Human islet T cells are highly reactive to preproinsulin in type 1 diabetes

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Cytotoxic CD8 T lymphocytes play a central role in the tissue destruction of many autoimmune disorders. In type 1 diabetes (T1D), insulin and its precursor preproinsulin are major self-antigens targeted by T cells. We comprehensively examined preproinsulin specificity of CD8 T cells obtained from pancreatic islets of organ donors with and without T1D and identified epitopes throughout the entire preproinsulin protein and defective ribosomal products derived from preproinsulin messenger RNA. The frequency of preproinsulin-reactive T cells was significantly higher in T1D donors than non-diabetic donors and also differed by individual T1D donor, ranging from 3 to over 40%, with higher frequencies in T1D organ donors with HLA-A*0201. Only T cells reactive to preproinsulin-related peptides isolated from T1D donors demonstrated potent autoreactivity. Reactivity to similar regions of preproinsulin was also observed in peripheral blood of a separate cohort of new-onset T1D patients. These findings have important implications for designing antigen-specific immunotherapies and identifying individuals that may benefit from such interventions.

Type 1 diabetes (T1D) results from the chronic immune-mediated targeting of insulin-producing beta cells within pancreatic islets with T cells playing a central role in disease pathogenesis (1, 2). Tissue specificity and a strong genetic association with the human leukocyte antigen (HLA) locus suggest that antigen specificity is necessary for T cells to attack beta cells and induce T1D (3). Therefore, considerable efforts have been undertaken to identify antigens for disease-associated T cells in order to understand the disorder's pathogenesis and develop therapies to prevent T1D (4–8). As such, many large well-controlled clinical trials evaluating antigen-specific immunotherapies have been conducted (e.g., oral, intranasal, subcutaneous, and DNA vaccines), especially with a focus on insulin- or preproinsulin-related epitopes. Unfortunately, none have achieved favorable clinical outcomes to date (9–14). However, several trials have identified subsets of responders who may potentially benefit from antigen-specific immunotherapy (13–18), thus suggesting the potential for heterogeneity of antigen specificity targeted by the adaptive immune system in individual T1D patients. Indeed, a recent report from the Environmental Determinants of Diabetes in the Young study demonstrated a trend of first appearing islet autoantibodies classified by HLA haplotypes (19), implicating that individual patients may have different antigen specificities that initiate and drive T1D development. To improve prevention efforts and direct tissue- and autoantigen-specific immunotherapy, an improved understanding of epitopes that activate T cells within inflamed pancreatic islets and subsequent reactivity in the peripheral blood is needed to optimally select patients for these therapies.

While the contribution of both CD4 and CD8 T cells to T1D development is evident, several lines of evidence have highlighted the importance of CD8 T cells as mediators of disease pathogenesis. First, CD8 T cells predominate within the immune infiltrates of inflamed pancreatic islets from T1D organ donors (7, 20). Second, as HLA class I molecules present peptides to activate CD8 T cells, beta cells within inflamed T1D islets up-regulate HLA class I molecules, which has the potential to enhance CD8 T cell–beta cell–specific interactions (20, 21). Finally, islet-specific CD8 T cells have been measured in the peripheral blood of T1D patients using fluorescent peptide/major histocompatibility complex (MHC) multimers (22, 23), with some of these specificities correlating to the functional loss of insulin secretion following the clinical onset of T1D (24, 25).

Fluorescent peptide/MHC multimer reagents have previously been used to identify self-reactive CD8 T cells within human T1D islets by in situ staining frozen pancreas sections (20, 26–28). These studies focused on islet peptides presented by the HLA-A2 variant, as it is most frequent in both T1D and the

Significance

Insulin is a major self-antigen in type 1 diabetes (T1D), and as such, insulin-based immunotherapies have been trialed to treat the underlying autoimmunity but with minimal clinical benefit. Here, we comprehensively assessed reactivity to insulin and its precursor, preproinsulin, by CD8 T cells obtained from the pancreatic islets of organ donors with and without T1D. CD8 T cells highly reactive to peptides throughout the entire preproinsulin protein were only found in T1D donors at varying frequencies. Our results suggest considering the use of preproinsulin rather than just insulin for intervention immunotherapies and have important implications for identifying individuals that may respond to antigen-specific therapies designed to treat autoimmune disorders.

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the presence of CD4 and CD8 T cells by flow cytometry (dispersed the isolated islets into single cells and analyzed them for been cultured over 10 d. Thus, the presence of T cells in islets one exception was the sample from T1D-1, from which islets had cating some residual beta cell function in these donors. Of note, three T1D donors who had a shorter diabetes duration to a few years, except one donor who had had diabetes for 15 y. adults and had T1D for relatively short periods of time, months (Table 1). The T1D donors were children, adolescents, or young transporter 8 antibodies

**Results**

**T Cells within Pancreatic Islets.** We analyzed islet samples obtained from seven T1D and seven nondiabetic organ donors for T cell infiltration and T cell receptor (TCR) sequence repertoires (Table 1). The T1D donors were children, adolescents, or young adults and had T1D for relatively short periods of time, months to a few years, except one donor who had had diabetes for 15 y. Of note, three T1D donors who had a shorter diabetes duration than the other study subjects had low but detectable levels of C-peptide as measured within 24 h prior to or at death, indicating some residual beta cell function in these donors.

We first assessed numbers of CD4 and CD8 T cells in islet samples within a few days after islets were isolated and cultured in media containing interleukin-2, interleukin-15, and interleukin-7; one exception was the sample from T1D-1, from which islets had been cultured over 10 d. Thus, the presence of T cells in islets were determined relatively soon after their isolation, when repertoire changes from in vitro culture could be minimized. We dispersed the isolated islets into single cells and analyzed them for the presence of CD4 and CD8 T cells by flow cytometry (SI Appendix, Fig. 1). While individual donors displayed a broad range of T cell numbers in the islets, T1D donors had significantly higher numbers of both CD4 and CD8 T cells compared to nondiabetic donors (Fig. 1A, $P = 0.002$ [CD4], $P = 0.003$ [CD8], T1D versus nondiabetic donors), and CD8 T cells tended to be more prevalent than CD4 T cells in both T1D and the nondiabetic donor groups (Fig. 1A, $P = 0.22$ [T1D donors], $P = 0.03$ [nondiabetic donors], CD4 versus CD8).

To further evaluate diversity and clonality, we examined TCR sequences expressed by tens to hundreds of T cells isolated from the islet samples. Certain proportions of TCR clonotypes were detected from multiple cells in both CD4 and CD8 subsets in the islets of T1D and nondiabetic donors, and some samples such as the CD4 subset of T1D-4 and CD8 subset of T1D-6 contained clonotypes that were expressed by over 20% of all T cells, suggesting expansion of specific islet-infiltrating T cells (Fig. 1B and C). When analyzing TCR repertoire diversity in each sample by calculating Shannon’s diversity index, all subsets were relatively diverse as index values of all samples were over 0.8, and there was no apparent difference between T1D and nondiabetic controls (Fig. 1D). However, CD4 repertoires were more diverse than CD8 repertoires (Fig. 1D, $P = 0.016$, CD4 versus CD8). This suggests that CD8 repertoires contained a higher proportion of clonally expanding cells than the CD4 repertoires, although it should be noted that the cell expansion may have occurred during in vitro culture for several samples such as T1D-1. Taken together, these results indicate that the pancreatic islets in T1D organ donors contain larger numbers of T cells than those in nondiabetic donors, and TCR repertoires in the islets of both T1D and nondiabetic donors were relatively diverse, yet there were clonally identical T cells especially within the CD8 subset.

**Screening Islet-Residing CD8 T Cells for Reactivity to Preproinsulin Peptides.** Next, we addressed the issue of which antigens are targeted by the T cells in the islets. To evaluate this question, we began by examining reactivity to preproinsulin, since this molecule is an abundant protein that is exclusively produced by pancreatic beta cells. We previously analyzed specificity to preproinsulin by CD4 T cells and identified several TCR clonotypes that responded to an insulin B chain, A chain, or C-peptide, a fragment that is cleaved from the A and B chains of insulin during production of the mature insulin molecule (8, 33). In the current study, we focused on CD8 T cells and sought to determine their specificity to preproinsulin-related peptides. To test functional autoreactivity of

| Table 1. Donor information |
|---------------------------|
| Donor ID | Donor reference | Sex | Age (years) | Duration of diabetes (years) | Islet-AABs* | C-peptide (ng/mL) | HLA-DRB1 | HLA-A | HLA-B | HLA-C |
| TID-1 | nPOD 69 | F | 6 | 3 | IAA | <0.05 | 04:01/07:01 | 02:01/26:01 | 35:01/50:01 | 04:01/06:02 |
| TID-2 | nPOD 6323 | F | 22 | 6 | GADA IA2A | <0.02 | 03:01/04:02 | 01:01/25:01 | 08:01/18:01 | 07:01/12:03 |
| TID-3 | nPOD 6342 | M | 14 | 2 | IAA 2A | 0.26 | 01:01/04:01 | 02:01/68:01 | 40:01 | 03:04 |
| TID-4 | nPOD 6367 | M | 24 | 2 | None | 0.39 | 04:01/07:01 | 02:01/29:02 | 18:01/44:03 | 07:01/16:01 |
| TID-5 | nPOD 6414 | M | 23 | 0.4 | IAA GADA ZnT8A | 0.16 | 03:01/09:01 | 01:01/23:01 | 07:02/08:01 | 07:01/07:02 |
| TID-6 | nPOD 6472 | M | 10 | 4 | IAA | 0.02 | 03:01/04:04 | 02:01/32:01 | 08:01/18:01 | 05:01/07:01 |
| TID-7 | VUMC/Pittsburgh | F | 28 | 15 | IAA | 0.03 | 08:04/09:01 | 02:05/23:01 | 35:01/41:02 | 04:11/17:01 |
| ND-1 | IIDP 9657 | F | 34 | No diabetes | Not tested | Not tested | 03:01/15:01 | 01:01/02:01 | 08:01/44:02 | 05:01/07:01 |
| ND-2 | IIDP 1726 | M | 21 | No diabetes | Not tested | Not tested | 07:01 | 02:01/29:02 | 27:05/44:03 | 02:02/16:10 |
| ND-3 | R300 | F | 30 | No diabetes | Not tested | Not tested | 03:01 | 01:01/30:02 | 08:01/18:01 | 05:01/07:01 |
| ND-4 | R301 | M | 18 | No diabetes | Not tested | Not tested | 11:01/13:02 | 02:01 | 14:02/49:01 | 07:04/08:02 |
| ND-5 | IIDP 5642 | M | 30 | No diabetes | Not tested | Not tested | 10:01/13:02 | 02:01/24:02 | 40:01/45:01 | 03:04/16:01 |
| ND-6 | IIDP 7400 | M | 30 | No diabetes | Not tested | Not tested | 03:01/15:01 | 01:01/30:02 | 07:02/18:01 | 05:01/07:02 |
| ND-7 | R283 | M | 22 | No diabetes | Not tested | Not tested | 1,13 | 1,2 | 51 (Bw4) | Cw15 |

*IIslet-autoantibodies: IAA = insulin antibodies; GADA = glutamic acid decarboxylase antibodies; IA-2A = insulinoma antigen-2 antibodies; ZnT8A = zinc transporter 8 antibodies

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Table 4); however, none of the 142 studied TCR transductants responded to those peptides, including the zinc-transporter 8 (ZnT-8) 186-194 peptide and islet-specific glucose-6-phosphatase catalytic subunit–related protein 265-273 peptide (SI Appendix, Table 5), and detected no responses to 173 peptide pools ranging from CD4 T cells in the residual pancreatic islets of T1D donors and open triangles: nondiabetic donors) indicate Shannon diversity indexes of TCR repertoires, in which 1 represents each T cell expresses a different TCR clonotype from all other T cells in a sample, and 0 represents all T cells in a sample express an identical TCR clonotype. Statistical significance for numbers of CD4 and CD8 T cells per islet and Shannon Diversity Index of TCR repertoires were assessed by the Wilcoxon rank sum test.

| TCR Clonotypes | Detected from multiple cells | Detected from single cells |
|----------------|----------------------------|---------------------------|
| TID            | 10                         | 0                         |
| Non-diabetic control | 10                         | 0                         |
| TID            | 10                         | 0                         |
| Non-diabetic control | 10                         | 0                         |
| TID            | 10                         | 0                         |
| Non-diabetic control | 10                         | 0                         |
| TID            | 10                         | 0                         |
| Non-diabetic control | 10                         | 0                         |
| TID            | 10                         | 0                         |
| Non-diabetic control | 10                         | 0                         |
| TID            | 10                         | 0                         |
| Non-diabetic control | 10                         | 0                         |
| TID            | 10                         | 0                         |
| Non-diabetic control | 10                         | 0                         |

Fig. 1. T cells in the islets of organ donors with and without T1D. (A) Numbers of CD4 (blue triangles) and CD8 (magenta inverse triangles) T cells detected per islet in each donor are shown. (B and C) Frequencies of TCR sequences detected from single and multiple cells are shown in green and white bars, respectively. White rectangles represent each unique TCR clonotype. Panels (B and C) show TCR sequencing results from CD4 and CD8 T cells, respectively. (D) Individual symbols (blue: CD4, magenta: CD8, filled triangles: TID donors, and open triangles: nondiabetic donors) indicate Shannon diversity indexes of TCR repertoires, in which 1 represents each T cell expresses a different TCR clonotype from all other T cells in a sample, and 0 represents all T cells in a sample express an identical TCR clonotype. Statistical significance for numbers of CD4 and CD8 T cells per islet and Shannon Diversity Index of TCR repertoires were assessed by the Wilcoxon rank sum test.

Table 1). Only seven TCRs failed to be expressed on the cell surface. These seven were derived from T cells that had two in-frame alpha chain clonotypes and may be intrinsically nonfunctional, leaving 144 TCR clonotypes that were subject to functional autoreactivity analysis.

To screen for antigen specificity, we adopted a multiplex assay system, in which 5KC T-hybridoma cells expressing each TCR clonotype and marked with unique fluorescent proteins were engineered to express a ZsGreen-1 fluorescent protein upon activation, thereby being capable of discretely assessing activation of multiple TCR transductants in a single culture well by flow cytometry (34). Using this assay system, we first tested responses to 103 preproinsulin-derived peptide pools in the presence of Epstein–Barr virus (EBV)-transformed autologous B cells. Each peptide pool consists of 8 to 11 mers of peptides ending at the same position of preproinsulin (SI Appendix, Table 2) and thus all peptide sequences that could be generated from preproinsulin was examined. After excluding two TCR transductants that showed nonspecific responses without antigen stimulation, 24 out of 142 TCR clonotypes (17%) reacted with one or more preproinsulin peptide pools (SI Appendix, Fig. 2A). As expected, many of these TCR transductants responded to peptide pools containing consecutive sequences (Fig. 2A).

In addition to preproinsulin peptides, it has been demonstrated that a defective ribosomal product (DRiP) mRNA (mRNA) (insulin DRiP) can be created. Insulin DRiP is formed from an alternate start codon in the INS gene and expressed by pancreatic beta cells, in particular under conditions of stress (35). CD8 T cells reactive to this product can be detected in peripheral blood of T1D patients and proved diabetogenic ex vivo (35, 36). Therefore, we further tested for insulin DRiP–reactive T cells in insulinic islets by surveying the same set of TCR transductants for responses to 36 peptide pools containing insulin DRiP–derived truncated peptides (SI Appendix, Table 3). Three TCR clonotypes derived from two T1D donors responded to insulin DRiP peptide pools in the presence of autologous EBV transformed B cells (Fig. 2B and SI Appendix, Fig. 2B). Additionally, we tested responses to 11 other islet protein–derived peptides that are known to be autoantigens for T1D (SI Appendix, Table 4); however, none of the 142 studied TCR transductants responded to those peptides, including the zinc-transporter 8 (ZnT-8) 186-194 peptide and islet-specific glucose-6-phosphatase catalytic subunit–related protein 265-273 peptide (SI Appendix, Fig. 2C). CD8 T cells in the islets could be specific to proteins derived from cells within islets other than beta cells. Therefore, we screened for a response to peptide pools derived from glucagon, a major protein expressed by pancreatic alpha cells (SI Appendix, Table 5), and detected no responses to 173 peptide pools ranging from the first through the last amino acid of glucagon (SI Appendix, Fig. 3). In sum, screening of 142 TCR clonotypes derived from CD8 T cells in the residual pancreatic islets of T1D donors identified 27 TCR clonotypes that are potentially reactive to beta cell protein products, preproinsulin or insulin DRiP.

**HLA Restriction for Preproinsulin-Responsive CD8 T Cells.** To confirm that the 27 TCR clonotypes truly respond to peptides identified by screening with peptide pools, individual peptides contained in each pool that provided positive responses were newly synthesized and used to stimulate cognate TCR transductants. Simultaneously, we sought to identify HLA molecules that present peptides to the TCRs by testing reactivity in the presence of antigen-presenting cell lines transduced with each HLA class I allele of the donors. We introduced each HLA class I gene into myelogenic K562 cells, which are devoid of endogenous HLA expression, and used them as antigen-presenting cells. TCR transductants were individually cultured with or without each single peptide in the presence of each HLA transductant line (Fig. 3 A and B and SI Appendix, Table 6). Overall, responsiveness to the newly synthesized peptides...
was confirmed in all TCR transductants except 1.B2, which responded to an insulin DRiP–truncated peptide pool only weakly in the screening. The remaining 26 TCR transductants reacted with one or more peptides presented by a particular HLA molecule, thus allowing identification of the HLA restriction molecules that present peptides to individual TCR clonotypes. All types of class I
Fig. 3. HLA restrictions of preproinsulin and insulin DRiP–reactive CD8 T cells. (A–C) TCR transductants were cultured with or without peptides in the presence of K562 antigen–presenting cells expressing each HLA class I molecule. Peptides contained in one or two peptide pools that induced the strongest response in the screening test were used as stimulus, and responses to each peptide are shown in the order listed in SI Appendix, Table 6a. Peptide sequences are shown in SI Appendix, Table 6b. Responses in the presence of antigen-presenting cells expressing each HLA-A, HLA-B, and HLA-C molecule are shown in red, blue, and green bars, respectively, and HLA alleles tested for each TCR transductant are indicated in SI Appendix, Table 6a. Results for first and second HLA alleles listed in the table are shown in dark and light colors in the figures, respectively. ZsGreen-1 expression levels in wells without peptides are indicated in the most left bars for each HLA allele. Results for preproinsulin-reactive TCR clonotypes derived from T1D donors, insulin DRiP–reactive TCRs derived from T1D donors, and preproinsulin-reactive TCR clonotypes derived from nondiabetic control donors are shown in panels A, B, and C, respectively. Mean values ± SEM obtained from three independent experiments are plotted, and a determined HLA allele is indicated in the upper right corner of each panel. (D) The numbers of clonotypes restricted by HLA-A, -B, and -C molecules are indicated. Individual colors represent TCR clonotypes derived from different donors. (E) Among 21 clonotypes derived from donors having HLA-A*02:01, the numbers of clonotypes restricted by A*02:01 and those restricted by alleles other than A*02:01 are shown. The dashed line indicates an expected probability of restriction by each allele (i.e., 21 divided by 6 possible alleles in each individual). An exact binomial test was used to test whether the proportion of TCR clonotypes restricted with HLA-A*02:01 was equal to 1/6, and this was not the case as 10/21 (48%) restricted to HLA-A*02:01 (P < 0.001).
molecules, HLA-A, -B, and -C, presented preproinsulin peptides to the tested TCR clonotypes (Fig. 3D). However, among donors having the HLA-A*02:01 allele, which is known to confer genetic risk for T1D (37, 38), 10 out of 21 TCR clonotypes (48%) recognized their cognate peptide presented by HLA-A*02:01. This is significantly more than those restricted by all other available class I molecules assuming equal use among a total of six possible presenting molecules (Fig. 3E, P < 0.001). Thus, polymorphic variants of HLA-A, -B, and -C class molecules are capable of presenting preproinsulin or DRiP peptides to islet-derived CD8 TCRs from T1D organ donors, with an enrichment for preproinsulin reactivity in T cells restricted by HLA-A*02:01.

**Defining Optimal Epitopes and Measuring Intensity of T Cell Response.**

The avidity between a TCR and a peptide-MHC complex is one factor that determines phenotype and cytotoxic activity of CD8 T cells (39, 40). To determine the preferred epitopes and intensity of responses to those peptides, we titrated peptide concentrations with each TCR transdant in the presence of K562 antigen-presenting cells expressing cognate HLA molecules (SI Appendix, Fig. 4 A and B). Epitopes were found throughout all regions of preproinsulin: signal peptide, B-chain, C-peptide, and A-chain; but there were three regions that were preferentially recognized by a number of TCR clonotypes derived from different T1D donors, two distinct regions in signal peptide and one in A-chain (Fig. 4A). Among TCR clonotypes recognizing peptides from the same region, the preferred peptide was identical when TCRs were restricted by the same HLA-I molecule. However, epitope sequences diverged between TCRs restricted by different HLA-I molecules within the same region of preproinsulin (Fig. 4B). For example, five TCR clonotypes that preferably responded to preproinsulin 1 to 11 were restricted by HLA-C*03:04, whereas those restricted by HLA-A*02:01 recognized peptides shifted toward the C terminus of preproinsulin. These results suggest that peptides from certain regions of preproinsulin are discriminatorily generated in either pancreatic beta cells or antigen-presenting cells and presented in a particular register by HLA class I molecules to activate T-cells.

Intensity of response by individual TCR transductants was diverse, as represented by half-maximal effective concentration (EC50) values ranging from low nM to over 100 μM (Fig. 4C and Table 2). Some A-chain–reactive TCR transductants reacted strongly, and responsiveness to the first half of the signal peptide region was very intense as well. Intriguingly, EC50 values of insulin DRiP–reactive TCR transductants are in the low nanomolar range, ranked among the highest avidities. This observation could relate to DRiP being a neoantigen expressed in either pancreatic beta cells or antigen-presenting cells and presented in a particular register by HLA class I molecules to activate T-cells.

**Islet-Residing Preproinsulin–Reactive CD8 T Cells Are Disease Specific.**

Some T cells were detectable in the islets of nondiabetic donors by flow cytometry, although the number of T cells in nondiabetic islets was significantly lower than those from T1D donors (Fig. 4F). To determine whether these islet-residing T cells in nondiabetic islets were specific to preproinsulin or insulin DRiP, TCR clonotypes that were detected from two or more CD8 T cells in nondiabetic islets were expressed on K562 hybridoma cell lines using the same method used to analyze TCR clonotypes found from T1D organ donors. We then tested them for response to the preproinsulin peptide pools, insulin DRiP pools, and 11 known islet peptides. Since peripheral immune tissues, such as spleen, were unavailable to generate autologous B cell lines from nondiabetic donors, responses were examined in the presence of K562 antigen–presenting cell lines transduced with each HLA allele present in a given donor. TCR transductants typically respond to antigens more strongly in the presence of K562 cells expressing a cognate HLA molecule than autologous EBV-transformed B cells. Therefore, assay sensitivity may be higher, but more false-positive responses would be detected in the screening of TCRs from nondiabetic donors compared to those from T1D donors. Among 71 TCR transductants that were successfully expressed and did not show nonspecific responses independent of peptide (SI Appendix, Table 1), four TCR transductants weakly responded to preproinsulin peptide pools, two derived from donor ND-1 and the other two from ND-2 (Fig. 4C and SI Appendix, Fig. 6). However, three of the four TCR transductants did not respond when tested with newly synthesized individual peptides. Only one TCR transdant from a nondiabetic organ donor (131.D5) responded to preproinsulin peptides presented by HLA-A*29:02 (Fig. 4C and SI Appendix, Table 6 and Fig. 4C). Notably, responsiveness of this TCR transdant was significantly lower than those from T1D donors (41, 42). Since the cell surface expression level of HLA molecules on antigen-presenting cells can contribute to TCR responsiveness, we examined HLA expression on our K562 antigen–presenting cells and the majority of the HLA class I variants are expressed at similar levels (SI Appendix, Fig. 5). Taken together, optimal epitopes recognized by the 26 CD8 TCR clonotypes spanned various regions of preproinsulin, and the intensity of response to these peptides differed by individual TCRs.
Table 2. Preproinsulin (PPI) and insulin DRiP–reactive T cell receptors

| Donor   | ID   | TCR ID | Freq | HLA          | Epitope         | EC50 (nM) | TRAV | TRAJ | CDR3a          | TRBV | TRBJ | CDR3b          |
|---------|------|--------|------|--------------|-----------------|-----------|------|------|----------------|------|------|----------------|
| T1D-1   | 1.C8 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.F3 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.B10 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.E9 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.F1 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.F3 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.B10 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.E9 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.F1 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.F3 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.B10 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.E9 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |
| T1D-1   | 1.F1 | PPI: 1-11/2-12/2-10 | 5.3% | A0201       | TRAV24/25 TRAJ58 CAFKRETSGLTFT | 68         | TRAV2        | TRAJ3        | DQ2.5        | CASTRLAGDEQF | TRBV13 | TRBJ2-1        | CASTRLAGDEQF |

Human islet T cells are highly reactive to preproinsulin in type 1 diabetes

extremely weak, with an EC50 value of about 1,000 μM (Fig. 4C and Table 2). Thus, the presence of CD8 T cells having strong reactivity to preproinsulin or insulin DRiP appears specific to the islets of T1D organ donors (26/142 TCRs [T1D donors] versus 1/71 [nondiabetic donors], P < 0.001).

**Heterogeneity in the Presence of Preproinsulin-Reactive CD8 T Cells.** As demonstrated in Islet-Residing Preproinsulin–Reactive CD8 T Cells Are Disease Specific, the presence of preproinsulin and insulin DRiP–specific TCR clonotypes in pancreatic islets is a unique feature of T1D organ donors. We further evaluated the frequency of CD8 T cells with these specificities within the pancreatic islets of the studied organ donors. As expected, the prevalence of preproinsulin or insulin DRiP–reactive T cells in T1D donors were significantly higher than those in nondiabetic donors (Fig. 5A–C, P < 0.01). However, there were also differences in the frequencies among donors with T1D, ranging from only 3 (T1D-5 and T1D-2) to 55% (T1D-1). To explore determinants that influenced the frequency of preproinsulin or insulin DRiP–specific T cells in the islets, we analyzed the association with serum C-peptide values (C-peptide is cleaved 1:1 from insulin when secreted from beta cells) and the presence of insulin autoantibodies. There was no association with either of these factors (Fig. 5D and E, P > 0.5 in both analyses). Thus, there was no evidence that the presence of functional beta cells or the presence of humoral immunity to insulin are associated with the presence of preproinsulin or insulin DRiP–reactive CD8 T cells in islets. Since preproinsulin or insulin DRiP–reactive TCR clonotypes were preferentially restricted by HLA-A*02:01 (Fig. 3E), we further investigated whether donors with the HLA-A*02:01 allele have a high frequency of preproinsulin or insulin DRiP–specific CD8 T cells in the islets. As shown in Fig. 5F, donors having HLA-A*02:01 had significantly higher proportions of preproinsulin or insulin DRiP–specific T cells in the islets compared to those not having A*02:01 (P = 0.034). These data provide a basis for particular HLA alleles being a determinant for individuals developing autoimmune responses directed against preproinsulin or insulin DRiP peptides.

**Preproinsulin Immune Responses in the Peripheral Blood of New-Onset T1D Patients.** We next sought to determine whether new-onset T1D patients have CD8 T cell reactivity to preproinsulin peptides similar to that observed in the islets of T1D organ donors. As the islet CD8 TCR clonotypes had optimal epitopes across regions of preproinsulin and 10 separate HLA class I molecules presented these epitopes, we elected to screen patients for peripheral blood responses using a cytokine enzyme-linked immunosorbent spot (ELISPOT) assay for IFN-γ, as opposed to using a tetramer-based assay. Similar to our studies screening islet TCR transductants, we used preproinsulin peptide pools consisting of 8 to 11 mers and grouped the pools into 10 separate conditions that spanned the entire protein. Peripheral blood mononuclear cells (PBMCs) from new-onset T1D patients and nondiabetic controls (Table 3 and Dataset S1) were tested for response to these pools. The groups were well matched in terms of age, sex, and the presence of HLA-A*02:01. All of the T1D patients were within less than 3 wk of onset and all the donors were nondiabetic. The results of the ELISPOT assay are shown in Fig. 6A and Table 3. Three pools (Pools 1, 2, and 3) corresponded to the HLA-A*02:01 peptides. The remaining seven pools corresponded to other HLA-A*02:01 alleles, and the response to the HLA-A*02:01 pool was significantly greater than to the other HLA-A*02:01 pools (P = 0.005). The results of the ELISPOT assay are shown in Fig. 6A and Table 3. Three pools (Pools 1, 2, and 3) corresponded to the HLA-A*02:01 peptides. The remaining seven pools corresponded to other HLA-A*02:01 alleles, and the response to the HLA-A*02:01 pool was significantly greater than to the other HLA-A*02:01 pools (P = 0.005).
diagnosis, with an average of only 3.5 d, such that exogenous insulin administration would not induce T cell responses.

IFN-γ ELISPOTs could be clearly delineated (Fig. 6A), and the majority of patients had a response to pooled viral peptides that are known to be CD8 T cell epitopes from cytomegalovirus, EBV, and measles (22) (SI Appendix, Fig. 7). To compare responses between subjects and groups, a stimulation index was calculated by dividing the number of ELISPOTs for a given preproinsulin peptide pool by those without any in vitro added antigen. A subset of new-onset T1D patients had responses to multiple preproinsulin peptide pools, 33% (10/30, Fig. 6B). Similar to our findings from islet-derived CD8 TCRs, the IFN-γ responses spanned all regions of the preproinsulin protein. Nondiabetic controls also had some responses with 22% (4/18) responding to two or more pools (Fig. 6C), which is a comparable frequency to one out of four nondiabetic organ donors having a preproinsulin reactive CD8 TCR (Fig. 6B). When comparing islet CD8 TCR reactivity from T1D organ donors to the blood of new-onset patients (Fig. 6D), there was remarkable similarity in the region of preproinsulin targeted when accounting for HLA associations. These data indicate that peripheral T cell responses to preproinsulin epitopes in new-onset T1D patients mirror islet-derived CD8 T cell reactivity from T1D organ donors.

Discussion

Cytotoxic CD8 T cells are implicated in tissue destruction of many autoimmune disorders through self-antigen presentation by HLA class I molecules. Here, we studied CD8 T cells in the pancreatic islets of organ donors with autoimmune T1D and those without diabetes without HLA bias and characterized the antigen receptors from these T cells for autoreactivity to insulin, its precursor preproinsulin, a major self-antigen in T1D, and the alternative preproinsulin mRNA peptide DRiP that is an islet neoantigen. Our findings indicate that 1) there are higher numbers of T cells within the residual islets of donors with T1D compared to those without diabetes, 2) CD8 T cells highly specific to preproinsulin are enriched within the islets of a subset of T1D organ donors, and 3) a number of different HLA class I molecules present epitopes throughout the entire preproinsulin protein (signal peptide, B-chain, C-peptide, and A-chain) to these islet-derived CD8 T cells.

Our data indicate a remarkable difference in T cell numbers and preproinsulin specificity between T1D and nondiabetic donors. The finding of CD8 T cell infiltration within the islets of T1D donors being greater than that for CD4 is consistent with previous studies analyzing pancreas histology sections (20, 26, 31, 43), several of these studies also identified islet reactive CD8 T cells using fluorescent peptide/MHC multimers (20). We also found CD8 T cells present in non-T1D islets, albeit in lower numbers, with a highly significant difference between preproinsulin reactive TCRs in T1D (26/142 TCR) versus non-T1D (1/71 TCR) islets. Multiple studies have demonstrated that islet-specific T cells are present in peripheral blood of individuals without T1D in a similar frequency to those with T1D, but such antigen-specific T cells are enriched in the pancreas of only those with T1D (27, 31). More specifically, von Herrath and colleagues recently showed that preproinsulin-specific T cells reside in the pancreatic exocrine compartment of nondiabetic organ donors, but over the course of disease development, they accumulate into insulin-containing islets (27). Our results analyzing preproinsulin specificity of T cells within the islets are consistent with these findings, and we further demonstrate that CD8 T cells in the islets of T1D donors exhibit high reactivity to preproinsulin. Important questions remain: Are T cells specific to insulin DRiP, which are not considered to be expressed in thymic epithelial cells, present in the exocrine tissue? Do pancreatic exocrine T cells move into the islets and what factors drives these T cells into the islets?

Tissue-resident CD8 T cells may not be sufficient to cause diabetes and may play a role in maintaining homeostatic tolerance (44, 45). In addition to preproinsulin-reactive CD8 T cells being infrequent in nondiabetic organ donor islets, we observed that the TCR transductants from those without diabetes required large concentrations of epitopes for stimulation compared to T1D organ donors. Whether or not these T cells reside in the pancreas due to coincidental tissue specificity, the fact that the majority of preproinsulin-reactive TCR transductants from T1D donor islets potently react with antigen at nanomolar concentrations implies that CD8 T cells in a diseased condition are clonally different from those without diabetes. It will be important to elucidate the timing within disease in which clonally different T cells that are highly preproinsulin reactive infiltrate the islets. Pancreases tissues isolated from prediabetic organ
reactive T cells in the pancreatic islets. HLA-A2+ T1D islet donors tended to have higher frequencies of preproinsulin-reactive T cells compared to those without this allele. This finding is comparable to those from a recent report showing a high frequency of T cells from pancreatic biopsies of recent onset T1D patients that were stained with a preproinsulin:15-24/HLA-A2 fluorescent multimer (28). Further studies analyzing the association between HLA haplotypes and preferred antigen specificity are warranted to dissect the heterogeneity of T cell responses 

Table 3. Demographic and immunologic characteristics of study participants with preproinsulin ELISPOT assays

| Age, years | New-onset T1D [n = 30] | Non-diabetic [n = 18] | P value* |
|------------|------------------------|-----------------------|----------|
| Mean (SD)  | 15.0 (6.1)             | 17.5 (9.0)            | 0.269    |
| Median     | 13.4                   | 14.5                  |          |
| Range      | 5.1 to 30.9            | 4.8 to 31.5           |          |
| Gender     |                        |                       | 0.127    |
| Female % [number] | 27% [n = 8] | 50% [n = 9] |          |
| Race/Ethnicity |                    |                       | 0.293    |
| Hispanic Black | 10% [n = 3] | 6% [n = 1] |          |
| Asian | 0% [n = 0] | 6% [n = 1] |          |
| Diabetes duration, days | | | |
| Mean (SD) | 3.5 (4.0) | NA |          |
| Median | 3.0 | NA |          |
| Range | 0 to 21 | NA |          |
| Islet autoantibodies, % [number] | | | |
| Insulin | 47% [n = 14] | NA |          |
| GAD65 | 70% [n = 21] | NA |          |
| IA-2 | 67% [n = 20] | NA |          |
| ZnT8 | 63% [n = 19] | NA |          |
| Positive for ≥1, % [number] | 87% [n = 26] | NA |          |
| HLA-A*02:01, % [number] | | | |
| Present | 62% [n = 18] | 50% [n = 9] | 0.546 |
| Absent | 38% [n = 11] | 50% [n = 9] |          |

SD = SD, NA = not applicable, GAD65 = glutamic acid decarboxylase 65, IA-2 = insulinoma antigen-2, ZnT8 = zinc transporter 8

*P values for continuous variables use Student’s t test, categorical variables use Fisher’s exact test.

donors (i.e., autoantibody positive but not yet develop clinical diabetes) will aid in answering this question. We also found heterogeneity in the frequency of preproinsulin-reactive CD8 T cells in the islets among T1D organ donors. Despite all seven donors having at least one TCR transductant responding to a preproinsulin epitope, there was a range of frequencies from 3 to over 40% of T cells responding to this self-antigen with three of seven donors having an abundant response. These frequencies were calculated within CD8 T cells for which antigen specificity analysis was conducted, and therefore, absolute frequencies could be over- or underestimated depending on the prevalence of preproinsulin or insulin DRIP-reactive T cells that were not analyzed for functional specificity. Nevertheless, it is likely that individual patients have different frequencies of CD8 T cells specific to preproinsulin or insulin DRIP in the islets. A key question remains how to identify individuals with frequent preproinsulin-reactive T cells in the pancreatic islets. HLA-A2+ T1D islet donors tended to have higher frequencies of preproinsulin-reactive CD8 T cells in the islets compared to those without this allele. This finding is comparable to those from a recent report showing a high frequency of T cells from pancreatic biopsies of recent onset T1D patients that were stained with a preproinsulin:15-24/HLA-A2 fluorescent multimer (28). Further studies analyzing the association between HLA haplotypes and preferred antigen specificity are warranted to dissect the heterogeneity of T cell responses in T1D.

Understanding antigen specificity of T cells within pancreatic islets has important implications for designing and testing antigen-specific immunotherapy. Many clinical trials have used preproinsulin- or insulin-based therapies in an attempt to prevent T1D onset and induce tolerance (16, 17). These approaches have included parenteral insulin, oral insulin, nasal insulin, CD4 T cell epitopes within proinsulin, and a DNA proinsulin vaccine (9–14, 18). Randomized-placebo-controlled trials using these therapies proved safe and showed heterogeneous efficacy with subsets of responders, and specific immune effects are apparent with the DNA proinsulin vaccine reducing the frequency of CD8 T cells reactive to proinsulin (12). Unfortunately, none of these insulin-based antigen-specific therapies demonstrated sufficient clinical benefit to date (46). The diversity of preproinsulin reactivity within the islets, in terms of individual patient response and epitopes utilized for CD8 T cells, may account for some of these heterogeneous results. Therapies that utilize insulin (parenteral, oral, and nasal) are composed of just the A and B chains of the protein, and from our studies, it is clear that there are CD8 T cell responses in the islets and blood to C-peptide and the signal peptide in addition to the A and B chains, highlighting consideration of using the whole preproinsulin protein rather than just insulin. In addition, clinical trials pursuing the relationship between HLA genotypes and responders to insulin-based antigen-specific therapies may allow us to classify individuals having clinical benefits from these immunotherapies.

A large number of T cell epitopes have been identified within preproinsulin as previously reviewed (4, 47, 48). Our assay system allowed us to screen reactivity to preproinsulin in a comprehensive manner and confirmed several known epitopes including preproinsulin:15-24 as a target for CD8 T cells in the islets (4, 48). We also identified additional epitopes presented by HLA class I molecules other than HLA-A*02:01. Our results indicate that several HLA class I molecules across HLA-A, -B, and -C can present epitopes of preproinsulin to activate islet-derived CD8 T cells. This has important implications for pathogenesis as well as biomarker development, as focusing on a single HLA class I allele will likely underestimate the total number of self-antigen-specific CD8 T cells. Importance of broad HLA coverage has been raised by a recent comprehensive review article about T cell epitopes for T1D (48), as several reports shed light on epitopes presented by various HLA class I
Fig. 6. Peripheral blood immune responses to preproinsulin (PPI) peptide pools. In each case, cryopreserved PBMCs were cultured in the presence or absence of peptides for 48 h, washed, and then cells transferred to an IFN-γ monoclonal antibody–coated plate for overnight culture followed by development and enumeration of ELISPOTs. (A) Representative IFN-γ ELISPOT images from PBMCs stimulated with no antigen, a viral mix of peptides, and preproinsulin peptide pools containing amino acids 11 to 20. Heat maps showing the response of PPI pools in (B) new-onset T1D patients (n = 30) and (C) nondiabetic subjects (n = 18) as a stimulation index (SI) (No. ELISPOTs in a PPI pool / No. ELISPOTs no antigen). (D) Comparison of islet-derived TCR clonotypes responding to PPI epitopes from T1D organ donors to those from the peripheral blood of new-onset T1D subjects by HLA class I association. Percentages depict the number of new-onset T1D subjects with a given HLA allele that had a SI ≥ 3 for peptide pools within a region of preproinsulin; n = number of new-onset T1D patients with a given HLA allele. White indicates no reactivity found in islets from T1D organ donors or peripheral blood of new-onset subjects.
molecules (49, 50). Additionally, we identified “hot spots” within the preproinsulin protein that were epitopes for islet-derived CD8 T cells. Assays such as cytokine ELISPOT and T cell proliferation assays with antigen are able to make use of all available HLA class I molecules in a patient (51, 52) and can be directed toward peptide sequences within “hot spots” of a self-antigen.

A proportion of nondiabetic individuals also have islet-reactive T cells in their blood (26, 30, 31), and we confirmed this through testing preproinsulin peptide pools in ELISPOT assays. We elected to use an IFN-γ ELISPOT assay to screen for reactivity in peripheral blood as there were many preproinsulin epitopes presented by 10 different HLA class I molecules in the islets. Although preproinsulin reactivity in PBMCs mirrored islet-derived CD8 T cell reactivity from T1D organ donors, we propose that additional T cell–based assays, markers, and signatures are likely required to determine disease specificity and possibly disease heterogeneity (36, 53). While it is still unresolved whether phenotypes of islet–specific T cells have different features in various stages of T1D development in terms of advancing from genetic risk to islet autoantibody seroconversion to clinical T1D onset, a recent study described exhausted self-reactive CD8 T cell phenotypes predict loss of beta cell function following clinical T1D onset (54). Thus, it will be important to characterize CD8 T cell phenotypes and fitness, in addition to TCR sequences and antigen specificity, in the islets across the stages of T1D development.

In addition to preproinsulin, other self-antigen specificities within T1D are recognized by CD8 T cells, including those to glutamic acid decarboxylase (GAD), insulinoma antigen 2 (IA-2), and zinc transporter 8 (23, 26, 48, 49, 55, 56). Translationally modified antigens include insulin DRiP peptides, with two such islet-derived TCRs responding to this antigen, and posttranslationally modified islet antigens such as fusion peptides within islet derived TCRs (26). Our current study analyzed responses to a limited number of peptides derived from islet proteins other than preproinsulin. Future studies are warranted to define the antigen specificity of the remaining TCR transductants to native and posttranslationally modified islet antigens as well as those unrelated to islet proteins but possibly involved in the T1D pathogenesis such as virus-derived peptides.

In conclusion, our findings demonstrate the presence of CD8 T cells strongly responding to preproinsulin is specific to islets of individuals with T1D. The frequency of such preproinsulin-specific CD8 T cells in the islets varies by individual and associates with certain HLA alleles. Understanding the self-antigens recognized by CD8 T cells within the target organ for autoimmune diabetes holds promise for improving therapies to prevent disease onset and identify those individuals that may benefit from immune intervention therapies.

Materials and Methods

Study Subjects and Study Approvals. Organ donors for islet isolation were identified from the Network for Pancreatic Organ Donors with Diabetes program (nPOD; Research Resource Identifiers [RRID]:SCR_014641) (57), a protocol at Vanderbilt University Medical Center/University of Pittsburgh (A.C.P. and Rita Bottino) (58), the Integrated Islet Distribution program (IIDP; RRID:SCR_014387) and the Albert Einstein College of Medicine (IIDP). Islet donors were identified from the Network for Pancreatic Organ Donors with Diabetes (nPOD) (RRID:SCR_014641) and being analyzed on a Cytocube flow cytometer (Beckman Coulter) (SI Appendix, Fig. 5).

Screening for Reactivity to Preproinsulin, Insulin DRiP, Glucagon, and Other Islet Antigen Peptides. We used a multiplex T cell stimulation assay system to test reactions to peptides and truncated peptide pools using a recently published protocol (34, 59). The details of methods were included in SI Appendix, Supplementary Methods and Materials.

Determining HLA Restriction and Potency of Responses to Optimal Epitopes. TCR transductants that responded to one or more truncated peptide pools were analyzed using peptides newly synthesized by Genemed Synthesis as described in SI Appendix, Supplementary Methods and Materials. Peptides and HLA class I alleles tested for each TCR clonotype are shown in SI Appendix, Tables 6a and 6b.

Cytokine ELISPOT Assays. Assays were conducted as previously described using the human IFN-γ ELISPOT kits (U/C/Tech Biosciences) (51). The details of methods were included in SI Appendix, Supplementary Methods and Materials.

Islet Autoantibody Measurements and Molecular HLA Typing. Serum obtained from peripheral blood was used to measure islet autoantibodies to insulin, GAD65, IA-2, and ZnT8 by fluid-phase radio-binding assays as previously described (60, 61). DNA obtained from peripheral blood or organ donor tissue was used to type HLA-DRB1, -A-, -B-, and -C alleles using oligonucleotide probes as previously described (62).

Statistical Analyses. Statistical analyses were performed using R software (R Core Team), GraphPad Prism 8 software (GraphPad Software), and SAS 9.4 (SAS Institute). The statistical tests used for each experiment are indicated in the corresponding table or figure legend. P < 0.05 was considered significant.

Data Availability. All study data are included in the article and/or supporting information.

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24. G. G. Pinkse et al.

16. M. A. Atkinson, B. O. Roep, A. Posgai, D. C. S. Wheeler, M. Peakman, The challenge of type 1 diabetes: Systematic analysis of T cell epitopes in autoimmunity. Diabet. Exper. Clin. Immunol. 148, 1–16 (2007).

25. B. O. Roep, M. Peakman, Antigen targets of type 1 diabetes autoimmunity. Cold Spring Harb. Perspect. Med. 2, a007781 (2012).

5. T. P. Di Lorenzo, M. Peakman, B. O. Roep, Translational mini-review series on type 1 diabetes. Diabetes 58, 172–182 (2009).

6. T. P. Di Lorenzo, M. Peakman, B. O. Roep, Translational mini-review series on type 1 diabetes. Diabetes Care 33, 658–670 (2010).

33. M. Nagata et al., Tissue distribution and clonal diversity of the T and B cell repertoire in type 1 diabetes. J. Immunol. 164, 1664–1669 (2000).

11. K. Näntö-Salonen et al., Preproinsulin-reactive CD8 T cells in the human pancreas through the PD-1/PD-L1 pathway. Diabetes Care 41, 2355–2361 (2018).

18. A. W. Michels et al., Islet-derived CD4 T cells targeting proinsulin in human autoimmune diabetes. Diabetes 66, 722–734 (2017).

29. A. Skowera et al., Conventional and neo-antigenic peptides presented by HLA-A2+ type 1 diabetic patients. J. Exp. Med. 209, 18425–18430 (2010).

30. A. Skowera et al., j-cell-specific CD8+ T cell phenotype in type 1 diabetes reflects chronic autoantigen exposure. Diabetes 64, 916–925 (2015).

31. S. Culina et al., ImmDialab Study Group, Islet-reactive CD8+ T cell frequencies in the pancreas, but not in blood, decrease with type 1 diabetic patients from healthy donors. Sci. Immunol. 3, eaao4013 (2018).

46. R. H. Srey et al., Tissue distribution and clonal diversity of the T and B cell repertoire in type 1 diabetes. JCI Insight 1, e88242 (2016).

49. M. G. Scotto et al., Proinsulin-reactive CD4 T cells in the islets of type 1 diabetes organ donors. Front. Endocrinol. (Lausanne) 12, 622647 (2021).

53. M. Battaglia et al., The Juvenile Diabetes Research Foundation Network for Pancreatic Organ Donors with Diabetes (nPOD) Program: Goals, operational model and emerging findings. Pediatr. Diabetes 15, 1–9 (2014).