

Detection and Enumeration of Beta Cell-Specific Cytotoxic T Lymphocytes in Type 1 Diabetes: Is Disease Prediction Possible?

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A B S T R A C T

Although millions of people worldwide with type 1 diabetes mellitus are successfully treated using daily insulin injections, this treatment often does not protect 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 treatment of newly diagnosed patients with drugs that interfere with the ongoing immune response. Unfortunately, at the time of diagnosis, it is believed that most patients have lost the majority of pancreatic beta cells as a result of a prolonged autoimmune response. The ability to predict those individuals that will develop diabetes prior to extensive beta cell loss would provide a larger window of opportunity for therapeutic intervention.

This paper describes ongoing research aimed at identifying the population(s) of autoreactive CD8⁺ T cells that are responsible for beta cell damage in the pre-diabetic period. First, using a mouse model of type 1 diabetes, we were able to quantify a highly pathogenic population of T cells in the peripheral blood over time, which enabled prediction of

R É S U M É

Même si le traitement par des injections quotidiennes d'insuline réussit bien chez des millions de personnes atteintes de diabète de type 1 dans le monde entier, souvent, ce traitement ne protège pas contre la mortalité précoce qui résulte des complications de la maladie. Parmi les stratégies prometteuses pour le traitement du diabète de type 1, citons la greffe d'îlots chez les patients dont le diabète est avéré et, chez ceux dont le diagnostic est récent, des médicaments qui entravent la réponse immunitaire continue. Malheureusement, au moment du diagnostic, on croit que la plupart des patients ont perdu la majorité des cellules bêta du pancréas par suite d'une réponse auto-immune prolongée. Si on pouvait prévoir quels patients présenteront un diabète avant qu'il ne se produise une perte importante des cellules bêta, on aurait plus de temps pour intervenir.

Ce compte rendu décrit la recherche en cours, qui vise à cerner la ou les populations de lymphocytes T CD8⁺ autoréactifs responsables de la perte des cellules bêta avant l'apparition du diabète. D'abord, au moyen d'un modèle murin de diabète de type 1, nous avons pu quantifier une population de lymphocytes T très pathogènes dans le sang périphérique au fil du temps, ce qui nous a permis de prédire quelles souris présenteraient un jour un diabète. Des techniques semblables ont été utilisées pour examiner le sang périphérique de patients présentant un diabète de type 1 de diagnostic récent ou de longue date. Les résultats ont démontré que le sang périphérique des patients atteints de diabète de type 1 de diagnostic récent contenait des lymphocytes T spécifiques des cellules bêta. Il n'est pas étonnant que les résultats obtenus chez ces patients aient aussi démontré qu'il est probable que de nombreuses populations de lymphocytes T soient importantes dans la destruction des cellules bêta, ce qui rend la prédiction de la maladie plus difficile.

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those mice that would eventually develop diabetes. Similar techniques were employed to study the peripheral blood of patients with recent-onset and longstanding type 1 diabetes. The results demonstrated that beta cell-specific T cells are present in the peripheral blood of patients with type 1 diabetes of recent-onset. Not surprisingly, the results from these patients also show that there are likely to be many populations of T cells that are important for beta cell destruction, rendering disease prediction more difficult.

INTRODUCTION

Type 1 diabetes mellitus is an autoimmune disease that culminates in the near-complete destruction of pancreatic beta cells and a life-long dependence on exogenous insulin. The disease appears clinically after a protracted period of silent beta cell destruction, during which cells of the immune system infiltrate the pancreatic islets and selectively destroy beta cells. There are many factors, both genetic and environmental, that determine susceptibility to type 1 diabetes, including at least 20 genes that are associated with development of the disease (1). Nonetheless, the concordance rate for disease among identical twins is only ~50% (2). The contributions to and interactions of genetic and environmental factors in disease pathogenesis and progression are not completely understood.

At the time of diagnosis, it is estimated that ~80% of an individual's insulin-producing beta cells have been destroyed by the body's immune system (3-5). While many different cells contribute to beta cell destruction, 2 major subsets of T lymphocytes, CD4⁺ T helper lymphocytes and CD8⁺ cytotoxic T lymphocytes (CTL), play critical roles and are required for disease progression (6,7). During the immune response to infection, these cells work together to target foreign pathogens and limit tissue damage. However, CD4⁺ and CD8⁺ T cells are inappropriately activated in type 1 diabetes, resulting in destruction of beta cells. Numerous studies of humans with type 1 diabetes and also of a murine model of spontaneous diabetes, the nonobese diabetic (NOD) mouse, have all shown that T cells are responsible for inducing a significant proportion of beta cell death. Indeed, T cell suppression by treatment with anti-T cell antibodies prevents disease in NOD mice and delays progression of disease in humans (8,9). In addition, NOD mice that lack antigen-specific lymphocytes (NOD^{scid} mice) do not develop immune cell infiltration of the islets (insulinitis) or diabetes (7).

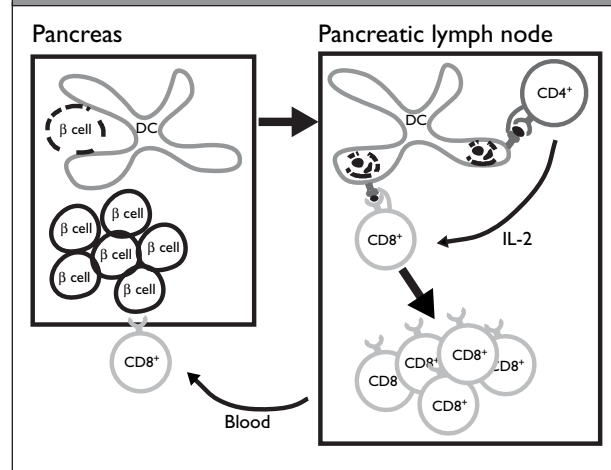
WHY DO BETA CELL-SPECIFIC T CELLS BECOME ACTIVATED?

During a typical immune response to infection, T cells undergo a series of coordinated events and receive a series of signals, each designed to alert the immune system to impending danger. These necessary signals are checkpoints to ensure that the immune system is not activated in response to self-antigens.

Nevertheless, the immune system is imperfect and may be inappropriately activated to produce exaggerated responses, as is the case with hypersensitivity reactions, or it may be triggered to destroy host tissue, as is the case with autoimmunity.

A primary role of T lymphocytes is to survey host cells for the presence of foreign pathogens. Intra- and extracellular proteins, both self and foreign, are continuously sampled and displayed on cell surfaces as small peptide fragments (antigens) in combination with major histocompatibility complex (MHC) class I or II molecules. (In humans, MHC molecules are known as human leukocyte antigens [HLA].) It is the combination of a specific peptide sequence and a particular MHC molecule (pMHC) that can potentially trigger

Figure 1. Bone marrow-derived dendritic cells acquire beta cell antigen in the pancreatic islets and activate T cells



Apoptotic beta cells can be acquired by local dendritic cells, which can move to the local draining lymph nodes and become ideal cells for activating T cells. Because dendritic cells are able to process beta cell antigen and present them on both MHC class I and class II molecules, they are able to activate both CD4⁺ and CD8⁺ beta cell-specific T cells. Populations of beta cell-specific T cells then traffic to the pancreas, where they can recognize and destroy beta cells.

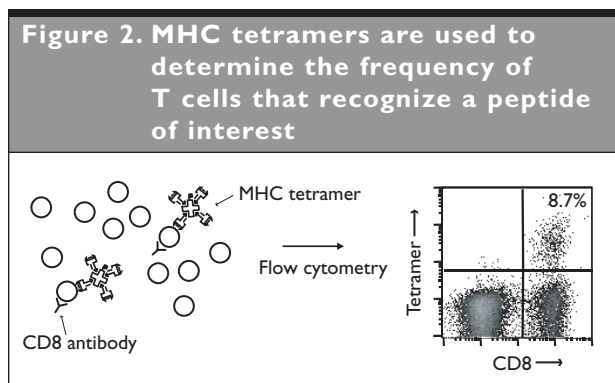
DC = dendritic cell

IL-2 = interleukin-2

MHC = major histocompatibility complex

the activation of individual T cells. By continually sampling the intracellular and extracellular milieu, T cells are alerted to cells that are infected with foreign pathogens, while possessing mechanisms that allow them to recognize self-tissue as benign. Self-peptides generally do not activate T cells because of tolerance mechanisms that eliminate or suppress T cells that recognize self-pMHC. Thus, for autoimmunity to arise, a failure must occur in 1 or more of these mechanisms that normally regulate the presence, activation and/or actions of autoreactive T cells.

Before clinical presentation of type 1 diabetes, the immune processes underlying beta cell destruction are subclinically active. Early in the pathogenesis of type 1 diabetes, autoreactive beta cell-specific T cells are likely activated in the pancreatic draining lymph nodes by 1 or more beta cell antigens presented on the surface of antigen-presenting cells, including dendritic cells (10,11). Upon recognition of beta cell antigen, activated T cells proliferate and return to the islets, where they destroy beta cells that express the inciting beta cell antigen. T cell-mediated killing of beta cells results in the release of additional beta cell antigens that may then be recognized by further subsets of T cells (Figure 1). It is therefore probable that the prolonged period of insulinitis before disease onset results in the generation of several subsets of autoimmune T cells, each recognizing a unique peptide derived from 1 or more beta cell proteins. Identification of the beta cell proteins and peptides that are recognized by the cellular immune response and of those T cell subsets that are important for disease development is a critical step in the design of T cell-specific diagnostics and therapeutic applications.



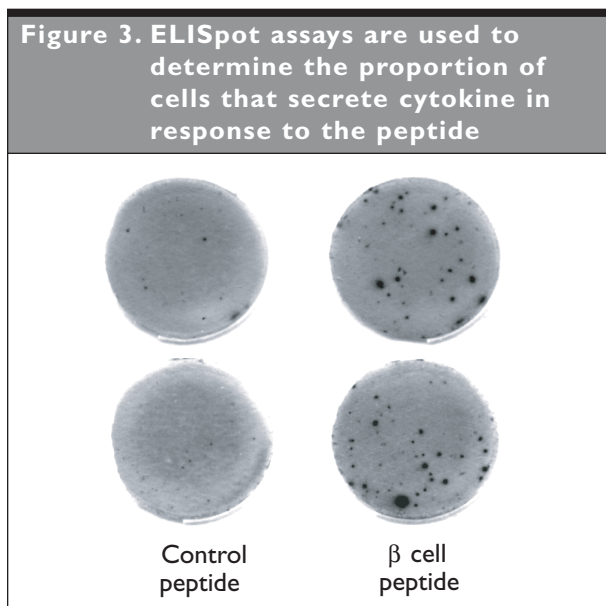
MHC tetramers may be used to determine the frequency of T cells that recognize a peptide of interest in a given cell population. In this example, cells isolated from the pancreatic islets of a prediabetic nonobese diabetic mouse were incubated with an antibody to CD8 (to detect CD8⁺ T cells) and an MHC class I tetramer that presents a beta cell peptide of interest (to determine whether any of the identified CD8⁺ T cells recognize the beta cell peptide). Flow cytometry was used to determine the proportion of cells that were bound to tetramer and antibody. In this example, 8.7% of cells that bound to the CD8 antibody (positive on the X axis) also bound to the tetramer (positive on the Y axis).

MHC = major histocompatibility complex

CAN BETA CELL-SPECIFIC T CELLS BE IDENTIFIED?

Is it possible to detect and quantify autoreactive T cells and thereby predict the occurrence of type 1 diabetes? Several research techniques have been developed for the detection of antigen-specific T cell responses, most notably enzyme-linked immunospot (ELISpot) assays and MHC tetramers. Each of these assays was designed to quantify the number of T cells within a given cell population that recognizes an antigen of interest. In ELISpot assays, the number of T cells that secrete an effector cytokine, commonly known as interferon gamma (IFN-gamma), is measured, while MHC tetramers quantify the frequency of T cells for a given antigen within a cell population. An example of typical results for these assays is shown in Figures 2 and 3 (techniques reviewed in references 12-14).

Together, these assays may be used to determine the frequency of a given antigen-specific T cell population of interest (MHC tetramers), while providing information regarding the function of these cells (ELISpot assays). The frequency of most antigen-specific T cell populations is very low (far below detectable limits) before their activation and proliferation (15). For example, before infection, the number of T cells in the body specific for a peptide fragment from a viral protein is low, <0.001% of T cells—far below the detection limits of most assays. However, upon acute viral



ELISpot assays may be used to determine the proportion of cells that responds to a peptide of interest in a given cell population. In this example, the ELISpot assay was used to determine the proportion of pancreatic islet cells (as in Figure 2) that secreted the effector cytokine IFN-gamma in response to stimulation with the beta cell peptide of interest. The cells on the right were stimulated with beta cell peptide, and the cells on the left were stimulated with a control peptide. The secretion of IFN-gamma by a cell was detected with antibodies and resulted in a dark spot.

IFN-gamma = interferon-gamma

infection, virus-specific T cells can account for as many as 40% of CD8⁺T cells (16), thereby facilitating their detection by both ELISpot and MHC tetramer assays.

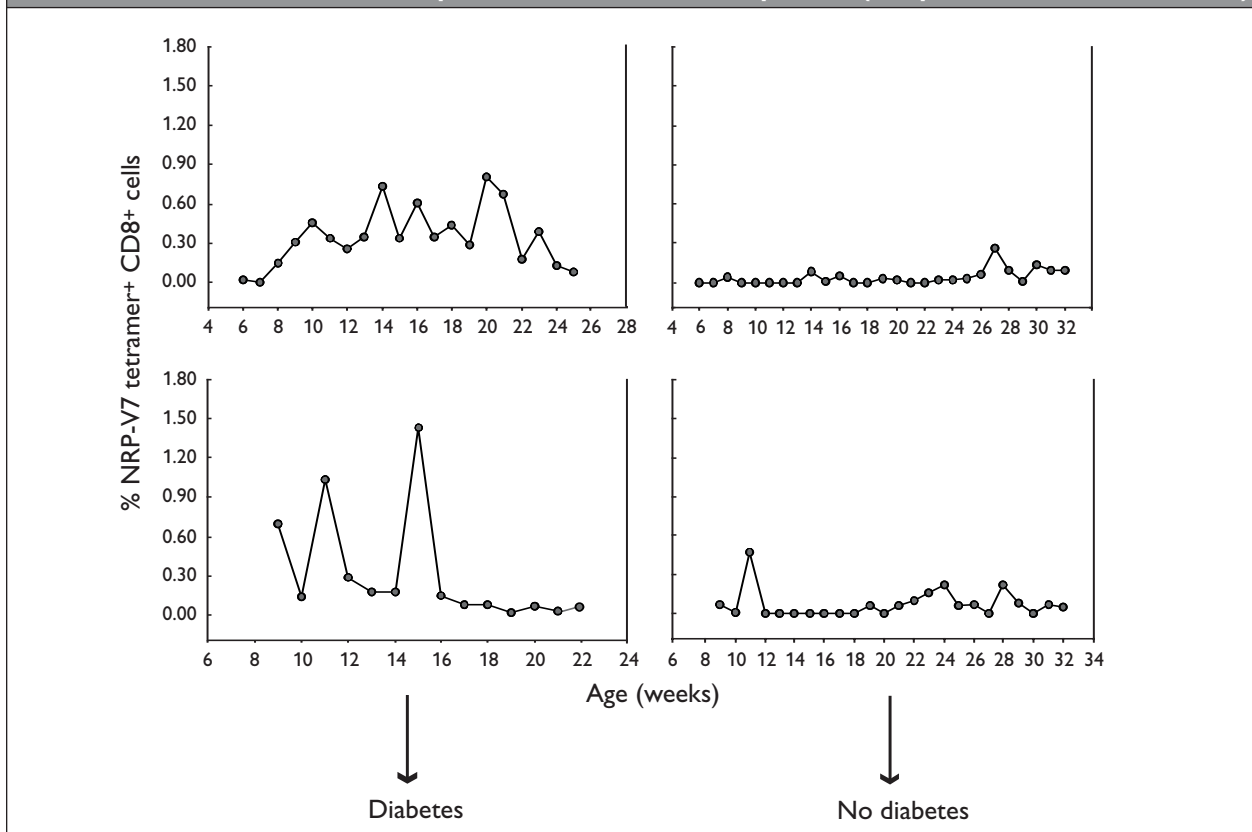
In order to examine beta cell-specific T cells using these methods, it is first necessary to identify beta cell antigens that are recognized by autoreactive T cells and, thus, are important for the progression of diabetes. Consequently, a major hurdle to the identification of beta cell-specific T cell subsets and their importance in diabetes development is the identification of beta cell antigens. Several beta cell peptides that may prove important in disease pathogenesis have been identified in the NOD mouse. NOD mice develop spontaneous autoimmune diabetes (in ~80% of females and ~50% of males) at 4 to 6 months of age and have many similarities with humans with type 1 diabetes (17,18). Like humans, NOD mice exhibit a prolonged period of immune cell infiltration (insulinitis) into the pancreatic islets that begins at approximately 4 weeks of age. Indeed, several beta cell proteins that are important in disease pathogenesis in NOD mice have been shown to be targeted in humans as well. Careful study of the islet-associated T cells derived from prediabetic and acutely diabetic NOD mice has permitted the identification

of several T cell antigens (19,20). These epitopes are primarily derived from beta cell proteins, such as insulin or glutamic acid decarboxylase (GAD), and peptide epitopes from these proteins have been shown to stimulate both CD4⁺ and CD8⁺ T cells. Studies investigating the roles of both CD4⁺ and CD8⁺ T cells that target insulin or GAD suggest that these beta cell antigens (and others) are likely involved in disease pathogenesis in the NOD mouse (21-24).

This review will focus on a peptide epitope derived from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP₂₀₆₋₂₁₄) (25), a beta cell protein of unknown function (26,27). Because IGRP₂₀₆₋₂₁₄ is recognized by an immunodominant population of autoreactive CD8⁺T cells in the NOD mouse, this epitope provides a particularly useful example of how detection and enumeration of autoreactive T cells before disease onset may aid in disease prediction.

Before the identification of IGRP, several peptide mimics capable of stimulating IGRP-reactive CTL had been described (28). Using 1 of these 9 amino acid peptide mimics, designated NRP-V7, we constructed MHC class I tetramers that enabled the detection and quantification of autoreactive CTL from the NOD mouse (29,30). NRP-V7-reactive cells

Figure 4. NRP-V7-specific T cells detected in the peripheral blood of nonobese diabetic mice can be used to predict diabetes development (adapted from reference 30)



Representative data from individual mice showing the proportion of NRP-V7 tetramer-positive cells present in peripheral blood. Mice with greater proportions of NRP-V7 tetramer-positive CD8⁺ cytotoxic T lymphocytes in the peripheral blood go on to develop diabetes. The mice depicted in the left top and bottom panels developed diabetes at 21 and 16 weeks of age, respectively.

were identified in pancreatic draining lymph nodes, spleen and peripheral blood and were found to constitute up to 37% of CD8⁺ T cells found in pancreatic islets (30). The high frequency of CTL specific for this 1 peptide suggests that IGRP₂₀₆₋₂₁₄ is a critical epitope, mediating a significant proportion of the autoimmune destruction of beta cells in the NOD mouse. Simultaneous analysis of peripheral blood and pancreatic islets showed that the presence of these beta cell-specific CTL in the peripheral blood was an effective surrogate marker for their infiltration into pancreatic islets. Consistent with this finding was the observation that serial weekly sampling of peripheral blood and analysis of the cumulative frequency of NRP-V7 tetramer-positive CTL allowed prediction of the NOD mice that would develop diabetes (Figure 4).

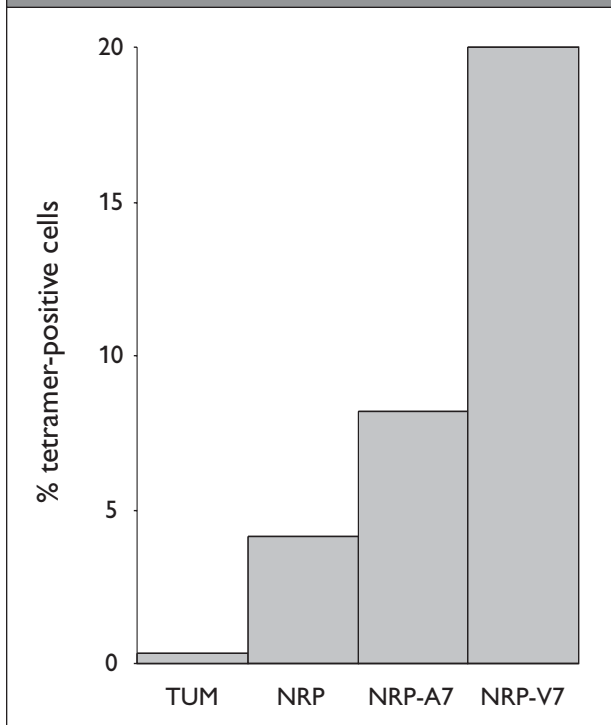
Autoreactive T cells are often more difficult to identify in ELISpot and MHC-tetramer assays because they generally recognize target antigen with less affinity than pathogen-

specific T cells (31,32). The use of high-avidity peptide mimics of natural autoepitopes is an effective way to circumvent this problem. For example, different modifications of the originally described peptide mimic NRP by substitution of amino acids at position 7 allowed for significantly improved recognition of autoreactive T cells using MHC class I tetramers or ELISpot assays (30-33). Of these different peptide mimics, NRP-V7 was able to detect the largest proportion of autoreactive T cells in the pancreatic islets and peripheral blood of prediabetic NOD mice (Figure 5). Presumably, the modified peptides bind to beta cell-specific T cells with greater avidity, increasing the half-life of T cell-peptide/MHC interaction, thus facilitating their detection. The use of a peptide mimic has also allowed for the identification of an important population of beta cell-specific CD4⁺ T cells in the NOD mouse (34,35).

GETTING OVER THE HURDLE IN HUMAN DIABETES

The identification of beta cell-specific T cell epitopes in humans has proven more difficult than in mice, largely because removal of islet-specific T cells from the immune cell-infiltrated pancreases of prediabetic humans is not feasible.

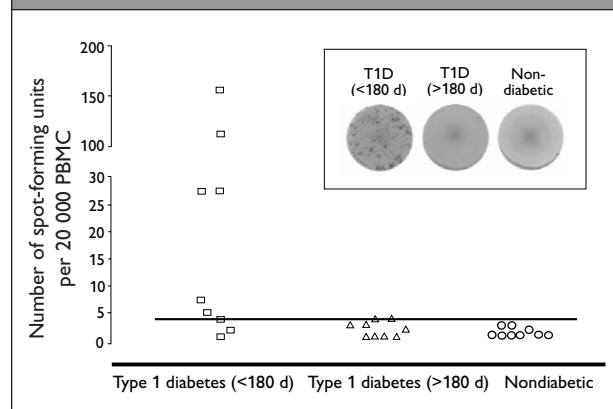
Figure 5. MHC class I tetramers with high-affinity peptide mimics bind to an increasing number of autoreactive T cells (adapted from reference 30)



A representative example of pancreatic islet cells (from an 8-week-old nonobese diabetic mouse) that were stained with a negative control tetramer (TUM) or with tetramers that stain beta cell-specific T cells with increasing affinity (NRP < NRP-A7 < NRP-V7). The data represent the number of tetramer-positive CD8⁺ cells found in the islets. This result was also seen with peripheral blood; however, the percentage of tetramer⁺ CD8⁺ cells was approximately one-tenth as high.

MHC = major histocompatibility complex

Figure 6. PreproIAPP₅₋₁₃-reactive CD8⁺ cytotoxic T lymphocytes are detectable in the peripheral blood of recent-onset type 1 diabetes patients (adapted from reference 38)



PBMC were isolated from the peripheral blood of recent-onset and established type 1 diabetes patients and from nondiabetic controls. PBMC were tested in IFN-gamma ELISpot assays for their ability to recognize preproIAPP₅₋₁₃ in the context of HLA*A201 (inset). The horizontal line indicates 3 SD above the mean for nondiabetic controls.

HLA = human leukocyte antigen

IAPP = islet amyloid polypeptide (amylin)

INF-gamma = interferon gamma

PBMC = peripheral blood mononuclear cells

SD = standard deviation

One approach involves the use of computer algorithms (36,37) to predict T cell epitopes from beta cell proteins of interest based on the sequence of amino acids that would preferentially bind the HLA haplotype of the individuals being investigated. Different HLA molecules prefer to bind certain amino acids at key anchor positions—for example, the common HLA-A201 molecule typically binds peptides with hydrophobic residues such as leucine at positions 2 and 9, limiting the number of possible peptides that can form a stable interaction with HLA-A201. Peptides predicted using these algorithms may be synthesized and screened for their potential relevance to type 1 diabetes using T cells obtained from the peripheral blood of patients with diabetes.

Using this approach, we recently identified a novel CTL epitope from the leader sequence of the beta cell protein islet amyloid polypeptide (IAPP or amylin) (38). The presence of autoreactive T cells specific for this epitope, preproIAPP₅₋₁₃, was assessed using peripheral blood T cells from patients with recent-onset or established type 1 diabetes as well as from nondiabetic control subjects. Using IFN- γ ELISpot assays, T cells specific for preproIAPP₅₋₁₃ were found in recent-onset patients, but were not detectable in patients with long standing (>6 months) disease or in nondiabetic controls (Figure 6) (38). These findings were consistent with those from our NOD mouse studies, in which we showed that after the onset of hyperglycemia, the number of beta cell-specific T cells detected using MHC class I tetramers decreased dramatically, presumably due to the loss of (beta cell) antigen necessary to drive proliferation of autoreactive CTL (30). These NOD mouse data suggest that after clinical presentation of type 1 diabetes in humans, the number of beta cell-specific T cells may be small, and the corresponding population of beta cell-specific T cells of interest would also be infrequent. In addition, they suggest that autoreactive T cells will be more readily detectable before disease onset, at a time when beta cell mass is greater and the disease process is more active.

PREDICTION OF TYPE 1 DIABETES: WHERE ARE WE NOW, WHERE DO WE GO?

Identification of the T cells responsible for beta cell damage during the development of diabetes is essential to understanding fully the roles of various T cell subsets in disease. The discovery of additional beta cell peptide epitopes—and identification of those that are most important in the progression of human type 1 diabetes—will be critical for understanding the T cell specificities that are important for initiating and propagating beta cell death. Identification of these populations of T cells and the epitopes that drive their expansion will greatly advance the goal of targeted immunotherapy of disease.

In addition to the discovery of endogenous epitopes, identification of their high-avidity peptide mimics should facilitate the detection of both CD8⁺ and CD4⁺ autoreactive T cells by allowing ex vivo analysis of low-frequency and/or low-avidity

T cell subsets. 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.

Prediction of autoimmune diabetes by visualization and enumeration of autoreactive T cells is currently possible in the NOD mouse model, but will likely prove to be more difficult in humans. The development of the autoreactive T cell response to beta cells in the prediabetic period is undoubtedly more variable in humans, both in timing and in diversity of the response. In addition, T cell epitopes for the many different HLA types that exist in humans with type 1 diabetes or in subjects at risk will need to be identified. We have also seen that the frequency and kinetics of beta cell-reactive T cells in the prediabetic period is variable among NOD mice and among different studies, strongly suggesting that T cell responses in humans are also quite variable (30).

Attempts to standardize T cell responses in humans to common beta cell antigens such as GAD and insulin have proven difficult, supporting the notion that the magnitude and timing of T cell responses varies considerably among individuals with type 1 diabetes (39,40). Prediction of diabetes in both the NOD mouse and prediabetic humans using autoantibody titers has seen great success. In humans, the presence of 3 beta cell antibodies—to insulin, to GAD and to IA-2 (a beta cell-specific protein tyrosine phosphatase)—predicts the development of diabetes within 5 to 10 years with a sensitivity ranging from 60 to 100% (41,42). Given that variability exists in both autoantibody and T cell prediction assays, it is likely that a future combination of T cell and autoantibody assays might improve prediction power.

At this time, a greater understanding of the T cell populations that are most important in beta cell destruction depends on a more complete description of beta cell autoantigens in humans. This in turn should lead to advances in the development of T cell-specific diagnostic and therapeutic applications for type 1 diabetes, in addition to more powerful prediction tools.

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