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Novel autoantigens for diabetogenic CD4 T cells in autoimmune diabetes

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Abstract

Autoreactive CD4 T cells play a central role in the development of type 1 diabetes. The BDC-panel of diabetogenic T cell clones was originally isolated from non-obese diabetic (NOD) mice and has been used to study the role of autoreactive CD4 T cells and T cell autoantigens in the development of diabetes. Recent studies by our group have led to the identification of two new target antigens for clones of this panel. This review describes the proteomic strategy used for antigen identification, the antigens identified, and the potential contribution of post-translational modification to autoantigen generation. In addition, we compare peptide epitopes for the T cell clones and discuss their potential applications in investigating the role of T cell autoantigens in the pathogenesis and regulation of disease.

Keywords

Autoreactive T Cells; Type 1 Diabetes; Autoantigens; Post-translational Modification

Autoreactive CD4 T cells in type 1 diabetes (T1D)

The destruction of pancreatic beta-cells is the key event that leads to the development of T1D. The autoimmune process driving beta-cell destruction is evident in the inflammatory infiltrates found in pancreatic islets, the presence of anti-islet antibodies, and a strong genetic association with loci of the class II major histocompatibility complex (MHC class II) [1]. Over half of the inherited predisposition to T1D maps to a chromosomal region that contains highly polymorphic class II genes [2] and importantly, the class II molecules are necessary for presentation of antigen to CD4 T cells. The non-obese diabetic (NOD) mouse is a well established animal model for the study of type 1 diabetes (T1D) and in order to investigate the role of T cells in disease, various autoreactive T cell clones have been isolated from NOD mice. The BDC panel of CD4 Th1 T cells, shown in Table 1, is the largest and best characterized panel of autoreactive T cell clones available [3]. These clones have been defined by their ability to induce diabetes upon transfer into young (<14 days old) NOD and NOD.scid recipients.

Recent work from our laboratory has led to the identification of two new autoantigens for diabetogenic T cells in T1D. Although nearly twenty different proteins have been identified as target antigens for T cells in the NOD mouse, and at least 12 of these are also autoantigens in human patients [4], the impact of most of these proteins on the disease process is not well understood, particularly with regard to antigens for CD4 T cells. The identification of autoantigens for T cells is essential, however, to understand their role in

pathogenesis of T1D and to develop strategies for antigen-specific tolerance induction. We review here our work to identify autoantigens and how we can apply this information to the investigation of T1D and its regulation.

Identification of autoantigens using a proteomic strategy

We have developed and applied a proteomic strategy to identify antigens for autoreactive T cells from our panel. For a successful application of this strategy, three critical components are required: 1) a highly sensitive T cell assay for tracking antigen, 2) an abundant source of antigenic starting material, and 3) a state-of-the-art proteomics facility. For the first component, we use T cell clones from the BDC panel which are maintained in culture and synchronized in their activation cycle through biweekly restimulation with irradiated NOD spleen cells and antigen extracted from beta-cell tumors of transgenic NOD-RIPTAg mice [5]. T cell activation through antigen/MHC is readily measured via IFN- γ ELISA. These T cell clones have been selected for high affinity responses to antigen, which is key to tracking small quantities of antigenic material during biochemical and proteomic purification. T cell hybridomas or T cells from T cell receptor transgenic (TCR-Tg) mice respond poorly to small concentrations of antigen in complex mixtures (cell lysates) and therefore generally do not provide sufficient sensitivity for detection of antigen during purification. As a source of antigen, we use beta-cell tumors obtained from NOD RIPTAg mice. Tumors harvested from a single NOD RIPTAg mouse can yield up to 1×10^7 beta-cells (compared to approximately 100,000 cells from the islets of one mouse) and contain a sufficient amount of antigen for sequential chromatographic purification (see below). A beta-cell membrane preparation (β -Membrane) containing the antigens for all of the T cell clones from our panel is prepared from the tumors. Lastly, the third component of our proteomics strategy involves a state-of-the-art mass spectrometry facility which is of great importance for the successful identification of antigen candidates. Our facility at National Jewish Health is an outstanding lab for this type of analysis and it is advantageous to be in direct contact with the mass spectrometry facility to aid with experimental design and sample preparation.

Figure 1 outlines the three steps necessary for our proteomic identification of T cell antigens, including 1) fractionation of antigen, 2) identification by mass spectrometry, and 3) validation of antigenicity. Fractionation of the antigen begins with a simple differential centrifugation of beta-cell tumors which yields a beta-cell membrane fraction (β -Membrane) enriched in antigens from the secretory granules [5]. This preparation is highly antigenic and is used as our control antigen in T cell cultures and assays. It can be solubilized using detergents and further fractionated by sequential chromatographic steps, including size exclusion chromatography (SEC), ion exchange chromatography (IEX), and/or reverse phase high performance liquid chromatography (RP-HPLC). After each chromatographic step, antigen-containing fractions are identified using the T cell assay (illustrated in Fig. 2 for the SEC of β -Membrane) and are pooled for further fractionation. In-solution digests of antigenic chromatographic fractions are usually prepared for mass spectrometry at this point, although a final purification step involving gel electrophoresis can be included.

Proteins present in antigenic fractions are subsequently identified by mass spectrometry, using, for example, an IonTrap mass spectrometer. Samples for the analysis can be obtained directly from chromatographic fractions (in-solution) or from a protein gel (in-gel) and should include fractions with varying degrees of antigenicity. Mass spectrometric analysis reveals proteins present in each fraction as well as spectral intensities for each protein. The intensity can be used as an indicator for individual protein abundance in a fraction, and comparison of antigenicity of a fraction with spectral intensity can be used to narrow down the list of potential antigen candidates. For in gel analysis, the protein gel is stained using a

sensitive staining protocol (e.g., silver stain [6]) and protein bands matching the antigen distribution are excised and analyzed by mass spectrometry.

Upon identification of likely antigen candidates, several confirmation steps should be performed to confirm the identity of the antigen. The traditional method of validating protein identity is through cloning and expression of the antigen in a cell line. For T cell antigens, it is possible that more rapid confirmation will be achieved through identification of peptide epitopes, especially when purified whole protein is not available. As T cells recognize peptides only after a protein (or peptide) is processed by antigen-presenting cells, ultimate identification of a T cell antigen is achieved through defining peptide ligands. This may require screening of a peptide library, or if the protein is small, a panel of overlapping peptides. Alternatively, other clues may exist such as amino acid motifs in a peptide mimotope [7]. If antigen-deficient mice are available, isolated tissues can be used for the purpose of confirmation; in our case we were able to test islets from antigen knock-out mice as a form of confirmation [7, 8]. For such validation tests to be convincing, it is important that control antigens and T cell clones reactive to other antigens are used.

Antigens for autoreactive CD4 T cells

Using the proteomic strategy described above, we recently identified the proteins chromogranin A (ChgA) and islet amyloid polypeptide (IAPP) as target antigens for several T cell clones from the BDC-panel (Table 1) [7, 8]. At least three T cell clones with different T cell receptors for antigen (BDC-2.5, BDC-10.1 and BDC-9.46) respond to the peptide WE14 (Table 2) of ChgA. WE14 is a naturally occurring cleavage product that is generated within the secretory granules of beta-cells as well as of other neuro-endocrine tissues [9]. We have also identified the peptide KS20 (Table 2) from IAPP as the epitope for the T cell clone BDC-5.2.9. The antigen targets for the T cell clones BDC-6.9, BDC-9.3 and BDC-6.3 are currently unknown. However, two of the clones, BDC-6.9 and BDC-9.3, share the TCR V β 4V α 13.1, and a previous genetic mapping study indicated that the BDC-6.9 antigen is IAPP [10], a finding supported by the fact that these T cell clones do not respond to antigen from islets of IAPP-deficient mice. Two other clones are reactive to the well-studied B9-23 epitope of insulin: BDC-4.38 (this line has two different V β populations responsive to insulin and one with verified diabetogenicity is listed) and PD-12.4.4 [11] are included in the panel. All three antigens, ChgA, IAPP, and insulin, are localized in the secretory granules of beta-cells and are processed by a common proteolytic mechanism catalyzed by at least one type of prohormone convertase as well as by a carboxypeptidase.

As shown in Fig. 3, the reactivity of T cell clones to the epitopes listed in Table 2 varies by several orders of magnitude. KS20 is the most antigenic peptide and the BDC-5.2.9 clone responds to this peptide at low nanomolar concentrations. The B9-23 epitope of insulin shows an intermediate level of antigenicity for the two T cell clones, PD-12.4.4 and BDC-4.38, stimulating at a low micromolar peptide concentration. WE14 is the poorest stimulator, with concentrations of around 100 mM being required to stimulate the ChgA-reactive T cell clones. The ED₅₀ values for the three peptides KS20, B9-23 and WE14 are approximately 0.02 mM, 10 mM and 70 mM respectively, spanning a concentration range of nearly three orders of magnitude.

Evidence for post-translational modifications in antigens for various T cell clones

Proteins are complex biomolecules susceptible to a broad range of post-translational modifications (PTMs), which can either occur spontaneously (e.g., spontaneous oxidation or glycation) or as a result of physiologic processes (e.g., enzymatically). Consequently, the

antigenicity and interactions of proteins with components of the immune system may be altered [12]. Modified self-antigens may have increased MHC class II binding affinity or an altered form of the antigen may be required for recognition by a specific T cell receptor. T cells recognizing epitopes of proteins modified as a result an autoimmune inflammatory process may circumvent negative T cell selection. PTMs have been associated with various autoimmune diseases and include citrullination of proteins in rheumatoid arthritis by the enzyme peptidyl arginine deiminase (PAD) [13–16], deamidation in celiac disease by the enzyme transglutaminase (TGase) [17], phosphorylation in systemic lupus erythromatosis [18–20] and oxidative damage in multiple sclerosis [21, 22]. Although PTM of proteins provides a mechanism by which neo-antigens are generated, there has been little description in the literature of protein alterations in T1D.

Demonstrating the existence of post-translationally modified proteins as autoantigens in T1D could have significant clinical implications. For example, modified proteins could play a critical role in the initial stages of the disease and therefore could be used as diagnostic markers before full onset of the disease. The detection of autoantibodies to citrullinated peptides in rheumatoid arthritis currently constitutes the most specific test for this disease with a high accuracy [23, 24]. Identification of PTMs as a key component for the development of diabetes could also open a broad field of prevention or treatment therapies for this disease. For example, antigen-specific immunotherapies [25] targeting autoreactive T cells or other immune components that recognize modified antigens could be applied to suppress or reverse disease. Furthermore, preventing the introduction of a disease-inducing PTM might halt development of disease; e.g., TGase-specific inhibitors may potentially be used to treat celiac disease, or PAD-inhibitors might be useful to prevent rheumatoid arthritis. Additionally, the reversal of a PTM after it has been introduced through the activation of repair pathways has been envisioned to suppress and reverse disease [12].

We have recently obtained evidence suggesting that PTMs could play a role in antigens for T cell clones from our panel. The peptide WE14 is currently the weakest epitope in the panel as very high peptide concentrations are required to stimulate T cells (Fig. 3). The natural form of the antigen found in beta-cell extracts is significantly more antigenic than this peptide, as demonstrated by T cell responsiveness to the β -membrane preparation in which the antigen is present at very low concentrations. This indicates that WE14 is not the natural ligand for the ChgA-reactive T cell clones. Additionally, mass spectrometric analysis of highly purified, chromatographic fractions containing the natural ligand for ChgA-reactive T cell clones did not lead to the identification of a peptide containing an amino acid sequence required for T cell recognition. This may indicate that the peptide is shielded from detection by mass spectrometry due to the presence of a currently unidentified modification. The strongest argument for PTM of this peptide, however, is our recent data indicating that WE14 can undergo modification in vitro by the enzyme transglutaminase, resulting in the formation of a highly antigenic converted product (Delong et al., manuscript submitted).

The peptide B9-23 is also a weak antigen. In the case of PD-12.4.4, approximately equal concentrations of peptide and beta-cell extract are required to observe a comparable T cell response by the insulin reactive T cell clone PD-12.4.4 [7]. It is possible that epitopes of insulin are post-translationally modified and at least one PTM of insulin has been reported in antigen responses of human T cells [26]. Because insulin is in such high abundance in beta-cells, it may be that a poorly antigenic epitope such as B9-23 is present at sufficient concentration to stimulate autoreactive T cells. Although the natural sequence of KS20 from IAPP is highly antigenic for one T cell clone, this peptide could result from an aberrant cleavage process in beta-cells. No antigenic peptides from IAPP have yet been identified for the T cell clones BDC-6.9 and BDC-9.3 and it may be that these epitopes of IAPP undergo PTM.

Why should we investigate autoantigens in T1D?

The discovery of new autoantigens for pathogenic T cells extends our areas of investigation of the causes and regulation of the autoimmune disease process in T1D. One objective we are pursuing is to define the role of new autoantigens in the spontaneous development of diabetes. A second goal is to investigate strategies for the induction of antigen-specific tolerance, with the view to discovering new methods to prevent or inhibit T1D in humans.

One way in which antigenic peptides have been used to investigate disease progression is through the development of tetramer reagents. MHC Class II tetramers have been used to detect antigen-specific CD4 T cells and have been particularly useful for understanding how CD4 T cells contribute to the immunological response in allergy, cancer and infectious disease, as recently reviewed [27]. However, the design and production of high affinity tetramer reagents for CD4 T cells in T1D has been challenging. Although insulin has been known to be an autoantigen for several decades, only recently has an I-Ag7 tetramer capable of detecting insulin-reactive CD4 T cells been available [28]. Because insulin is such a weak antigen, this team had to use a combination of two different tetramers loaded with the insulin peptide B9-23 in two different MHC binding registers. Another way to design class II tetramers is to use peptide mimotopes instead of the natural ligands. In 2003, Stratmann et al. used this approach to detect “BDC-2.5-like” CD4 T cells in NOD mice [29]. An interesting new area of tetramer development is the use of MHC tetramers loaded with post-translationally modified peptides. Two groups have been able to effectively use such tetramers to detect antigen-specific cells in celiac disease and rheumatoid arthritis [30] [31]. We have recently developed a new I-Ag7 tetramer that contains the KS20 peptide. Preliminary data indicate that this reagent may be highly sensitive as IAPP-reactive T cells are readily detected in pancreas of pre-diabetic NOD mice (Baker et al., unpublished data). Reagents such as these will lead to a better understanding of the autoimmune response in T1D.

Perhaps the ultimate reason for identifying autoantigens is to develop new strategies for the induction of antigen-specific tolerance; i.e., antigen-specific treatments that inhibit the reaction of specific subsets of pathogenic T cells to self-antigen without global immunosuppression. The use of peptide-coupled splenocytes has been demonstrated to be a safe and efficient process for inducing antigen-specific tolerance in multiple animal models of autoimmunity, including experimental autoimmune encephalitis (EAE) and the NOD mouse model of T1D [32–34]. The fixation of donor cells with a chemical cross-linker, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI), in the presence of antigen, results in the formation of covalent isopeptide bonds, linking the peptide to the cells and generating a tolerogenic antigen carrier system [32]. We have recently used the ChgA peptide, WE14, to investigate whether disease induced by the ChgA-specific T cell clone BDC-2.5 could be prevented with peptide-coupled splenocytes. Our preliminary data indicate that WE14-coupled splenocytes are highly effective and can block disease by the relevant T cell clone, but not by irrelevant clones or with the use of irrelevant peptide antigens (He et al., unpublished data). We are further investigating this system with peptides from IAPP and combinations of peptides to determine whether polyreactive T cell transfer can be prevented or spontaneous disease in NOD mice can be reversed.

Concluding remarks

Islet-reactive T cell clones have been very useful in studies on disease pathogenesis and regulation in T1D. A panel of diabetogenic CD4 T cell clones has also proved to be valuable in the detection and characterization of autoantigens. Proteomic approaches coupled with sensitive T cell reagents have been key to identifying antigens and through such an

approach, we recently identified two beta-cell secretory granule proteins as new autoantigens in T1D. Evidence suggests that beta-cell proteins may undergo post-translational modification to become autoantigens in T1D. Other recent work indicates the utility of antigenic peptides in exploring both the contribution of CD4 T cells to disease and new strategies for antigen-specific tolerance induction.

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Figure 1. Proteomic strategy for identification of T cell antigens

Antigen is purified from beta-cell tumors using several sequential purification steps such as differential centrifugation, chromatography and gel electrophoresis. Proteins in fractions are identified by mass spectrometry and likely antigen candidates are selected by comparing antigen distribution over fractions with individual protein abundances. Validation of antigen can be accomplished through antigen testing of expressed protein, identification of peptide ligands, or testing of antigen-deficient tissue.

Figure 2. Purification of T cell antigens by size exclusion chromatography (SEC)
Antigen was purified from the beta-cell extract by SEC. Protein absorbances were measured at 280 nm. Small aliquots of collected fractions were assayed by ELISA with the T cell clones BDC-2.5, BDC-5.2.9 or BDC-6.9. IFN- γ responses above 50 ng/ml are not shown, as measurements were above the valid detection range of the assay.

Figure 3. Comparison of peptide antigenicities for various T cell clones

Representative T cells (BDC-5.2.9, PD-12.4.4 and BDC-2.5) were incubated in the presence of APCs as well as increasing concentrations of peptide (KS20, B9-23, WE14). IFN- γ in cellular supernatants was measured by ELISA after 48 hr of incubation. ED50 values, described in the text, were determined by reading the peptide concentration in the graph at a T cell response of 25 ng/ml.

Table 1
Diabetogenic CD4+ Th1 T Cell Clones

T Cell	TCR	Diabetogenicity	Antigen	Epitope
BDC-2.5	Vβ4Vα1	+	ChgA	WE14
BDC-10.1	Vβ15Vα13	+	ChgA	WE14
BDC-9.46	Vβ8.3	n.d. *	ChgA	WE14
BDC-4.38	Vβ12	+	Insulin	B9-23
PD-12.4.4 ^a	Vβ1	+	Insulin	B9-23
BDC-5.2.9	Vβ6Vα12	+	IAPP	KS20
BDC-6.9	Vβ4Vα13.1	+	n.d.	n.d.
BDC-9.3	Vβ4Vα13.1	+	n.d.	n.d.
BDC-6.3	Vβ4Vα3.1	+	n.d.	n.d.

^aDaniel, et al. [11]

*
n.d. not determined

Table 2

Peptide epitopes recognized by autoreactive T cell clones

Epitope	Amino Acid Sequence *
WE14	WSRMDQLAKELTAE
KS20	KCNTATCATQRALNFLVRSS
B9-23	SHLVEALYLVCGERG

* one letter code for amino acids