Vimukthi Pathiraja,[1] Janine P. Kuehlich,[1] Peter D. Campbell,[1] Balasubramanian Krishnamurthy,[1,2] Thomas Loudovaris,[1] P. Toby H. Coates,[3] Thomas C. Brodnicki,[1,2] Philip J. O'Connell,[4] Katherine Kedzierska,[5] Christine Rodda,[6] Philip Bergman,[7] Erin Hill,[7] Anthony W. Purcell,[8] Nadine L. Dudek,[8] Helen E. Thomas,[1,2] Thomas W.H. Kay,[1,2] and Stuart I. Mannering[1,2]

Proinsulin-Specific, HLA-DQ8, and HLA-DQ8-Transdimer–Restricted CD4$^+$ T Cells Infiltrate Islets in Type 1 Diabetes

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Type 1 diabetes (T1D) develops when insulin-secreting β-cells, found in the pancreatic islets of Langerhans, are destroyed by infiltrating T cells. How human T cells recognize β-cell-derived antigens remains unclear. Genetic studies have shown that HLA and insulin alleles are the most strongly associated with risk of T1D. These longstanding observations implicate CD4$^+$ T-cell responses against (pro)insulin in the pathogenesis of T1D. To dissect the autoimmune T-cell response against human β-cells, we isolated and characterized 53 CD4$^+$ T-cell clones from within the residual pancreatic islets of a deceased organ donor who had T1D. These 53 clones expressed 47 unique clonotypes, 8 of which encoded proinsulin-specific T-cell receptors. On an individual clone basis, 14 of 53 CD4$^+$ T-cell clones (26%) recognized 6 distinct but overlapping epitopes in the C-peptide of proinsulin. These clones recognized C-peptide epitopes presented by HLA-DQ8 and, notably, HLA-DQ8 transdimers that form in HLA-DQ2/-DQ8 heterozygous individuals. Responses to these epitopes were detected in the peripheral blood mononuclear cells of some people with recent-onset T1D but not in HLA-matched control subjects. Hence, proinsulin-specific, HLA-DQ8, and HLA-DQ8-transdimer–restricted CD4$^+$ T cells are strongly implicated in the autoimmune pathogenesis of human T1D.

Type 1 diabetes (T1D) is an autoimmune disease caused by the CD4$^+$ and CD8$^+$ T-cell-mediated destruction of pancreatic insulin-producing β-cells (1). β-Cell destruction leads to primary insulin deficiency, which is treated by exogenous insulin therapy, and currently there is no cure. The pathogenesis of T1D has been well characterized using the NOD mouse model, but the immune basis of T1D in humans is less clear.

Genetic association studies have provided powerful insights into the etiology of human T1D (2,3). The HLA class II region has the strongest impact on risk of T1D. Some HLA alleles—DQB1*06:02 for example—dominantly protect against T1D (4). In contrast, of all alleles, HLA-DQ2 (DQA*05:01, DQB*02:01) and DQ8 (DQA*03:01, DQB*03:02) confer the greatest risk of developing T1D (5). Remarkably, individuals heterozygous for HLA-DQ2 and HLA-DQ8 are at greater risk of developing T1D than those

[1]Immunology and Diabetes Unit, St. Vincent's Institute of Medical Research, Fitzroy, Victoria, Australia
[2]Department of Medicine, University of Melbourne, St. Vincent's Hospital, Fitzroy, Victoria, Australia
[3]Department of Medicine, University of Adelaide, Adelaide, South Australia, Australia
[4]National Pancreas Transplant Unit, University of Sydney at Westmead Hospital, Sydney, New South Wales, Australia
[5]Department of Microbiology and Immunology, University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Parkville, Victoria, Australia
[6]University of Melbourne, NorthWest Academic Centre, Sunshine Hospital, St. Albans, Victoria, Australia
[7]Department of Paediatrics, Monash University, Clayton, Victoria, Australia
[8]Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia

Corresponding author: Stuart I. Mannering, smannering@svi.edu.au.

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T.W.H.K. and S.I.M. contributed equally to this work.

with either HLA-DQ2 or -DQ8 alone (6). The basis of this observation is unclear. However, HLA-DQ2 or -DQ8 heterozygous antigen-presenting cells (APCs) can form two extra functional HLA molecules: a DQ8 transdimer composed of the DQ2α chain paired with the HLA-DQ8β chain (DQA*05:01; DQB*03:02) and, similarly, a DQ2 transdimer in which the DQ8β chain pairs with DQ2α (DQA1*03:01; DQB1*02:01) (7). These transdimers may promote β-cell autoimmunity by presenting unique diabetogenic epitopes, or the high density of T1D-promoting HLA molecules (DQ2, DQ8, DQ2trans, and DQ8trans) may promote autoimmune $CD4^+$ T-cell responses against β-cell antigens (7,8). Because the epitopes presented by HLA-DQ2/-DQ8 transdimers have not been identified, these possibilities remain speculative. Nonetheless, the function of HLA-DQ is to present antigens to $CD4^+$ T cells; hence these genetic associations are strong evidence that $CD4^+$ T-cell responses against β-cell antigens play a pivotal role in the development of T1D in humans.

Because insulin-specific autoantibodies were detected before the onset of T1D (9), insulin and its precursor proinsulin have been candidate autoantigens in T1D. It is now clear that T-cell responses to (pro)insulin are essential for the development of T1D in the NOD mouse (10,11), but the role of (pro)insulin in human T1D has not been confirmed. Genetic association studies have implicated proinsulin because a T1D susceptibility locus, IDDM2, maps to a variable number of tandem repeats (VNTR) upstream of the insulin gene (12,13). This polymorphism is believed to modulate proinsulin expression in the thymus, affecting central tolerance (12–14). This observation provides circumstantial evidence that proinsulin plays a crucial role as an autoantigen in T1D. Many studies have attempted to detect proinsulin-specific $CD4^+$ T-cell responses in the peripheral blood mononuclear cells (PBMCs) of patients with T1D and healthy control subjects (15). Using sensitive methods capable of detecting rare T cells, some investigators detected weak responses to proinsulin peptides (16). However, these $CD4^+$ T cells could not be analyzed in detail. Furthermore, T cells isolated from the pancreatic lymph nodes of deceased organ donors who had T1D were reported to be insulin specific but HLA-DR4 restricted (17).

These genetic associations between HLA and insulin alleles predict that HLA-DQ2- and HLA-DQ8-restricted, proinsulin-specific $CD4^+$ T cells will infiltrate human islets, where they play a pathogenic role in T1D. Although predicted, these $CD4^+$ T cells have never been isolated from human islets, leaving a crucial piece missing from the chain of autoimmune causation in T1D. This gap in our knowledge has led some to question about the autoimmune basis of human T1D (18,19).

Here we show that $CD4^+$ T cells isolated from within the islets of an organ donor who had T1D recognize proinsulin epitopes presented by HLA-DQ8 and HLA-DQ8 transdimers. This work reveals how molecules—HLA-DQ8 and proinsulin—implicated by genetic association studies lead to autoimmune response against human β-cells and provides new evidence to support the notion that human T1D is an autoimmune disease.

RESEARCH DESIGN AND METHODS

Ethical Approvals

The isolation of pancreatic islets from deceased organ donors was approved by the St. Vincent's Hospital Human Research Ethics Committee (approval no. SVH HREC-A 011/04). Collection of peripheral blood samples from patients with T1D was approved by Southern Health Human Research Ethics Committee-B (approval no. 12185B). Collection of blood from healthy donors was approved by St. Vincent's Hospital Human Research Ethics Committee (approval no. SVH HREC-A 135/08).

Islet Isolation

After obtaining informed consent from the next of kin, the pancreas was removed from a brain-dead donor and sent to the Tom Mandel Islet Transplant Program. Islets were purified by intraductal perfusion and digestion of the pancreas with collagenase followed by purification using Ficoll density gradients, as previously described (20). Islet purity was assessed by light microscopy after staining with dithizone (Sigma Chemicals, St. Louis, MO). Thirty thousand islet equivalents were isolated from the tail of the pancreas. Approximately 1,000 islets were cultured in CMRL 1066 overnight then stained to detect T cells by flow cytometry. All the remaining islets were cultured directly after isolation in a 24-well plate in 1.0 mL of RPMI medium/5% pooled human serum containing interleukin (IL)-2 (20 U/mL) and IL-15 (5 ng/mL) for 7–10 days. After this time a halo of lymphocytes was visible surrounding 3–4 islets. Cells from wells containing growing T cells were collected and stained with anti-CD3-PerCP (UHCTI), CD4-phycoerythrin (OKT4), and anti-CD8α-Alexa Fluor 647 (OKT8). Dead cells were excluded with propidium iodide staining. $CD3^+/CD4^+$ cells were cloned by sorting a single $CD4^+$ T cell into wells of a 96-well plate containing irradiated allogeneic PBMCs, anti-CD3, and cytokines, as described elsewhere (21,22). After 2–3 weeks, growing clones were collected and subjected to another round of CD3-stimulated growth in the presence of IL-2 (20 U/mL) and IL-4 (5 ng/mL), as described previously (21,22). In most cases this expansion yielded >100 million cloned $CD4^+$ T cells. Aliquots of five million expanded T cells were cryopreserved in 10% DMSO/FCS, as described previously (23), and stored over liquid nitrogen until required.

Histology

Specimens from the head of the pancreas were taken before perfusion with collagenase and were snap frozen in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) Frozen sections were fixed in acetone for 5 min at room temperature and then washed in PBS. Endogenous peroxidases were quenched by incubating sections with 0.3% hydrogen peroxide (Merck, Whitehouse

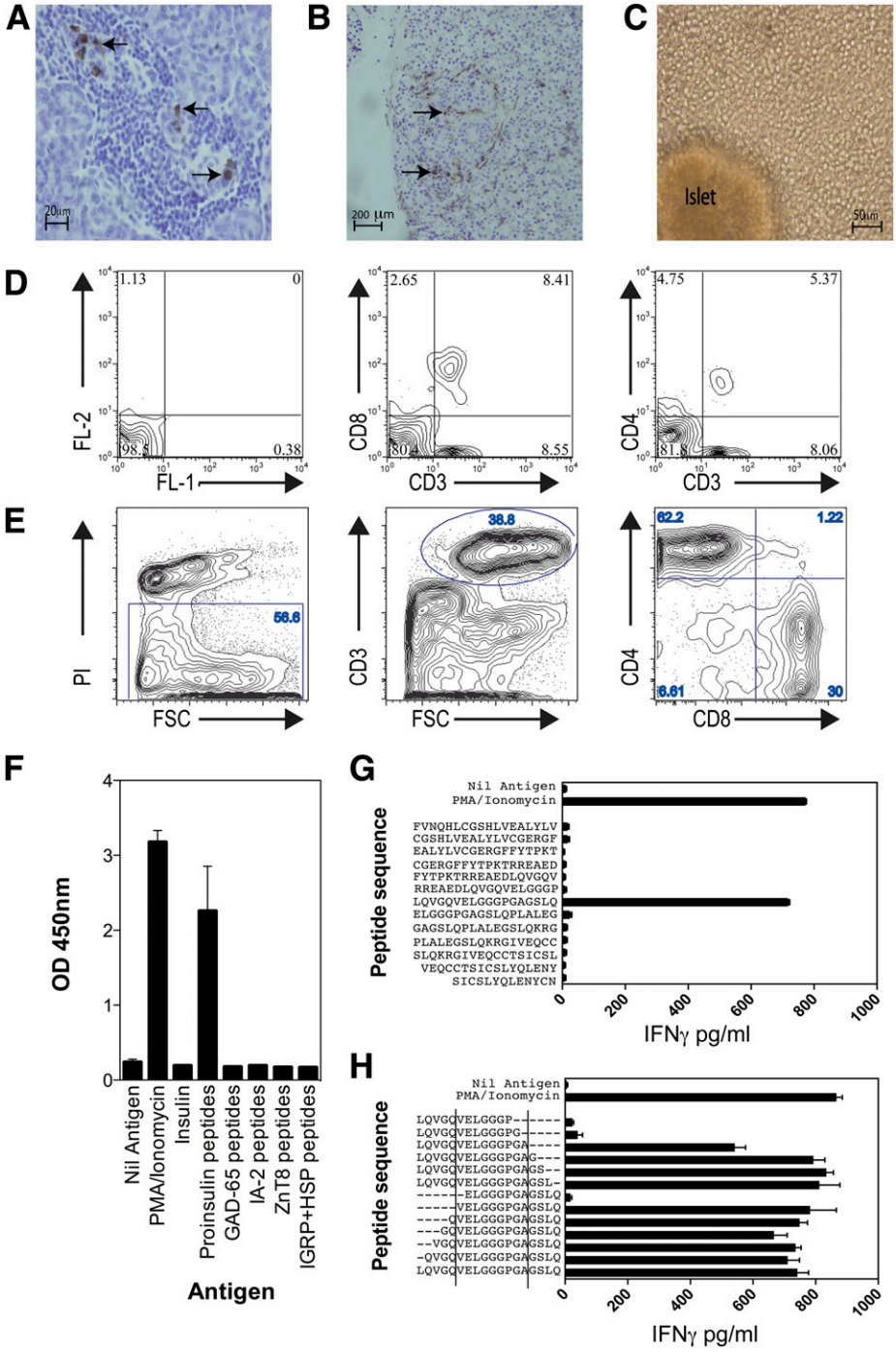

Figure 1—Isolation and analysis of islet-infiltrating CD4$^+$ T cells. *A*: A section of the subject's pancreas stained for insulin (brown), indicated by the arrows. *B*: A section of pancreas containing an islet infiltrated with CD4$^+$ cells. The CD4 staining is brown, and some CD4$^+$ cells are indicated by the arrows. *C*: A micrograph of an islet after 10 days of culture in IL-2 and IL-15. *D*: These graphs display the results from flow cytometry analysis, showing dispersed islets 12 h after isolation from the donor. From left to right, the plots show results of an unstained control, staining with anti-CD3-fluorescein isothiocyanate (*x*-axis) and anti-CD8-phycoerythrin (*y*-axis), and staining with anti-CD3 (*x*-axis) and anti-CD4 (*y*-axis). The percentage of each population are shown in each quadrant. *E*: These panels show the results of similar flow cytometry analysis of the cells that emerged after 10 days of culture. Dead cells were excluded by propidium iodide (PI) staining. PI$^-$/CD3$^+$ cells then were gated, and the percentage of CD3$^+$, CD4$^+$, and CD3$^+$/CD8$^+$ cells are shown. The percentage of gated cells for each population is shown in the regions (blue). A similar gating and staining strategy was used to clone these cells by single-cell sorting. Cloned islet-infiltrating CD4$^+$ T cells were screened for antigen specificity against a panel of peptides. *F*: Clones were tested in duplicate against pools of peptides. The mean optical densities (ODs) and SEM of an IFN-γ ELISA for one representative clone (6.15) are shown. HSP, heat shock protein; PMA, phorbol 12-myristate 13-acetate. *G*: "Coarse" epitope mapping using a panel of 18mer peptides overlapping by 12 amino acids for clone 6.15. *H*: "Fine" epitope mapping of clone 6.15. The mean IFN-γ concentrations and SEM of triplicate measurements are shown. A representative of duplicate experiments is shown. Similar results were obtained for the other proinsulin-specific clones (not shown).

Station, NJ) for 15 min. Nonspecific protein–binding sites were blocked by incubating sections with 2.5% milk powder and 10% FCS in PBS for 15 min. Sections were incubated with mouse anti-human CD4 or guinea pig anti-insulin and then horseradish peroxidase (HRP)-rabbit antimouse IgG or HRP–rabbit anti–guinea pig antibodies (Dako, Glostrup, Denmark), followed by staining with a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. HRP staining was detected with diaminobenzidine tablets (Sigma-Aldrich, St. Louis, MO), and sections were counterstained with hematoxylin. To confirm labeling specificity, tissue sections were incubated with isotype antibodies, no primary antibody, or diaminobenzidine alone (controls). Sections were viewed on a Leitz Laborlux K microscope and photos were taken with an Olympus CKX41 camera.

Synthetic Peptides

Peptides were synthesized by GL Biochem (Shanghai, China) using 9-fluorenylmethoxycarbonyl chemistry. Peptides were purified by reversed-phase high-performance liquid chromatography to at least 85% purity and lyophilized. Peptides were reconstituted in 40% acetonitrile, 0.5% acetic acid, and water to 5 mmol/L, aliquoted, and stored at −80°C. A complete list of peptides is shown in Supplementary Tables 4–9.

T-Cell Assays

Cloned CD4$^+$ T cells were thawed and used directly in functional assays. To identify the epitopes recognized by the cloned CD4$^+$ T cells, they were incubated with a class II HLA-matched Epstein Barr virus–transformed B-cell line (KJ; HLA-DRB1*03:01, 04:04; DQB1*02:01, 03:02) and synthetic peptides that mimic the entire sequence of human proinsulin or a total of 26 peptides reported to be epitopes recognized by CD4$^+$ T cells from patients with T1D (Supplementary Tables 4–9). Cloned CD4$^+$ T cells (10–50,000/well) were cultured with 5–10,000 APCs with and without peptides, as indicated in the figures. The response of a T cell to antigen was measured as interferon (IFN)-γ secretion into the culture media. The IFN-γ concentration in the culture media was determined by ELISA (BioLegend, San Francisco, CA). In preliminary screening experiments an IFN-γ standard curve was omitted; in these cases the results are plotted as optical densities at 450 nm.

HLA Restriction

HLA restriction was determined in two steps, as described previously (24–26). First, monoclonal antibodies specific for HLA-DR (clone L243), -DP (clone B7/21), and -DQ (clone SPV-L3) were added to the peptide-stimulated T-cell cultures to a final concentration of 1–10 μg/mL. Second, the HLA alleles were determined using a panel of T2 cells transduced with a retrovirus encoding for HLA-DQA and -DQB alleles (specific alleles are indicated in the figure legends).

TCR Sequencing

RNA was extracted from one to two million cloned T cells using an RNA extraction kit (Macherey Nagel, Duren,

Table 1—Summary of the epitope mapping of human islet-infiltrating CD4$^+$ T-cell clones

Proinsulin (B-chain: 1–30; C-peptide: 31–65; A-chain: 66–86)	HLA[a]	No. of clones (clone names)
FVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKR**GIVEQCCTSICSLYQLENYCN**		
VELGGGPGA	DQ8	4 (1.9, 2.1, 3.14, 4.6)
VELGGGPGA	DQ8	3 (5.8, 5.9, 6.15)
VELGGGPGA	DQ8	1 (2.13)
QVELGGGPGAG	DQ8	1 (4.13)
QVELGGGPG	DQ8	1 (5.5)
AGSLQPLALE	DQ8	1 (1.1)
AGSLQPLAL	DQ8	1 (1.2)
SLQPLALEGSL	DQ8trans	2 (2.4, 3.15)

Boldface indicates parts of a chain. [a]DQ8 = HLA-DQA1*03:01; DQB1*03:02; DQ8trans = HLA-DQA1*05:01; DQB1*03:02.

Germany) and converted to cDNA using SMARTScribe Reverse Transcriptase (Clontech, Mountain View, CA). TRA and TRB genes were amplified using pools of reverse primers using Taq polymerase (Invitrogen, Mulgrave, Victoria, Australia), as described by Wang et al. (27). PCR product was sequenced and the T-cell receptor (TCR) genes identified by alignment with the IMGT database (www.imgt.org/IMGT_vquest/vquest) (28).

Insulin VNTR Genotyping

Genomic DNA was purified from five million cloned T cells using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genotyping of the single nucleotide polymorphism, rs689, was performed using an inventoried TaqMan single nucleotide polymorphism genotyping assay obtained from Applied Biosystems (Life Technologies, Carlsbad, CA). The assay was run on a Roche LightCycler 480 II using Roche Probe Master Mix (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions.

CFSE Proliferation Assays

The CFSE (5,6-carboxylfluorescein diacetate succinimidyl ester) proliferation assays were performed as described previously (15,22). Briefly, PBMCs were labeled with 0.1 μmol/L CFSE (Invitrogen, Grand Island, NY) and cultured (0.5×10^6 cells in 0.5 mL) in sterile 5.0-mL tubes. CFSE-labeled PBMCs were cultured with either no antigen, proinsulin 40–52 peptide, proinsulin 48–62 peptide, or tetanus toxoid (10 LfU/mL). After 7 days of culture the cells were washed in PBS and stained on ice with anti-human CD4-Alexa Fluor 647 (clone OKT4, conjugated in house). Optimal compensation and gain settings for the flow cytometer for each experiment were determined based on unstained and single-stained samples. Propidium iodide was used to exclude dead cells. $CD4^+$ T-cell proliferation was measured by determining the number of $CD4^+/CFSE^{dim}$ cells for every 5,000 $CD4^+/CFSE^{bright}$ cells. The results are presented as a cell division index (CDI), which is the ratio of the number of $CD4^+$ cells that proliferated in the presence of antigen to the number that proliferated without antigen, as described previously (29).

RESULTS

To characterize human $CD4^+$ T-cell responses associated with T1D, we obtained the pancreas from an organ donor who suffered from T1D. The organ donor was diagnosed with T1D at the age of 16 years. At diagnosis his blood glucose was 612 mg/dL (34 mmol/L; normal, 4.0–6.0 mmol/L) and C-peptide was 1.2 ng/mL (0.4 nmol/L). He had autoantibodies to GAD-65 and tyrosine phosphatase–related islet antigen 2. He was treated with short- (insulin glulisine) and long-acting insulin (insulin detemir). Death occurred from hypoglycemia 3 years after diagnosis, and organs were retrieved for donation after ~24 h in critical care. Before organ donation his blood glucose was 144 mg/dL (8.0 mmol/L) and C-peptide was 0.3 ng/mL (0.1 nmol/L). His HLA type was HLA-A*01:01, 02:01; B*08:01, 51:01; DRB1*03:01, 04:04; DQA1*03:01, DQB1*03:02; DQA1*05:01, DQB1*02:01. Genetic analysis of the insulin promoter revealed that the donor was homozygous for the allele predisposing to T1D (class I VNTR).

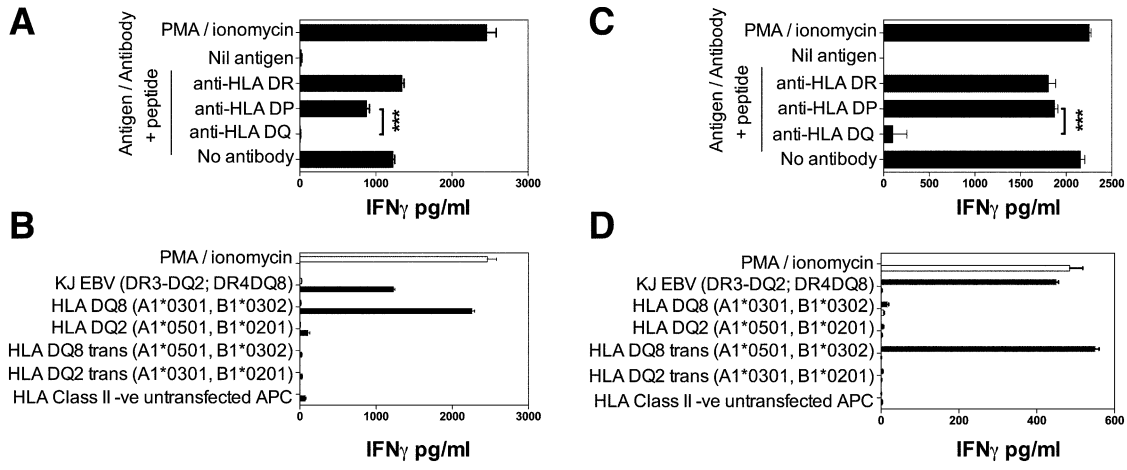

Figure 2—Analysis of the clone's HLA restriction. Antibodies against HLA-DR, -DP, and -DQ were included in the peptide stimulation assays. *A* and *B*: The HLA restriction analysis of clone 6.15. *A*: Responses to proinsulin peptide are inhibited by a monoclonal antibody to HLA-DQ (clone SPV-L3), but not HLA-DP (clone B7/21) or HLA-DR (clone L243) at 1.0 μg/mL. Anti-HLA-DQ significantly inhibited IFN-γ secretion (***$P < 0.0001$, two-tailed *t* test). *B*: IFN-γ secretion in response to antigen-presenting cells (APC; T2) expressing the HLA molecules indicated. *C* and *D*: HLA restriction analysis for clone 3.15. *C*: This clone's response to proinsulin peptide (proinsulin 43–60) was also significantly blocked by a monoclonal antibody to HLA-DQ (***$P < 0.0001$, two-tailed *t* test). *D*: Clone 3.15 (and clone 2.4; see Supplementary Figs. 3 and 4), responded to proinsulin peptide presented by antigen-presenting cells that expressed the HLA-DQ8 transdimer (DQA1*05:01, DQB1*03:02). All graphs show the mean of triplicate IFN-γ concentration measurements, and the bars represent the SEM. Each experiment was performed at least twice, with similar results. PMA, phorbol 12-myristate 13-acetate.

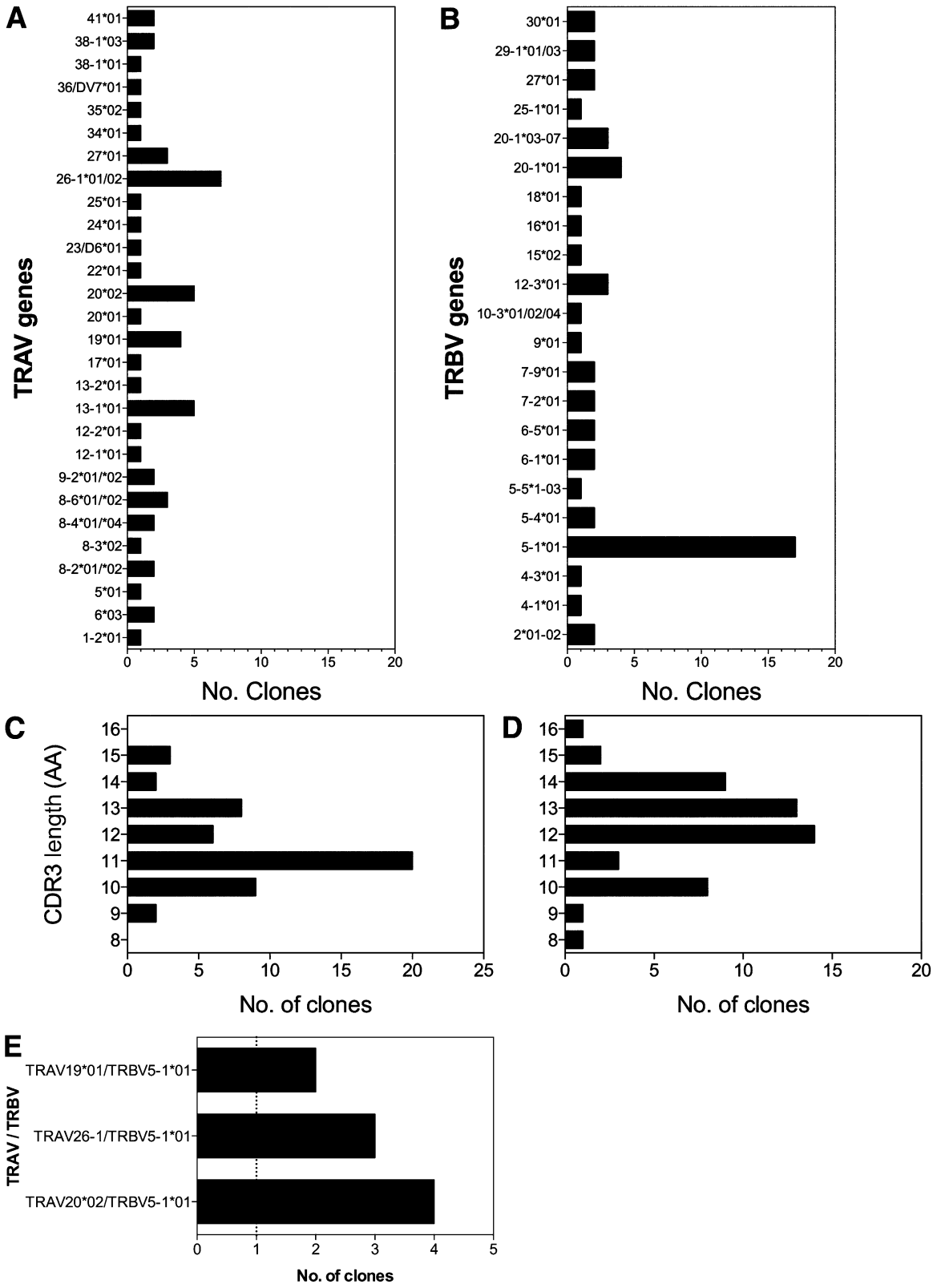

Figure 3—Analysis of the islet-infiltrating CD4+ T-cell clone's TCR gene usage. The TCR genes are from each of 53 islet-infiltrating $CD4^+$ T cells. Sequences were analyzed using IMGT/V-quest, and the number of clones expressing a *TRAV* (*A*) or *TRBV* gene (*B*) are plotted. Each gene was sequenced at least twice. The CDR3 regions were identified, and the TCRs with a CDR3 region of each length is plotted for both TRAV (*C*) and TRBV (*D*). *E*: Three groups of clones with identical TCRs were identified. The graph shows the *TRA* and *TRB* genes on the vertical *x*-axis and the number of clones that express these TCRs on the horizontal *y*-axis. The TCR gene sequences are shown in Supplementary Spreadsheet 1.

Histological analysis of the donor's pancreas revealed a wide range of islet appearance, including apparently normal, heavily infiltrated with residual insulin, and pseudoatrophic islets without insulin or infiltrate. Of the islets, 39% (143 of 371) had residual insulin staining; 70% (100 of 371) of insulin-positive islets were infiltrated by one or more T cells, and 25% of insulin-positive islets were infiltrated by >15 T cells (Fig. 1*A*). The infiltrate included $CD4^+$ and $CD8^+$ T cells (Fig. 1*B*), which was confirmed by flow cytometry (Fig. 1*D*). Thirty thousand islet equivalents were isolated to >90% purity. After overnight culture in CMRL 1066, the islets were cultured in RPMI medium/pooled human serum with IL-2 and IL-15. After 1 week cells were visible around 3–4 islets (Fig. 1*C*). These cells were $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells (Fig. 1*E*), which were cloned and grown in vitro (21,26). No cells grew with islets isolated from two organ donors without T1D.

We isolated 62 $CD4^+$ T-cell clones, of which 53 could be characterized in detail. Their antigen specificity was investigated by testing against a panel of synthetic peptides. All clones produced IFN-γ in response to phorbol 12-myristate 13-acetate/ionomycin stimulation, except four clones (1.5, 5.1, 4.16, and 5.8). An example of one clone (6.15) that responded to the pool of proinsulin peptides is shown (Fig. 1*F–H*). Of 53 clones, 14 (26.4%) responded to proinsulin peptides (Supplementary Fig. 1), but none of the clones responded to any of the other epitopes tested (data not shown). Clones that responded to the pool of 13 proinsulin peptides (Fig. 1*F*) were retested separately against each peptide. Ten clones, including 6.15, responded to proinsulin 37–54 (Fig. 1*G*). The remaining clones responded to proinsulin 43–60 or proinsulin 49–66. Fine epitope mapping (Fig. 1*H*) revealed that all 10 clones that responded to proinsulin 37–54 recognized the same core epitope (proinsulin 42–50, VELGGGPGA), but differences in their fine specificity were noted (Table 1 and Supplementary Fig. 2). Clone 4.13 responded to a slightly longer epitope (proinsulin 41–51, QVELGGGPGAG), and clone 5.5 responded to an epitope shifted one amino acid to the N-terminus (proinsulin 41–49, QVELGGGPG). Four clones specific for proinsulin 43–60 (1.1, 1.2, 2.4, and 3.15) recognized three distinct but overlapping epitopes: clone 1.1 recognized proinsulin 50–59 (AGSLQPLALE), 1.2 recognized proinsulin 50–58 (AGSLQPLAL), and clones 2.4 and 3.15 both recognized proinsulin 52–62 (SLQPLALEGSL) (Table 1 and Supplementary Fig. 2).

We next characterized the HLA restriction of the proinsulin-specific clones. The donor had the high T1D-risk HLA type: HLA-DR3,-DR4;-DQ2-DQ8. The responses of all 14 clones to proinsulin peptides were inhibited by a monoclonal antibody specific for HLA-DQ. Two examples, clone 6.15 (Fig. 2*A*) and clone 3.15 (Fig. 2*C*), are shown. (Other clones are shown in Supplementary Fig. 3.) The restricting HLA alleles were determined using a panel of cell lines transduced with individual HLA-DQ genes. Twelve clones responded to proinsulin peptide presented by HLA-DQ8 (DQA1*03:01, DQB1*03:02). Clone 6.15 is shown as an example (Fig. 2*B*). Clones 2.4 and 3.15 did not respond to HLA-DQ8 (DQA1*03:01, DQB1*03:02), but they responded to proinsulin peptides when presented by an HLA-DQ8 transdimer (DQA1*05:01; DQB1*03:02). Clone 3.15 is shown as an example in Fig. 2*D*. Data for all clones are shown in Supplementary Fig. 4. One clone (1.2) responded strongly to peptide presented by HLA-DQ8 but also responded to the same peptide presented by the HLA-DQ8 transdimer (DQA1*05:01; DQB1*03:02). Hence, all islet-infiltrating, proinsulin-specific $CD4^+$ T-cell clones are restricted by HLA-DQ8 or the HLA-DQ8 transdimer.

To examine the clonal diversity of the islet-infiltrating $CD4^+$ T cells, the TCR genes expressed by the clones were sequenced. The clones used a range of *TRAV* and *TRBV* genes (Fig. 3*A* and *B*). The most frequently expressed *TRAV* gene was *TRAV26–1*, which was expressed by 7 of 53 clones (13%). In contrast, usage of *TRBV* genes was more skewed; *TRBV5–1**01 was expressed by 16 of the clones (30%). Translated TCR sequences for all clones are shown in Supplementary Table 3. No skewing of the CDR3 length was detected for *TRAV* and *TRBV* genes (Fig. 3*C* and *D*). In total, 47 unique clonotypes were expressed. We identified three sets of sister clones with identical TCRs (Fig. 3*E*). The first set comprised four clones: 1.9, 2.1, 3.14, and 4.6. These clones all used *TRAV20**02 and *TRBV5–1**01 and recognized proinsulin 42–50. The second set comprised three clones (5.8, 5.9, and 6.15) that used *TRAV26–1**01 and *TRBV5–1**01 and recognized proinsulin 42–50. The final set comprised two clones (2.4 and 3.15) that used *TRAV19**01 and *TRBV5–1**01 and responded to proinsulin 52–62. The remaining 44 clones expressed unique TRA/TRB combinations. The CDR3 DNA sequences of the proinsulin-specific TCRs are shown in Table 2.

Analysis of the clones' antigen specificity showed that the clones isolated more than once all recognized proinsulin epitopes. Interestingly, the TCRβ chain, *TRBV5–1**01, was used by four of five clone families specific for epitopes falling in proinsulin 40–51. Three of four *TRVB5–1**01-expressing clones also expressed *TRAV26–1**01. Collectively, the 14 clones specific for proinsulin C-peptide epitopes used eight distinct TRA/TRB combinations (Table 2). Although several clones used the same *TRAV* and *TRBV*, there was no evidence of conserved amino acids in CDR3 (Table 2 and Supplementary Table 3). This analysis revealed that several independent, proinsulin-specific clones infiltrated the islets of this donor.

Finally, we used a CFSE-based proliferation assay (29) to determine whether $CD4^+$ T-cell responses to these defined epitopes were detectable in PBMCs from HLA-$DQ8^+$ individuals with recent-onset (<3 months) T1D. Two of eight subjects had $CD4^+$ T-cell responses (CDI ≥2.0) to proinsulin 40–52, and three of eight had responses to proinsulin 48–62 (Fig. 4*A* and *B*). No responses were detected from six HLA-$DQ8^+$ subjects without T1D. $CD4^+$ T-cell proliferation to tetanus toxoid was detected in all donors (Supplementary Tables 1 and 2). This concurs

with previous reports using longer peptides, showing that $CD4^+$ T-cell responses to this region of the C-peptide are detectable in the PBMCs of people with T1D (16).

DISCUSSION

We made the following discoveries from our analysis of the islet-infiltrating $CD4^+$ T cells from the pancreas of a deceased organ donor who had T1D: *1*) $CD4^+$ T-cell responses were directed against six distinct but overlapping epitopes from the C-peptide of proinsulin; *2*) all proinsulin-specific islet-infiltrating $CD4^+$ T-cell clones recognized peptides presented by HLA-DQ; *3*) a pair of clones responded to a proinsulin epitope presented by the HLA-DQ8 transdimer; and *4*) most (five of eight) proinsulin-specific clones expressed *TRBV5–1**01.

To our knowledge, this is the first report to reveal that HLA-DQ8-restricted, proinsulin-specific $CD4^+$ T cells infiltrated the pancreatic islets of a patients with T1D. More than one in four of the clones were specific for proinsulin, but we did not detect any clones specific for insulin or any clones specific for any of the other 26 epitopes tested. Our results suggest that HLA-DQ-restricted $CD4^+$ T cells specific for proinsulin are abundant within the islets and central to the pathogenesis of T1D. The absence of insulin-specific T-cell clones is consistent with reports that HLA-DQ-restricted regulatory T-cell responses toward insulin are detectable in individuals with T1D and their unaffected siblings (30).

The donor's homozygous type 1 insulin VNTR genotype may have promoted the broad T-cell response against proinsulin. HLA-DR4-restricted $CD4^+$ T cells specific for proinsulin 52–66 were most frequently detected in the blood of subjects who were VNTR I homozygous (14). Our analysis of the TCR genes expressed by human islet-infiltrating $CD4^+$ T cells revealed that the 53 clones expressed 47 unique clonotypes. We cannot exclude the possibility that the isolation of multiple clones with unique TCRs arose from skewing of the repertoire of infiltrating T cells occurred while the islets were cultured in the presence of cytokines, rather than in vivo before islet isolation. Nonetheless, eight unique proinsulin-specific clonotypes were present, arguing against the possibility that proinsulin-specific $CD4^+$ T cells were grossly favored during the isolation.

The T cells we isolated were not selected based on their antigen specificity, yet the epitopes recognized fall in a narrow region of the C-peptide of proinsulin. Several clones recognize epitopes containing the sequence—GGGPGA (see Table 2)—which is rich in amino acids with very small side chains. It remains to be determined whether this very "bland" epitope is recognized by $CD4^+$ T cells with degenerate specificity, as has been reported for proinsulin-specific $CD8^+$ T cells (31). This degenerate specificity may allow these proinsulin-reactive T cells to escape thymic deletion yet become activated in the islets, where the concentration of proinsulin is very high. The antigen specificity of many of the clones was

Table 2—Comparison of TCR sequences from proinsulin-specific islet-infiltrating $CD4^+$ T-cell clones

Clone	TRAV	TRAJ	TRA CDR3	CDR3§	TRBV	TRBJ	TRBD	TRB CDR3	CDR3§	Epitope HLA
1.9*	20*02	7*01	CAVQAGGNNRLAF	11	5–1*01	1–2*01	1*01	CASSLERDGYTF	10	VELGGGPGA DQ8
6.15†	26–1*01	21*01	CIAIYNFNKFYF	10	5–1*01	1–6*02	2*01	CASSLEASSYNSPLHF	14	VELGGGPGA DQ8
2.13	26–1*01	39*01	CIVSHNAGNMLTF	11	5–1*01	2–5*01	2*01	CASSLERETQYF	10	VELGGGPGA DQ8
4.13	6*02	36*01	CALKYGANNLFF	10	18*01	1–1*01	1*01	CASSPTTGGDEAFF	12	QVELGGGPGAG DQ8
5.5	26–1*01	54*01	CIVRVEIQGAQKLVF	13	5–1*01	2–5*01	1*01	CASSLGPGQRETQYF	13	QVELGGGPG DQ8
1.1	25*01	16*01	CAGGFSDGQKLLF	11	20–1*03–07	2–7*01	1*01	CSARTEAYEQYF	10	AGSLQPLALE DQ8
1.2	20*02	58*01	CAVIETSGSRLTF	11	20–1*01	2–3*01	2*01	CSARDQQRVDTQYF	12	AGSLQPLAL DQ8
2.4‡	19*01	49*01	CALSRAGTGNQFYF	12	5–1*01	2–4*01	2*01	CASSLGLRGENIQYF	13	SLQPLALEGS DQ8trans

*Four clones have these identical TCR sequences; a single example is shown. †Three clones have these identical TCR sequences; a single example is shown. ‡Two clones have these identical TCR sequences; a single example is shown. §Number of amino acids comprising the CDR3.

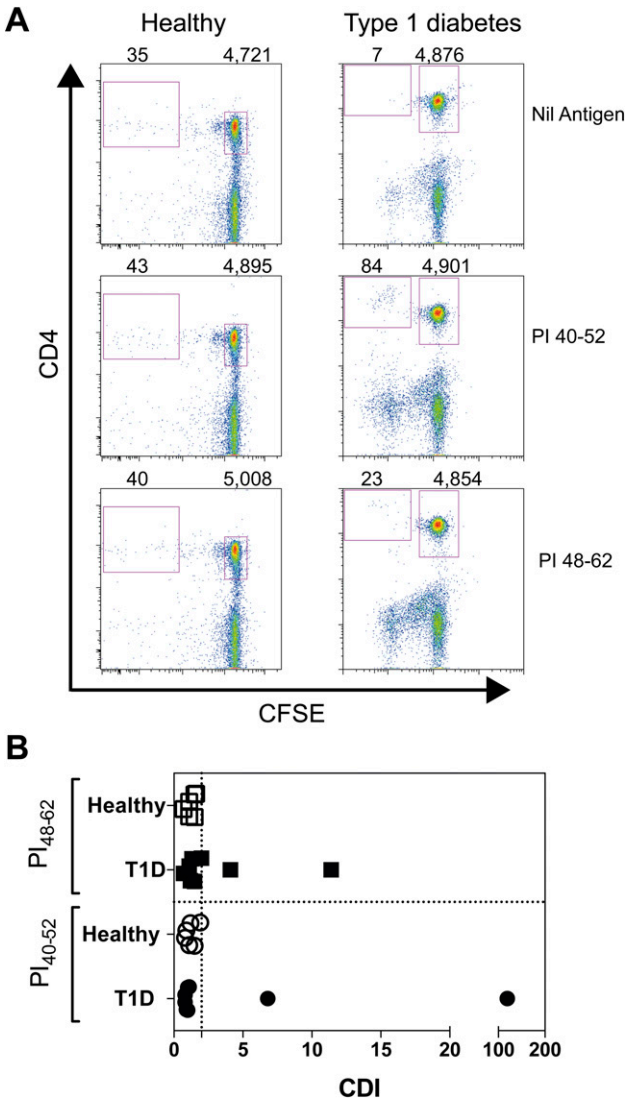

Figure 4—CD4$^+$ T-cell responses to proinsulin peptides in peripheral blood mononuclear cells. *A*: Two groups were tested: PBMCs from individuals without T1D ("healthy") and PBMCs from donors with recent-onset (<3 months) T1D ("type 1 diabetes"). A CFSE-based proliferation assay was used to detect proinsulin-specific CD4$^+$ T-cell responses. Flow cytometry was performed using a BD LSRFortessa. A representative plot, gated on viable (propidium iodide negative) lymphocytes stained with Alexa Fluor 647 anti-CD4 (clone OKT4; conjugate in house) and CFSE, is shown. Two gates are shown in each plot. The upper right-hand gate includes CD4$^+$ CFSE^{bright} cells that did not proliferate. The number of events in this gate is shown above the plot. The second gate included the CD4$^+$, CFSE^{dim} cells that did proliferate. The number of events in this gate is shown above the plot. CD4$^+$ T-cell proliferation is quantified by standardizing the number of CD4$^+$, CFSE^{dim} events for each sample to 5,000 CD4$^+$, CFSE^{bright} events (29). The standardized number of CD4$^+$ CFSE^{dim} events is expressed as a ratio, known as the CDI of the number of events with antigen (proinsulin [PI] 40–52 or proinsulin 48–62) to the number of events without antigen ("nil antigen"). Each antigen, including nil, was tested in triplicate. *B*: A summary of responses to proinsulin peptides. The mean (of triplicate) CDI for responses to proinsulin 40–52 and proinsulin 48–62 (both peptides were tested at 10 μM) for eight HLA-DQ8$^+$ subjects with recent-onset (<3 months) T1D and six HLA-DQ8$^+$ subjects who do not have T1D are shown. The differences between responses from donors with and without T1D did not reach statistical significance ($P > 0.05$, unpaired *t* test with Welch's correction). The dotted vertical line is a CDI of 2.0, which is the cutoff between positive and negative responses. All subjects' CD4$^+$ T cells responded to tetanus toxoid in these assays.

not defined. Most of these clones may respond to other as yet undefined β-cell antigens. It remains possible that the four clones that did not make IFN-γ respond to some of the peptides tested here but were not detected because they secrete cytokines other than IFN-γ.

There have been few previous reports of HLA-DQ8-restricted responses against β-cell antigens (32). All 14 T-cell clones reported in this study recognized proinsulin peptides presented by HLA-DQ8 or HLA-DQ8 transdimer. There was no evidence of a unique HLA-DQ8-transdimer–restricted epitope; the HLA-DQ8-transdimer–restricted epitope overlaps almost completely with two HLA-DQ8-restricted epitopes. Hence, these data support the hypothesis that HLA-DQ2; DQ8 heterozygous individuals are at the greatest genetic risk of T1D because they have a relatively high density of T1D-associated HLA molecules on the surface of their APCs.

Pancreatic sections from the donor studied here were included in the analysis of human islet-infiltrating CD8$^+$ T cells by Coppieters et al. (33). This analysis revealed that HLA-A2-restricted CD8$^+$ T cells specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), GAD-65, preproislet amyloid polypeptide, and preproinsulin were present in this donor's islets. It is believed that HLA class I–restricted CD8$^+$ T cells that recognize β-cell autoantigens are responsible for β-cell destruction (34). In the NOD mouse model, spreading of the CD8$^+$ immune response from proinsulin to IGRP only requires CD4$^+$ T cells specific for proinsulin (11), which could also be the case in humans. Identification of HLA-DQ8-restricted epitopes strongly implicated in β-cell autoimmunity will facilitate the analysis of CD4$^+$ T-cell responses in T1D.

We did not attempt to characterize the memory/activation status of the islet-infiltrating CD4$^+$ T cells. To obtain sufficient cells to characterize, we stimulated the T cells with anti-CD3 in the presence of cytokines. This treatment activates the cells and drives their proliferation, precluding an analysis of their activation status. We expect these cells would have an effector memory phenotype in vivo, consistent with their role in mediating β-cell destruction. This issue could be addressed using HLA-DQ8/C-peptide tetramers to stain proinsulin-specific CD4$^+$ T cells, combined with antibody staining for activation/memory markers. However, this analysis will be limited by the small number of cells that can be recovered from human islets.

Here we report an analysis of islet-infiltrating CD4$^+$ T cells from a single donor. Further work will be required to determine the variability of islet-infiltrating CD4$^+$ T cells in different individuals. Analysis of T-cell responses in

PBMCs underscores the variability inherent in human studies. This presumably reflects differences in the specificity of pathogenic $CD4^+$ T-cell response between individuals. The capacity to detect $CD4^+$ T-cell responses may allow antigen therapy to be targeted toward individuals who have an existing response to these epitopes.

Antigen-specific therapies are a very attractive approach to treating T1D. Toward this goal, an 18mer proinsulin peptide (proinsulin 51–68), encompassing the epitopes recognized by clones 1.1, 1.2, 2.4, and 3.15, has been tested in a phase 1 human trial (35). Our work, showing that proinsulin-specific $CD4^+$ T cells are a major component of the islet $CD4^+$ T-cell infiltrate, adds further support to the notion that $CD4^+$ T cells against proinsulin are pathogenic and therefore are attractive candidates for use in antigen-specific therapy protocols.

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