



Immune Recognition of β -Cells: Neopeptides as Key Players in the Loss of Tolerance

Eddie A. James,¹ Massimo Pietropaolo,² and Mark J. Mamula³

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Prior to the onset of type 1 diabetes, there is progressive loss of immune self-tolerance, evidenced by the accumulation of islet autoantibodies and emergence of autoreactive T cells. Continued autoimmune activity leads to the destruction of pancreatic β -cells and loss of insulin secretion. Studies of samples from patients with type 1 diabetes and of murine disease models have generated important insights about genetic and environmental factors that contribute to susceptibility and immune pathways that are important for pathogenesis. However, important unanswered questions remain regarding the events that surround the initial loss of tolerance and subsequent failure of regulatory mechanisms to arrest autoimmunity and preserve functional β -cells. In this Perspective, we discuss various processes that lead to the generation of neopeptides in pancreatic β -cells, their recognition by autoreactive T cells and antibodies, and potential roles for such responses in the pathology of disease. Emerging evidence supports the relevance of neopeptides generated through processes that are mechanistically linked with β -cell stress. Together, these observations support a paradigm in which neopeptide generation leads to the activation of pathogenic immune cells that initiate a feed-forward loop that can amplify the antigenic repertoire toward pancreatic β -cell proteins.

Type 1 diabetes (T1D) is an organ-specific autoimmune disease in which pancreatic β -cells are selectively destroyed. The precise events that initiate disease remain unknown, but the most current evidence indicates that antibodies that recognize either insulin or glutamic acid decarboxylase (GAD65) are the earliest evidence of loss of self-tolerance (1,2). Disease progression is characterized by an accumulation of autoantibodies against additional β -cell antigens (3)

and the activation of autoreactive T cells, which have been shown to infiltrate pancreatic islets (4,5). The genetic risk factors associated with autoimmune diabetes share significant overlap with other organ-specific autoimmune diseases, implying common disease mechanisms and pathways. Among these, genetic predisposition is most strongly associated with susceptible HLA class II haplotypes (6). The most likely contribution of HLA class II proteins to disease is through selection of a potentially autoreactive CD4⁺ T-cell repertoire (7). T-cell responses, in turn, provide help to autoreactive B cells and facilitate affinity maturation of antibodies that recognize β -cell antigens. It has been clearly shown that autoantibodies and autoreactive T cells recognize multiple β -cell antigens (8). However, important questions remain about the events that lead to the loss of B- and T-cell tolerance and the inadequacy of regulatory mechanisms to restrain β -cell-specific responses. Although the timing of such responses remains unclear, mounting evidence implicates the formation of neopeptides as a relevant means of disrupting β -cell tolerance. In this article, we discuss diverse processes that can generate neopeptides, their recognition by autoantibodies and T cells, environmental factors and pathways that promote their formation, and evidence for their role in the pathogenesis of T1D.

POSTTRANSLATIONAL PROCESSES GENERATE NEOPEPTIDES

Human proteins are translated from mRNA into polypeptides composed of 20 standard amino acids. Several of these standard amino acids can be posttranslationally modified by enzymatic processes, many of which are part of normal physiology, or can be altered through spontaneous (non-enzymatic) biochemical reactions (9). Far more than half of all self-proteins (as many as 90%) bear one or more

¹Translational Research Program, Benaroya Research Institute at Virginia Mason, Seattle, WA

²Diabetes Research Center, Department of Medicine, Baylor College of Medicine, Houston, TX

³Section of Rheumatology, Department of Medicine, Yale School of Medicine, New Haven, CT

Corresponding author: Eddie A. James, ejames@benaroyaresearch.org.

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posttranslational modification (PTM) (9). These modifications lead to more than 140 unique amino acid structures that in some cases are essential for their biological functions and may be required to establish immune tolerance during positive and negative selection and in the periphery. In some cases, PTMs occur through abnormal processes that can alter protein function or recognition by the immune system. Tissue-specific PTMs that arise as a consequence of inflammation or reactive oxygen species (ROS) are unlikely to be well represented in the thymus, bone marrow, or healthy tissue (9). Consequently, PTM represents a likely means of undermining self-tolerance. However, due to a lack of data regarding modified amino acids, available algorithms that predict peptide/protein binding to immune receptors (HLA, T-cell receptor, or B-cell receptor) are not ideally suited to assess the impact of PTMs. Furthermore, peptides with specific PTM residues cannot be readily synthesized in some cases. Therefore, ongoing studies will be necessary to identify the characteristics of neoantigens in disease-relevant tissue, to elucidate the mechanisms of their development, and to assess their recognition by immune cells.

ENZYMATIC POSTTRANSLATIONAL PROCESSES

Enzymatic posttranslational processes that have been implicated in neopeptide generation include acetylation (10), citrullination (11), glycosylation (12), hydroxylation (13), methylation (either protein or DNA methylation) (14), phosphorylation (15), and transglutamination (16). Among these, citrullination and transglutamination are most clearly implicated as processes that generate neoantigens in human disease, but evidence suggests that others also play a role in neopeptide formation (Table 1).

Citrullinated Autoantigens

Citrulline, which is among the most studied PTMs in the context of autoimmunity, is a diagnostic biomarker of rheumatoid arthritis (RA). Citrulline modification results

from the deimination of arginine residues by peptidylarginine deiminase (PAD). The family of PAD enzymes is upregulated during inflammation and activated by influx of Ca^{2+} ions (17). Anticitrulline antibodies are among the earliest immune responses that are diagnostic of RA and often correlate with disease severity (18). We have recently documented the biological consequences of citrulline modifications and autoimmunity that arise from pancreatic β -cell proteins in the development of T1D (19). In particular, citrullinated GAD65 and glucose-regulated protein (GRP78) elicit antibody and T-cell responses in human T1D and in NOD diabetes, respectively (20,21). The latter study also observed a dramatic upregulation of PAD2 in the pancreatic islets of NOD mice.

Deamidated Self-epitopes

We and others have demonstrated a role for protein deamidation by tissue transglutaminase (TTG) in eliciting autoimmune recognition of β -cell proteins. Specifically, deamidated insulin, GAD65, IA-2, ZnT8, and phogrin peptides are more efficiently presented on the surface of antigen-presenting cells (APCs) by disease-susceptible HLA-DQ proteins (22). Deamidated β -cell epitopes derived from these proteins are targeted by autoreactive T cells in human subjects with T1D (23,24). Furthermore, TTG activity is amplified by ROS or cytokine stress imparted on the endoplasmic reticulum (ER) of β -cells, suggesting that formation of deamidated epitopes can occur within disease-relevant tissues (25).

NONENZYMATIC POSTTRANSLATIONAL PROCESSES

Several nonenzymatic processes have also been implicated in neopeptide generation, including oxidation (26), carbonylation (27), isoaspartic acid (isoAsp) formation (28), and carbamylation (29). Among these, oxidation and carbonylation are implicated as processes that generate neoantigens in T1D (Table 1). The first published neopeptide derived from a β -cell antigen was an oxidized proinsulin peptide (26). Protein carbonylation represents another major PTM product of ROS and/or inflammatory cytokine stress of β -cells. Increased levels of nonenzymatic PTMs, such as carbonylation, arise from defects in cellular antioxidant defenses by the increase of ROS synthesis or by the failure of catalases, peroxidases, or superoxide dismutase to remove or repair oxidized self-proteins (30).

Carbonyl Modifications

Carbonylation is an irreversible, iron-catalyzed oxidative modification of the side chains of lysine, arginine, threonine, or proline. Mitochondrial functions are particularly sensitive to carbonyl modification, which also has detrimental effects on other intracellular enzymatic pathways (30). A number of diseases have been linked with altered carbonylation of self-proteins, including Alzheimer and Parkinson diseases and cancer (27). There is some data to support that carbonyl PTM is a mechanism that directs unstable self-proteins into cellular degradation pathways. It is hypothesized

Table 1—Neopeptide mechanisms implicated in T1D

Modification	Mediator	Relevant antigens	References
Citrullination	PAD enzymes	GRP78 GAD65 IAPP	(20,21)
Deamidation	TTG2 enzyme Spontaneous	Proinsulin IA-2 ZnT8	(22,24)
Oxidation	ROS	Insulin	(26,46)
Carbonylation	ROS	Serca2a P4Hb	(27)
Peptide fusion	Unknown	Insulin + free peptides	(34)
Alternative splicing	Cytokines Tissue-specific effects	IGRP	(41)
DRiP	Unknown	Insulin	(23)

that carbonyl PTM self-proteins that fail to be properly degraded in pancreatic β -cells are autoantigens that are targeted in T1D. Recently submitted studies have identified several carbonylated pancreatic β -cell neoantigens in human and murine models of T1D (27). Among these neoantigens are chaperone proteins that are required for the appropriate folding and secretion of insulin. These studies imply that although some PTM self-proteins may be direct targets of autoimmunity, others may alter, interrupt, or disturb downstream metabolic pathways in the β -cell. In particular, these studies indicated that upstream PTMs resulted in misfolding and/or metabolic disruption between proinsulin and insulin production, which provides one explanation for recent observations of increased proinsulin-to-insulin ratios in the progression of T1D (31).

IsoAsp Modifications

Another nonenzymatic, spontaneous PTM that has gained attention in the context of autoimmune diseases is the conversion of aspartic acid residues to isoAsp. IsoAsp PTM confers immunogenicity to self-peptides and proteins that normally do not elicit an immune response in their unmodified aspartic acid form (28). IsoAsp modification occurs most often through the spontaneous deamidation of Asn-Gly/Ser linkages or by the isomerization of Asp-Gly/Ser linkages. A great number of both intracellular and extracellular proteins are found with isoAsp PTMs. Notably, isoAsp formation is amplified in stressed or aged cells and often alters or destroys normal protein function (32). Inflammatory diseases processes, such as systemic lupus erythematosus (SLE), have several known isoaspartyl PTM autoantigens, including histones and snRNPs (33). Inflamed pancreatic islets have vastly elevated levels of intracellular isoAsp PTMs (M.-L. Yang and M.J.M., unpublished data).

Hybrid Peptide Autoantigens

Peptide fusion is an additional posttranslational process that has been shown to generate neoepitopes through the combination of peptide fragments within β -cell secretory granules (34). Although the mechanisms that lead to the generation of such hybrid peptide fusion products remain unclear, the existence of hybrid insulin peptides has been verified through mass spectrometry. Furthermore, specific hybrid insulin sequences were demonstrated to be potent agonists for pathogenic T-cell clones isolated from the pancreatic infiltrates of NOD mice and were also recognized by CD4⁺ T cells isolated from the islets of individuals with T1D (5). Ongoing work seeks to clarify the role of responses to hybrid insulin peptides in human disease.

ADDITIONAL PROCESSES

Recent publications raise the possibility that neoepitope generation might also occur through processes that include epigenetics, RNA splicing, and protein translation. Current evidence that these mechanisms represent important drivers for neoepitope formation in T1D and other autoimmune diseases remains limited. However, we mention these areas

as frontiers for ongoing research. Establishing the importance of immune responses to epitopes formed through these mechanisms has the potential to provide new options for developing biomarkers of disease that reflect distinct forms of tissue pathology.

DNA and Protein Methylation in T1D Autoimmunity

Epigenetic changes in the transcription and translation of genes independent of DNA sequence occur as a consequence of specific DNA and protein modifications (35). Such epigenetic changes are mediated through small noncoding RNA transcripts by established pathways of DNA methylation and through histone acetylation/methylation leading to nucleosome remodeling. DNA and protein methylation are catalyzed by specific methyltransferases (DNA methyltransferases [DNMTs] and protein arginine methyltransferases, respectively), whose activity is significantly altered by inflammatory cytokines and tissue ROS. For example, the oxidation of DNA sequences is a prerequisite for methylation by ten-eleven translocases. Significant hypomethylation of DNA has been linked with several classic autoimmune diseases, such as SLE, multiple sclerosis, RA, Addison disease, Graves disease, and mixed connective tissue disease (36). Therefore, there is rationale to consider the possible influence of epigenetic changes on protein expression and immune recognition in T1D. Relevant to T1D, epigenetic modifications occur in pancreatic β -cells during progression of diabetes in NOD mice (37). In particular, cytokine inflammation (IL-1 β , IL-6, and IFN γ) of the islets triggered increased DNMT3a-mediated methylation of exons in *Ins1* and *Ins2* genes. Consequently, DNMTs and protein arginine methyltransferases are likely to play a role in the regulation of β -cell differentiation and insulin gene expression, both of which are pathways that are altered in the presence of inflammatory cytokines.

Alternative Splicing Products

Eizirik et al. (38) reported that exposure of human islets to proinflammatory cytokines leads to modulation of transcript levels and increases in alternative splicing for a number of putative candidate genes for T1D. Their findings suggest a mechanism through which alternative splicing may lead to the generation of neoantigens and subsequent presentation of novel β -cell epitopes (39). As a specific example of this mechanism, isoforms of IGRP-lacking exons 2, 3, and 4 are present in the pancreas but are rarely detected in the thymus or spleen (40). These novel protein junctions were shown to be recognized by immune cells, illustrating that alternative splice variants can exhibit altered immunogenicity (41). In another important study, it was shown that an INS-IGF2 transcript (which contains the preproinsulin signal peptide, the B-chain, and eight amino acids of the C-peptide) is formed through alternative splicing, expressed in human islets, and recognized by antibodies from the sera of T1D patients, many of which cross-reacted with insulin (42). Given its sequence and apparent coregulation with insulin, this protein is thought to engender immune reactivity that targets the insulin signal peptide and C-peptide. Given these

two examples, it seems plausible that tissue-specific splice variation could also give rise to unique antigenic determinants within other β -cell proteins.

Defective Ribosomal Initiation Products

Translation of defective ribosomal initiation products (DRiPs) was recently reported as a possible source of neopeptides in β -cells (23). Unconventional translational products originate from initiation complexes that assemble at non-AUG codons, resulting in polypeptides that represent alternative cryptic reading frames (43). Such DRiPs are rapidly degraded and presented by HLA molecules on the cell surface, eliciting potent cytotoxic responses, which can aid clearance of intracellular pathogens and tumor surveillance. In a similar fashion, aberrantly transcribed proteins from an out-of-frame alternative start codon within the proinsulin gene generate unique peptides that were shown to activate CD8⁺ T cells that had potent cytotoxic activity (23).

ANTIBODY RECOGNITION OF NEOPEPTIDES

The phenomenon of neopeptide recognition by autoantibodies has been shown to be relevant in a variety of autoimmune diseases. For example, in RA, antibody responses directed against various citrullinated synovial proteins are remarkably disease-specific and routinely used as a diagnostic test in the clinic (18). Appearance of the first anticitrullinated protein antibodies occurs years prior to disease onset, and accumulation of additional autoantibody specificities correlates closely with the imminent onset of clinical arthritis (44). There is analogous evidence supporting a hierarchical emergence of autoantibody specificities and multiple waves of autoimmune damage in T1D (3,45). Substantial data from longitudinal studies indicate that insulin and GAD65 autoantibodies appear at the earliest time points during progression, followed by additional antibody specificities directed at IA-2 and ZnT8. Emerging data also demonstrate the presence of autoantibodies that recognize modified forms of established β -cell antigens and additional self-proteins. For example, a recent study demonstrated that oxidative-PTM insulin autoantibodies are present in subjects at risk for T1D development, suggesting a possible early role for this PTM (46). This initial observation holds promise for the use of oxidative-PTM insulin autoantibodies as a predictive biomarker of disease progression. Recently, we showed that serum reactivity is present in T1D patients toward determinants within the extracellular domain (IA-2ec-PTM), including deamidated IA-2 epitopes. IA-2ec-PTM autoantibodies can be detected in a higher percentage of T1D patients as compared with serum reactivity against the native IA-2ec (24) (M.P., personal communication). However, these observations were in the context of established disease, suggesting an intermediate or late role for this PTM.

Multiple autoimmune diseases often cluster within families (or even within one person), implying shared etiology. Consequently, relevant insights can be gleaned from studies of more traditional autoantibody-mediated systemic autoimmune diseases, such as SLE and RA, where inter- and

intramolecular epitope spreading are clearly paradigms for disease progression (47). In general, early autoimmunity is marked by restricted B- and T-cell epitopes, followed by an expanded repertoire coinciding with the onset of more significant tissue pathology, as demonstrated through the pioneering murine studies of Sercarz and colleagues (48,49) and later validated in human disease. Notably, cryptic self-peptides that trigger autoimmunity are clearly generated by what we now have characterized as PTMs of intact self-proteins. Akin to T1D, other autoimmune syndromes tend to cluster to subcellular tissues or tissue components that share biological or biochemical properties. For example, SLE is marked by autoimmunity to nucleic acid-bearing macromolecules (snRNPs, histones, chromatin, dsDNA, Ro/SSA, La/SSB). Unlike tissue-specific autoimmunity in T1D, the origins of autoimmunity in SLE are more mysterious in that the autoantigens reside in every somatic cell. Although early B- and T-cell responses exhibit limited clonality, they evolve to include multiple determinants within the self-protein and to other related proteins in the tissue microenvironment, features that are only recently being appreciated in human T1D (50,51). Unlike other systemic autoantibody-mediated diseases, such as RA and SLE, there is no clear evidence that T1D-related autoantibodies play a pathogenic role. Autoantibodies against citrulline-containing neopeptides of proteoglycan are thought to trigger or intensify arthritis by forming immune complexes with this autoantigen in the joints of RA patients with anticitrullinated protein antibodies. In a similar manner, autoantibodies and immune complexes are hallmarks of tissue pathology in SLE. Therefore, it remains likely that autoantibodies or the B cells that produce them contribute to the pathogenesis of T1D.

PTMs in Antigen Processing and Presentation

PTMs of self-protein may also indirectly control autoantibody and T-cell autoimmunity via altered negative selection of the immune repertoire. It is clear that the accurate processing of self-proteins is the major factor for peptides that control negative selection. The specificity of intracellular processing enzymes, including cathepsins, is altered by the presence of PTM residues at or near cleavage motifs. These PTMs alter both the lengths and sequences of self-peptides generated, as well as the rate and concentrations in which they are made. Thus, PTMs not present at the time of T cell-negative selection may allow the escape of an autoimmune repertoire that will respond to neoantigens and/or cryptic peptides now present by the processing of PTM self-proteins. Many proteases do not recognize the peptide linkage between isoAsp residues and adjacent carboxyl side amino acids (52). Likewise, the spontaneous deamidation of asparagine residues prevents proteolytic processing by asparagine endopeptidase (53). Simply put, recognition and proteolytic cleavage of PTM self-proteins can generate a new repertoire of peptides during antigen presentation and the induction of immune tolerance. As noted earlier, the presence of specific PTMs within a processed peptide also may change its binding affinity to HLA.

Impact of APC Type on Antigen Processing and Presentation

Generation and presentation of self-peptides can be strongly influenced by the specific APC subsets in which self-protein is processed. For example, autophagy was required in B cells for the presentation of a citrullinated peptide (54). Our laboratory and others have demonstrated the unique APC functions of B cells in presenting antigen to T cells, including transfer of processed antigens by B cells to other APCs, including macrophages and dendritic cells. Thus, different APC subsets themselves may control the determinants generated and eventually presented by the immune system (55).

T-CELL RECOGNITION OF NEOEPITOPES

As we have already discussed, multiple posttranscriptional and posttranslational processes can alter the primary sequence of self-proteins in ways that can promote immune recognition. Alteration of discrete amino acids has the potential to increase the affinity of HLA/peptide interactions by placing a favorable modified residue within a binding pocket or to modulate HLA/peptide–T-cell receptor interactions by positioning an altered residue in a T-cell contact position. Indeed, the influence of specific PTMs on the binding of antigenic peptides to autoimmune-associated HLA proteins is well documented. Conversion of glutamine by TIG2 into glutamate greatly enhances peptide binding to HLA-DQ8 and HLA-DQ2 (16). Likewise, conversion of arginine to citrulline by PAD enzymes enhances peptide binding to HLA-DR “shared epitope” alleles, such as HLA-DR0401.

Disease-Associated T-Cell Responses to β -Cell Neopeptides

Early evidence that T cells specifically target PTM neopeptides in T1D was described by Mannering et al. (26) in defining T cells specific to a vicinal disulfide bond region within the oxidized insulin A chain. Subsequent reports have described the enhanced recognition of neopeptides by CD4⁺ T cells from patients with T1D (20,22). Likewise, recent published work demonstrated that deamidated tyrosine phosphatase-related IA-2 peptides are naturally processed and presented on dendritic cells (22) and are recognized by T cells in the context of HLA-DQ8 (24). In these studies, T cells specific for neopeptides were more frequent in patients with established T1D than in control subjects and exhibited a disease-associated Th1-like phenotype. Furthermore, for neopeptides generated through PTMs, T cells generally discriminated between the native peptide and its modified counterpart. In summation, the existing literature demonstrates that oxidation, citrullination, and deamidation can have a direct impact on T-cell recognition that contributes to loss of tolerance.

BIOCHEMICAL PATHWAYS OF NEOEPITOPE FORMATION

Published findings support a paradigm in which neopeptide formation is potentiated through inflammatory insults (including pathogens such as viruses and bacteria), irritants

(including cigarette smoke), and biochemical stresses (including ER and oxidative stress) (56). These diverse processes initiate a cascade that culminates in autoimmune recognition of neopeptides with the potential to drive additional inflammation, further neopeptide formation, and acceleration of autoimmunity (Fig. 1). As an example, published evidence establishes a clear link between the extrusion of neutrophil extracellular traps, which occurs when neutrophils encounter microorganisms, and citrullination of self-proteins, such as histones in rheumatic disease (57). Immune recognition of these proteins may be further enhanced by their colocalization with extruded DNA, which can act as a potent Toll-like receptor agonist. Likewise, there is an increasingly apparent link between ER stress and neopeptide generation in β -cells. For example, a recent study demonstrated that chemically induced ER stress generates

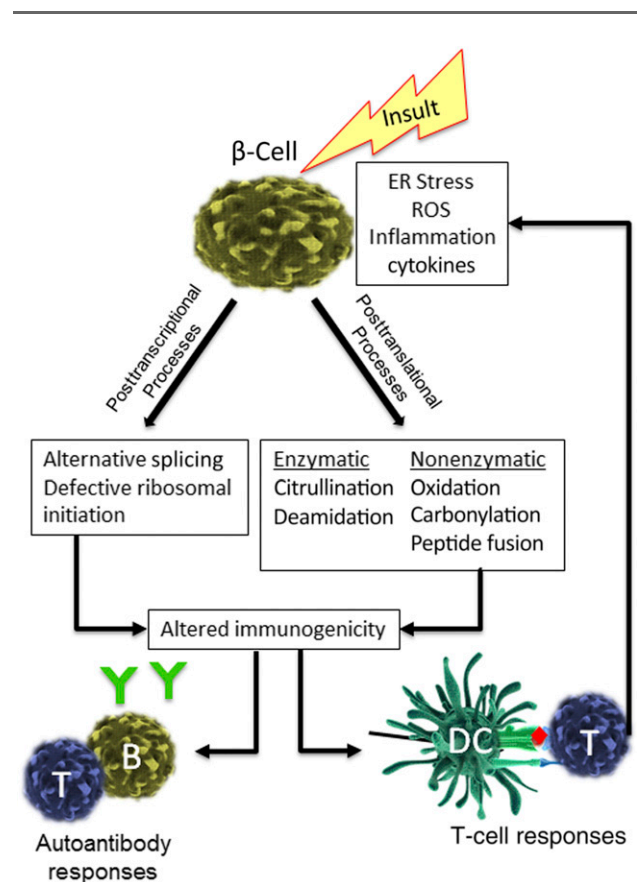


Figure 1—Mechanisms of neopeptide formation. The formation of neopeptides arises as a consequence of various types of insults that generate ER stress, ROS, and/or inflammatory cytokines in the affected tissue. These stresses elicit posttranscriptional (including alternative splicing and DRiP) and posttranslational (both enzymatic and non-enzymatic) processes that lead to the generation and release of neopeptides with altered immunogenicity that can be presented to autoreactive T cells by APCs or recognized by autoreactive B cells, which have escaped negative selection in the thymus and bone marrow. These autoreactive cells become activated and carry out autoimmune effector functions, perhaps leading to additional inflammation, further release of self-epitopes, and an expansion and acceleration of autoimmunity. DC, dendritic cell.

sufficient cytosolic Ca^{2+} to activate TTG2 to modify self-peptides (25). As PAD enzymes are also calcium dependent, it seems probable that protein citrullination would also be upregulated in response to ER stress. Likewise, an inflammatory cytokine milieu (as would be expected to be generated in response to pathogens or early autoreactive responses to a primary autoantigen) would be sufficient to elicit alternative splicing of β -cell proteins (38) and translation of DRiPs (43). These data, then, support the hypothesis that interactions between environmental insults and the unique physiology of β -cells contribute to the generation of modified autoantigens (58), providing a mechanism by which modified self-peptides may arise and provoke autoimmunity.

SINGLE NUCLEOTIDE POLYMORPHISM CAN INFLUENCE PTM

Recent advances in next-generation sequencing technologies and proteomics have yielded a wealth of data for both single nucleotide polymorphisms (SNPs) and PTMs. Although available data are distributed across a number of different databases, researchers can now match genome-wide association study data sets with PTM-SNP databases in an effort to identify PTM-related effects of disease-associated SNPs (59). This approach may provide insights into unveiling new PTMs related to disease and potentially finding novel disease biomarkers.

ENVIRONMENTAL FACTORS AND PTMS

Multiple environmental factors are thought to play a role in the sequence of events leading to T1D. Factors that have been implicated include viral, microbial, diet-related, anthropometric, and psychosocial factors. In particular, viruses have been shown to cause diabetes in animal models either by directly infecting and destroying β -cells or by triggering an autoimmune attack against these cells (60). Likewise, proof-of-concept studies conducted in NOD mice suggest that changes in the composition of intestinal microbiota

prevent or reduce the incidence of disease (61). Some evidence suggests direct links between infectious pathogens and the initiation of protein modification and neopeptide formation. Analogous to host-derived proteolysis and PTMs of self-proteins, microbes are also a source of proteases and modifying enzymes that can contribute to the generation of neopeptides (62). Furthermore, the physiological stress that is induced by environmental factors such as viral infection may enhance neoantigen formation, contributing to the loss of peripheral tolerance and hastening disease onset (63). Ongoing observational cohort studies, such as The Environmental Determinants of Diabetes in the Young (TEDDY) study, aim to identify environmental determinants that may trigger islet autoimmunity and either accelerate or slow progression in subjects with evidence of islet autoimmunity.

A ROLE FOR NEOPEPTIDES IN T1D PATHOGENESIS

There is a general consensus that the pathogenesis of T1D is initiated when individuals who possess a high level of genetic risk (e.g., susceptible HLA, insulin VNTR, PTPN22 genotypes) are exposed to environmental factors (e.g., enteroviruses, diet, microbiome) that precipitate a loss of tolerance that manifests through the appearance of insulin and/or GAD autoantibodies. This early autoimmunity is followed by epitope spreading, increasing both the number of antigenic targets and the diversity of epitopes within these targets. These processes create a feed-forward loop antigen release that induces increasing inflammation and increasing numbers of distinct T-cell specificities (64). The formation and recognition of neopeptides represents one mechanism through which epitope spreading can occur. Indeed, published studies have demonstrated increased immunogenicity of β -cell peptides following oxidative or enzymatic PTM. Nevertheless, in spite of recent data supporting the presence of T cells that recognize various classes of neopeptides, the stage of disease during which such epitopes are recognized remains unclear, mainly due

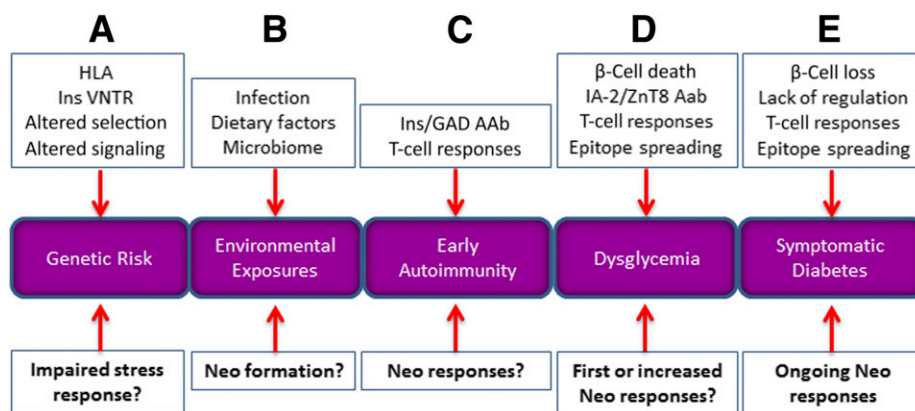


Figure 2—Roles for neopeptides in T1D pathogenesis. Neopeptides can be envisioned to play a role at multiple stages of T1D pathogenesis. *A*: Genetic risk may confer an impaired response, increasing neopeptide formation. *B*: Environmental insults may cause neopeptide formation. *C*: Early autoimmunity may include neopeptide responses in addition to responses to insulin and GAD65. *D*: Progression to dysglycemia is likely to include the emergence of new classes of neopeptide responses. *E*: Neopeptide responses are clearly present as part of ongoing autoimmunity that is present during established T1D. Aab, autoantibody; Ins, insulin; Neo, neopeptide.

to a lack of longitudinal data sets. As depicted in Fig. 2, mechanisms related to neopeptide formation and recognition can be envisioned at multiple stages of T1D pathogenesis. At the level of genetic risk, susceptible individuals may exhibit a genetically driven impairment of their stress response, increasing the likelihood of neopeptide formation. At the level of environmental exposure, many of the insults that are thought to initiate T1D are known to cause neopeptide formation. During the window of β -cell destruction that encompasses early autoimmunity through dysglycemia and diagnosis of T1D it remains unclear when neopeptide responses appear in relation to “classic” responses to insulin and GAD65. However, by the time of onset, neopeptide responses are clearly present and remain as part of the ongoing autoimmunity that is present during established T1D.

CONCLUSIONS

In instances of stress and inflammation, neopeptides can arise and have been linked with autoimmunity of T1D and key examples from other autoimmune syndromes. In particular, inflammatory cytokine stress and/or ROS-mediated stress triggers a number of both direct and indirect biochemical pathways leading to the generation of neoantigenic epitopes. Direct effects include the presentation of novel PTM determinants on HLA class I or II by APCs. PTMs may also directly affect the biochemical processing of self-proteins, ultimately altering both negative selection and the autoimmune B- and T-cell repertoire. PTMs may directly break B- or T-cell tolerance but indirectly initiate mechanisms of epitope spreading to other self-proteins over the progression of disease. For example, B-cell responses that originate to PTM self-proteins often spread in an intra- and intermolecular manner to include determinants associated with the original antigenic stimulus. More indirect effects of modifications include those found to alter DNA methylation or histone acetylation/methylation and chromatin structure, leading to downstream changes in gene expression. As we noted, these indirect effects are most often amplified by inflammation in the pancreatic islets. Other direct effects may include neopeptide generation through novel mechanisms such as DRiP formation and alternative splicing. Other indirect effects include PTMs of β -cell proteins that may lead to downstream alterations in insulin biogenesis. The ultimate product of both direct and indirect generation of neopeptides is an accumulation of robust and diverse autoimmune B- and T-cell responses, accelerating the pathological destruction of pancreatic islets. Clearly, the emergence of sophisticated methods of tissue and single-cell proteomics will identify novel neopeptides, including some that occur at near the earliest stages of disease. A detailed mechanistic understanding of the pathways that lead to specific classes of neopeptides will certainly suggest targets of therapeutic manipulation and intervention that would be hoped to impede the progression of disease.

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References

1. Krischer JP, Liu X, Lemmark Å, et al.; TEDDY Study Group. The influence of type 1 diabetes genetic susceptibility regions, age, sex, and family history on the progression from multiple autoantibodies to type 1 diabetes: a TEDDY study report. *Diabetes* 2017;66:3122–3129
2. Krischer JP, Lynch KF, Lemmark Å, et al.; TEDDY Study Group. Genetic and environmental interactions modify the risk of diabetes-related autoimmunity by 6 years of age: the TEDDY study. *Diabetes Care* 2017;40:1194–1202
3. Yu L, Rewers M, Gianani R, et al. Antiislet autoantibodies usually develop sequentially rather than simultaneously. *J Clin Endocrinol Metab* 1996;81:4264–4267
4. Coppieters KT, Dotta F, Amirian N, et al. Demonstration of islet-autoreactive CD8 T cells in insulinitic lesions from recent onset and long-term type 1 diabetes patients. *J Exp Med* 2012;209:51–60
5. Babon JA, DeNicola ME, Blodgett DM, et al. Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes. *Nat Med* 2016;22:1482–1487
6. Noble JA, Valdes AM, Varney MD, et al.; Type 1 Diabetes Genetics Consortium. HLA class I and genetic susceptibility to type 1 diabetes: results from the Type 1 Diabetes Genetics Consortium. *Diabetes* 2010;59:2972–2979
7. Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW. Autoreactive T cells in healthy individuals. *J Immunol* 2004;172:5967–5972
8. Pietropaolo M, Towns R, Eisenbarth GS. Humoral autoimmunity in type 1 diabetes: prediction, significance, and detection of distinct disease subtypes. *Cold Spring Harb Perspect Med* 2012;2:2
9. Doyle HA, Mamula MJ. Autoantigenesis: the evolution of protein modifications in autoimmune disease. *Curr Opin Immunol* 2012;24:112–118
10. Juarez M, Bang H, Hammar F, et al. Identification of novel acetylated vimentin antibodies in patients with early inflammatory arthritis. *Ann Rheum Dis* 2016;75:1099–1107
11. Kinloch A, Tatzler V, Wait R, et al. Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis. *Arthritis Res Ther* 2005;7:R1421–R1429
12. Dzhambazov B, Holmdahl M, Yamada H, et al. The major T cell epitope on type II collagen is glycosylated in normal cartilage but modified by arthritis in both rats and humans. *Eur J Immunol* 2005;35:357–366
13. Batsalova T, Lindh I, Bäcklund J, Dzhambazov B, Holmdahl R. Comparative analysis of collagen type II-specific immune responses during development of collagen-induced arthritis in two B10 mouse strains. *Arthritis Res Ther* 2012;14:R237
14. Brahm H, Raymackers J, Union A, de Keyser F, Meheus L, Lührmann R. The C-terminal RG dipeptide repeats of the spliceosomal Sm proteins D1 and D3 contain symmetrical dimethylarginines, which form a major B-cell epitope for anti-Sm autoantibodies. *J Biol Chem* 2000;275:17122–17129
15. van Stipdonk MJ, Willems AA, Amor S, et al. T cells discriminate between differentially phosphorylated forms of alphaB-crystallin, a major central nervous system myelin antigen. *Int Immunol* 1998;10:943–950
16. van de Wal Y, Kooy Y, van Veelen P, et al. Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. *J Immunol* 1998;161:1585–1588
17. Machold KP, Stamm TA, Nell VP, et al. Very recent onset rheumatoid arthritis: clinical and serological patient characteristics associated with radiographic progression over the first years of disease. *Rheumatology (Oxford)* 2007;46:342–349
18. Klareskog L, Rönnelid J, Lundberg K, Padyukov L, Alfredsson L. Immunity to citrullinated proteins in rheumatoid arthritis. *Annu Rev Immunol* 2008;26:651–675
19. Nguyen H, James EA. Immune recognition of citrullinated epitopes. *Immunology* 2016;149:131–138

20. McGinty JW, Chow IT, Greenbaum C, Odegard J, Kwok WW, James EA. Recognition of posttranslationally modified GAD65 epitopes in subjects with type 1 diabetes. *Diabetes* 2014;63:3033–3040
21. Rondas D, Crèvecoeur I, D'Hertog W, et al. Citrullinated glucose-regulated protein 78 is an autoantigen in type 1 diabetes. *Diabetes* 2015;64:573–586
22. van Lummel M, Duinkerken G, van Veelen PA, et al. Posttranslational modification of HLA-DQ binding islet autoantigens in type 1 diabetes. *Diabetes* 2014;63:237–247
23. Kracht MJ, van Lummel M, Nikolic T, et al. Autoimmunity against a defective ribosomal insulin gene product in type 1 diabetes. *Nat Med* 2017;23:501–507
24. Acevedo-Calado M, James EA, Morran MP, et al. Identification of unique antigenic determinants in the amino terminus of IA-2 (ICA512) in childhood and adult autoimmune diabetes: new biomarker development. *Diabetes Care* 2017;40:561–568
25. Marré ML, Profozich JL, Coneybeer JT, et al. Inherent ER stress in pancreatic islet β cells causes self-recognition by autoreactive T cells in type 1 diabetes. *J Autoimmun* 2016;72:33–46
26. Mannering SI, Harrison LC, Williamson NA, et al. The insulin A-chain epitope recognized by human T cells is posttranslationally modified. *J Exp Med* 2005;202:1191–1197
27. Yang ML, Doyle HA, Clarke SG, Herold KC, Mamula MJ. Oxidative modifications in tissue pathology and autoimmune disease. *Antioxid Redox Signal*. 11 December 2017 [Epub ahead of print]. DOI: <http://doi.org/10.1089/ars.2017.7382>
28. Mamula MJ, Gee RJ, Elliott JL, et al. Isoaspartyl post-translational modification triggers autoimmune responses to self-proteins. *J Biol Chem* 1999;274:22321–22327
29. Yu HC, Lai PH, Lai NS, Huang HB, Koo M, Lu MC. Increased serum levels of anti-carbamylated 78-kDa glucose-regulated protein antibody in patients with rheumatoid arthritis. *Int J Mol Sci* 2016;17:17
30. Nyström T. Role of oxidative carbonylation in protein quality control and senescence. *EMBO J* 2005;24:1311–1317
31. Rodriguez-Calvo T, Zapardiel-Gonzalo J, Amirian N, et al. Increase in pancreatic proinsulin and preservation of β -cell mass in autoantibody-positive donors prior to type 1 diabetes onset. *Diabetes* 2017;66:1334–1345
32. Teshima G, Porter J, Yim K, Ling V, Guzzetta A. Deamidation of soluble CD4 as asparagine-52 results in reduced binding capacity for the HIV-1 envelope glycoprotein gp120. *Biochemistry* 1991;30:3916–3922
33. Doyle HA, Aswad DW, Mamula MJ. Autoimmunity to isomerized histone H2B in systemic lupus erythematosus. *Autoimmunity* 2013;46:6–13
34. Delong T, Wiles TA, Baker RL, et al. Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. *Science* 2016;351:711–714
35. Lu Q. The critical importance of epigenetics in autoimmunity. *J Autoimmun* 2013;41:1–5
36. Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. *Nat Rev Genet* 2012;13:679–692
37. Rui J, Deng S, Lebastchi J, Clark PL, Usmani-Brown S, Herold KC. Methylation of insulin DNA in response to proinflammatory cytokines during the progression of autoimmune diabetes in NOD mice. *Diabetologia* 2016;59:1021–1029
38. Eizirik DL, Sammeth M, Bouckennooghe T, et al. The human pancreatic islet transcriptome: expression of candidate genes for type 1 diabetes and the impact of pro-inflammatory cytokines. *PLoS Genet* 2012;8:e1002552
39. Juan-Mateu J, Villate O, Eizirik DL. Alternative splicing: the new frontier in diabetes research. *Eur J Endocrinol* 2016;174:R225–R238
40. Dogra RS, Vaidyanathan P, Prabakar KR, Marshall KE, Hutton JC, Pugliese A. Alternative splicing of G6PC2, the gene coding for the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), results in differential expression in human thymus and spleen compared with pancreas. *Diabetologia* 2006;49:953–957
41. de Jong VM, Abreu JR, Verrijn Stuart AA, et al. Alternative splicing and differential expression of the islet autoantigen IGRP between pancreas and thymus contributes to immunogenicity of pancreatic islets but not diabetogenicity in humans. *Diabetologia* 2013;56:2651–2658
42. Kanatsuna N, Taneera J, Vaziri-Sani F, et al. Autoimmunity against INS-IGF2 protein expressed in human pancreatic islets. *J Biol Chem* 2013;288:29013–29023
43. Starck SR, Ow Y, Jiang V, et al. A distinct translation initiation mechanism generates cryptic peptides for immune surveillance. *PLoS One* 2008;3:e3460
44. Sokolove J, Bromberg R, Deane KD, et al. Autoantibody epitope spreading in the pre-clinical phase predicts progression to rheumatoid arthritis. *PLoS One* 2012;7:e35296
45. Orban T, Sosenko JM, Cuthbertson D, et al.; Diabetes Prevention Trial-Type 1 Study Group. Pancreatic islet autoantibodies as predictors of type 1 diabetes in the Diabetes Prevention Trial-Type 1. *Diabetes Care* 2009;32:2269–2274
46. Strollo R, Vinci C, Napoli N, Pozzilli P, Ludvigsson J, Nissim A. Antibodies to post-translationally modified insulin as a novel biomarker for prediction of type 1 diabetes in children. *Diabetologia* 2017;60:1467–1474
47. Shlomchik MJ, Craft JE, Mamula MJ. From T to B and back again: positive feedback in systemic autoimmune disease. *Nat Rev Immunol* 2001;1:147–153
48. Kaufman DL, Clare-Salzler M, Tian J, et al. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 1993;366:69–72
49. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 1992;358:155–157
50. Seay HR, Yusko E, Rothweiler SJ, et al. Tissue distribution and clonal diversity of the T and B cell repertoire in type 1 diabetes. *JCI Insight* 2016;1:e88242
51. Hinman RM, Cambier JC. Role of B lymphocytes in the pathogenesis of type 1 diabetes. *Curr Diab Rep* 2014;14:543
52. Johnson BA, Aswad DW. Fragmentation of isoaspartyl peptides and proteins by carboxypeptidase Y: release of isoaspartyl dipeptides as a result of internal and external cleavage. *Biochemistry* 1990;29:4373–4380
53. Moss CX, Matthews SP, Lamont DJ, Watts C. Asparagine deamidation perturbs antigen presentation on class II major histocompatibility complex molecules. *J Biol Chem* 2005;280:18498–18503
54. Ireland JM, Unanue ER. Autophagy in antigen-presenting cells results in presentation of citrullinated peptides to CD4 T cells. *J Exp Med* 2011;208:2625–2632
55. Harvey BP, Quan TE, Rudenga BJ, Roman RM, Craft J, Mamula MJ. Editing antigen presentation: antigen transfer between human B lymphocytes and macrophages mediated by class A scavenger receptors. *J Immunol* 2008;181:4043–4051
56. Catrina AI, Ytterberg AJ, Reynisdottir G, Malmström V, Klareskog L. Lungs, joints and immunity against citrullinated proteins in rheumatoid arthritis. *Nat Rev Rheumatol* 2014;10:645–653
57. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* 2013;5:178ra40
58. Marré ML, James EA, Piganelli JD. β cell ER stress and the implications for immunogenicity in type 1 diabetes. *Front Cell Dev Biol* 2015;3:67
59. Kim Y, Kang C, Min B, Yi GS. Detection and analysis of disease-associated single nucleotide polymorphism influencing post-translational modification. *BMC Med Genomics* 2015;8(Suppl. 2):S7
60. Zipris D, Lien E, Nair A, et al. TLR9-signaling pathways are involved in Kilham rat virus-induced autoimmune diabetes in the biobreeding diabetes-resistant rat. *J Immunol* 2007;178:693–701
61. Hansen AK, Hansen CH, Krych L, Nielsen DS. Impact of the gut microbiota on rodent models of human disease. *World J Gastroenterol* 2014;20:17727–17736
62. Opendakker G, Proost P, Van Damme J. Microbiomic and posttranslational modifications as preludes to autoimmune diseases. *Trends Mol Med* 2016;22:746–757
63. Marré ML, Piganelli JD. Environmental factors contribute to β cell endoplasmic reticulum stress and neo-antigen formation in type 1 diabetes. *Front Endocrinol (Lausanne)* 2017;8:262
64. von Herrath M, Sanda S, Herold K. Type 1 diabetes as a relapsing-remitting disease? *Nat Rev Immunol* 2007;7:988–994