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Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes

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AUTHOR CONTRIBUTIONS

S.C.K., D.M.H. and J.A.B.B. designed the study. J.A.B.B., M.E.D., D.M.B. and S.C.K. performed experiments. T.S.B., W.E., R.H., M.B. and M.C.-T. performed experiments. R.M., I.C., E.A.J., L.O., C. Mathieu, T.D. and K.H. generated and supplied reagents. R.B., A.N., J.K., A.P., C. Mathews, M.A.A., M.B., R.H., A.C.P. and D.M.H. provided islets. S.C.K. wrote the manuscript, and all authors edited the manuscript.

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Abstract

A major therapeutic goal for type 1 diabetes (T1D) is to induce autoantigen-specific tolerance of T cells. This could suppress autoimmunity in those at risk for the development of T1D, as well as in those with established disease who receive islet replacement or regeneration therapy. Because functional studies of human autoreactive T cell responses have been limited largely to peripheral blood-derived T cells^{1–3}, it is unclear how representative the peripheral T cell repertoire is of T cells infiltrating the islets. Our knowledge of the insulinitic T cell repertoire is derived from histological and immunohistochemical analyses of insulinitis^{4–8}, the identification of autoreactive CD8⁺ T cells *in situ*, in islets of human leukocyte antigen (HLA)-A2⁺ donors⁹ and isolation and identification of DQ8 and DQ2–DQ8 heterodimer-restricted, proinsulin-reactive CD4⁺ T cells grown from islets of a single donor with T1D¹⁰. Here we present an analysis of 50 of a total of 236 CD4⁺ and CD8⁺ T cell lines grown from individual handpicked islets or clones directly sorted from handpicked, dispersed islets from nine donors with T1D. Seventeen of these T cell lines and clones reacted to a broad range of studied native islet antigens and to post-translationally modified peptides. These studies demonstrate the existence of a variety of islet-infiltrating, islet-autoantigen reactive T cells in individuals with T1D, and these data have implications for the design of successful immunotherapies.

Information on the specificity and function of the T cell repertoire that infiltrates human islets in T1D is limited^{9–14}. Therefore, we examined the lymphocytic infiltrate from handpicked islets from nine donors with T1D (2–20 years disease duration), seven donors without T1D and two donors with type 2 diabetes (T2D). The disease history, islet cellular infiltrate detected by immunohistochemistry, insulin content, lymphocytes detected by flow cytometry and the number of T cell lines and clones grown from the islets are summarized (Table 1 and Supplementary Table 1). Five of nine T1D donors showed insulin-positive islets. The features of control samples (Is.1–Is.9) are shown in Supplementary Table 2. The schema of islet handling is shown in Figure 1a. To recover the maximum number of T cells from islets, two methods were used. First, T cells were sorted by flow cytometry directly from enzymatically dispersed, handpicked islets. Given that carryover of T cells in the dispersed acinar tissue can occur in handpicking, we developed a second method, a gel-based culture protocol (Online Methods) for islets to visualize T cell outgrowth directly from individual islets. We recovered both CD4⁺ and CD8⁺ T cells from nine of nine islet donors with T1D, and CD4⁺ T cells from one donor without T1D from the two methods combined. Representative flow cytometry profiles of isolated, handpicked islets from donor

nPOD69 (with T1D) and Is.7 donor (without T1D) are shown in Figure 1b,c. For the islets from donors with T1D, an average of 221 ± 471 CD4⁺ T cells and 155 ± 210 CD8⁺ T cells were detected (average CD4:CD8 ratio, 1.4:1). A greater frequency of CD8⁺ T cells ($P = 0.03$) was detected from the islets of donors with T1D than from those of donors without T1D (Table 1 and Supplementary Table 1). These results are expected as seen from immunohistochemistry studies of pancreas tissue from donors with and without T1D^{5,6}.

Cellular outgrowth from handpicked islet remnants was seen after 5–10 d with T cell stimulation and growth factors (Fig. 1d), with outgrowth size increasing over time. We detected CD4⁺ T cell lines in all islet samples from donors with T1D ($P = 0.003$), as compared to the islets from the control donors or with T2D. From six of nine donors with T1D, T cell lines grown directly from islets contained both CD4⁺ and CD8⁺ T cells (designated ‘mixed’), ranging from CD4:CD8 percentages of 85:15 to 45:55 (Supplementary Fig. 1), that require further sorting and analyses, given that CD4⁺ T cell growth bias occurs over CD8⁺ T cells. CD8⁺ T cell lines were detected by flow cytometry or grown from eight of nine donors with T1D; no CD8⁺ lines were grown from the islets of donors without T1D. No T cell outgrowth from islets of donors without T1D or donors with T2D was observed except for five CD4⁺ T cell lines from donor Is.7. These data, combined with the *ex vivo* sorting data, suggest that these T cells are islet infiltrates from donors with T1D and are not from lymphocytes in islet capillaries.

Islet infiltration in individuals with T1D is heterogeneous: adjacent pancreatic lobes can differ in the presence or absence of insulitis, and infiltrated islets can exist in close proximity to noninfiltrated, normal-appearing islets¹⁵. In agreement with this heterogeneity, a range of 10–1,178 T cells from 100 handpicked islets was detected by flow cytometry, and we detected cellular outgrowth from an average of 26% islets plated (outgrowth from a range of 9–45 individual islets for 100 islets per sample) (Supplementary Table 1). A meta-analysis of immunohistochemistry studies to define insulitis described islet infiltration as 15 CD45⁺ cells per islet, and insulitis as infiltration in <10% islets, although greater numbers of islet-infiltrating CD45⁺ lymphocytes have been detected in some nPOD sample pancreata from donors with T1D^{5,6}. Neither immunohistochemistry nor the isolation of live, islet-infiltrating T cells can measure all infiltrated T cells because sectioning and staining through entire islets or pancreas is not possible, and the use of all isolated islets from an entire pancreas for autoimmunity studies is generally not possible. Owing to the heterogeneous nature of the insulitis and the rarity of pancreatic tissue from donors with T1D, sampling might not be equivalent in any given islet preparation. On the basis of average recovery of islets from donors with T1D, we calculated that we examined 0.56% of recovered islets for T cell infiltration (Supplementary Table 3).

Next we tested the autoreactivity of CD4⁺ T cell lines and clones grown and sorted from islets of donors with T1D by using peptide panels derived from islet-associated proteins known either to bind to HLA-DR3, HLA-DR4 or HLA-DQ8 and/or to elicit T cell responses from the peripheral blood of individuals with these risk alleles¹. For each T cell line or clone, either autologous or HLA-matched Epstein–Barr virus (EBV)-transformed B cells (Figs. 2 and 3) were pulsed with peptides for 2 h and then cultured with T cell lines or clones for 48 h. Reactivity was measured by cytokine secretion, as detected by an enzyme-

linked immunosorbent assay (ELISA) and or Luminex. We detected CD4⁺ T cell responses to glutamic acid decarboxylase 65 (GAD) GAD_{555–567} (Fig. 2a), proinsulin_{76–90} (Fig. 2b), GAD_{274–286} (Fig. 2c), GAD_{115–127} (Fig. 2d) and islet antigen (IA)-2_{545–562} (Fig. 2e).

To screen for T cell reactivity from processed whole protein, we used a panel of Priess B cells that were transduced with constructs encoding autoantigens that have been identified as targets of autore-active T cells in T1D¹. We detected a CD4⁺ T cell line grown from an islet from donor nPOD6323 that responded to Priess B cells expressing chromogranin A (Fig. 2f), and from individual islets from donor nPOD69, two CD4⁺ T cell lines recognized Priess B cells expressing (pre)proinsulin (Fig. 2g,h), and one recognized the Priess B cells expressing chromogranin A (Fig. 2i). As compared to the parent B cells, T cell lines had positive stimulation indices to the B cells expressing autoantigen over their response to B cells alone (stimulation index (SI) = 2–4), but they were not as robust as other lines that responded to some peptides (e.g., Fig. 2b; SI = 16.8).

Three expanded CD8 β ⁺ T cell lines from donor nPOD6268 were probed for autoreactivity through the use of pooled HLA-A2 pentamers loaded with known islet peptide targets³. All three lines had increased numbers of CD8 β ⁺ T cells bound with the pool of HLA-A2 pentamers loaded with insulin B_{10–18}, IA-2_{797–805} and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)_{265–273} peptides, as compared to the numbers of CD8⁺ T cells bound by the HLA-A2 multimer loaded with the malaria peptide (Fig. 2j–l). After two rounds of expansion, 99% of the CD8 β ⁺ T cells in these lines were of unknown reactivity, which indicates a broader range of CD8⁺ autoreactivity than has been previously reported for T1D³.

The autoreactivity of T and B cells in autoimmune diseases with post-translationally modified peptides is a potential mechanism by which tolerance to autoantigens can be broken. T and B cell reactivity to post-translationally modified epitopes¹⁶ has been detected in the context of rheumatoid arthritis¹⁷, celiac disease¹⁸ and T1D^{19–26}; for example, (pre)proinsulin and IA-2 peptides in the context of the high-risk DQ8-*trans* class II molecule^{10,27}. In previous studies, human peripheral autoreactive T cells responded to epitopes containing post-translational modifications such as a vicinal disulfide bond in insulin²⁰, citrullination or deamidation^{19,21–27}, or islet-derived T cells with hybrid insulin–peptide fusions of proinsulin C peptide with islet amyloid polypeptide (IAPP) or neuropeptide Y²⁸ (Supplementary Fig. 2). Among the T cells studied here, a CD4⁺ T cell line grown from an islet from donor nPOD6323 recognized glucose-regulated protein 78 (GRP78)_{292–305} with an arginine to citrulline modification at position 297 (Fig. 3a). A CD4⁺ T cell clone sorted from islets from donor T1D.7 recognized IAPP_{65–84} with arginine to citrulline modifications at positions 73 and 81 (Fig. 3b). From donor nPOD6367, a CD4⁺ T cell line recognized the hybrid insulin–peptide fusion with an insulin-A chain peptide (Fig. 3c). From donor nPOD6323, two CD4⁺ T cell lines recognized different hybrid insulin–peptide fusions of different two peptides from IAPP (Fig. 3d,e).

Autoreactive CD4⁺ T cells detected here produced interferon (IFN)- γ ; all lines and clones secreted different patterns of cytokines upon peptide stimulation, with some additionally secreting tumor necrosis factor (TNF- α) and/or interleukin (IL)-2 with variable secretion of

IL-9, C-C motif chemokine ligand 20 (CCL20) or granulocyte- macrophage colony-stimulating factor (GM-CSF). Two lines secreted IL-13 in a nonpeptide-specific manner. IL-4 and IL-5 were not detected from any autoreactive T cell line or clone (Supplementary Fig. 3). These data indicate an inflammatory islet microenvironment in donors with T1D.

Both insulin secretion, as measured by C-peptide in the circulation²⁹, and islet autoimmunity, as measured by islet infiltrate⁵, persist for decades after diagnosis in many patients with T1D. Moreover, the chronic destructive function of islet autoimmunity is also observed in partial pancreas transplants between identical twins who are discordant for T1D³⁰, and even in some immunosuppressed pancreas-transplant recipients from unrelated donors³¹. For islet replacement or regeneration to be successful, the islet-infiltrating lymphocytic response must be understood and prevented or suppressed, ideally by using antigen-specific therapies (as opposed to general immunosuppression with its attendant toxicities).

This work defines and extends¹⁰ our knowledge of autoreactive T cell responses in human T1D, which is needed to target islet- infiltrating T cell autoreactivity. Study of human samples is important, because therapies in rodent models of T1D have not translated well to durable clinical benefit^{32,33}. An important aspect of defining the autoreactive T cell response of infiltrating islets is the determination of the *ex vivo* frequency of autoreactive T cells; this requires the development and validation of numerous multimer reagents, especially for hybrid fusion peptides and modified peptides. Additional studies exploring the reactivity of this large bank of islet-infiltrating T cells are ongoing, especially with new epitopes—native or modified— guided by epitope-discovery efforts, and this T cell bank is a valuable resource for the T1D community. Future studies of islet-infiltrating T cells from donors with circulating autoantibody, but with no clinical diabetes diagnoses, are planned. A complete knowledge of the diversity of the islet infiltrating, autoreactive, pro-inflammatory response in human T1D is crucial for the successful design of durable autoantigen-specific tolerance induction therapies for those at risk of developing, and those with established, T1D.

ONLINE METHODS

Islet donors

We studied isolated human islets from donors with established T1D ($n = 9$) and from donors without a diagnosis of T1D ($n = 7$) or with T2D ($n = 2$). These donors were recovered through the highly collaborative and coordinated efforts of Vanderbilt University (five donors with T1D, including nPOD69, and one donor with T2D and all control donors without T1D) and from the Network of Pancreatic Organ Donors with Diabetes (nPOD, four donors with T1D) via collaborative agreements with the National Disease Research Interchange (NDRI), the International Institute for the Advancement of Medicine (IIAM), from the Integrated Islet Distribution Program at the City of Hope (IIDP) or from Prodo Labs (one donor with T2D). All institutions have current internal review board (IRB) approval for these studies of de-identified and discarded tissue. The availability of islets isolated from donors with T1D is rare, and these studies reflect the isolated islets (donors with T1D, with T2D and without T1D) that were received over a 2-year period. Donors with T1D (five males, four females; mean age, $19.7 \pm \text{s.d. } 7.7$ years; Table 1) were older than

donors without diabetes (six males, one female; mean $5.8 \pm \text{s.d. } 3.4$ years; Supplementary Table 2) ($P = 0.0006$). Patients with T2D were predictably older (one male, one female; 51 and 25 years old, respectively; Supplementary Table 2) than the other donors. As expected, in comparison with the islets from donors with T1D, control donors and donors with T2D harbored few T cells in their islets⁶. The small amount of blood drawn after donor demise is used for autoantibody testing.

Human islets

In general standardized methods, the pancreata are perfused briefly with Belzer UW solution to clear the vasculature. Islet isolation³⁴ and islet handling³⁵ have been described. The average (mean $\pm$ s.d.) islet equivalents (IEQ) recovered from six of the islet isolations from donors with T1D was 35,883 ($\pm 32,757$) (Supplementary Table 3). A schema of islet handling from receipt to T cell sorting and growth is shown (Fig. 1a). Islets were received for experimental use 2–5 d after donor's brain death. We received ~500–1,000 islet equivalents per donor of variable purity (10–80%). Islets were handpicked under a dissecting microscope for increased purity. For the two methods of analysis of islet-infiltrating T cells, we assayed 200 IEQs, corresponding to an average of 0.56% of recovered IEQ examined (Supplementary Table 3). Handpicking of islets under a dissecting microscope for increased purity occurred over 2–3 h. 100 handpicked islets were enzyme-dispersed, washed and stained with viability dye and lymphocyte markers (see '*Ex vivo* detection and sorting of islet-infiltrating lymphocytes') (~75 min). Cells were detected and sorted within 6 h of islet receipt. Sorted cells were cultured for 12–14 d with 1–2 rounds (a total of 4–6 weeks) of stimulation (see '*Ex vivo* detection and sorting of islet-infiltrating lymphocytes' below) and then characterized in the same manner as the T cell lines. All clones and lines were cryopreserved. Tallies of CD4⁺ and CD8⁺ T cells detected by *ex vivo* flow cytometry from dispersed islets is shown in Supplementary Table 1 and from donors without T1D (Supplementary Table 2).

Another aliquot of 100 handpicked islets was immediately put into culture on gel-based culture with T cell stimulation and growth factors (see 'Direct growth of T cells from islets'). These islets remained in culture for 5–10 d with daily microscopic inspection. When cellular outgrowth was observed from individual islets, cells were collected by pipette under a dissecting microscope and placed in culture as individual lines with cytokine for expansion. An aliquot of each line was tested for T cell subset by flow cytometry. Tallies of the numbers of CD4, CD8, and CD4/CD8 *mixed* T cell lines grown from individuals plus the number of CD4 or and CD8 T cell clones sorted *ex vivo* from dispersed islets are shown in Supplementary Table 1 for the donors with T1D and for the donors without T1D (Supplementary Table 2).

Ex vivo detection and sorting of islet-infiltrating lymphocytes

Within 4–6 h of islet receipt, 100 handpicked islets were enzymatically dispersed (TrypLE, Thermo Fisher), washed and stained with vital dye (1:100 dilution in a 100- μ l volume, Zombie Violet, BioLegend)³⁶, anti-CD45, anti-CD3 (BioLegend) and anti-CD19, anti-CD4 and anti-CD8 (BD BioSciences). Anti-CD3 and anti-CD45 were used at a dilution of 1:100 and anti-CD4, anti-CD8 and anti-CD19 were used at a dilution of 1:25 in a 100- μ l volume.

To retain all islet-infiltrating T cells, CD4⁺ and CD8⁺ T cells were sorted (BD FACSARIA) onto irradiated, allogeneic peripheral blood mononuclear cells in complete media (HL-1 media, supplemented with 2 mM -glutamine, 5 mM HEPES and 100 U/ml penicillin and 100 µg/ml streptomycin, 0.1 mM each nonessential amino acids, 1 mM sodium pyruvate (all from Lonza) and 5% heat-inactivated human male AB serum (Omega Scientific) with 4.5 µg/ml PHA-P (Thermo Scientific) for T cell stimulation plus IL-2 (20 U/ml) (Proleukin), IL-4, IL-7 and IL-15 (all at 10 ng/ml) (Peprotech), a blocking anti-Fas antibody (eBiosciences), anti-PD-1 antibody (BD Biosciences) (all antibodies at 1 µg/ml) and mifepristone (100 nM) (Invitrogen)³⁷. For rounds of restimulation, T cell lines or clones were stimulated with irradiated allogeneic feeders, PHA-P, IL-2, IL-7, IL-4 and IL-15 in complete media with 5% human serum, as described above. In all figures, labeling of a clone denotes that the T cell was single-cell sorted from dispersed islets; labeling as a line denotes growth from an individual islet.

Direct growth of T cells from islets

Immunohistochemical examination of pancreata has shown that acinar tissue can contain lymphocytes^{38,39}. Although we enriched for islets by handpicking, carryover is possible; therefore, we developed a gel-based method for visualization, growth and retrieval of cellular outgrowth from individual islets. Another 100 handpicked islets were plated on tissue culture plates (CoStar) coated with Matrigel (Corning Life Sciences) in media with growth factors described above (without PHA-P) with anti-CD3 and anti-CD28 (both at 5 µg/ml) (BD BioSciences), a blocking anti-Fas antibody (eBiosciences), anti-PD-1 antibody (BD Biosciences) (all antibodies at 1 µg/ml) and mifepristone (100 nM) for T cell stimulation. Cellular outgrowths were collected under a dissecting microscope after 5–10 d, and expanded with growth factors described (without T cell stimulation), and assayed by flow cytometry for CD4 and CD8β expression (Coulter). Both CD4 and CD8 T cell lines are considered polyclonal (e.g., Fig. 2j–l) and require cloning. In addition, 102 T cell lines grown from individual islets from six of nine islet donors with T1D were mixtures of CD4 and CD8 T cells, which require separation and cloning by fluorescence-activated cell sorting (FACS) (Supplementary Table 1 and Supplementary Fig. 1). We have noted an islet-derived CD4⁺ T cell growth bias over islet-derived CD8⁺ T cells, indicating the importance of separation of CD4 and CD8 T cells at the sorting step, or as soon as cellular outgrowth is detected from the islets, to have a fair representation of the T cell composition of the insulinitis.

Antigen-presenting cells (APCs)

Spleen single-cell suspensions from matched islet donors were used to generate EBV-transformed B cells for use as autologous APCs by standard methods using virus-containing supernatants (American Type Culture Collection). Priess B cells (HLA class II molecules, DRB1*04, DRB4*101, DQB1*03, DR53) were also used as APCs in some experiments (Sigma-Aldrich). Class II negative B cells (BLSs) that are stably transfected with HLA-DRB1*04:01 and HLA-DRA1*01:01 were used as APCs. Priess B cells were transduced with individual lentiviral vectors with open reading frames (ORFs) for the gene products for chromogranin A (ORFeome internal ID 3610), pre-proinsulin (ORFeome internal ID 5628), ZnT8 (aka Slc30A8, ORFeome internal ID 54579) and glutamic acid decarboxylase 65

(*GAD2*) (ORFeome internal ID 53270) obtained from the ORFeome5.1 collection (Dana-Farber Cancer Institute, <http://horfdb.dfci.harvard.edu/hv5/>). Each ORF was confirmed by sequencing. All B cells are grown in media with Plasmocin (InvivoGen). To confirm the ability of the transduced Priess cells to function as antigen-presenting cells, we tested their reactivity with known T cell clones. T cell clone 164 (reactive with GAD65_{555–567} in the context of DRB1*0401) secreted IFN- γ when co-cultured with Priess B cells expressing the ORF for *GAD2*, but not when cultured with the Priess B cells expressing the ORF for *MOG*. T cell clone Ob.1A12 secreted IFN- γ when co-cultured with Priess B cells expressing the ORF for myelin basic protein (MBP), but not when cultured with the Priess B cells expressing the *MOG* ORF. This clone responds to MBP_{85–99} in the context of DRB1*15:01 and DRB1*04 (Supplementary Fig. 4).

Peptides

Autoreactive peptides with reported reactivity to HLA-DR3, HLA-DR4 or HLA-DQ8 (ref. 1) described in the figures were synthesized by New England Peptide or Sigma-Aldrich (>95% purity). Peptides showing binding or reactivity in the context of DR17, DR4 and DQ8 were chosen from GAD65, IGRP, proinsulin, insulin, IA-2 and IAPP. Modified peptides used were hybrid insulin peptides²⁸, native and citrullinated peptides from glucose-regulated protein 78 (GRP78)²² that were designed by using a prediction algorithm program for binding to HLA-DQB1*03:02 or HLA-DRB1*04:01 (a panel of 60 peptides) and citrullinated peptides of GAD65 and IAPP.

Detection of autoreactivity

CD4⁺ T cell lines and clones were tested for peptide reactivity by pulsing irradiated B cells with peptides (50 μ g/ml) for 2 h at 37 °C and then washing. In addition, irradiated parent Priess and autoantigen-expressing Priess B cells were used as APCs (Fig. 2f–i). For both peptide-pulsed B cell and autoantigen-transduced B cell as antigen-presenting cell experiments, B cells were plated at $7.5\text{--}10 \times 10^3$ cells/well with 2.5×10^4 T cells in triplicate wells of round-bottom 96-well plates (CoStar). After culture for 48–72 h, supernatants were collected and IFN- γ was detected by standard ELISA (BD BioSciences). After one or two rounds of stimulation, reactivity of the T cell lines was re-tested and cytokine secretion was analyzed by Luminex (MILLIPLEX MAP Human Th17 Magnetic Bead Kit). Blocking anti-HLA-DR (L243, BD BioSciences) or anti-HLA-DQ (SPV-L3, Abcam) was added in some experiments (antibodies at 20 μ g/ml) to confirm HLA restriction. Of note, 218 lines/clones are under continuing investigation, with additional panels of modified peptides. For detection of CD8⁺ T cell autoreactivity, CD8⁺ T cell lines were treated with dasatinib⁴⁰, stained with Zombie Violet and either with pools of HLA-A2 pentamers (ProImmune) loaded with insulin B chain_{10–18}, IA-2_{797–805}, IGRP_{265–273} or pre-proinsulin_{15–24}, GAD65_{114–123}, IAPP_{5–13} multimers³ or individually, with malaria circumsporozoite protein_{319–327} as a negative control and gates were drawn on the basis of this staining. Events were detected by flow cytometry (LSRII, Becton Dickinson).

Statistical analysis

Significant differences were calculated using Prism 4.0a software. Two-tailed paired or unpaired Student's *t*-tests and 95% confidence intervals were used. For comparison of ages

of the groups, donors with T1D, $n = 9$, and for donors without diabetes, $n = 7$. For all comparisons of the numbers of CD4 or CD8 T cells detected by flow cytometry from dispersed islets from donors with and without T1D, $n = 6$ for all groups. For all comparisons of the numbers of CD4 or CD8 or mixed T cell lines grown from individual islets from donors with and without T1D, $n = 9$ for all groups. For all cytokine measurements, the supernatant from triplicates of each cell culture condition ($\pm$ peptide) was assayed, and results are presented as the mean $\pm$ s.d. (SD) of the triplicates. Each experiment was replicated three times, and a representative experiment is shown with individual values in a scatter plot with error bars (SD).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic of islet handling and *ex vivo* isolation and growth of T cells from islets. **(a)** Isolated islets were received and handpicked to increase purity. To capture the maximum number of islet-infiltrating T cells, an aliquot of 100 handpicked islets was dispersed with enzyme, stained for CD45, CD3, CD19, CD4, CD8 and Zombie Violet viability dye. All detectable CD45⁺CD3⁺CD4⁺ and CD45⁺CD3⁺CD8⁺ T cells were single-cell sorted by flow cytometry (Supplementary Table 1) and cultured for 1–3 rounds (4–6 weeks) with irradiated allogeneic feeders, PHA-P and IL-2, IL-7 and IL-15. *The frequency of CD4⁺ and CD8⁺ T cells detected by *ex vivo* flow cytometry from dispersed islets is shown in Supplementary Tables 1 and 2. Alternatively, aliquots of 100 handpicked islets were cultured on a gel matrix with soluble anti-CD3, anti-CD28, anti-Fas, anti-PD-1, IL-2, IL-4, IL-7, IL-15 and mifepristone. After 5–10 d of culture, cellular outgrowth from islets was recovered under a dissecting microscope and cultured as above with irradiated allogeneic feeders, PHA-P and IL-2, IL-7 and IL-15. Surface expression of CD4⁺ or CD8⁺ was determined or confirmed by flow cytometry. **Numbers of T cell lines and clones grown are shown in Supplementary Tables 1 and 2. **(b,c)** Representative flow cytometric analysis of dispersed islets from a donor with T1D (nPOD69) **(b)** and a donor without T1D (Is.7) **(c)**. Not shown, forward scatter (FSC) versus side scatter (SSC) panels with cells stained with viability dye. Frequency of cell subsets is shown. In the FSC and SSC panels, the colored cells indicate the origin of the positively sorted cells shown in the subsequent panels (CD8, red; CD4, green; and CD4⁺CD8⁺, purple). **(d)** Outgrowth of T cells from an islet remnant from nPOD69, cultured as described on the top line of the schematic in **a**. Scale bar, 60 μ m.

Figure 2.

Detection of reactivity to known autoreactive targets of CD4⁺ and CD8⁺ T cell lines and clones sorted or directly grown from islets from donors with T1D. T cell lines and clones were tested for reactivity against known autoreactive peptide and protein targets presented by B cells (described below for each panel) as described in Online Methods. The sample source and designation of each line or clone are listed, along with the HLA restriction, if determined (in *italics*). (a) A CD4⁺ T cell line grown directly from an islet from donor T1D.6 recognized GAD₅₅₅₋₅₆₇ in the context of a bare lymphocyte syndrome (BLS) B cell expressing only HLA-DRA1*01:01 and HLA-DRB1*04:01. (b) A CD4⁺ T cell line grown directly from an islet from donor nPOD69 recognized proinsulin₇₆₋₉₀ in the context of a BLS B cell expressing only HLA-DRA1*01:01 and HLA-DRB1*04:01. (c) A CD4⁺ T cell clone sorted from islets from donor nPOD6342 recognized GAD₂₇₄₋₂₈₆ by the secretion of IFN-γ in the context of Priess B cells. (d) A CD4⁺ T cell clone sorted from the islets from donor T1D.7 recognized GAD₁₁₅₋₁₂₇ in the context of autologous EBV-transformed B cells. (e) A CD4⁺ T cell clone sorted from the islets from donor T1D.7 recognized IA₂₋₅₄₅₋₅₆₂ in the context of autologous EBV-transformed B cells. Priess B cells were transduced with

lentiviral vectors containing open-reading frames (ORFs) of autoantigens were used as antigen-presenting cells (Supplementary Fig. 4 and Online Methods). The autoantigens represented by the ORFs were myelin oligodendrocyte glycoprotein (MOG), GAD65, preproinsulin, zinc transporter 8 (ZNT8), myelin basic protein (MBP) and chromogranin A (ChgA). **(f)** A CD4⁺ T cell line grown from an islet from donor nPOD6323 reacted with a Priess B cell transduced with the ChgA ORF. **(g,h)** Two CD4⁺ T cell lines grown from separate islets from donor nPOD69 reacted with a Priess B cell transduced with a preproinsulin ORF. **(i)** A CD4⁺ T cell line grown from an islet from donor nPOD69 reacted with a Priess B cell transduced with the ChgA ORF. Three CD8⁺ T cell lines were grown from handpicked islets on a gel matrix from donor nPOD6268. **(j–l)** After two rounds of polyclonal stimulation and expansion with cytokines, lines were stained with pools of HLA-A2 pentamers loaded with peptides insulin B_{10–18}, IA-2_{797–805} and IGRP_{265–273} **(j)** or GAD_{114–123}, PPI_{15–24} and IAPP_{5–13} **(k)** and line 6268.6 **(l)** stained with an HLA-A2 pentamer loaded with a malaria circumsporozoite protein_{319–327} as a negative control. One of three similar experiments is shown with data presented as the mean $\pm$ s.d. (SD) of triplicates of cell culture wells. *P* values as determined by two-tailed paired Student's *t*-tests and 95% confidence intervals are shown in the figures.

Figure 3.

Detection of autoreactivity of CD4⁺ T cell lines and clones sorted or directly grown from islets from donors with T1D with modified peptides. CD4⁺ T cell lines and clones were tested for autoreactivity with panels of peptides synthesized with substitutions corresponding to post-translational modifications with co-cultures, as described in Figure 2. **(a)** A CD4⁺ T cell line grown from an islet from donor nPOD6323 responded to an HLA-matched, EBV-transformed B cell (DR3⁺, DR4⁺, DQ2⁺, DQ8⁺) pulsed with GRP78₂₉₂₋₃₀₅ with an arginine (Arg)-to-citrulline (Cit) modification at amino acid position 297. **(b)** A CD4⁺ T cell clone sorted directly from islets from donor T1D.7 secreted IFN- γ in response to an autologous EBV-transformed B cell line pulsed with IAPP₆₅₋₈₄ with two Arg-to-Cit modifications at amino acid positions 73 and 81. **(c)** A CD4⁺ T cell line grown directly from an islet from donor nPOD6367 responded to HLA-matched, EBV-transformed B cells pulsed with a hybrid insulin peptide (insulin C peptide and insulin A chain, hEGGG:A chain—GQVELGGG:GIVEQCC). **(d)** A CD4⁺ T cell line grown directly from an islet from donor nPOD6323 secreted IFN- γ in response to HLA-matched, EBV-transformed B cells pulsed with hybrid insulin peptide (insulin C peptide and IAPP, hEGGG:IAPP1 (GQVELGGG:TPIESHQ)). **(e)** A CD4⁺ T cell line grown directly from an islet from donor nPOD6323 secreted IFN- γ in response to HLA-matched, EBV-transformed B cells pulsed with hybrid insulin peptide (insulin C peptide and IAPP, hEGGG:IAPP2 (GQVELGGG:NAVEVLK)). One of three similar experiments is shown, with data presented as the mean $\pm$ s.d. of triplicates of cell culture wells. *P* values shown are determined by two-tailed paired Student's *t*-tests and 95% confidence intervals.

Table 1

Summary of characteristics of donors with T1D.

Donor case ID	Duration of T1D (years)	HLA	Positive autoantibodies (on demise)*	Histology and/or immunohisto- chemistry
14-year-old female nPOD6342	2	A2, A68 DR1, DR4 DQ5, DQ8	IA-2A ⁺	Insulin ± islets with ± insulinitis
24-year-old male nPOD6367	2	A2, A29 DR4, DR7 DQ2, DQ8	Negative	Insulin ± islets with no insulinitis observed
12-year-old female nPOD6268	3	A2, A68 DR17, DR13 DQ2, DQ6	#	Rare insulin ± islets with ± insulinitis
6-year-old female nPOD69	3	A2, A26 DR4, DR7 DQ2, DQ8	#	Insulin-negative islets with no insulinitis observed
22-year-old female nPOD6323	6	A1, A25 DR4, DR17 DQ2, DQ8	GADA ⁺ IA-2A ⁺	Rare insulin-positive islets ± insulinitis
20-year-old male T1D.6	7	A2, – DR17, DR4 DQ2, DQ8	IA-2A ⁺	Numerous insulin-positive islets
27-year-old male T1D.7	17	A1, A3 DR17, DR4 DQ2, DQ8	Not available	Not available
30-year-old male T1D.8	20	A1, A3 DR1, DR4	#	No insulin-positive islets observed
22-year-old male T1D.9	20	A2, – DR4, DR13	Not available	Not available

The characteristics of donors with T1D, HLA, autoantibody status at demise and histology and/or immunohistochemistry of pancreatic islets are shown. A flow chart of the handling of the islets from receipt to T cell detection and growth is shown in Figure 1. Asterisk (*), presence of serum autoantibody on demise. Pound sign (#), anti-insulin antibodies are not listed for donors with T1D because they could not be distinguished as autoantibodies versus antibodies arising from insulin treatment.