The Evolving Landscape of Autoantigen Discovery and Characterization in Type 1 Diabetes

Anthony W. Purcell,[1] Salvatore Sechi,[2] and Teresa P. DiLorenzo[3,4,5,6]

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Type 1 diabetes (T1D) is an autoimmune disease that is caused, in part, by T cell–mediated destruction of insulin-producing β-cells. High risk for disease, in those with genetic susceptibility, is predicted by the presence of two or more autoantibodies against insulin, the 65-kDa form of glutamic acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2), and zinc transporter 8 (ZnT8). Despite this knowledge, we still do not know what leads to the breakdown of tolerance to these autoantigens, and we have an incomplete understanding of T1D etiology and pathophysiology. Several new autoantibodies have recently been discovered using innovative technologies, but neither their potential utility in monitoring disease development and treatment nor their role in the pathophysiology and etiology of T1D has been explored. Moreover, neoantigen generation (through posttranslational modification, the formation of hybrid peptides containing two distinct regions of an antigen or antigens, alternative open reading frame usage, and translation of RNA splicing variants) has been reported, and autoreactive T cells that target these neoantigens have been identified. Collectively, these new studies provide a conceptual framework to understand the breakdown of self-tolerance, if such modifications occur in a tissue- or disease-specific context. A recent workshop sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases brought together investigators who are using new methods and technologies to identify autoantigens and characterize immune responses toward these proteins. Researchers with diverse expertise shared ideas and identified resources to accelerate antigen discovery and the detection of autoimmune responses in T1D. The application of this knowledge will direct strategies for the identification of improved biomarkers for disease progression and treatment response monitoring and, ultimately, will form the foundation for novel antigen-specific therapeutics. This Perspective highlights the key issues that were addressed at the workshop and identifies areas for future investigation.

The Centers for Disease Control and Prevention reports that about 9.4% of the U.S. population has diabetes and about 5% of the people with diabetes have type 1 diabetes (T1D) (1). Although T1D has a significant genetic component, most diagnosed people do not have a known family history of the disease. The causes that lead to T1D are not fully established, but in individuals with genetic susceptibility (determined in large part by the expression of certain class II MHC molecules [2]), the development of the disease can usually be predicted by the presence of two or more autoantibodies with different specificities (3,4). Autoantibodies against insulin, the 65-kDa form of glutamic acid decarboxylase (GAD65), insulinoma-associated protein 2 (IA-2), and zinc transporter 8 (ZnT8) are commonly known as the major specificities in T1D, but their role in the pathophysiology of the disease is not clear.

[1]Infection and Immunity Program and Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, Victoria, Australia
[2]Division of Diabetes, Endocrinology, and Metabolic Diseases, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD
[3]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY
[4]Division of Endocrinology, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY
[5]Einstein-Mount Sinai Diabetes Research Center, Albert Einstein College of Medicine, Bronx, NY
[6]Fleischer Institute for Diabetes and Metabolism, Albert Einstein College of Medicine, Bronx, NY

Corresponding authors: Anthony W. Purcell, anthony.purcell@monash.edu, and Teresa P. DiLorenzo, teresa.dilorenzo@einstein.yu.edu

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Recently, several new antigens and epitopes and their corresponding humoral and/or T cell–mediated responses have been reported. However, their potential utility in monitoring disease development, progression, and treatment and their role in the pathophysiology and etiology of T1D have been explored only in a very limited manner. What leads to the loss of tolerance and autoimmunity in T1D is certainly one of the key questions to be answered for understanding the pathogenesis of the disease. Although imperfect (5), central tolerance leads to the deletion of some proportion of self-reactive lymphocytes. However, lymphocytes specific for epitopes generated only in a tissue- and/or disease-specific context will not be subject to central tolerance mechanisms (6). Thus, the idea that a neoantigen or a modified self-antigen (e.g., arising from a tissue-specific posttranslational modification) can lead to the breakdown of tolerance is a compelling hypothesis (7) that warrants investigation.

For assessing the state of the art in elucidating the potential role of neoepitopes and neoantigens in the pathophysiology of T1D, the National Institute of Diabetes and Digestive and Kidney Diseases convened a group of scientists with different expertise at the Autoantigens Discovery and Characterization in Type 1 Diabetes workshop in Bethesda, MD, 31 October–1 November 2017 (www.niddk.nih.gov/news/meetings-workshops/2017/autoantigens2017). In particular, experts from other autoimmune diseases, cancer immunotherapy, and cutting-edge technologies for T-cell and antigen characterization and discovery were brought together. The workshop was organized around three main themes: characterization of the autoimmune response in T1D and other disease contexts, identification of new autoantigens and epitopes in T1D, and novel technologies in T-cell response and autoantigen identification and characterization. This report highlights the main points that were discussed at the workshop and reflects on possible future developments that might be needed for moving toward a better understanding of the autoimmune response in T1D.

EMERGING CONSIDERATIONS FOR THE CHARACTERIZATION OF THE IMMUNE RESPONSE TO β-CELL ANTIGENS

In the 35 years since the discovery of insulin as the first autoantigen in human T1D, over 30 additional ones have been reported (8), though a substantial fraction of these putative autoantigens have only been sparsely studied and/or have not withstood the test of time. In contrast, insulin, GAD65, IA-2, and ZnT8 are well accepted by the field as major autoantigens (Table 1), due primarily to the utility of their corresponding autoantibodies in T1D risk assessment and diagnosis (3,4). Several additional antigens have also established their place in human T1D (Table 2). Examples include islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (9,10), chromogranin A (ChgA) (11,12), and islet amyloid polypeptide (IAPP) (13–15). Still others (Table 3), e.g., peripherin (16), tetraspanin-7 (17), prolyl-4-hydroxylase β (P4Hb) (18), glucose-regulated protein 78 (GRP78) (19), urocortin-3 (20), and insulin gene enhancer protein isl-1 (20), have only more recently been discovered and warrant further exploration.

Islet-Infiltrating Cells

Until recently, nearly all of the knowledge concerning T-cell reactivity to islet antigens in humans was obtained using T cells from peripheral blood rather than from islets themselves. Some of the earliest evidence for the presence of islet-reactive T cells in the blood of T1D patients and at-risk individuals was reported in the early 1990s (21). Since then, more than 100 epitopes, derived from over 10 antigens, have been identified using peripheral blood as the T-cell source (22,23). It should be noted, however, that the majority of these epitopes have not yet been rigorously proven to be naturally processed and presented by relevant antigen-presenting cells. Hopes of similarly examining the antigenic reactivities of T cells isolated from islets were dampened by the problem of tissue accessibility, coupled with the notion that β-cells and, thus, β-cell–specific T cells, were unlikely to be present in the pancreata of long-standing T1D patients. However, the Joslin Medalist Study, which examined pancreata from T1D donors who had lived with the disease for 50 years or more, revealed that β-cells were indeed still present in such individuals, as were islet-infiltrating T cells (24). This finding led to the realization that there was much to be learned about human T1D, and it was surely one of the stimuli for continued investigator-initiated studies of the human T1D pancreas as well as the growth of the Network for Pancreatic Organ Donors with Diabetes (nPOD), an initiative which procures and distributes T1D pancreata to the research community (25). These efforts have recently made possible the first investigations of the antigenic specificities (26–29) and T-cell receptor repertoire (30) of human islet-infiltrating T cells in T1D. Some of the specificities previously identified using peripheral blood have now been validated using islet T cells, supporting the continued and complementary use of peripheral blood T cells for antigen identification efforts, and new specificities have also been uncovered. Whether some specificities will be found only in the islets, and not also in peripheral blood, remains to be determined. However, it now appears that T cells specific for islet antigens are enriched in the pancreas, but not in the blood or pancreatic lymph nodes, of donors having T1D compared with donors without diabetes (20,31). Another important open question relevant to both antigen identification and T-cell receptor repertoire analyses is what proportion of the human islet-infiltrating T cells are truly specific for islet antigens. Findings from several mouse studies support the idea that islet-infiltrating T cells may be largely β-cell–specific (32,33), but this remains controversial (34,35) and ideally would be addressed using human samples. These open questions

Table 1—Major autoantigens used in human T1D diagnosis and risk assessment

			Human T1D		
Autoantigen	Expression	Subcellular location	Antibodies	$CD4^+$ T cells	$CD8^+$ T cells
Insulin	β-cell	Secretory granule	+	+	+
GAD65	Neuroendocrine	Synaptic-like microvesicles	+	+	+
IA-2	Neuroendocrine	Secretory granule	+	+	+
ZnT8	β-cell	Secretory granule	+	+	+

notwithstanding, the discovery of previously unknown specificities using islet T cells has breathed new life into the antigen identification efforts of the T1D community and has encouraged collaboration and consultation with those studying other related disease entities.

Modified Epitopes

Celiac disease is an enteropathy mediated by a T-cell response to gluten peptides in which tissue transglutaminase 2 has converted at least one glutamine to glutamic acid (36). The deamidation of glutamine residues in gliadin and other wheat proteins generates high-affinity peptide ligands for the disease-associated HLA-DQ alleles (36). This reflects the preference of HLA-DQ2 and HLA-DQ8 for peptides with acidic residues, and the resultant conversion of glutamine to glutamic acid facilitates the binding of these peptides to the disease-associated alleles. Unlike in T1D, in celiac disease an immune response with a defined onset can be experimentally induced in humans with an oral gluten challenge, thus greatly facilitating the antigen identification efforts that led to these discoveries. Despite this important difference, T1D investigators continue to draw inspiration from their celiac disease colleagues, especially since the two diseases share a genetic association with the same HLA-DQ alleles. Deamidated peptides of several classical T1D antigens, i.e., insulin (37,38), GAD65 (37,39), and IA-2 (26,40), have recently been shown to be recognized by peripheral blood and/or islet T cells from T1D patients.

The association of citrullination of arginine residues of joint autoantigens with rheumatoid arthritis was first suggested by a genetic association with a single nucleotide polymorphism that affects stability of the PADI4 transcript, encoding protein-arginine deiminase type-4 (41). Subsequent studies have indeed shown citrullinated joint autoantigens to be the target of both autoantibodies (42,43) and also $CD4^+$ T cells restricted by the rheumatoid arthritis–associated alleles HLA-DR4 and -DR1 (44,45). Citrullination, at least in part, appears to dictate the binding of autoantigen-derived peptides to disease-associated HLA-DR molecules as well as affecting T-cell recognition by some patient-derived T-cell clones. As in rheumatoid arthritis, autoantibodies (19,46) and T cells from patients with T1D, including islet-infiltrating ones, have also been shown to respond to citrullinated autoantigens including GRP78 (19,26), GAD65 (39), and IAPP (26).

Another recent advance has been the identification of so-called hybrid insulin peptides, which comprise peptide fragments derived from both insulin and other insulin secretory granule proteins that are fused together to form the hybrid peptide. Though first identified as the cognate antigens for pathogenic $CD4^+$ T-cell clones derived from NOD mice, their potential importance in human T1D was suggested by the finding that they are also recognized by islet-infiltrating T cells obtained from patients (26,27).

These discoveries support the contention that antigen identification efforts in T1D must continue, as novel and important insights are still arising from such work. They also suggest the cautionary note that antigen identification efforts should consider posttranslationally modified peptides and other forms of neoepitopes (e.g., ones generated by translation of RNA splicing variants [20] or alternative open reading frame usage [47]) whenever feasible. For example, recently it was reported that a defective ribosomal product, or DRiP (48), can be translated from the human insulin mRNA when an out-of-frame downstream AUG serves as a translation initiation site. This leads to usage of an alternative reading frame that includes the 3′ untranslated region and the synthesis of a product having 43 amino acids (47). A nonapeptide derived from this was predicted to bind well to the human class I MHC molecule HLA-A*02:01 and was found to be recognized by $CD8^+$ T cells from HLA-A*02:01–positive T1D patients (47).

Table 2—Select additional established autoantigens in human T1D

			Human T1D		
Autoantigen	Expression	Subcellular location	Antibodies	$CD4^+$ T cells	$CD8^+$ T cells
IGRP	β-cell	Endoplasmic reticulum		+	+
ChgA	Neuroendocrine	Secretory granule		+	+
IAPP	β-cell	Secretory granule	+	+	+

Table 3—Examples of recently identified autoantigens in human T1D

			Human T1D		
Autoantigen	Expression	Subcellular location	Antibodies	$CD4^+$ T cells	$CD8^+$ T cells
Peripherin	Neuroendocrine	Filaments	+		
Tetraspanin-7	Neuroendocrine	Plasma membrane	+		
P4Hb	Not restricted	Endoplasmic reticulum	+		
GRP78	Not restricted	Endoplasmic reticulum	+	+	
Urocortin-3	β-cell, α-cell	Secretory granule			+
Insulin gene enhancer protein isl-1	Not restricted	Nucleus			+

The burgeoning area of neoepitopes in T1D is ushering in the idea of the β-cell contributing to its own demise (49), in the sense that neoepitope formation, including alternative open reading frame usage, can be enhanced under conditions of endoplasmic reticulum stress and inflammation (47,50,51), with the resulting neoepitopes potentially contributing to the breakdown of immunological self-tolerance. Furthermore, β-cells have recently been shown to release peptides derived from insulin catabolism into the circulation, and these peptides can subsequently activate pathogenic insulin-specific T cells (52).

Disease Heterogeneity

It is now becoming appreciated that, whether designing antigen identification strategies or clinical trials, T1D should not simply be viewed as a single disease but rather as a heterogeneous entity. Some aspects of heterogeneity, e.g., age at onset, have long been known and appreciated, while others, such as the pattern of autoantibody appearance (53,54) and pancreatic immune cell presence (both among individuals and among islets in a single individual) (25,55,56), have only been ushered in relatively recently. From studies of insulitic lesions, among other approaches, investigators are now working to identify T1D endotypes, or subsets of the disease likely sharing a common pathogenic mechanism (57). Characterization of the spectrum of antigens and epitopes recognized in each case will likely help in these efforts. This is important work, as disease heterogeneity has been blamed, at least in part, for the failure of the field to identify a robust preventive or reversal strategy for T1D, despite years of earnest and exhausting efforts (58).

IDENTIFICATION OF NEW AUTOANTIGENS AND EPITOPES IN T1D

With multiple established autoantigens well accepted by the field (Tables 1 and 2), the quest to identify new autoantigens may seem redundant on first inspection. However, additional autoantigens may not only prove to be powerful targets of immunomodulatory therapies but also shed light on the pathogenesis of T1D. Moreover, given the noted heterogeneity of the disease, a more personalized approach to immune profiling will be facilitated through the validation of a broader spectrum of disease-relevant autoantigens. Additional autoantigens may also help to further stratify treatment modalities and provide diagnostic or prognostic tests that go beyond current clinical management of individuals with T1D. Perhaps more importantly, there is still an immediate requirement to identify the HLA class I– and class II–restricted epitopes recognized by autoreactive T cells in T1D, as the vast majority of identified and validated T-cell epitopes are restricted to a mere handful of HLA alleles (22,23). Given the independent associations of HLA class I and class II alleles with disease (2), understanding the T-cell reactivity on a personalized basis will herald in a new era of T1D treatments and diagnostics.

In addition to a requirement to identify additional autoantigens of relevance to different stages of the disease, understanding the role of posttranslational modifications of both new and established autoantigens is critical for the launching of new therapies and for providing a molecular basis of the disease. Posttranslational modification of antigens can impact the liberation of immunogenic epitopes during antigen processing (59), altering the spectrum of presented peptides. Modification of peptide antigens can also affect their binding to different HLA alleles, with some modifications enhancing binding to disease-associated allomorphs (37,49,59,60) and others providing novel targets for T-cell recognition (61–70).

Besides modification of antigens by processes such as deamidation and citrullination, a more novel class of neoepitopes has also been implicated in T1D. This class is potentially generated through transpeptidation, a reverse proteolysis reaction, that can generate spliced or hybrid peptide antigens such as the hybrid insulin peptides recognized by $CD4^+$ T cells discussed above (27). Likewise, recent studies have also emphasized the contribution of proteasomal or other posttranslational splicing reactions to the class I MHC antigen processing pathway (71–75), estimated in multiple studies (71,72), though not in all (73), to contribute an astonishing 30% of peptides to the peptide repertoire of antigen-presenting cells. While their role in T1D is not yet apparent, such peptides may be targets of the autoimmune response in T1D. Consistent with this notion is the recent finding that a peptide derived from noncontiguous parts of IAPP is recognized by islet-infiltrating $CD8^+$ T cells from T1D patients (20).

NOVEL TECHNOLOGIES IN T-CELL RESPONSE AND AUTOANTIGEN IDENTIFICATION AND CHARACTERIZATION

T1D has seen a recent uptake of new and novel technologies for the characterization of T-cell responses and the identification of autoantigens. Prominent among these has been recent progress in the development of autoimmune-prone mice "humanized" to express HLA molecules for use in epitope mapping and pathogenicity studies (76–78). Similarly, the generation of tissue repositories has facilitated the production of islet-derived T-cell clone libraries and related resources for the validation and discovery of novel T-cell targets (25,26,29,30,79). Such resources can be interrogated with synthetic peptides, antigen preparations from islets and other sources, or with relevant peptide-MHC multimers. Though not limited to T1D-related antigens, the Immune Epitope Database (iedb.org) is a critical resource for the selection of candidates for such screening efforts (80). In each case, appropriate posttranslational modifications can be introduced, as is particularly evident by recent studies with HLA class II tetramers in T1D (19,40) and other autoimmune diseases (45,81,82). Multiplexing these assays, particularly tetramers with either coded fluorophores (83,84), mass cytometry tags (85), or DNA bar codes (86,87), significantly extends the use of this screening technology and interfaces with single-cell genomic studies to study gene expression in autoreactive T-cell clones (86,87). Indeed, advances in single-cell analysis have led to extrapolation of T-cell and B-cell reactivity profiles and the identification of additional modifiers of disease. At least for class I MHC–restricted T-cell epitopes, coupling peptide/MHC multimer technology with other analyses may be of particular importance, given the recent finding that the frequency of tetramer-binding islet-reactive $CD8^+$ T cells in peripheral blood does not differ between T1D patients and healthy control subjects (31).

Finally, continued development of unbiased approaches for antigen and epitope identification is also urgently needed. In one such strategy, small molecules are used as "epitope surrogates" to enrich for T1D-specific autoantibodies from patient sera (16). The enriched antibodies are then used to identify the target protein(s), the approach that yielded the identification of phosphorylated peripherin as an autoantigen in T1D (Table 3) (16). Likewise, serum screening of a nucleic acid–programmable, cell-free protein array, designed in an unbiased manner (i.e., without regard to pancreas expression level), recently revealed close to 20 previously unidentified targets of the autoantibody response in T1D (88). These targets await further study. It should be noted that each autoantigen discovery approach has its own characteristic strengths and weaknesses (89). Thus, the different strategies are best viewed as complementary rather than competing or redundant.

In the quest for unbiased approaches for antigen and epitope identification, mass spectrometry has certainly risen to the fore. Improvements in sensitivity and speed of this instrumentation now make peptidome profiling of HLA-bound peptides relatively routine and have opened up the possibility to work with limited patient-derived material (90,91). Such analysis also allows for unambiguous definition of posttranslational modifications of epitopes (92–95) or from proteome extracts (19,96–98) of patient-derived material. To date, characterization of the HLA class I–bound peptides from human β-cells has been limited, primarily due to difficulties in obtaining sufficient material (20). Islets harvested from cadaveric donors with T1D have very few β-cells remaining, and those from donors without diabetes have naturally low levels of cell surface HLA expression in the absence of inflammation, making direct detection of presented peptides challenging. To circumvent these issues, various approaches have been used to discover epitopes of relevance to T1D including the direct biochemical isolation and characterization of naturally presented autoantigen-derived peptides from murine β-cell lines (99), stably transfected human non–β-cell lines expressing autoantigen(s) and cell surface HLA allotypes of interest (100,101), or human β-cell lines generated by targeted oncogenesis (20). These latter approaches take advantage of the cellular antigen processing machinery and can directly identify the antigenic peptides sampled for cell surface presentation by disease-associated MHC molecules, although questions remain as to whether such approaches faithfully represent natural presentation on primary human β-cells. Peptidomics was recently combined with transcriptomics to identify peptides derived from two new autoantigens, insulin gene enhancer protein isl-1 and urocortin-3 (Table 3), for which the cognate T cells are enriched in the pancreata of T1D donors compared with those without diabetes (20).

CONCLUSION

A more complete knowledge of the specificities of T and B cells in T1D will assist in the development of targeted immune tolerance as well as in diagnosis, patient characterization, and pre- and posttherapy immune monitoring. Exhilarating recent discoveries, such as T-cell recognition of hybrid and other posttranslationally modified peptides, have demonstrated that much remains to be discovered. Targeted immune system tolerance remains a highly sought-after yet elusive goal for the prevention and treatment of T1D. The need is urgent, given that the incidence of the disease is on the rise, and T1D associated with immune checkpoint inhibitor therapy for cancer is an emerging entity also requiring our focused and immediate attention.

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