REVIEW

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Neoepitopes: a new take on beta cell autoimmunity in type 1 diabetes

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Abstract

Type 1 diabetes is an autoimmune disease caused by T cell-mediated destruction of pancreatic insulin-producing beta cells. The epitopes recognised by pathogenic T cells in human type 1 diabetes are poorly defined; however, a growing body of evidence suggests that T cell responses against neoepitopes contribute to beta cell destruction in type 1 diabetes. Neoepitopes are formed when self-proteins undergo post-translational modification to create a new epitope that is recognised by T- or B cells. Here we review the role of human T cell responses against neoepitopes in the immune pathogenesis of type 1 diabetes. Specifically, we review the different approaches to identifying neoepitopes relevant to human type 1 diabetes and outline several advances in this field that have occurred over the past few years. We also discuss the application of neoepitopes to the development of antigen-specific therapies for type 1 diabetes and the unresolved challenges that need to be overcome before the full repertoire of neoepitopes recognised by pathogenic human T cells in type 1 diabetes can be determined. This information may then be used to develop antigen-specific therapies for type 1 diabetes and assays to monitor changes in pathogenic, beta cell-specific T cell responses.

Keywords Antigen-specific therapy · Beta cells · Hybrid insulin peptides · Neoepitopes · Post-translational modification · Review · T cells · Type 1 diabetes

Abbreviations
GRP   Glucose-regulated protein
HIP   Hybrid insulin peptides
IAPP Islet amyloid polypeptide
TCR   T cell receptor

Type 1 diabetes

Type 1 diabetes is an autoimmune disease caused by the autoimmune destruction of pancreatic insulin-producing beta cells [1, 2]. The molecular basis of this autoimmune beta cell destruction remains poorly understood. Autoantibodies specific for beta cell proteins arise early in life. The greater the number of antigen specificities recognised by these

autoantibodies, the greater an individual's risk of developing type 1 diabetes [3]. It is clear that CD4^{+} and CD8^{+} T cells are primarily responsible for beta cell destruction in type 1 diabetes. Many genetic loci have been described that are associated with increased risk of developing type 1 diabetes [4]. Of these, the strongest risk alleles are found in the HLA class II region, specifically *HLA-DQ* [5] and the insulin gene promoter [6]. The natural history of type 1 diabetes has been classified into three stages: stage 1 is characterised by the presence of autoantibodies specific for beta cell proteins; in stage 2, dysglycaemia develops in addition to autoantibody presence; at stage 3, an individual has symptomatic type 1 diabetes [7].

Autoimmunity, post-translational modifications and neoepitopes

Over the past decades, T cell responses against neoepitopes have become increasingly implicated in the pathogenesis of autoimmune diseases, including type 1 diabetes (reviewed in [1, 8–10]). Autoimmune T cell responses that are specific to neoepitopes formed in peripheral tissues may explain how and why T cell responses are not subject to the usual tolerance mechanisms; specifically, thymic deletion of self-reactive T

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cells and peripheral tolerance. The corollary of the 'neoepitope hypothesis', is that neoepitopes formed during cellular stress will challenge the immune system's capacity to distinguish self from non-self. It may be that, when the cell is under physiological stress, these mechanisms are attenuated, leading to destructive autoimmunity in genetically susceptible individuals [11]. For example, endoplasmic reticulum (ER) stress leads to the modification of tyrosine phosphatase-related islet antigen 2 (IA-2), creating neoepitopes recognised by human CD4^{+} T cells [12].

Why is it important to define neoepitopes associated with type 1 diabetes?

Identification of the epitopes recognised by pathogenic human T cells in type 1 diabetes is important for both translational and basic research. For translational research, this information will support the development of assays that can monitor changes in the function and frequency of antigen-specific T cells from a blood sample. Neoepitopes that are known targets of pathogenic T cell responses will be prime candidates for incorporation into antigen-specific therapies to restore immune tolerance to beta cells. These goals go hand-in-hand. A suitably robust and sensitive assay to monitor changes in T cell numbers and function is essential for monitoring changes induced by experimental antigen-specific therapies [13].

The identification of epitopes recognised by pathogenic T cells in type 1 diabetes is also an important goal for basic research scientists. The elucidation of targets of the autoimmune T cell response against beta cells will address enduring questions about the immune pathogenesis of human type 1 diabetes [1]. Currently, it is not known why immune tolerance towards beta cells is lost in people who develop type 1 diabetes. Identifying the relevant T cell epitopes will help to reveal how pathogenic T cells avoid thymic deletion and peripheral tolerance mechanisms. These insights may explain many of the enigmatic features of the aetiology of type 1 diabetes, such as the long-known association between *HLA-DQ2/DQ8* alleles and increased risk of developing type 1 diabetes [5, 14].

How to identify neoepitopes: approaches and challenges

Identifying neoepitopes recognised by T cells in people with, or at risk of type 1 diabetes continues to be difficult. The problem has three facets: to find (1) beta cell specific T cells, (2) the appropriate antigen and (3) the appropriate modification of that antigen.

Beta cell-specific T cells have been identified using three broad approaches; the first of these being functional analysis of human T cells. For example, T cells specific for relevant antigens/epitopes have been isolated from peripheral blood mononuclear cells (PBMCs) using the carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assay [15], or HLA tetramers complexed with candidate neoepitopes [16]. In addition, human T cell-based approaches have recently been used to identify relevant T cells by their location within islets [17–19] (discussed in more detail below). Second, proteomic approaches, including peptide elution and mass spectrometry, have been used to identify potentially relevant modifications of beta cell proteins. The advantage of these approaches is that they identify modified proteins in a relatively unbiased manner, enabling unanticipated modifications to be identified (reviewed in [20]). For example, using 2D gel electrophoresis, glucose-regulated protein (GRP) 78 was found to be citrullinated in NOD mouse islets [21]. More recently, peptide elution from human beta cell lines has identified HLA-A2-restricted neoepitopes, formed by mRNA splice variants of beta cell antigens [22]. Third, some neoepitopes recognised by human T cells have first been identified in NOD mice. The best recent example of this is the discovery of hybrid insulin peptides (HIPs) [23], which were first identified as the targets of the pathogenic BDC T cells from NOD mice and then shown to be recognised by human islet-infiltrating CD4^{+} T cells.

Autoantibody responses against neoepitopes

While the analysis of immune responses to neoepitopes has focused on T cells, there is some evidence that autoantibodies specific for neoepitopes can be detected in people with type 1 diabetes. For example, autoantibodies specific for reactive oxygen species (ROS)-modified type II collagen were detected in HLA-DR4 positive people with type 1 diabetes [24]. A higher titre autoantibody response was detected against oxidised insulin compared with unmodified insulin in serum from people with type 1 diabetes [25]. Moreover, in a follow-up study, it was found that autoantibodies specific for oxidised insulin were detected in children before they developed type 1 diabetes (in stage 2 of development); more than 90% of children who had oxidised-insulin-specific autoantibodies went on to develop type 1 diabetes [26]. Together, these studies imply that oxidatively modified forms of insulin may arise and prime autoantibody responses in people who develop type 1 diabetes.

An overview of neoepitopes in type 1 diabetes

The neoepitopes currently implicated in type 1 diabetes development (summarised in Table 1) can result from the following processes: (1) modifications arising during antigen processing and presentation [27]; (2) conversion of glutamine to

Table 1 Summary of neoepitopes recognised by human T cells implicated in type 1 diabetes

Modification	Antigen[a]	Neoepitope[a,b]	HLA restriction	Identification approach	References
Vicinal disulfide bond	Insulin	GIVEQ**CC**TSICSL	HLA-DR4	Antigen-specific T cell analysis	[27]
Glutamine to glutamic acid	Proinsulin	EDL**E**VG**E**VE	HLA-DQ8	HLA binding	[28]
	IA-2	SLSYEPALL**E**PYLFH**E**FGS	HLA-DQ8	HLA binding; HLA tetramers	[12]
	GAD-65	MNILL**E**YVVKSFD; KGMAALP**X**LIAFTSEHSHFS	HLA-DR4	HLA binding; HLA tetramers	[16]
Arginine to citrulline	GRP78	VEKAK**X**ALSSQHQA	ND	Proteomics; human islet-infiltrating T cells	[18]
	IAPP	VGSNTYGK**X**NAVEVLKREP	ND	Proteomics; human islet-infiltrating T cells	[18]
	IAPP	VGSNTYGK**X**NAVEVLK**X**EPL	HLA-DQ8	HLA binding; HLA tetramers	[12]
Peptide fusion	Proinsulin–IAPP2	GQVELGGG**NAVEVLK**	HLA-DQ8	Murine and human islet-infiltrating T cells	[18, 23]
	IAPP–IAPP	**VAL**K**LQVFL**	HLA-A2	Peptidomics; transcriptomics	[22]
Alternative reading frame	Insulin	**MLYQHLLPL**	HLA-A2; HLA-DQ8	Alternative start codon analysis; HLA binding; HLA tetramers	[29]

[a] A representative example(s) of antigen/neoepitope for each modification is listed. Modified residues are shown in bold

[b] X represents the amino acid citrulline

IA-2, islet antigen 2; ND, no data

glutamate, or arginine to citrulline [12, 16, 18, 28]; (3) peptide fusion [18, 22, 23]; or (4) aberrant mRNA translation [22, 29]. It is already clear that multiple mechanisms lead to the development of neoepitopes recognised by type 1 diabetes-associated T cell responses.

Recent advances in type 1 diabetes-associated T cell neoepitopes

Neoepitopes implicated in the pathogenesis of human type 1 diabetes have been reviewed elsewhere [8–10, 30, 31]. Here we focus on some recent discoveries that reveal new mechanisms for the formation of neoepitopes recognised by T cells from individuals with type 1 diabetes.

HIPs In 2016, Delong et al [23] reported that diabetes-associated CD4^{+} T cells from NOD mice recognised HIPs. These neoepitopes are formed by the fusion of two peptide fragments in beta cells. The HIPs reported by Delong and colleagues were all fusions of parts of proinsulin C-peptide with other beta cell-granule components, such as islet amyloid polypeptide (IAPP) 2 (reviewed in [10, 32]). The mechanism that leads to the formation of HIPs is unclear, but it is believed to result from transpeptidation (in a similar manner to the formation of 'spliced' CD8^{+} T cell epitopes) (reviewed in [32]). Although CD4^{+} T cells that responded to HIPs were first discovered in the NOD mouse, two laboratories have isolated human islet-infiltrating CD4^{+} T cells that respond to HIPs, including HLA-DQ8-restricted CD4^{+} T cell clones [23]. More recently, it has been reported that T cell responses to HIPs

predict onset of diabetes in the NOD mouse model [33]. It will be of great interest to determine if this is also true in humans.

Neoepitopes recognised by islet-infiltrating T cells Building on our technique for isolating human islet-infiltrating T cells [17], Babon et al [18] reported that islet-infiltrating CD4^{+} T cells recognise a variety of neoepitopes. T cell lines recognised HIPs formed by the fusion of proinsulin C-peptide with peptides from insulin A-chain, IAPP1 and IAPP2. In addition, they found islet-infiltrating CD4^{+} T cells that responded to neoepitopes incorporating citrulline in place of arginine, specifically, GRP78[292–305] with citrulline at position 297. This finding provides evidence that this neoepitope, which was first identified in the NOD mouse [21], may play a role in the pathogenesis of type 1 diabetes in humans. CD4^{+} T cell responses were also detected against IAPP[65–84] with citrulline at positions 73 and 81. In almost all cases, the responses to a particular neoepitope were only found in a single individual (i.e. the neoepitope was only defined with the analysis of islet-infiltrating T cells from one person), suggesting that each individual may respond to a unique or restricted set of neoepitopes. Data on HLA restriction was not reported in this study, so it is unclear if these neoepitopes are presented by type 1 diabetes-associated HLA molecules [18]. Extensive T cell receptor (TCR) sequencing from human islet-infiltrating T cells has revealed a wide range of TCR genes used by these cells [34]. Pairing TCR sequencing with the analysis of neoepitope specificity will facilitate the dissection of neoepitope-specific T cell responses in human islets.

Alternative reading frames Kracht et al [29] described neoepitopes derived from the insulin gene (*INS*) encoded by

a non-canonical reading frame. CD4$^+$ and CD8$^+$ T cell responses were described against peptides that were formed by translation initiation 341 nucleotides downstream (3′) of the canonical start codon. Using this non-canonical start codon results in a different reading frame allowing translation to 'read through' the canonical stop codon. In this way the *INS* gene encodes a new polypeptide and, as a consequence, potential neoepitopes. Importantly, Kracht et al reported type 1 diabetes-associated CD4$^+$ cells against the first nine amino acids (MLYQHLLPL) of the alternative reading frame *INS* gene product and HLA-A2-restricted responses against the same epitope. This study was the first to describe neoepitopes formed by translation from an alternative start codon. It will be of great interest to determine if non-canonical start codons result in the expression of neoepitopes from other beta cell-associated genes.

Elution and analysis of epitopes from HLA class I Gonzalez-Duque et al [22] used transcriptomics and peptide elution to thoroughly characterise beta cell-derived epitopes presented by HLA class I. After identifying candidate epitopes, antigen-specific T cells were identified by HLA-A2 tetramer staining. From this work, in addition to many conventional epitopes, an HLA-A2-restricted hybrid epitope was identified, which was formed by the fusion of IAPP$_{15–17}$ with IAPP$_{5–10}$. Interestingly, several epitopes that resulted from mRNA splice variants of beta cell proteins were also identified. In the majority of cases, alternatively spliced mRNA levels were not increased after exposure to inflammatory cytokines (IFN-γ, TNF-α and IL-1β).

Opportunities and challenges for therapeutic intervention

From our growing understanding of the natural history of human type 1 diabetes, it is now clear that autoimmune T- and B cell responses arise early in life (stage 1 of type 1 diabetes development) [35], often many years before clinical disease (stage 3) manifests. The timing of the emergence of autoimmune T cell responses to neoepitopes is not yet known. The hypothesis that pathogenic T cell responses against neoepitopes arise very early in the course of type 1 diabetes, or even initiate the autoimmune responses against beta cells, is attractive. However, currently there is no experimental evidence to support this hypothesis. Recent evidence suggests that changes in the gene expression pattern of naive proinsulin- and GAD-responsive CD4$^+$ T cells may precede the appearance of autoantibodies [35]. These observations suggest that responses to unmodified antigens arise very early in the course of disease. How T- and B cell responses to neoepitopes evolve, and the capacity of these cells to respond to unmodified antigens, remains to be elucidated. A more

detailed understanding of the dynamics of beta cell antigen-specific autoimmune responses may support the development of antigen-specific therapies.

With a growing list of neoepitopes, how can those best for T cell assays and antigen-specific therapies be identified? Ideally, the focus should be on the epitopes for which there is the strongest evidence for recognition by pathogenic T cells. At this stage, as discussed above, it is not known if any neoepitopes are the targets of pathogenic T cell responses in stage 1 or stage 2 of type 1 diabetes [7]. However, circumstantial evidence, such as being the targets of islet-infiltrating T cells, is also helpful; in particular those neoepitopes that are also restricted by HLA-DQ2/DQ8 should be on the 'short list'. We suggest that there will not be a single 'magic bullet' but, instead, a panel of epitopes (both neoepitopes and conventional ones) will be required to measure and attenuate the full range of T cell responses against beta cells present in people who develop, or who are at risk of, type 1 diabetes.

Determining which neoepitopes are clinically relevant, i.e. the targets of pathogenic T cell responses, is difficult. Ideally, immunologists will be able to fulfil Koch's postulates [36], by showing that T cells that recognise a particular neoepitope cause type 1 diabetes. Clearly, this is not possible in humans. One option is to develop a murine model of the human T cell response to beta cell antigens. While there have been some attempts [37, 38], to our knowledge there are no mouse models where a human TCR, specific for the human form of a beta cell antigen presented by HLA molecules, causes autoimmune diabetes. Hence, the pathogenicity of neoepitope-specific T cells remains to be directly determined.

The ultimate goal is to use neoepitopes in a form of antigen-specific therapy to curb the autoimmune T cell responses that drive beta cell destruction. Some allergic diseases have been treated with antigen-specific therapies [39]. Although type 1 diabetes has a different aetiology, in principle, progress in the field of allergy gives support to the notion that delivery of a disease-causing antigen/epitope in the appropriate format can be clinically beneficial. In type 1 diabetes, it is likely that giving antigen-specific therapy during stage 1 or stage 2 of the disease would be most effective because therapy delivered at these early stages may attenuate T cell responses against a presumably small number of (neo)epitopes, diffusing the autoimmune response before it can 'spread' to other antigens/epitopes. However, there are two barriers to this approach: (1) identifying the neoepitopes that are pivotal for autoimmunity during stages 1 and 2 of type 1 diabetes (it is more feasible to search for responses against neoepitopes in stage 3 of the disease); and (2) identifying individuals who are likely to benefit from such therapy before they show any symptoms of type 1 diabetes (stage 1 and 2 of the disease are asymptomatic). Alternatively, antigen-specific therapy could be given shortly after diagnosis of type 1 diabetes. In this scenario a panel of (neo)epitopes should be

delivered to comprehensively suppress pathogenic T cell responses. Once the appropriate neoepitopes are identified, there are many emerging approaches that may be used to deliver these epitopes to induce or restore immunological tolerance (reviewed by [40, 41]).

Future directions in identifying and validating neoepitopes

Our challenge now is to develop high-throughput systems to identify neoepitopes and determine whether responses against neoepitopes drive autoimmune beta cell destruction in type 1 diabetes. New approaches for the identification of neoepitopes will need to accommodate the inconvenient truth that these molecules can be formed in a variety of ways [23, 29, 32], making neoepitope prediction almost impossible.

Although many challenges remain, neoepitopes have emerged as major new components in the type 1 diabetes puzzle over the past 15 years. Exciting new discoveries of novel mechanisms for neoepitope generation have greatly expanded our thinking and the possibilities for therapeutic intervention. With this progress, there is no doubt that the next 10–15 years will see a great increase in our knowledge of neoepitopes and the way in which they can be applied for improving the care of people with, or at risk of, type 1 diabetes.

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