempts to detect circulating GAD-specific $CD4^+$ T cells in NOD mice (135) or other naturally-occurring autoepitopes in different autoimmune diseases (134, 136, 137) using MHC class II tetramers has required expansion of T cells either by repeated immunizations with the target peptide or prolonged *in vitro* culture. A

previous study using MHC class I tetramers to examine autoreactive $CD8^+$ T cells in pancreatic islets *ex vivo* found that up to 80% of islet-associated T cells were specific for a peptide derived from the insulin B chain, INS_{15-23} (111). In the present study, INS_{15-23}-specific $CD8^+$ cells were only modestly detectable in the pancreatic islets of young NOD mice, and not detectable in the peripheral blood. Identification of a significant number of INS_{15-23}-specific $CD8^+$ cells occurred only on one occasion. Eight 4-week-old mice were pooled for analysis and 5 of the 50 cells that were $CD8^+$ were identified also as being INS_{15-23}- $tetramer^+$. Unfortunately, those five positive cells did not form a convincing tetramer positive population (Figure 13a, top row). On all other occasions, no measurable INS_{15-23}-tetramer staining was found. Although INS-reactive cells were either not abundant enough or of too low avidity to be detected with the INS_{15-23}- tetramer, there was indication that INS-reactive cells were potentially important during diabetes development, as IFN-γ ELISpot analysis revealed a small but consistent population of INS-specific $CD8^+$ T cells in pancreatic islets.

In contrast, the proportion of NRP-reactive CTL identified in this study was much greater than that previously reported (63). Although in the previous study a small number of NRP-reactive cells were identified *ex vivo* (63), they chose to expand those populations *in vitro* in order to identify them in significant numbers. The work presented here resulted in identification of a higher number of NRP-reactive cells in the pancreatic islets (up to 37% of $CD8^+$ cells). In our laboratory, *in vitro* culture of pancreatic islets *decreased* the proportion of NRP-V7-$tetramer^+$ cells, likely as a result of death of memory T cells in response to incubation with IL-2 (data not shown). Initial data following the recent identification of the endogenous source of the NRP mimotope as

IGRP, has shown that pancreatic islet cells stained with IGRP-tetramer have a similar proportion of positive cells as compared to staining with the NRP-V7-tetramer. Cells that stain with the IGRP tetramer do so with significantly reduced fluorescence intensity suggesting that as might be expected, the endogenous peptide is of lower affinity than the NRP-V7 mimotope (Federation of Clinical Immunology Societies, May 15-19, 2003). It also suggests however, that with a large enough autoreactive response to a given autoepitope, it is possible, at least for $CD8^+$ T cells, to measure the response directly *ex vivo*. That such a large proportion of $CD8^+$ cells within pancreatic islets at any given time are specific for this one 9 amino acid sequence suggests that these T cells, and potentially T cells reactive to other epitopes on the IGRP protein are very important for the development of diabetes, at least in the NOD mouse model. These data also suggest that a useful strategy to maximize identification of autoreactive cells, may be to modify endogenous epitopes and create high affinity mimics of autoepitopes.

It is interesting that the only instance where autoreactive T cells have been identified *ex vivo* from peripheral blood using MHC class I tetramers prior to this study is in the case of vitiligo, a chronic autoimmune skin disease (133). In vitiligo, CTL specific for a melanocyte antigen were found in the peripheral blood of patients and the frequency of melanocyte-specific CTL was proportional to the severity of disease. These findings support the hypothesis that autoreactive T cells are difficult to detect in the peripheral blood prior to symptoms of autoimmune disease because of both their low avidity and low frequency. In a disease such as vitiligo, where the target antigen in skin is maintained for a prolonged period, continual stimulation of T cell populations is likely to generate highly avid autoreactive T cells that are easier to detect. Moreover, because autoreactive

CTL were visualized in blood obtained from patients with ongoing disease, it is not surprising that the frequency of autoreactive cells would also be high. This is not the case in autoimmune diabetes, where at the time of clinical presentation the bulk of the cognate antigen (β-cell proteins) is lost, and the frequency of autoreactive T cells is low (Figures 17, 18b, 20, and 21b).

In the majority of animals studied (27/30), when there was infiltration of the pancreatic islets with NRP-reactive cells, NRP-reactive cells were also detected in the peripheral blood, providing an effective means of non-invasively monitoring ongoing β-cell damage. Although a significant correlation between the number of NRP-V7-reactive $CD8^+$ cells in the peripheral blood and pancreatic islets was not observed, this was not unexpected since recently activated autoreactive $CD8^+$ T cells in the peripheral blood were likely in the process of trafficking to pancreatic islets. In support of this notion, tetramer positive autoreactive cells in the blood also expressed the T cell activation markers CD69 and CD44 (data not shown). The finding that non-diabetogenic, H2-K^d-expressing Balb/c mice contained a small, but convincing population of NRP-V7-reactive cells in the peripheral blood suggests that β-cell-reactive T cells are present in the periphery in non-autoimmune mouse strains, but are kept under control by peripheral tolerance mechanisms. This is supported also by findings that humans without autoimmune disease have small populations of autoreactive T cells in the periphery (159).

NOD mice destined to develop diabetes had a significantly higher number of β-cell-specific T cells in the peripheral blood, and accordingly, quantification of these cells resulted in an effective means of predicting diabetes outcome. The ability to analyze the

proportion of NRP-V7 tetramer positive cells in peripheral blood on a weekly basis during the pre-diabetic period revealed novel information regarding circulating autoreactive T cells during the development of autoimmune diabetes. For the female cohort, those mice destined to develop diabetes not only had a significantly larger proportion of NRP-V7-specific $CD8^+$ T cells in the peripheral blood, but the appearance of these autoreactive cells occurred in distinct cycles (Figure 16). This finding raises the possibility that each cycle represented a round of clonal proliferation of autoreactive T cells undergoing avidity maturation (63, 160, 161). Previous work by Amrani *et al.* (63) has demonstrated that as NOD mice age, the NRP-reactive cells within the pancreatic islets increase in affinity, thereby becoming more effective at destroying β-cells. The data observed are consistent with this model, whereby the final expansion seen in the peripheral blood prior to disease onset would be the T cell population with the highest β-cell avidity, capable of the most efficient β-cell destruction.

A similar pattern of cyclic expansion was not seen for the male mice. There were consistently large fractions of NRP-V7-reactive cells that appeared in the peripheral blood of male mice, but unlike the situation in female mice, the cells did not flux (appear and disappear). This might suggest that in the male mice, the NRP-V7-reactive cells are not leaving the peripheral blood (and not homing to the pancreatic islets in as great of numbers as seen in female mice). This situation seems unlikely, as the presence of these cells in the peripheral blood of male mice predicts disease outcome also, suggesting that they are doing β-cell damage. Alternatively, this finding suggests that there are simply greater numbers of NRP-V7-reactive cells that appear more frequently in male mice.

Importantly, the quantification of autoreactive T cells in the peripheral blood each week permitted prediction of diabetes occurrence. A cumulative sum of 0.75 or 2.00% NRP-V7-reactive cells in the peripheral blood between 9 and 16 weeks of age predicted diabetes outcome with increasing sensitivity for female and male mice respectively, as the animals approached hyperglycemia. It was also observed that in the context of weekly monitoring, any mouse with greater than 0.50% NRP-V7-reactive cells at any one time became diabetic. However, even for mice that went on to develop diabetes, particularly for females, the majority of their weekly samples contained less than 0.50% NRP-V7-reactive $CD8^+$ cells (Table 3-4). Thus, for any prospective study to predict diabetes occurrence in mice, our experience suggests that weekly monitoring, or at least multiple samplings, would be required and that the cumulative sum of autoreactive T cells (rather than a single measurement) will be the most useful and accurate predictor.

Although the appearance and quantification of NRP-V7-reactive cells in the peripheral blood was able to also predict disease occurrence in male mice, there were many interesting differences between the sexes. Although NRP-V7-reactive cells initially accumulated in the male circulation at the same frequency and rate as in female NOD mice (between 6 and 15 weeks of age), male NOD mice did not develop diabetes until a significantly greater number of autoreactive CTL had been detected in the peripheral blood (Figure 24). This finding suggests that there are other factors – apart from the absolute number of NRP-reactive CTL –that affect progression of disease. As mentioned previously, it is possible that β-cell-reactive $CD8^+$ cells do not home as efficiently to pancreatic islets. Alternatively, it is possible that the NRP-reactive cell population identified in the male mice are of lower affinity than those in female mice, and as a result

are not as efficient at destroying β-cells, thereby requiring more autoreactive cells to eventually result in diabetes development. In addition, it is also interesting to note that all mice destined to develop diabetes, regardless of sex, reached the calculated cumulative predictive threshold in between 9 and 15 weeks of age (average age of 11.2 and 13.5 weeks for females and males, respectively). The age at which this threshold is reached however, is *independent* of diabetes onset (Figure 23). This finding strongly suggests that there are other factors that control the timing and duration until disease onset. It also very strongly suggests that if animals do not achieve a certain calculated cumulative threshold of autoreactive cells by 15 weeks of age, then they will not develop diabetes. These data imply that there may be a critical rate of accumulation of autoreactive cells required within a particular time frame (in between 9 and 15 weeks of age) for disease to occur. As seen in Figure 24, animals that are non-diabetic at 32 weeks of age, have on average, a calculated cumulative threshold above that used to predict diabetes. However, the rate of "accumulation" was perhaps too slow to result in significant β-cell damage. Analysis of the intra-islet lymphocytes of non-diabetic mice revealed that a significant fraction of NRP-reactive $CD8^+$ T cells exist, albeit a fraction much lower that that seen at earlier ages (Figure 13b). Future experiments can hopefully determine whether those autoreactive cells within the pancreas of non-diabetic mice are of lower T cell affinity.

Because tetramers directly detect β-cell specific effector T cells, tetramer screening offers a complementary approach for identification of pre-diabetic individuals. As IAA appear on average, first at 8 weeks of age, and the presence of NRP-reactive T cells appear on average at 9 weeks of age, these two assays together provide a very powerful tool for diabetes prediction in the NOD mouse. While the utility of human

leukocyte antigen (HLA) tetramers for predicting human disease cannot be assessed until more human CTL autoepitopes are identified, they may eventually permit earlier and more specific prediction of disease. In particular, tetramers might be used to identify pre-diabetic individuals well before disease onset, potentially allowing for interventions aimed at preserving β-cell mass.

Finally, peripheral blood measurements of autoreactive $CD8^+$ T cells using tetramers may offer possibilities beyond type 1 diabetes. These findings suggest that it will be possible to detect and quantify autoreactive T cells in other autoimmune diseases that involve $CD8^+$ T cells, like experimental autoimmune encephalomyelitis or primary biliary cirrhosis (162). Indeed, a high priority should now be placed on the identification of human $CD8^+$ T cell epitopes in diabetes, as well as other autoimmune disorders, in order to exploit HLA tetramers for prediction of disease.

Chapter 4:

Analysis of autoreactive CTL in pancreatic islet transplants

Introduction

Until recently, success in human islet transplantation, defined as the time that a patient remains free of insulin injections post-transplantation, has been low. Of the 267 islet transplants completed between 1990 and 1998, only 12.4% remained insulin-free for greater than one week, and 8.2% for greater than one year (163). Whole pancreas transplantation has shown greater success (70 – 80%) in reversing insulin dependence for at least 3 years; however the risk of morbidity associated with both surgery and long-term immunosuppression has limited the use of this procedure, an exception being combined pancreatic/kidney transplantation (164). In the recent past, islet transplantation has been accomplished with much greater success through the use of the "Edmonton Protocol". With this method, two transplantation procedures are performed over 2 – 10 weeks, during which approximately 850 000 human islets from at least two different cadaveric donors are infused into the portal vein (163). Success with this method is attributed to improvements in islet isolation and delivery and glucocorticoid-free immunotherapy. As of 2002, 80% (of >40 transplants) of patients remained free from insulin therapy at one year post-transplantation. By 34 months post-transplantation, the maximum follow-up period for any of the patients, there was indication that prolonged insulin independence

might be achieved following islet transplantation (165). The Edmonton islet transplantation protocol is a less-invasive procedure that promises to solve many of the limitations associated with whole pancreas transplantation and an international multi-centre trial is currently underway in order to test the efficacy of this methodology in other centres.

Despite significant improvement in islet transplantation, grafts continue to fail in some patients, making repeated transplant procedures necessary (165, 166). Failure of transplanted islets is thought to be mediated primarily by the action of T cells, including both T cells that recognize allogeneic graft antigen, and/or autoreactive T cells that recognize β-cell antigen (167). Graft rejection resulting from ineffective suppression of one or both of these T cell mechanisms is difficult to identify without tools that directly examine the damaging T cells. Currently, graft health is assessed using surrogate markers of β-cell function. The ability to assay for T cell populations that may be causing graft damage might provide an earlier, more accurate indicator of graft damage or rejection. This type of assay would be useful also for analysis of new immunosuppressive regimens or other therapies both in human transplant recipients and in animal models.

Prediction of pancreatic islet graft failure

Current assessment of islet graft health/survival relies on the measurement of secondary outcomes of graft damage. Elevated levels of serum autoantibody and blood glucose levels, insulin requirements, as well as HbA_{1c}, and/or loss of C-peptide secretion are all indicative of damage to grafted islets (163). These markers are not sensitive readouts of islet damage or impending graft failure. For example, recurrent β-cell

autoimmunity resulting from rejection of a pancreas segment transplanted from a non-diabetic twin to a diabetic twin was not accompanied by an increase in serum autoantibody levels (168). However, examination of T cells from the peripheral blood of islet transplant recipients revealed that graft failure is preceded by T cell allo- and/or auto-reactivity (169). Simple assays of T cell reactivity toward islet grafts would be useful for signaling impending graft damage and monitoring T cell-directed immunotherapy. In human islet transplant recipients, donated islets are most often a mix of HLA classes, and rejection of the grafted tissue is likely to involve a combination of recurrent autoimmunity and allogeneic rejection. Defining the T cell subsets that are causing graft damage in the many HLA classes of recipients is likely to be challenging. Fortunately however, the HLA genetic association with T1D is high, and the expansion of autoreactive memory T cells will likely be limited to a subset of HLA types.

The principles of diabetes prediction outlined in chapter 3 were used to determine whether an MHC class I tetramer based-approach could be used to predict islet graft failure following islet transplantation in a mouse model. The importance of NRP-V7-reactive CTL in the rejection of both syngeneic and allogeneic islet transplants was also determined.

Results

Examination of autoreactive CTL in the peripheral blood and islet grafts following islet transplantation

Having established that NRP-reactive cells detected in the peripheral blood of pre-diabetic NOD mice were a surrogate marker for pancreatic islet infiltration, and were

predictive of diabetes development, it was reasonable to hypothesize that the rejection of islet transplants could also be predicted by the appearance of NRP-reactive cells in the peripheral blood. It was shown in Chapter 3 that following the onset of hyperglycemia in NOD mice, the proportion of NRP-reactive cells decreased dramatically (Figure 17 and 20). These data suggested that following diabetes onset in the NOD mouse, when the majority of β-cells were lost, lack of β-cell antigen led to a waning of the β-cell-specific CTL population. In this case, re-introduction of β-cells by islet transplantation would provide β-cell antigen (particularly as the graft is being destroyed and the β-cells are undergoing apoptosis) that could stimulate the proliferation of previously activated NRP-reactive memory CTL. The size and proliferation rate of this population could potentially provide a means of monitoring the immune response to the transplanted islets. It is possible that similar to spontaneous disease, increased numbers of autoreactive T cells in the peripheral blood could act as a surrogate marker for insulitis and ongoing β-cell damage. In order to test this hypothesis, the peripheral blood of diabetic NOD mice was monitored following transplantation of syngeneic pancreatic islets.

Diabetic female NOD mice were maintained on insulin (by insulin implant or by daily injection) for a minimum of 4 weeks prior to transplantation. The proportion of NRP-V7-reactive cells in the peripheral blood prior to transplantation was determined. The number of NRP-reactive cells in the peripheral blood one week prior to transplantation ranged from 0.03 – 0.09% of $CD8^+$ cells (after subtraction of TUM-tetramer$^+$ $CD8^+$ cells) (Figure 25a and 25b). Islets (approximately 550) from syngeneic NOD*scid* mice were then transplanted under the left kidney capsule and peripheral blood was sampled on days 1, 3, 5, 6, 7, and 9 post-transplantation and the proportion of NRP-

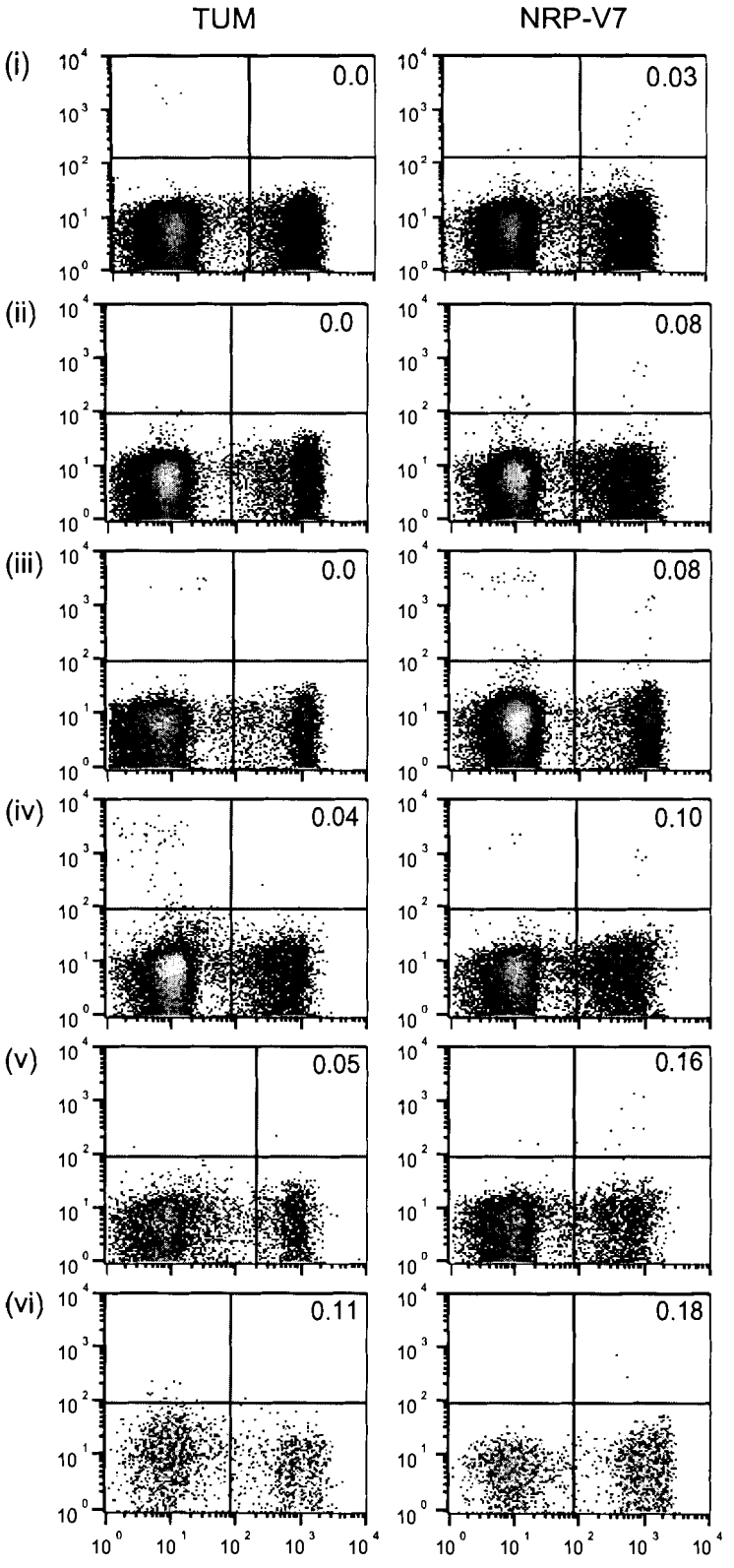

Figure 25a. *NRP-V7-reactive cells do not appear in the peripheral blood prior to rejection of islet transplants.* Shown is a representative example of the proportion of NRP-V7-tetramer$^+$ CD8$^+$ cells in the peripheral blood of female diabetic NOD mice that had been maintained on exogenous insulin for approximately four weeks (i). 550 NOD*scid* islets were then transplanted under the kidney capsule of diabetic NOD mice, and the proportion of NRP-V7-tetramer$^+$ CD8$^+$ cells in the peripheral blood was measured on days 1 (ii), 3 (iii), 5 (iv), 7 (v), and 9 (vi). One representative experiment (of six) is shown. Numbers (top right quadrant) indicate the percentage of CD8$^+$ B220$^-$ tetramer$^+$ cells.

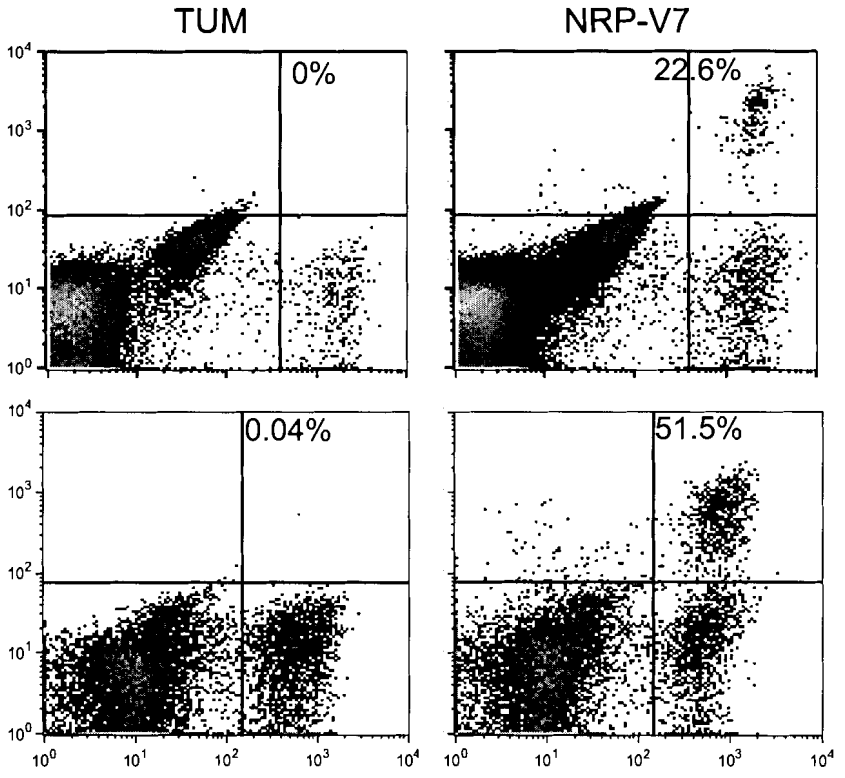

Figure 25b. *A large proportion of the CD8+ cells that infiltrate transplanted islets are NRP-V7-reactive.* Shown are 2 representative examples of NOD*scid* islet grafts that have been removed from NOD recipients and stained with NRP-V7 tetramer, and antibodies to CD8 and B220. Numbers (top right quadrant) indicate the percentage of $CD8^+$ $B220^-$ $tetramer^+$ cells. Both of the grafts shown were stained on the 6th day post-transplantation. The top row is a graft that was taken from a mouse that still had normal glucose levels, while the bottom row represents a graft that was taken from a mouse on the day of graft failure.

V7 tetramer$^+$, CD8$^+$ cells was analysed. Blood glucose was monitored daily. There was no significant increase in the number of NRP-reactive CD8$^+$ T cells in the blood following transplantation of islets (Figure 25a and 25b). There was an indication that the number of autoreactive CTL increased in the peripheral blood just prior to graft rejection, however this increase was small and not consistently seen.

Upon removal and inspection of the graft however, there was a significant proportion of NRP-V7 tetramer$^+$ CD8$^+$ within the lymphocytes invading the islet graft. Grafts were removed on the first or second day of graft failure, and up to 50% of the CD8$^+$ cells within the graft stained intensely with the NRP-V7 tetramer (Figure 25b and Table 4-1). Summary data are shown for the percentage of NRP-V7 tetramer$^+$ CD8$^+$ cells in the peripheral blood pre- and post-transplantation, and also for the cells within the islet graft (Figure 25c). Tetramer positive cells within the islet graft were positive for the memory T cell marker CD44$^+$ (170-172). They did not however, stain as intensely with CD69, a marker of recent T cell activation, confirming that the graft T cells are of memory phenotype (173, 174) (Figure 26).

NRP-V7-reactive CD8$^+$ T cells are present during the rejection of partially allogeneic islet grafts

Transplantation of human islets often involves several cadaveric donors with no attempt to match the HLA types of donor and recipient (163). Thus, it is therefore likely that the immune response to the grafted tissue in some cases involves expansion of not only allogeneic T cells, but also autoreactive memory T cells. Using a simple animal model of transplantation, the involvement of autoreactive CD8$^+$ T cells in the rejection of partially matched donor islets was investigated. NOD mice express the MHC class I

Table 4-1. Percentage of NRP-V7$^+$ CD8$^+$ T cells in syngeneic islet transplants.

Days to graft failure	Day of analysis	% NRP-V7+ CD8$^+$ cells (- control)
0	6	6.0
0	6	14.5
0	6	34.9
4	8	14.5
6	6	51.1
7	9	18.3
8	9	11.1
9	9	25.4
>6	6	22.6
>6	6	9.8

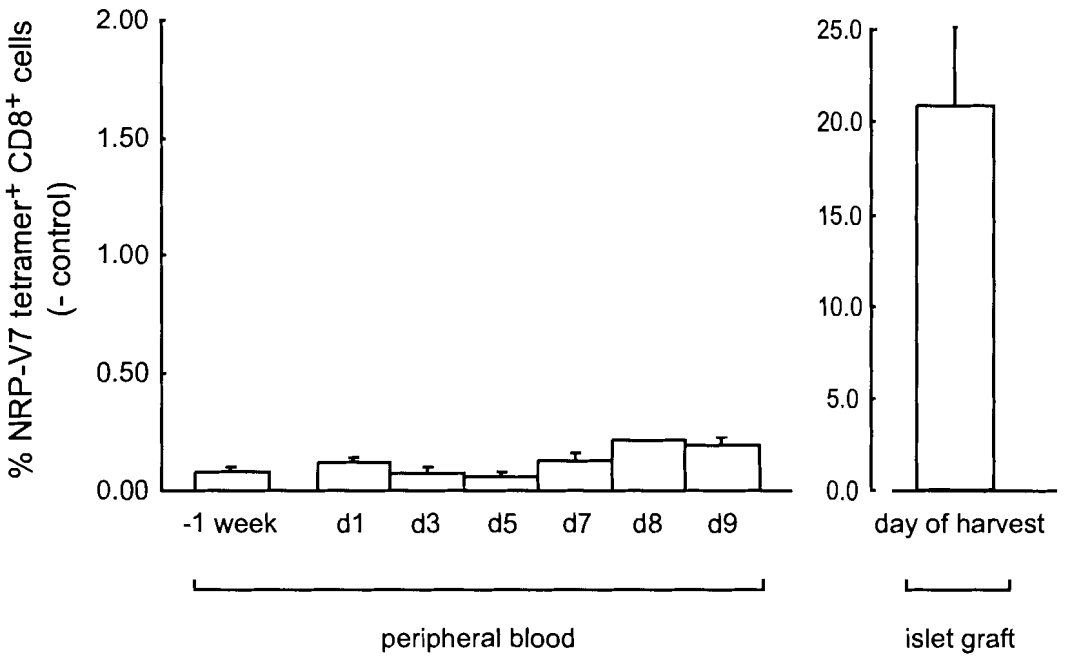

Figure 25c. *NRP-V7 reactive cells are present in large numbers in islet grafts, but not in the peripheral blood following syngeneic islet transplantation.* The percentage of NRP-V7- tetramer+ CD8+ cells was determined in the peripheral blood pre- (1 week before transplant, n=8) and post- (day (d)1, n=8; d3, n=4; d5, n=7; d7, n=4; d8, n=1; d9, n=3) transplantation, and infiltrating the islet grafts (n=10). The data shown are the percent NRP-V7- tetramer+ CD8+ B220- (- control) +/- SEM.

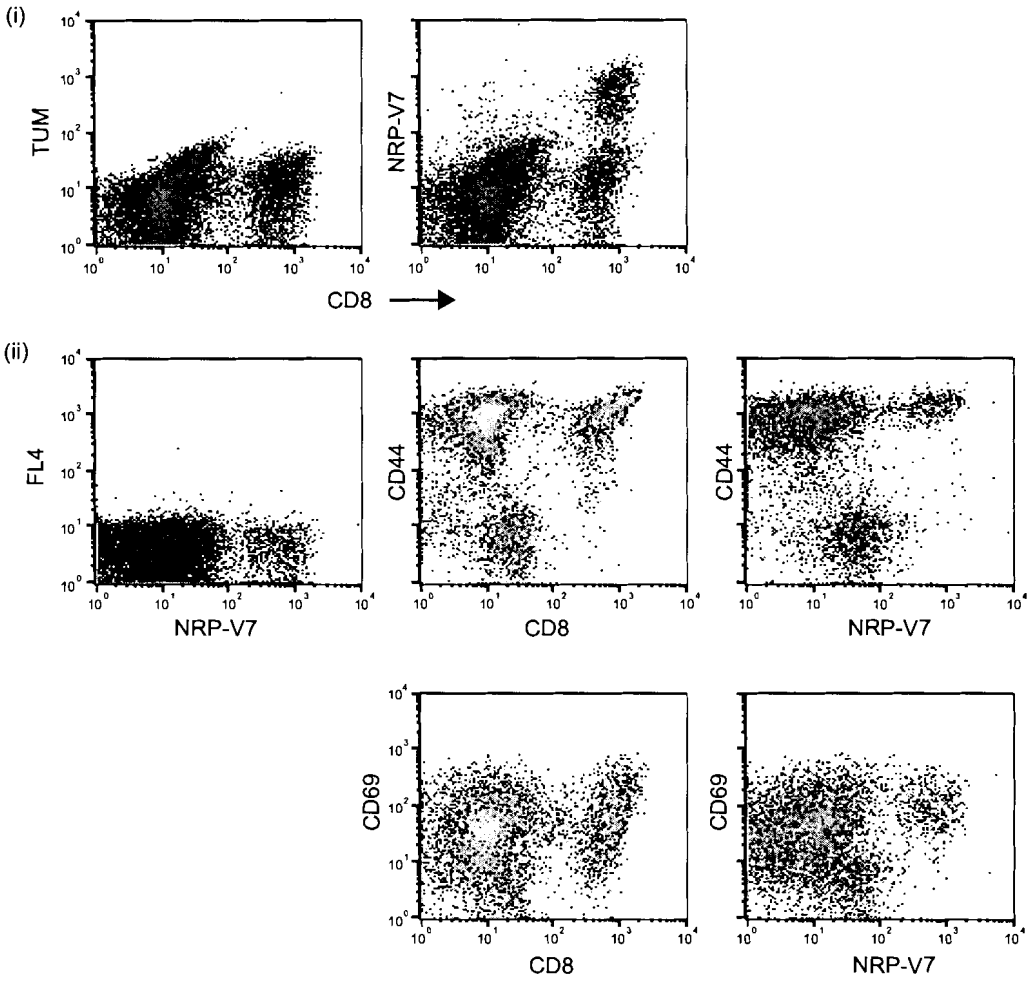

Figure 26. *The NRP-V7 tetramer+ CD8+ cells that infiltrate islet grafts express CD44.* Shown is a graft removed on day 6 post-transplantation that was stained for the T cell activation markers CD44 and CD69. (i) NRP-V7 and TUM tetramer staining (34.9% of $CD8^+$ $B220^-$ cells were NRP-V7-$tetramer^+$). (ii) Top row: CD44 expression on all $CD8^+$ cells (middle panel) and on NRP-$V7^+$ $CD8^+$ cells only (right). Bottom row: CD69 expression on all $CD8^+$ cells (left panel) and on $CD8^+$ NRP-$V7^+$ cells only.

molecules H2-K^d and H2-D^b, and have an unusual MHC class II genotype (I-A^{g7}, I-E^O). In these experiments, the MHC class I molecules of the donor mice were varied, and the resulting expansion of the H2-K^d-restricted NRP-V7-reactive subset of CTL in recipient NOD mice was examined. First, 550 Balb/c islets (MHC class I molecules: H2-K^d, D^d, L^d, MHC class II molecules mismatched) were transplanted under the kidney capsule of diabetic female NOD mice. The blood glucose of 4 of 6 transplant recipients did not normalise completely. The other 2 recipients had normal blood glucose by 24 hours post-transplant, with the transplants failing on day 5 and day 6. All of the transplants were removed on day 7 and assessed for infiltration of the graft by NRP-V7-reactive CTL (Figure 27 and Table 4-2). In 5/6 cases there were NRP-V7-reactive cells within the graft (range 1.6 – 14.3%).

In order to determine whether the proliferation of H2-K^d-restricted NRP-V7-reactive cells in the partially allogeneic transplants (Balb/c islets transplanted in diabetic NOD) was a result of the common H2-K^d allele between these two strains, C57Bl/6 mice (MHC class I molecules: H2-K^b, D^b, MHC class II molecules mismatched) were used as islet donors. Even in the absence of the MHC class I molecule H2-K^d, a significant population of H2-K^d-restricted NRP-V7-reactive cells was found within the islet grafts of recipient mice (Figure 28 and Table 4-3). Of the 4 transplants completed, all recipients achieved normal glucose levels by 24 hours post-transplantation. Grafts were analyzed 1-2 days after failure, except in one case, where the graft was removed before failure (at 13 days post-transplant) to determine whether the number of autoreactive cells would be higher before graft failure. All of the grafts contained a significant number of NRP-V7-

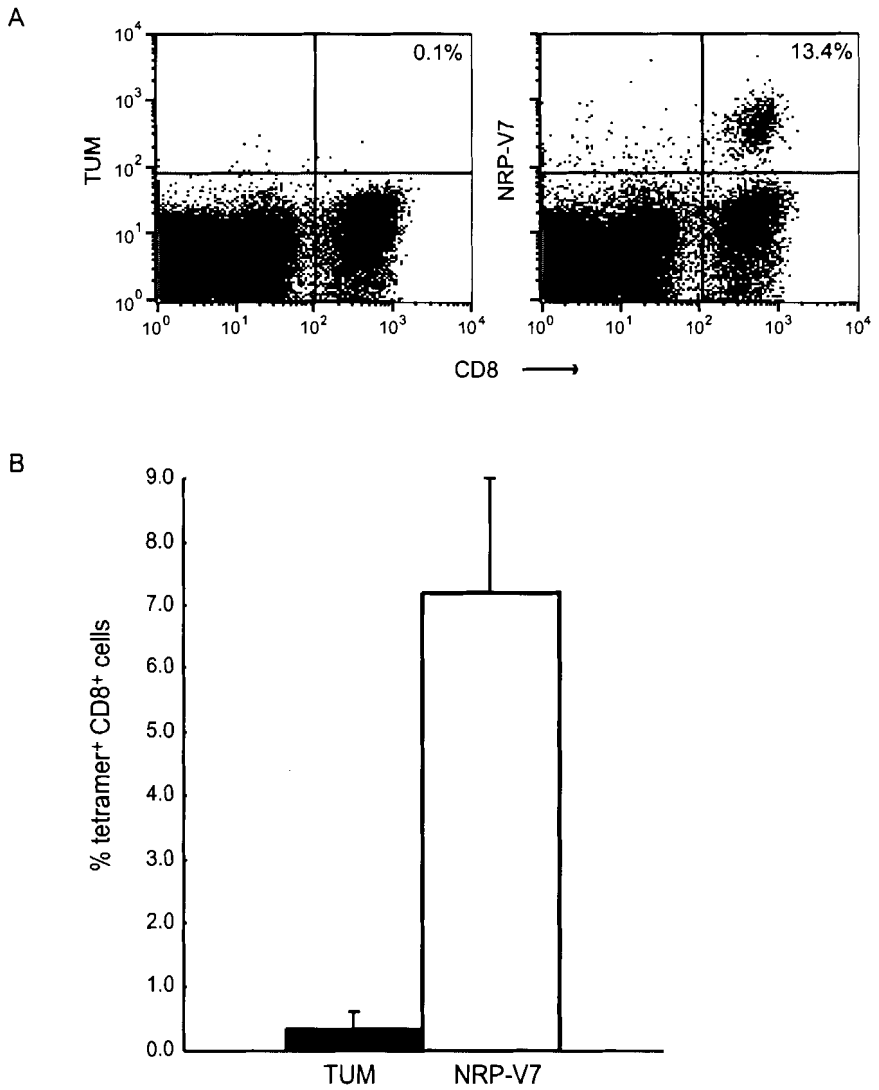

Figure 27. *NRP-V7-reactive cells participate in the rejection of H2-K^d-matched, partially allogeneic islet transplants.* 550 islets from Balb/c mice (H-2^d) were transplanted under the kidney capsule of diabetic female NOD mice (H-2K^d, D^b, I-Ag^7). Islet grafts were removed and assessed for the proportion of NRP-V7-reactive cells. A. A representative example of a graft that failed on day 5 post-transplant, and was analyzed on day 7. Numbers (top right quadrant) indicate the percent tetramer$^+$ CD8$^+$ B220$^-$ cells. B. The mean (+/-SEM) percentage of tetramer$^+$ cells for all of the grafts analyzed (n = 6).

Table 4-2. Percentage of NRP-V7$^+$ CD8$^+$ T cells in partially allogeneic islet transplants (H-2^d donor).

Days to graft failure	Day of analysis	% NRP-V7+ CD8$^+$ cells (- control)
0	7	14.3
0	7	1.6
0	7	0.0
0	7	2.7
5	7	13.3
6	7	9.1

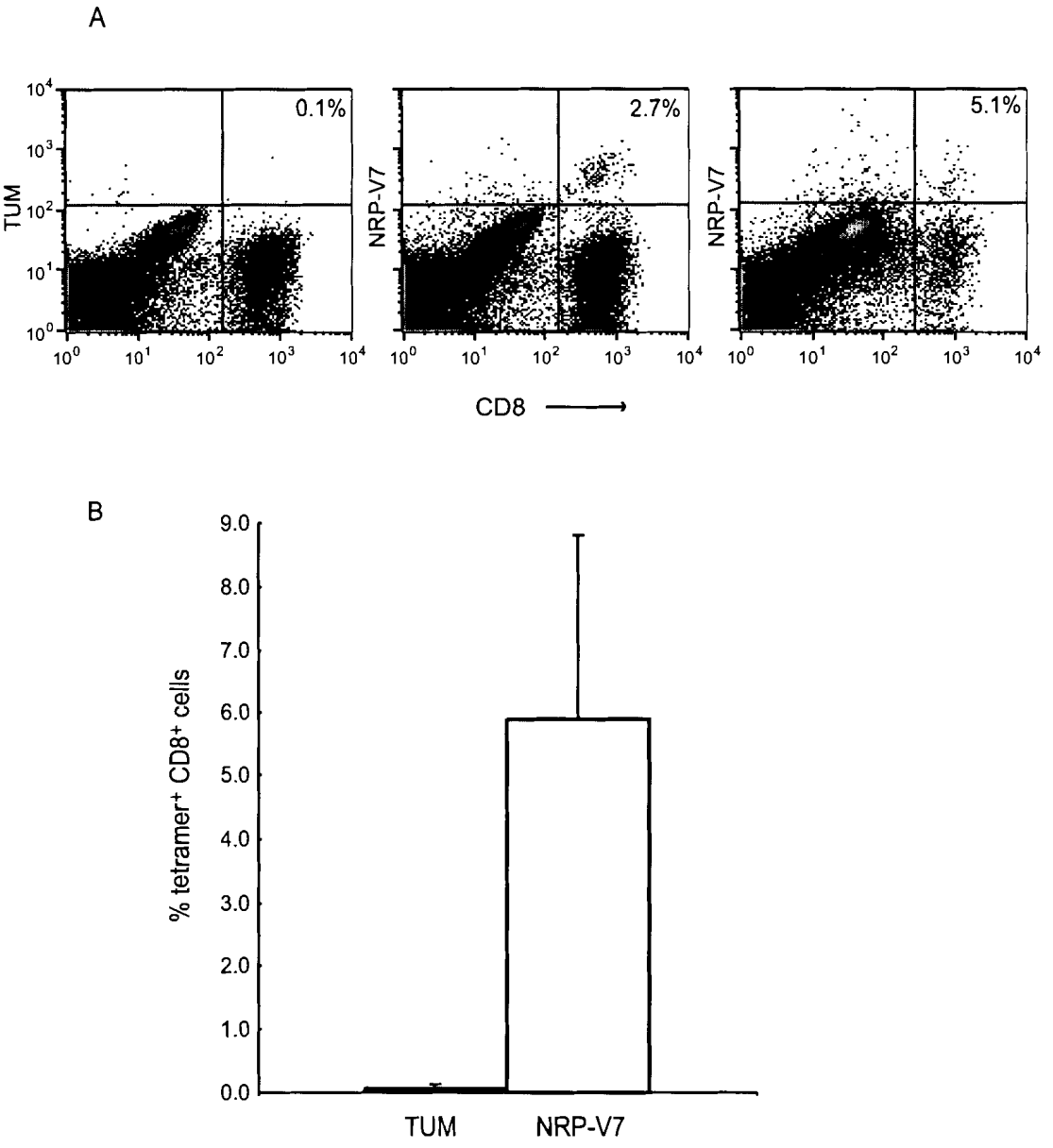

Figure 28. *NRP-V7-reactive cells participate in the rejection of H2-K^d-mismatched, partially allogeneic islet transplants.* 550 islets from C57Bl/6 mice (H-2^b) were transplanted under the kidney capsule of diabetic female NOD mice (H-2K^d, D^b, I-A^{g7}). Islet grafts were removed and assessed for the proportion of NRP-V7-reactive cells. A. Representative examples of grafts that were assessed on day 13 (middle) and day 7 (right) post-transplant. The midlde graft was still functional at the time of analysis, while the graft on the right failed on day 7. Numbers (top right quadrant) indicate the percent tetramer$^+$ CD8$^+$ B220$^-$ cells. B. The mean (+/-SEM) percentage of tetramer$^+$ cells for all of the grafts analyzed (n = 4).

Table 4-3. Percentage of NRP-V7$^+$ CD8$^+$ T cells in partially allogeneic islet transplants (H-2^b donor).

Days to graft failure	Day of analysis	% NRP-V7+ CD8$^+$ cells (- control)
6	7	14.3
7	7	4.9
13	13	1.4
>13	13	2.6

reactive $CD8^+$ cells (range of 1.4 – 14.3%), although the brightness and definition of the $tetramer^+$ population varied (see Figure 28). The graft that was examined prior to graft failure contained a relatively low percentage of NRP-V7-reactive cells as compared to the animals that were examined immediately upon graft rejection. The same observation was seen with the Balb/c donor grafts, but less consistently seen in the syngeneic transplants (Tables 4-1, 4-2, and 4-3). Interestingly, the mean fluorescence intensity (MFI) of the $CD8^+$ $tetramer^+$ population was consistently lower in the partially allogeneic Balb/c (mean MFI = 573 +/- 37) and C57Bl/6 (mean MFI = 458 +/- 109) grafts, as compared to the syngeneic NOD*scid* grafts (mean MFI = 885 +/- 186) (compare Figures 25b, 27 and 28).

Discussion

The ability to detect β-cell reactive T cells in the peripheral blood in the post-transplantation period would be advantageous for monitoring graft health and the T cell response to immune-mediated therapies. Despite the fact that autoreactive T cells are clearly detectable in the peripheral blood prior to hyperglycemia during the development of spontaneous autoimmune diabetes, the autoimmune rejection of syngeneic islet grafts is not preceded by the appearance of these cells in the peripheral blood, at least in the NOD mouse.

One possible mechanism to explain this finding is that as a result of inflammation caused by the surgical procedure, immune cells were recruited to the graft site. Once at the graft site, the NRP-V7-reactive memory CTL (prior to transplantation, only a small percentage of $CD8^+$ T cells) can be induced to proliferate by the H2-K^d-expressing β-

cells, some of which are likely to be undergoing apoptosis as a result of the transplantation procedure (175, 176). These autoreactive T cells then expand at the graft site, and are able to destroy the grafted tissue. In this scenario, significant numbers of autoreactive CTL would not be trafficking through the peripheral blood on the way to the graft site. Alternatively, it is also possible that large numbers of autoreactive T cells were not detected in the peripheral blood because the time points examined were not optimal, or that these cells traffic through the blood to the graft site in an asynchronous manner, both scenarios preventing detection of a large population of NRP-V7-reactive CTL in the peripheral blood at any given time.

The phenotype of the memory CTL may also determine whether these cells are identifiable in the peripheral blood. It is thought that "central memory" cells require re-stimulation by APC in secondary lymph organs for reactivation, while "effector memory" cells are capable of proliferating in response to antigen presented on MHC class I bearing target cells, in the absence of APC (42, 177, 178). Therefore, if NRP-V7-reactive memory CTL were of the effector memory phenotype, and trafficking through blood was required to reach the antigen site, this likely occurred prior to significant proliferation.

Investigation of the T cell subsets within the graft itself revealed that a significant fraction of the $CD8^+$ cells were NRP-V7-reactive. In fact, the proportion of $CD8^+$ cells that were NRP-V7-reactive in syngeneic islet grafts following transplantation (up to 52%, and on average 20% of $CD8^+$ cells, Figure 25c) was higher than that seen during the development of spontaneous disease (up to 36%, and on average 10% of $CD8^+$ cells, Figure 13b), supporting the notion that these cells were an avid population of β-cell-

reactive CTL involved in the final stages of β-cell destruction. Moreover, the staining intensity of the tetramer$^+$ T cells from islet grafts was higher than the intensity of T cells taken *ex vivo* from islets during the pre-diabetic period (compare Figures 13a and 25b), suggesting that it was the most avid autoreactive CTL that were allocated to the memory T cell pool.

To assess whether H2-K^d-restricted autoreactive CTL would proliferate in response to H2-K^d matched and mismatched allogeneic islet grafts, Balb/c (K^d, D^d) and C57Bl/6 (K^b, D^b) islets were transplanted into NOD (K^d, D^b) mice, and the proliferation of NRP-V7-reactive cells in the grafts of recipient mice was measured. In response to transplantation with H2-K^d-expressing Balb/c islets, there was a large expansion of NRP-V7-reactive CD8$^+$ cells. The presence of NRP-V7-reactive CTL in the graft site was not surprising in this model, as it is possible that β-cells or APC from Balb/c mice would process β-cell antigen for H2-K^d just as NOD mice do, supporting a model where autoreactive T cells from NOD mice are proliferating in response to H2-K^d on Balb/c β-cells or APC (a direct pathway of graft antigen presentation). It is likely that a larger population of NRP-V7-reactive cells would have been detected from recipient grafts that did not restore normal glucose levels had the grafts been analysed at an earlier time point (rather than on day 7 post-transplantation).

Following transplantation with H2-K^b islets from C57Bl/6 mice however, there was an equally large proliferative response of the NRP-V7-reactive CTL population in the recipient NOD mice. These findings do not support the direct pathway for antigen presentation (as above), as it is not possible for H2-K^d-expressing autoreactive T cells

from recipient NOD mice to be reactivated by H2-K^b-expressing APC or β-cells. This model suggests that NRP-V7-reactive CTL proliferate following presentation of C57Bl/6 (donor) β-cell antigen by NOD (recipient) APC that have invaded the graft (an indirect pathway of graft antigen presentation). It seems surprising that such a large population of autoreactive CTL would occupy the graft despite their presumed inability to kill grafted β-cells, because they do not possess the MHC class I molecule H2-K^d. Alternatively, NRP-V7-reactive T cells, or a subtly different population of T cells that stain with the NRP-V7 tetramer may have proliferated as a result of bystander activation. Cytokine-mediated bystander activation of non-antigen-specific memory $CD8^+$ T cells has been documented following virus infection (179-182). It is not known whether this process can be induced by the islet graft milieu.

It is interesting that the autoreactive CTL that are responding to the partially allogeneic grafts appear to be of lower affinity than those participating in the rejection of syngeneic grafts (compare Figures 25b, 27 and 28). Presumably, the NRP-V7-reactive memory CTL that remain following spontaneous diabetes are those with the highest avidity for β-cells (63). The expansion of NRP-V7-reactive T cells in syngeneic grafts from NOD*scid* mice seems to support this idea, as their tetramer-bound fluorescence intensity was brighter than that of islets taken from pre-diabetic mice (compare Figures 25b and 13a). The MFI of the $tetramer^+$ cells proliferating in response to the partially allogeneic grafts however, appears lower, suggesting that these are a different population of less avid $CD8^+$ T cells.

It is not known whether the proliferation of autoreactive CTL in the syngeneic versus the partially allogeneic grafts occurs by the same mechanism. If antigen presentation were occurring indirectly in the recipient lymph nodes (by recipient APC), then CTL would have to traffic through the blood to reach the graft site. However it does not appear, at least in the syngeneic mouse model, that a significant number of autoreactive memory CTL traffic through the blood. It is possible that in human recipients, where graft rejection occurs over a much longer time period, T cell trafficking may be more succinct and thus amenable to measurement. Alternatively, expansion of T cells *in vitro* for examination at various time points post-transplantation is another option that appears promising (169).

NRP-V7-reactive (IGRP specific) CTL appear to play a critical role in the rejection of syngeneic islet grafts in the NOD mouse. It is interesting that this population of autoreactive CTL may also be involved in the rejection of islet grafts that are only partially MHC matched. That such a large population of autoreactive CTL should be present at a graft site where they are not capable of eliciting β-cell damage directly is intriguing, and further studies of this T cell population will likely add important information regarding transplantation of islets with unmatched HLA type into autoimmune recipients.

Chapter 5:

General Conclusions

The studies in this thesis show that autoreactive T cells can be detected *ex vivo* using MHC class I tetramers with high affinity altered peptide ligands of natural autoepitopes. In some cases, tetramers bearing a natural autoepitope will detect autoreactive CTL, albeit at a lower frequency. $CD8^+$ T cells specific for the β-cell protein IGRP were detected in the pancreatic islets, peripheral blood, pancreatic draining lymph nodes and spleen. T cells responsive to the IGRP protein are likely very important in β-cell destruction in the NOD mouse, as they make up a large proportion of the T cells within the islets, particularly in the weeks prior to disease onset. The presence of these T cells in the peripheral blood was cyclical, and in the majority of animals, was a surrogate marker for pancreatic inflammation. Importantly, weekly analysis of the peripheral blood of both female and male NOD mice performed during the pre-diabetic period, particularly between the ages of 9 – 15 weeks, could accurately predict those mice that would go on to develop diabetes.

IGRP-reactive T cells are important also for the rejection of islet transplants. Up to half of the $CD8^+$ cells that invade syngeneic islet grafts recognize the IGRP epitope, suggesting again that this is a population of very avid, pathogenic T cells. $CD8^+$ T cells specific for IGRP seem to be involved also in the rejection of allogeneic grafts, although their exact role is not yet known.

Given the genetic heterogeneity of humans with type 1 diabetes, the identification of additional endogenous $CD8^+$ and $CD4^+$ β-cell-specific epitopes, as well as a better understanding of their relative immunodominance and pathogenic role are critical prerequisites for antigen-specific disease prediction and prevention. As part of this process, it may also be critical to modify key residues within these epitopes in order to increase the utility of these peptides. The ability of high avidity MHC tetramers to visualize low avidity T cell populations in the peripheral blood of humans would provide a simple, minimally invasive method for assessing the presence of autoreactive T cells within infiltrated tissues, and might be useful for the early prediction of diabetes. Likewise, the discovery of peptides that greatly improve or diminish TCR affinity may form the basis of preventive or therapeutic vaccines in humans.

Appendices: Animal care certificates

The University of British Columbia

ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: **A99-0031**

INVESTIGATOR OR COURSE DIRECTOR: **Tan, R.**

DEPARTMENT: **Pathology & Laboratory Med**

PROJECT OR COURSE TITLE: **Autoreactive CTL in diabetes**

ANIMALS: **Mice 234**

START DATE: **99-05-01** APPROVAL DATE: **01-05-28**

FUNDING AGENCY: **Canadian Diabetes Association**

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

Approval of the UBC Committee on Animal Care by one of:
Dr. D. W. Rurak, Chairman
Dr. J. Love, Director, Animal Care Centre
Ms. L. Macdonald, Manager, Animal Care Committee

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
323-2194 Health Sciences Mall, Vancouver, V6T 1Z3
Phone: 604-822-8155 FAX: 604-822-5093

The University of British Columbia

ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: **A01-0111**

INVESTIGATOR OR COURSE DIRECTOR: **Tan, R.**

DEPARTMENT: **Pathology & Laboratory Med**

PROJECT OR COURSE TITLE: **Autoreactive Cytotoxic T Lymphocytes (CTL) in Type I Diabetes**

ANIMALS: **Mice 200**

START DATE: **01-06-01** APPROVAL DATE: AUG 16 2001

FUNDING AGENCY: **B.C. Children's Hospital**

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

Approval of the UBC Committee on Animal Care by one of:
Dr. D. W. Rurak, Chairman
Dr. J. Love, Director, Animal Care Centre
Ms. L. Macdonald, Manager, Animal Care Committee

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
323-2194 Health Sciences Mall, Vancouver, V6T 1Z3
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The University of British Columbia

ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: **A02-0054**

INVESTIGATOR OR COURSE DIRECTOR: **Tan, R.**

DEPARTMENT: **Pathology & Laboratory Med**

PROJECT OR COURSE TITLE: **Autoreactive cytotoxic T lymphocytes (CTL) in type 1 diabetes**

ANIMALS: **Mice 600**

START DATE: **01-10-01** APPROVAL DATE: JUN 1 0 2002

FUNDING AGENCY: **Canadian Diabetes Association**

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

Approval of the UBC Committee on Animal Care by one of:
Dr. W.K. Milsom, Chair
Dr. J. Love, Director, Animal Care Centre
Ms. L. Macdonald, Manager, Animal Care Committee

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

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ANIMAL CARE CERTIFICATE

PROTOCOL NUMBER: **A01-0111**

INVESTIGATOR OR COURSE DIRECTOR: **Tan, R.**

DEPARTMENT: **Pathology & Laboratory Med**

PROJECT OR COURSE TITLE: **Autoreactive Cytotoxic T Lymphocytes (CTL) in Type I Diabetes**

ANIMALS: **Mice 200**

START DATE: **01-06-01** APPROVAL DATE: **February 24, 2003**

FUNDING AGENCY: **B.C. Children's Hospital**

The Animal Care Committee has examined and approved the use of animals for the above experimental project or teaching course, and have been given an assurance that the animals involved will be cared for in accordance with the principles contained in Care of Experimental Animals - A Guide for Canada, published by the Canadian Council on Animal Care.

Approval of the UBC Committee on Animal Care by one of:
Dr. W.K. Milsom, Chair
Dr. J. Love, Director, Animal Care Centre
Ms. L. Macdonald, Manager, Animal Care Committee

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

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