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Review

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Type 1 diabetes (T1D) is a chronic autoimmune disease that causes severe loss of pancreatic β cells. Autoreactive T cells are key mediators of β cell destruction. Studies of organ donors with T1D that have examined T cells in pancreas, the diabetogenic insulinitis lesion, and lymphoid tissues have revealed a broad repertoire of target antigens and T cell receptor (TCR) usage, with initial evidence of public TCR sequences that are shared by individuals with T1D. Neopeptides derived from post-translational modifications of native antigens are emerging as novel targets that are more likely to evade self-tolerance. Further studies will determine whether T cell responses to neopeptides are major disease drivers that could impact prediction, prevention, and therapy. This Review provides an overview of recent progress in our knowledge of autoreactive T cells that has emerged from experimental and clinical research as well as pathology investigations.

Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease resulting in severe loss of pancreatic β cells (1) due to the targeting of islet cell autoantigens. Autoantibody and T cell responses to autoantigens are detected in at-risk individuals during the asymptomatic period preceding T1D diagnosis and at clinical onset. Autoantibodies are robust predictive and diagnostic biomarkers (2); autoreactive T cells are considered the main effectors of β cell destruction. Accordingly, 50%–60% of the genetic risk for T1D derives from HLA alleles encoding molecules involved in the presentation of antigen peptides to T cells (3). An individual's HLA variants influence peptide binding and signal transduction after T cell receptor (TCR) engagement. These influences on antigen presentation are key during thymic selection processes and peripheral activation of the immune response.

Strong predisposition for T1D derives from selected HLA class II (HLA-II) haplotypes, especially *HLA-DRB1*04* (DR4), *DQA1*03:01-DQB1*03:02* (DQ8), *HLA-DRB1*03:01* (DR3), and *DQA1*05:01-DQB1*02:01* (DQ2). Approximately 80%–90% of patients carry at least one high-risk haplotype, and 30%–50% have both (4). The heterozygous genotype confers the strongest predisposition due to the formation of a *trans*-complementing HLA-DQ heterodimer, consisting of the DQ2 α -chain (*DQA1*05:01*) and the DQ8 β -chain (*DQB1*03:02*), which effectively presents autoantigenic epitopes to T cells (5). The HLA-DRB1 chain is also involved in the presentation of diabetogenic epitopes, but *DRB1*04* variants differ in their disease association even when in *cis* with the high-risk *DQA1*03:01-DQB1*03:02* (4). Selected HLA-I variants such as HLA-A2, HLA-A24, HLA-B39, HLA-B57, and HLA-B18 contribute to T1D risk (3, 6). HLA-A2 is also common in the general population, being present in about 50% of individuals of European descent. Most studies of autoreactive CD4⁺ and CD8⁺ T cell responses in T1D have focused on those restricted by these

HLA types (7). The ability to detect and phenotype autoreactive T cells in circulation, where they are present at extremely low frequencies, has greatly improved. For example, HLA-II tetramers or HLA-I multimers/monomers allow measurement of autoreactive T cells in the circulation *ex vivo*, without *in vitro* amplification that might alter phenotypic features (8–10).

This Review integrates experimental, human pathology, and clinical research studies and identifies key outstanding questions related to autoreactive T cells in T1D.

T cells in the T1D pancreas

For decades, pathology studies relied on sporadic access to T1D pancreata (11). Early efforts to recover T1D pancreata include collecting autopsy specimens from recently diagnosed patients in the United Kingdom (12) and limited percutaneous biopsies from living patients in Japan (13). In the last decade, the DiViD study from Norway (14) obtained laparoscopic biopsies from 6 adult patients near diagnosis, and the JDRF Network for Pancreatic Organ Donors with Diabetes (nPOD) in the USA recovers pancreatic and other tissues from T1D donors to support diabetes researchers worldwide and engage investigators in collaborative studies (15). nPOD enables examination of T1D pancreata from donors with a wide range of disease durations (16).

The pathologic hallmark of T1D is insulinitis, an inflammatory lesion of the islet associated with β cell loss (17–19). Inflammatory cells are observed in the islet periphery (peri-insulinitis) or within the islet parenchyma. Peri-insulinitis is the predominant lesion in the human pancreas (16, 20) and is less severe than insulinitis in the NOD mouse model (16, 20, 21). Insulinitis is defined by at least 15 CD45⁺ cells/islet present in three or more islets, with concomitant evidence of insulin-negative islets, dubbed pseudo-atrophic islets (18). Only 10%–30% of islets show insulinitis at any time, even when tissue is obtained at diagnosis, including in nPOD and DiViD specimens (14, 16, 17, 21). George Eisenbarth dubbed the lobular and patchy distribution of insulinitis “vitiligo of the pancreas” in his Banting lecture (22). The lesion typically affects insulin-positive islets, suggesting that T cells leave islets after destroying β cells.

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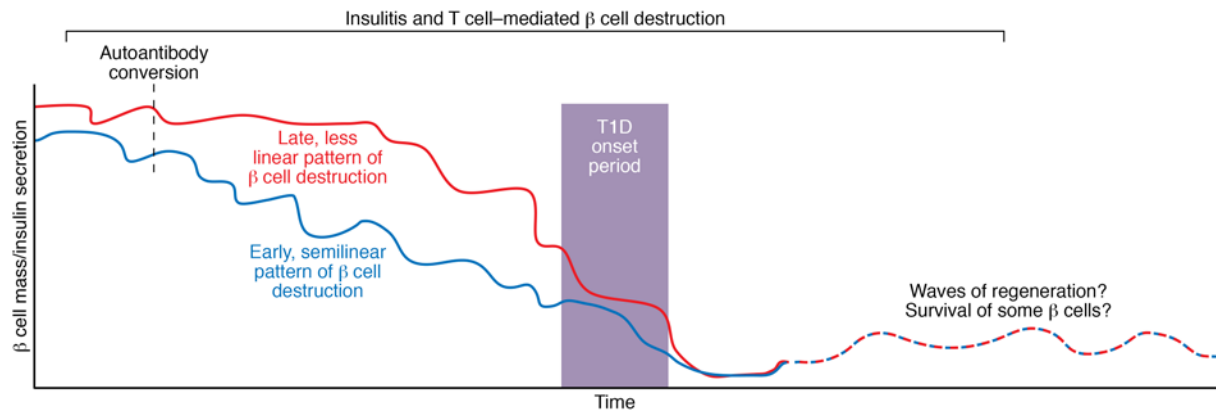


Figure 1. The natural history of islet autoimmunity. To illustrate the chronic nature and heterogeneity of islet autoimmunity and highlight critical gaps in knowledge, this figure depicts two hypothetical patterns of T cell-mediated β cell destruction and loss of insulin secretion: early triggering of insulinitis and semi-linear β cell destruction (blue line); or late start of the autoimmune β cell destruction, closer to clinical diagnosis (red line). This is a simplified schematic representation: loss of β cell mass and insulin secretion may not always correlate and may vary based on the relative importance of autoimmune destruction and β cell dysfunction during disease progression in a given patient. Seroconversion for T1D-associated autoantibodies to native antigens is considered the earliest step in the triggering of islet autoimmunity; it occurs in early life in children at genetic risk of T1D, but it may occur later in life in many individuals. As only a minority of islets are affected at any given time, the process proceeds asynchronously. Residual β cell mass at onset may be a function of the severity of autoimmune destruction and initial mass, which are impacted by age. It is often incomplete at diagnosis, but it continues afterward, sometimes for years. Low levels of insulin secretion are detected in many patients even decades after diagnosis: some β cell regeneration occurs after diagnosis, and it has been hypothesized that regeneration may occur during the preclinical stage. Critical questions remain unanswered: To what extent and when does the detection of humoral and cellular responses in the circulation reflect insulinitis and β cell destruction, and which responses are relevant? At what time are insulinitis and β cell destruction triggered during the progression of islet autoimmunity? Is there a preferred order for autoantibody and T cell responses to native antigens and neopeptides, and what are their relative roles in insulinitis, β cell destruction, and disease onset (trigger/driver responses versus secondary responses)?

Eventually, most islets become pseudo-atrophic remnants of islet autoimmunity. In studies of 80 nPOD T1D donors (16, 23), 17 donors with up to 12 years of disease duration exhibited insulinitis; thus, islet autoimmunity may persist for years after diagnosis. Residual β cells were present in all T1D donors with insulinitis, and their β cell mass was higher than in T1D donors without insulinitis. T1D patients with more than 50 years of diabetes and residual insulin secretion also displayed insulinitis and residual β cells (24).

CD8⁺ T cells are the predominant T cell population and most abundant inflammatory cell type in insulinitis. Using HLA multimers, investigators demonstrated the antigen specificity of autoreactive CD8⁺ T cells in insulinitis lesions of nPOD T1D donors (23). The infiltrating CD8⁺ T cells' antigen repertoire increased in diversity with longer disease duration (23). Thus, antigen/epitope spreading occurs or continues after diagnosis (Figure 1).

Hyperexpression of HLA-I molecules by endocrine cells in insulin-containing islets is another key feature of the T1D pancreas (25). This observation was confirmed with various methodologies in multiple patient cohorts (26) (Figure 2). Hyperexpression of HLA-I molecules helps explain the predominance of CD8⁺ T cells in insulinitis. Earlier studies also reported aberrant expression of HLA-II molecules by β cells in 22 of 26 and 6 of 12 pancreata from patients with recent-onset and long-standing disease, respectively (25, 27, 28). Expression of HLA-I/II molecules may be triggered by viruses linked to T1D (29, 30). Inflammatory cytokines (IFN- γ plus TNF- α or lymphotoxin) induce HLA-I/II molecules on β cells in vitro (31, 32). The significance of HLA-II expression by cells was investigated using transgenic expression of MHC class II (MHC-II) molecules in β cells, which did not drive immune infiltration of mouse islets (33–35). However, these studies were not conducted in diabetes-prone mice, and the role of MHC-II expression in the presence of defec-

tive self-tolerance to β cell antigens and predisposing MHC variants remains controversial. Thus, HLA-II expression by β cells in the T1D pancreas should be reassessed with modern methodology and experimental models given the hypothetical implication that β cells may directly present self-antigens to autoreactive CD4⁺ T cells (Figure 3) (36).

T cells in the prediabetic pancreas

Clinical onset of T1D is preceded by an asymptomatic period lasting months to years during which autoimmunity causes progressive β cell destruction (Figure 1). Autoantibody screening identifies individuals at risk for T1D among patients' relatives; those with a single autoantibody have low risk, while about 40%–80% of relatives with multiple autoantibodies develop T1D within 5–10 years, respectively (2). However, the extent to which humoral and cellular autoimmune responses in blood reflect ongoing pancreas pathology during the prediabetic period are unknown, as is the relationship of autoantibody positivity with insulinitis and the features of islet-infiltrating T cells. Despite progress in identifying autoantibody-positive non-diabetic donors (15, 37–39), recoveries have been too few; we advocate that pancreata from these rare donors should be allocated to research rather than transplant whenever possible (40).

The limited data available from nPOD (16) and European studies (41, 42) suggest that insulinitis is found only in donors with high-risk HLA types and multiple autoantibodies. Higher T1D risk associated with multiple autoantibodies may reflect ongoing insulinitis, and low risk associated with a single autoantibody appears consistent with lack of insulinitis in pancreata of organ donors with a single autoantibody (mostly against GAD65). Since these donors are identified in the general population and many lack T1D-predisposing HLA types (16, 43), only some may be representa-

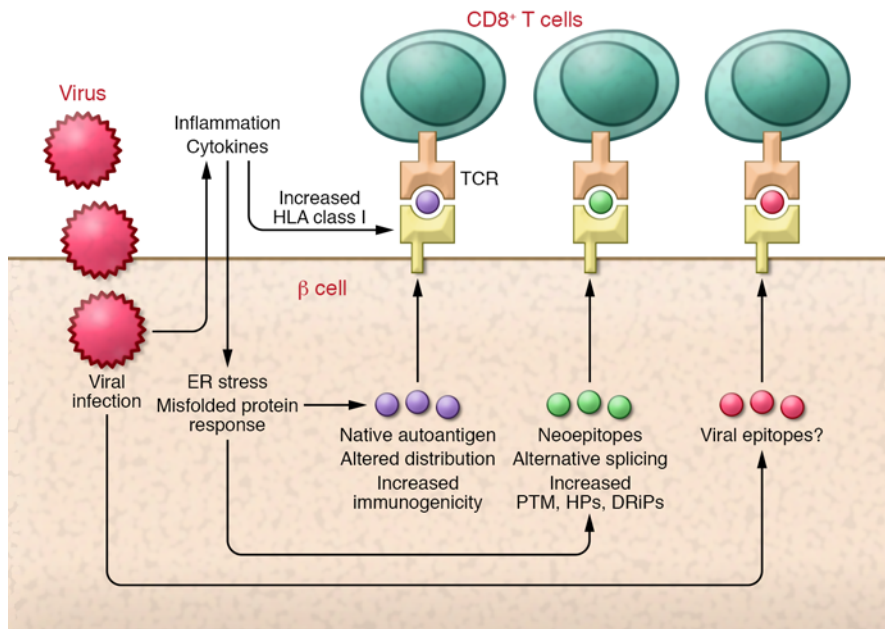


Figure 2. CD8⁺ T cell responses to pancreatic β cells. Schematic representation of major autoantigen classes that may be targeted by CD8⁺ T cells, the predominant immune cell type in the insulinitis. As described in the main text, these may include native antigens and neopeptides. The generation of these epitopes may be promoted by β cell inflammation and stress. Insulinitis is often associated with an interferon response with hyperexpression of HLA class I molecules, which may be induced by viral infections. Hyperexpression of HLA class I molecules facilitates presentation of autoantigen epitopes to CD8⁺ T cells. Whether islet-infiltrating CD8⁺ T cells also target viral epitopes remains to be investigated.

tive of pre-diabetes. However, abnormalities are reported in the pancreas of donors with single autoantibodies (15): accumulation of proinsulin-containing vesicles in β cells observed in nPOD organ donors with single and multiple autoantibodies may reflect impaired proinsulin conversion, disrupted vesicular trafficking, and possibly increased metabolic requirements and stress. Consistent with the increases in size and number of β cells/islet observed in autoantibody-positive donors (44), there is no evidence of reduced β cell mass in independent analyses of autoantibody-positive nPOD donors (44, 45). Based on these findings, Figure 1 illustrates two theoretical patterns of β cell destruction: insulinitis may begin early, with destruction proceeding in a semi-linear fashion; or insulinitis and β cell destruction may occur closer to diagnosis. The latter fits the observations that (i) the proportion of infiltrated islets is modest near diagnosis, and insulinitis is only present in non-diabetic donors with multiple autoantibodies (16, 21, 23); (ii) β cell loss near diagnosis is partial in many patients (16, 21, 23), and no appreciable reduction in β cell mass has been reported in autoantibody-positive donors (44, 45); and (iii) impaired glucose metabolism, abnormalities of insulin secretion (46, 47) and insulin sensitivity (48), and increased β cell death (49) usually manifest shortly before diagnosis in longitudinal studies of at-risk relatives.

Loss of immunological tolerance to β cells

Several islet cell molecules were identified as targets of autoimmunity in T1D, including native proteins and epitopes of proinsulin (9, 50–63), GAD65 (64–72), tyrosine phosphatase-like insulinoma-associated antigen 2 (IA-2) (67, 73–75), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (76–80), the cation

efflux transporter ZNT8 (7, 81–85), chromogranin (86, 87), islet-amyloid polypeptide (IAPP) (8), and more. Many are present in secretory granules, and some are unique to β cells (insulin, IGRP, ZNT8), while some are expressed in other cells and tissues, including neuroendocrine cells (GAD65, IA-2) (88, 89).

Autoimmune responses to islet cell self-molecules evidence a specific loss of immunological self-tolerance. Most self-molecules, including those with tissue-restricted expression, are expressed in the thymus early in life to establish central tolerance (90). Yet several mechanisms may determine suboptimal thymic expression and, in turn, imperfect self-tolerance. For T1D autoantigens, allelic variation and epigenetic regulation affect the selection and levels of self-epitopes presented to developing T cells during thymic selection. A polymorphic variable nucleotide tandem repeat (VNTR) sequence at the 5' of the insulin gene influences thymic expression of insulin, and alleles that confer resistance to T1D are associated with higher insulin thymic transcription than predisposing variants (91, 92). Epigenetic regulation may suppress thymic transcription of a parental copy of the insulin

gene, especially for T1D-protective alleles associated with higher transcription (91–93). These mechanisms may allow autoreactive T cells to escape thymic selection, as reported for insulin in mouse models (94–98) and patients (99, 100).

T1D-predisposing HLA molecules may contribute to suboptimal thymic presentation of autoantigens. Weak interactions between a preproinsulin peptide and HLA-A2 lead to suboptimal presentation to the TCR of responding CD8⁺ T cells (101), which may more easily survive thymic selection. Of note, HLA-A2 is found also in about 50% of the general European-descent population; the often-reported detection of circulating autoreactive T cells in healthy individuals (102) may derive from negative selection processes that are inherently imperfect for islet cell self-molecules. However, autoreactive T cells in T1D patients are reportedly enriched in memory cells versus a naive phenotype in healthy subjects (103, 104). The positive effects of treatment with an anti-memory cell agent in patients with recently diagnosed T1D supports the importance of memory autoreactive T cells in this disease (105, 106). Memory autoreactive T cells were linked to T1D recurrence in transplanted patients, both in islet (107, 108) and pancreas transplantation (109), despite immunosuppression.

Autoreactive T cells exhibit proinflammatory cytokine profiles in T1D patients and regulatory profiles in healthy subjects (85, 110, 111). T1D patients exhibit defects in peripheral tolerance, including impaired Treg function (112, 113) and effector T cell (Teff) resistance to Treg suppression (114). Many T1D risk genes predispose to impaired immune regulation (115); the best studied are *PTPN22* (encoding protein tyrosine phosphatase, non-receptor type 22), *CTLA4* (encoding cytotoxic T lymphocyte-associated protein 4),

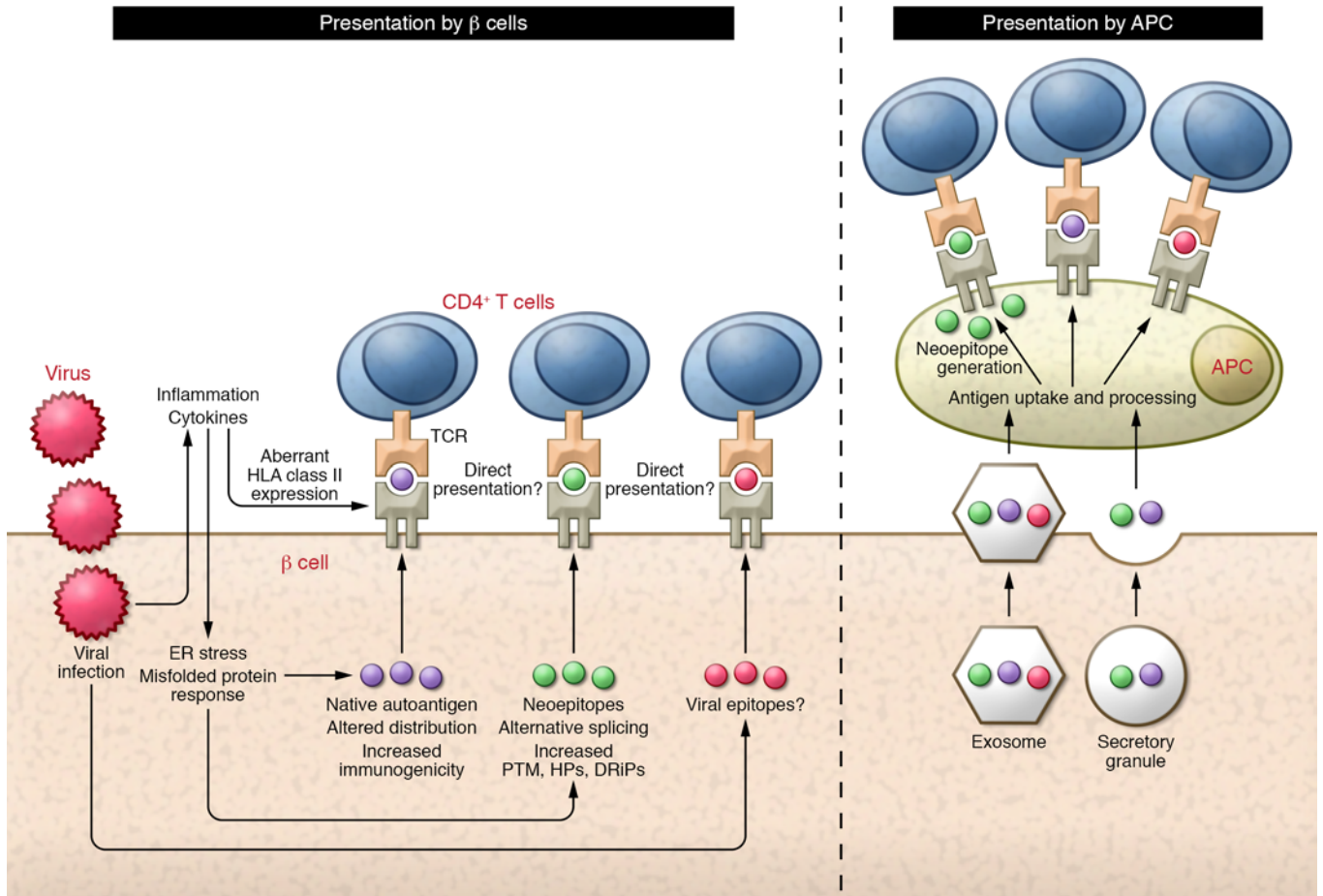


Figure 3. CD4⁺ T cell responses to pancreatic β cells. Schematic representation of major autoantigen classes that may be targeted by CD4⁺ T cells. Similar to CD8⁺ T cells, these may include native antigens and neopeptides, which may be formed under conditions of β cell inflammation and stress. β Cells can produce several neopeptides: for example, peptides originating from alternative splicing, insulin hybrid peptides (HP), and DRiP insulin peptides are reportedly produced in β cells (for DRiPs, the evidence is from studies of cell lines). Many autoantigens are secretory granule proteins, which may be released by β cells and acquired by APCs. Exosomes released by β cells also contain autoantigens. CD4⁺ T cells may react with antigens captured, processed, and presented by APCs in the pancreatic lymph node and in the islets. The B:9–23 epitope is produced in the secretory granules, captured, and presented by APCs, at least in the NOD mouse. The generation of neopeptides in β cells further raises the question of whether the previously reported aberrant expression of HLA class II molecules by β cells might allow CD4⁺ T cells to recognize antigens on their surface directly.

and *IL2RA* (encoding the IL-2 receptor α-chain, IL-2Rα), which control TCR signaling, inhibition of T lymphocyte responses, and Treg development and function (116, 117), respectively.

Environmental exposures may promote loss of tolerance. Along with their epidemiological associations (29, 30), enterovirus infections of β cells impair insulin secretion (118), alter mRNA/miRNA expression (119, 120), and induce interferon responses (121, 122) that promote β cell stress, dysfunction, and apoptosis (123). Enteroviruses may also trigger autoimmunity via presentation of self-molecules in an inflammatory context (bystander activation) (124) and/or by molecular mimicry (125–127). Cross-reactivities with viral proteins were reported for autoantibody (128–134) and T cell responses (73, 132, 135–144) with GAD65, IA-2, and insulin autoantigens. β Cell inflammation and ER stress promote β cell dysfunction and immunogenicity, and protein misfolding is associated with abnormal autoantigen presentation (145–147). Cytokine-induced ER stress enhances β cell release of exosomes loaded with autoantigens and immunostimulatory chaperones, which are taken up by antigen-presenting cells (APCs) and may be presented to T cells (Figure 3)

(148). Dendritic cells' efficiency at cross-presentation is important for self-tolerance (149–151) and viral responses (152) but is also linked to T1D in NOD mice (153–155). In the NOD mouse, self-antigen cross-presentation to CD8⁺ T cells is also mediated by B lymphocytes and promotes disease progression (156).

Modified T cell autoantigens (neopeptides)

Increasing evidence suggests that islet autoimmunity also targets neopeptides expressed by the target cells, which may not be available for negative thymic selection. Differential expression of *IA-2* and *IGRP* mRNAs in thymus and pancreas (157, 158) may promote autoimmunity, as the immune system may not be tolerant to alternatively spliced variants expressed in pancreas but not thymus (159). Many autoantigens in autoimmune disease are post-translationally modified (PTM) (160). Inflammation and stress are likely factors in the generation of PTM antigens, which derive from both normal and abnormal processes in cells. Predisposing HLA types are critical for the presentation of these epitopes to T cells (161, 162). Below, the major neopeptide classes associated with T1D are described.

Neopeptides generated by PTM. A PTM epitope exists in the insulin A chain (A1-A13): T cell recognition requires oxidized cysteine residues at A6 and A7, with the formation of a vicinal disulfide bond between them (60). Other forms of PTM include citrullination (163) and transglutamination, which enhance GAD65 peptide binding to HLA-DRB1*04:01 and modulate recognition by modifying amino acids at TCR contact positions (164). Memory T cells reacting with these PTM epitopes were detected in blood at a higher frequency in T1D patients than controls. The same T cells had weak or no reactivity toward the native peptides. Tissue transglutaminase mediates the deamidation reaction and generates PTM antigens in celiac disease, which shares HLA-II susceptibility with T1D (111). The HLA-DQ *trans*-dimer formed in DQ8/DQ2 heterozygotes exhibits binding preference for negatively charged peptides generated by deamidation. Van Lummel et al. (111) identified CD4⁺ T cells against proinsulin peptides that preferentially bound to the HLA-DQ *trans*-dimer in DQ8/DQ2 heterozygotes after deamidation, and other deamidated peptides that preferentially bound to the HLA-DQ8 *cis*-dimer. While healthy controls and T1D patients displayed similar reactivities, autoreactive T cells from most patients produced proinflammatory IFN- γ in response to cognate antigen stimulation, in contrast to regulatory IL-10 responses in controls.

Neopeptides generated by differences in MHC binding registers. Insulin is considered a key autoantigen in the NOD mouse model of autoimmune diabetes, as genetic manipulation to abolish response to the insulin B chain B:9-23 peptide prevents diabetes (165). Unanue and Eisenbarth/Kappler/Michels defined key molecular interactions of insulin peptides with the NOD mouse I-Ag7 MHC-II molecule, which is remarkably similar to HLA-DQ8 (166) in both sequence and binding features for insulin peptides (167). Unanue's group (161, 162, 168) identified two registers in which peptides encompassing the insulin B:9-23 epitope bind to I-Ag7: register 1 (B:12-20) and register 2 (B:13-21). Type A CD4⁺ T cells recognize insulin presented by APCs, and these T cells are deleted in the thymus through presentation via register 2. Type B insulin-reactive T cells do not react with insulin protein processed by APCs but respond to soluble B chain peptide when weakly bound by I-Ag7 in register 1. Type B CD4⁺ T cells are not deleted in the thymus and become activated by APCs in islets (162). Thus, a single amino acid shift of the B chain peptide bound to I-Ag7 determines whether CD4⁺ T cells recognize peptides generated by insulin processing and allows escape from negative selection. In essence, differences in MHC register usage determines whether a peptide is presented as a "neopeptide," as the same peptide was not presented in this register in the thymus.

Kappler's group described a register 3 in which two peptides containing the critical amino acid residues of insulin B:9-23 (B:9-20 and B:9-21) bind poorly to the I-Ag7 molecule (86, 169). According to these authors, most, if not all, NOD mouse CD4⁺ T cells reacting against B:9-23 target this peptide in the low-affinity register 3. These T cells can be divided into two types based on whether their response is improved or inhibited by glycine substitution at the B21 glutamic acid at the peptide's p8 position. Modifying the wild-type insulin B:9-23 peptide amino acid sequence at positions p8 and/or p9 (p8E/p9R) into p8G/p9E- and p8E/p9E-generated mimotopes that bind to I-Ag7 in register 3 increased the binding affinity with insulin-specific TCRs and favored the activation of

higher-affinity insulin-specific T cells in NOD mice (169). Register 3 presentation may not occur in the thymus, possibly explaining how insulin-specific CD4⁺ T cells escape negative selection. Despite unresolved differences related to registers used in these studies, these observations support the concept that differences in MHC register use are key to disease development and lead to the presentation of native antigens as neopeptides. Similar mechanisms may be operative in patients: HLA-DQ8-restricted CD4⁺ T lymphocytes may target insulin peptides in a low-affinity binding register similar to the NOD register 3 (170, 171). Ultimately, differences in HLA-peptide complex interactions can impact responding T cell activation and phenotypes and synergize with the reduced insulin expression in the thymus that is associated with predisposing insulin gene variants. Moreover, CD4⁺ T cells from T1D patients display abnormal immunological synapsis associated with escape from negative selection and enhanced effector responses upon encounter with cognate antigen (172).

Neopeptides generated by peptide fusion. Neopeptides are also formed by fusion of peptides from two proteins, termed hybrid peptides, that are not genetically encoded and are high-affinity T cell targets (173). Hybrid peptides include those resulting from the fusion of proinsulin C-peptide fragments and other β cell secretory granule proteins, such as chromogranin, IAPP, or neuropeptide Y (NPY). Perhaps the formation of hybrid peptides explains the essential role of insulin and chromogranin in diabetes development in NOD mice (165, 174). Hybrid peptides may result from proteolytic hydrolysis of peptide bonds in the presence of naturally occurring cleavage products. Insulin and other secretory proteins are packaged together in secretory granules, which may favor reversed proteolytic transpeptidation. CD4⁺ T cells that react against hybrid peptides were identified in NOD mice as well as islet-infiltrating cells isolated from deceased T1D patients (173, 175).

Neopeptides generated by aberrant translational products. The generation of defective ribosomal products (DRiPs) from the proinsulin gene is another recently discovered mechanism leading to production of T cell-targeted β cell neopeptides. β Cells generate DRiP neopeptides using an alternative initiation site for translation and by translating the 3' UTR (176). T cell reactivity against proinsulin DRiPs was tested in peripheral blood samples from T1D patients, and proliferative responses were observed in most. Strong T cell responses to DRiPs were detected in individuals with predisposing HLA-DQ types, such as HLA-DQ8/DQ2, who can express HLA-DQ *trans*-dimers (HLA-DQ8trans, DQA1*05:01/DQB1*03:02 or HLA-DQ2trans, DQA1*03:01/DQB1*02:01). T cell responses to DRiPs were not observed in rare patients carrying T1D-protective HLA-DQ or insulin gene haplotypes, consistent with the concept that both genes impact thymic selection of insulin-reactive T cells.

The proinsulin UTR includes two SNPs in tight linkage disequilibrium with insulin VNTR variants associated with T1D risk and the modulation of insulin gene transcription in the thymus (91, 92). The presence of these SNPs in the UTR produces several DRiP variants. T cell responses against DRiPs containing amino acid residues encoded by predisposing or protective haplotypes were strongly correlated, suggesting that the UTR SNPs did not influence immunogenicity and may not be critical for HLA-DQ binding. Dendritic cells processed and presented proinsulin DRiPs,

preferentially via the HLA-DQ8 trans, and patient T cells exclusively responded by producing IFN- γ and granzyme B. HLA-A2 can also present DRiPs, and T1D patients had higher frequencies of DRiP-specific CD8⁺ T cells than healthy subjects. DRiP-specific CD8⁺ T cells killed HLA-A2–positive human islet cells in vitro, and pretreating the islet cells with high glucose and proinflammatory cytokines potentiated the cytotoxic action. Proinsulin DRiPs were formed under thapsigargin-induced experimental ER stress, a form of stress characterized by calcium depletion in the ER and increased cytoplasmic calcium levels, in contrast to classical ER stress caused by accumulation of misfolded proteins. Thus, in the T1D pancreas, ER stress and inflammation impair β cell function by increasing production of autoantigens (147), and DRiPs could trigger and enhance T cell–mediated killing of β cells (176).

Ex vivo studies of T cells in T1D-relevant tissues

Investigations of antigen-specific T cells from T1D-relevant tissues are rare. Earlier studies detected CD4⁺ T cells against insulin and/or GAD65 in the pancreatic lymph nodes (PLNs) or islets obtained from a few deceased T1D patients (57). GAD65-reactive CD4⁺ T cells were detected in the circulation and lymph nodes draining the transplanted pancreas in a T1D recipient who lost graft function due to recurrent autoimmunity (109), and GAD65-reactive CD4⁺ T cells from pancreas transplant recipients killed cells when co-transplanted with human islets in immunodeficient mice (109). Autoimmune β cell damage was reported in humanized mice grafted with immune cells from a T1D patient (177, 178). Proinsulin-specific (61, 63) and IGRP-reactive CD8⁺ T cells (109) are also cytotoxic in vitro. Insulin-reactive CD4⁺ T cells from T1D donor islets targeted a PTM epitope in the insulin A chain (60), which was presented by HLA-DR4 (DRB1*04:01, but could also be presented by *04:04 and *04:05). Of 53 CD4⁺ T cell clones (179), 47 unique clonotypes were identified, including 8 recognizing proinsulin presented by HLA-DQ8; 26% of these clones targeted 6 distinct but overlapping epitopes in the C-peptide of proinsulin. These were presented by the HLA-DQ *trans*-heterodimer that forms in HLA-DQ2/-DQ8 heterozygotes using DQA1*05:01 and DQB1*03:02. More recently, collaborative efforts supported by nPOD have allowed expanded analyses of islet-infiltrating T cells from T1D organ donors. Islets were purified using standard isolation protocols and distributed to several laboratories, which isolated islet-infiltrating T cells and examined antigen specificities, phenotypic features, and TCR sequences.

Babon et al. (175) obtained islet-infiltrating T cells from 9 donors between 6 and 30 years old with 2–20 years since T1D diagnosis. Representing the largest analysis to date of islet-infiltrating T cells from T1D donors, the study explored the largest number of antigen specificities. More than 250 T cell lines or clones were derived. Lines were obtained from all T1D donors, but islet-infiltrating T cells were not recovered from control donors, with one exception. Thus, unlike autoreactive T cells detected in blood, lymphocytic islet infiltration is highly specific for T1D. Of 50 lines examined for autoreactivity so far, 19 reacted against a variety of known autoantigens, including PTM epitopes (180) and hybrid peptides (173). These autoreactive T cells secreted IFN- γ and other inflammatory cytokines, consistent with an effector phenotype. This study's major finding is the broad repertoire and heterogeneity

of the T cell responses, which may reflect differences in HLA restriction, environmental exposures, and disease stage. However, a small fraction of islet-infiltrating T cell lines and clones reacted to known autoantigen epitopes. Given the broad range of disease duration, these findings match pathology studies showing that islet autoimmunity continues for years after diagnosis.

Michels et al. (181) analyzed islet-infiltrating T cells from nPOD donors, focusing on proinsulin-specific CD4⁺ T cells. The study included 3 nPOD T1D donors overlapping with those of Babon et al. (175) and nPOD donor 6323 examined by Seay et al. (182), described below. These donors were 6, 14, and 19 years old, had T1D for 2, 3, and 6 years, respectively, and displayed insulinitis in the pancreatic islets. Sequencing of infiltrating T cells' TCR genes revealed diverse repertoires, but certain TCRs were repeatedly detected, suggesting some level of clonal expansion. Many of these TCR sequences were found in multiple islets. TCRs expressed by islet-infiltrating T cells reacted against the insulin B:9–23 epitope and were restricted by HLA-DQ8. Reactivity to a 19–35 epitope of C-peptide reported in the islets of a previously discussed T1D donor (179) was reproduced. However, the majority of the TCRs examined did not respond to proinsulin/insulin epitopes, consistent with the broad reactivity reported by Babon et al. (175).

Seay et al. (182) studied pancreas, PLN, spleen, irrelevant lymph nodes, and peripheral blood from 18 nPOD donors diagnosed with T1D 4–32 years prior to their death at ages 11–44 years. The researchers conducted high-throughput immunosequencing of the TCR β -chain (TRB) to investigate TCR repertoire diversity; TCR sharing among blood and various tissues in T cell subsets (CD4⁺ conventional T [Tconv] cells; CD4⁺ Tregs; CD8⁺ T cells); TCR clonal expansion in selected compartments; and whether any T1D-associated TCRs were public, or shared, among patients. TRB sharing across compartments and TCR diversity were similar in patients and controls, revealing limited evidence for receptor biases. Moreover, there was low CD3 β sharing across tissues, and shared sequences were not known to target T1D autoantigens. In both groups, there was minimal TCR overlap between PLN Tregs and Tconv cells, which may not support T-lineage instability and plasticity contributing to T1D through the interconversion of Tregs to Tconv effector cells. However, those TCRs matching sequences with previously reported autoreactive T cells were highly enriched in the T1D donors. These included autoreactive T cells directed against epitopes of proinsulin C-peptide 19–35 previously identified in the islets of a T1D donor (179) and several GAD65-reactive clones reported in blood of prediabetic individuals and patients (183). These proinsulin and GAD65 TCR sequences were found in the PLN of some T1D donors. CD4⁺ T cells targeting the GAD65 555–567 epitope were described in peripheral blood of patients (68, 184).

Seay et al. (182) described a TCR sequence matching a previously reported GAD65 clone (GAD4.13) that represented approximately 25% of the TCR sequences found in the PLN of a T1D donor. In this donor, the CDR3 β sequence was the most abundant in PLN Treg and CD8⁺ T cell subsets, providing initial evidence of strong clonal dominance. The GAD4.13 CDR3 β sequence was detected in several tissue compartments in about 40% of T1D donors and in islet-infiltrating T cells in nPOD donor 6323. Approximately 14% of 399 CD3 β sequences identified in this donor's islet-infiltrating CD8⁺ T cells were also in the PLN, and most of these

shared sequences were detected in blood, yet there was minimal overlap between the islet and PLN compartment and no overlap with circulating CD4⁺ Tconv cells. Together with the data of Michels et al. on the same donor (181), these findings support the existence of public TCRs shared among patients. Additional evidence for public TCRs derives from the observation that TCR sequences of GAD65 autoreactive CD4⁺ T cells from a patient with T1D recurrence in the transplanted pancreas (109) overlapped with sequences from a large number of the nPOD T1D donors studied by Seay et al. (182).

Closing remarks

Autoreactive T cells targeting a broad repertoire of antigens and epitopes are considered the main mediators of β cell death in T1D. Given the diversity of islet autoimmune responses and patient heterogeneity, it is challenging to discern whether any responses are more pathogenic than others. Growing evidence supports the targeting of “neopeptides” that result from post-translational modification of native antigens, fusion of peptides derived from different proteins, alternative splicing, and defective ribosomal intermediate products. T cells reacting with neopeptides may have higher affinity and stronger pathogenic potential if central tolerance mechanisms do not apply to these epitopes, which may not be expressed by tolerogenic thymic medullary epithelial cells or tolerogenic stromal cells and APCs in peripheral lymphoid tissues (185–187). Many neopeptides may only be expressed in the target organ; in particular, neopeptides involving insulin may be limited to β cells, and stress and inflammation may influence their expression. The role of neopeptides is a critical research area where we may learn more about impaired tolerance mechanisms and whether responses to neopeptides are primary autoimmune responses and major disease drivers. If future studies can identify key disease driver epitopes, there would be major implications for disease prediction, prevention, and therapy, especially for antigen-specific therapies.

It is currently unknown if a proportion of autoreactive T cells can react with viral epitopes, whether or not they cross-react with islet antigens. Given the extensive literature associating T1D with enterovirus infection of the islets (30), the association of insulinitis with hyperexpression of HLA-I molecules, and the predominance of CD8⁺ T cells in islet infiltrates, it is reasonable to hypothesize that a significant proportion of islet-infiltrating CD8⁺ T cells may react against viral epitopes (Figures 2 and 3). If so, CD8⁺ T cells against viruses may contribute to T1D pathogenesis by eliminating infected β cells presenting viral epitopes in the context of HLA-I hyperexpression. New investigations should attempt to identify CD8⁺ T cells against T1D-associated viruses, especially for epitopes that are good binders for T1D-predisposing HLA molecules; determination of cross-reactivity with autoantigens should be also explored. Moreover, antiviral responses may be depressed in T1D patients, at least temporarily. For example, impaired responses were linked to subsequent development of insulin autoimmunity in early life (127). Perhaps responses to viral antigens that cross-react with islet autoantigens are more susceptible to regulation: weaker antiviral responses may favor the persistence of viral infections. Addressing these major unanswered questions could impact our understanding of the potential role of viruses and overall disease pathogenesis, and design of future therapies.

The ability to detect autoreactive T cells in the circulation has improved dramatically, but their common presence in healthy subjects requires assessing autoreactive T cell phenotypes and functions to aid in interpretation of positive results. Recent studies of T1D and prediabetic organ donors have examined pathology and autoreactive T cells in the pancreas, focusing on islet-infiltrating T cells and those in lymphoid tissues. These studies provide insight into the T cells associated with insulinitis and allow comparisons with peripheral blood readouts. TCR analysis in blood, pancreas, and lymphoid tissues may help firmly establish whether detection of certain autoreactive T cells with particular phenotypes can be a biomarker of ongoing insulinitis. Initial evidence for limited TCR sharing among patients could represent key responses and perhaps therapeutic targets. Further studies investigating TCR sharing between antigen-specific Tconv cells and Tregs reacting to the same epitope with identical HLA restriction could help define to what degree Tregs compete with Tconv cells for binding to a shared cognate epitope. If TCR sharing is low, opportunities may also open for selective therapeutic targeting of Tregs or Tconv cells. For example, there may not be natural Tregs specific for neopeptides.

Ongoing pathology studies of pancreata obtained near T1D onset or afterward are revealing that only a proportion of islets are affected at any given time. It remains unclear when T cells begin infiltrating the islets relative to the initiation of autoimmunity as marked by autoantibody conversion. There is interest in defining which classes of autoimmune responses are detected first, under the hypothesis that initial responses are triggers and possibly major disease drivers. Perhaps responses to neopeptides represent the earliest and most pathogenic responses, with responses to native epitopes playing a secondary role. An alternative hypothesis is that more benign, initial responses are directed toward native antigens, are fairly regulated, and produce modest inflammation and little damage to β cells; however, persisting and increasing islet inflammation, β cell stress, and perhaps viral infections could lead to the generation of neopeptides and trigger more aggressive autoimmunity. Generation of novel reagents that track autoantibody and T cell responses against neopeptides will enable interrogation of archived blood samples from at-risk relatives in natural history studies, some of which begin at birth. Studies of clinical cohorts and expanded numbers of organ donors will be critical to addressing outstanding questions about autoreactive T cells in T1D.

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