



One in Ten CD8⁺ Cells in the Pancreas of Living Individuals With Recent-Onset Type 1 Diabetes Recognizes the Preproinsulin Epitope PPI₁₅₋₂₄

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In type 1 diabetes (T1D), a lifelong autoimmune disease, T cells infiltrate the islets and the exocrine pancreas in high numbers. CD8⁺ T cells are the main cell type found in the insulitic lesion, and CD8⁺ T cells reactive against β-cell antigens have been detected in peripheral blood and in the pancreas of patients with short- or long-term disease. In the Diabetes Virus Detection (DiViD) study, researchers collected pancreatic tissue, by pancreatic tail resection, from living patients with recent-onset T1D. These tissues have been extensively studied by the scientific community, but the autoreactive nature of the T-cell infiltrate has remained unexplored. Our objective was to determine the number and localization of these cells in pancreas samples obtained through the DiViD study. Here, we demonstrate the presence of high frequencies of CD8⁺ T cells reactive against a highly relevant epitope derived from the preproinsulin signal peptide in pancreatic tissue samples from these donors. We also show the heterogeneity of islet distribution and CD8⁺ T-cell infiltration. Our findings contribute to the current limited existing knowledge of T-cell reactivity in the pancreas of donors with recent-onset T1D and indicate that antigen-specific therapies directed toward preproinsulin could have high clinical impact.

Researchers involved in the Diabetes Virus Detection (DiViD) study collected samples of pancreatic tissue by

pancreatic tail resection from six living, adult individuals newly diagnosed with type 1 diabetes (T1D) (1). These pancreatic biopsy specimens were laparoscopically obtained 3–9 weeks after diagnosis of T1D. The DiViD study was the first of its kind, and pancreatic tissue from all the individuals has been widely shared with the scientific community. Numerous studies of these extremely valuable samples have provided seminal contributions to our understanding of T1D. Notably, a detailed analysis of these samples showed the presence of a high number of remaining insulin-containing islets often in proximity to insulin-deficient islets (2). These islet pools were highly heterogenous; some seemed to be unaffected by the disease process, whereas others presented evident peri-islet and intraislet insulinitis (2). This observation confirmed, on one hand, the presence of apparently intact β-cell mass left shortly after diagnosis and, on the other hand, the heterogenous distribution of the insulitic lesion in humans.

CD8⁺ T cells are the most abundant cell type in islets with insulinitis and are associated with β-cell destruction (3). In the human diabetic pancreas, CD8⁺ T cells infiltrate the exocrine tissue as well as the islets (4), where they are most frequently found in the periphery of, rather than within, the islet parenchyma. Complete understanding of their phenotype, specificity, and function is still lacking. CD8⁺ T cells reactive against β-cell antigens can be detected in the blood of patients with T1D. However, similar cell

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frequencies of autoreactive T cells have been reported in people without diabetes (5), further underlining the importance of identifying these cells in the target tissue, the pancreas. Previous studies have demonstrated the presence of cells reactive against β -cell antigens in infiltrates from isolated islets (6) and in the pancreas from organ donors with short and long duration of T1D (7,8). These cells seem to be antigen experienced and sequestered in the pancreas in high numbers in patients with T1D (5,8).

Preproinsulin (PPI) is considered a prominent source of epitopes for multiple HLA class I alleles and a major autoantigen in T1D (9). β -cells undergo substantial endoplasmic reticulum stress in response to glucose stimulation (10), increasing PPI translation 50-fold and reaching a production rate of 1 million PPI molecules/min (11). This increase in production upregulates PPI presentation, increasing the likelihood of immune recognition in the context of autoimmunity (12). Consequently, cloned PPI-specific CD8⁺ T cells can kill human β -cells in vitro, especially if they are exposed to high glucose concentrations (12). We and others have described the presence of β -cell reactive CD8⁺ T cells in the pancreas of individuals with T1D (7,8,13). However, until recently, to our knowledge, the number and distribution of these cells had not been studied. Therefore, our objective was to determine the number and localization of these cells in pancreas samples obtained through the DiViD study. Here, we demonstrate the presence of high frequencies of CD8⁺ T cells reactive against a highly relevant epitope derived from the PPI signal peptide in the pancreas of living individuals with recent-onset T1D.

RESEARCH DESIGN AND METHODS

Patient Characteristics

Four individuals (DiViD patients 1, 2, 4, and 5; age range, 24–31 years) were included in this study. Patients were selected on the basis of the presence of the HLA-A*02:01 haplotype. Detailed information is listed in Table 1. Pancreatic biopsy samples were collected 3–5 weeks after diagnosis of T1D as part of the DiViD study (1). HbA_{1c} at biopsy ranged from 6.7% to 10.3%. All donors were autoantibody-positive as shown in Table 1. Previous basic histologic characterization showed the presence of remaining insulin-containing islets, some of them with insulinitis. The DiViD study was approved by the Norwegian Government's Regional Ethics Committee (Oslo, Norway) and written informed consent was obtained from participants. All experimental procedures were approved by the La Jolla Institute for Immunology Institutional Review Board (protocol no. DI-054–0218).

Tissue Processing and Immunofluorescence Staining

Pancreatic tissues were embedded in optimal cutting temperature compound, and 5 μ m-thick sections were processed and labeled using in situ tetramer and immunofluorescence staining. Tetramers were obtained from the Fred Hutchinson Cancer Research Center, Immune

Monitoring Core (Seattle, WA). Quality control of the tetramers was done using peripheral blood mononuclear cells spiked with cytomegalovirus (CMV)_{495–503} or PPI_{15–24} T-cell clones and have been shown elsewhere (13). Tyramide Signal Amplification was used for tetramer visualization (Thermo Fisher Scientific, Waltham, MA). Briefly, 1 μ g of allophycocyanin (APC)-labeled tetramer was diluted in 500 μ L of PBS and then spun at 15,800g to avoid aggregation. After overnight incubation at 4°C and gentle washing, tissue sections were fixed with 1% paraformaldehyde for 15 min at room temperature and incubated with 3% H₂O₂ for 15 min. Sections were then washed and blocked with 10% goat serum for 30 min at room temperature.

After washing, sections were incubated with a purified mouse-anti-APC antibody (1/50, clone APC003; BioLegend) and rabbit-anti-glucagon (1/300; ab10988; Abcam) for 1 h at room temperature. Then, sections were washed and incubated with poly-goat-anti-mouse-horseradish peroxidase (TSA SuperBoost Kit; Thermo Fisher Scientific) and goat-anti-rabbit Alexa Fluor 488 (1/1,000; catalog no. A11070; Thermo Fisher Scientific). After a blocking step using 2% mouse serum, sections were washed and incubated with mouse-anti-human CD8 conjugated in house to Alexa Fluor 555 (1/100; catalog no. 555631; BD). Sections were then washed and incubated with tyramide-Alexa Fluor 647 following the manufacturer's instructions (TSA SuperBoost Kit; Thermo Fisher Scientific). Finally, sections were counterstained with Hoechst (1/5,000; Life Technologies) and mounted with Prolong Gold antifade mounting medium (Invitrogen, Carlsbad, CA).

In total, five consecutive sections were used to detect antigen-specific cells against PPI (PPI_{15–24}), CMV (CMV_{495–503}), Epstein-Barr virus (EBV; EBV_{426–434}), influenza A virus (FLU; FLU matrix protein 58–66), and the enterovirus coxsackievirus B3 (EV; EV_{1,587–1,596}). An additional section was included as a negative control and incubated without tetramer. Stained tissue sections were scanned using a Zeiss AxioScan.Z1 slide scanner and images were analyzed using Zen software (Zeiss, Jena, Germany).

Data and Resource Availability

The data sets generated during and/or analyzed during this study are available from the corresponding author upon reasonable request. No applicable resources were generated or analyzed during the current study.

RESULTS

First, for each section, the tissue area was measured and the islets were identified and counted on the basis of glucagon staining (representative images are shown in Supplementary Fig. 2). The number of islets was highly variable between patients, and ranged from 8.2 to 25.4 islets per section (Fig. 1A). Then, the total number of CD8⁺ cells per section (expressed as CD8 T-cell density), and the number of CD8⁺ cells located in the islet periphery or inside the

Table 1—Patient characteristics

	DiViD-1	DiViD-2	DiViD-4	DiViD-5
Age, years	25	24	31	24
BMI, kg/m ²	21.0	20.9	25.6	28.6
HbA _{1c} at biopsy, % (mmol/mol)	6.7 (49.7)	10.3 (89.1)	7.4 (57.4)	7.4 (57.4)
Diabetes duration, weeks	4	3	5	5
T1D-associated autoantibodies	IA-2A ⁺ ZnT8A ⁺ GADA ⁺ mIAA ⁺	IA-2A ⁺ ZnT8A ⁺ GADA ⁺	IA-2A ⁺ GADA ⁺ mIAA ⁺	IA-2A ⁺ GADA ⁺ mIAA ⁺
HLA risk for T1D	A*01:01, 02:01 B*08:01, 40:01 DRB1*01:03, 03:01 DQB1*05:01, 02:01	A*02:01, 11:01 B*18:01, 40:01 DRB1* 04:01, 13:01 DQB1*03:02, 06:03	A*02:01 B*15:01, 35:01 DRB1*04:01 DQB1*03:02	A*02:01, 03:01, B*18:01, 40:01 DRB1*03:01, 04:01 DQB1*02:01, 03:02

GADA, GAD antibodies; IA-2A, insulinoma-associated protein antibodies; mIAA, insulin antibodies; ZnT8A, islet-specific zinc transporter 8 antibodies.

islets were calculated (Fig. 1 and Supplementary Fig. 1). CD8 T-cell density ranged from 27.2 to 63.8 cells/mm² (Fig. 1B). The number of CD8⁺ cells per islet was also

heterogenous. It varied between islets and between patients despite the small difference in disease duration (3–5 weeks) and ranged from 0.7 to 5.1 CD8⁺ cells/islet,

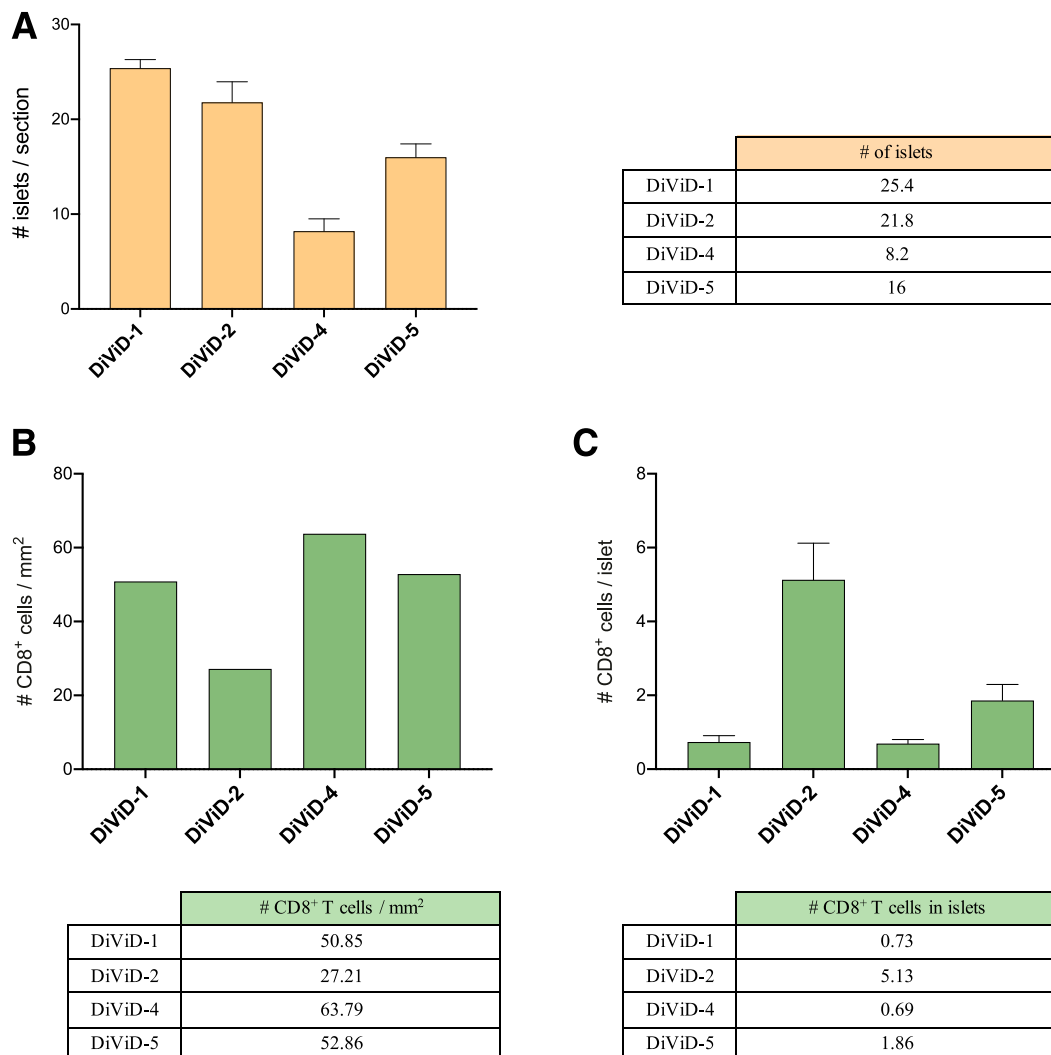


Figure 1—Heterogeneity of islet distribution and CD8⁺ T-cell infiltration in the DiViD cases. Bar graphs and tables show: the mean number of islets per section (# islets/section) (A); CD8 T-cell density in the whole tissue section expressed as number of CD8⁺ cells/mm² (B); and mean number of CD8⁺ cells per islet (# CD8⁺ cells/islet) (C). Error bars represent the SD.

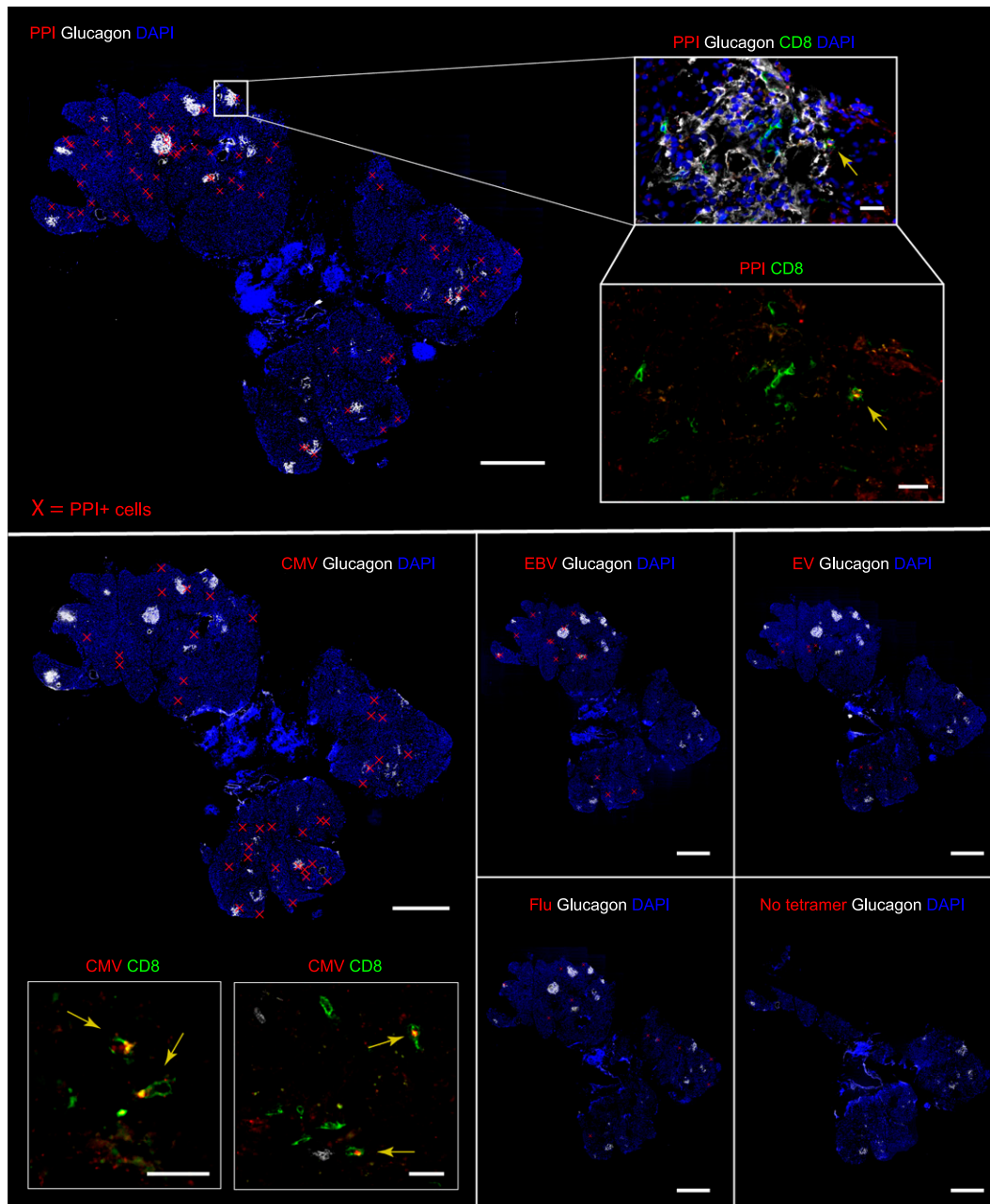


Figure 2—Representative images from immunofluorescence in situ tetramer staining of pancreas sections from DiViD-2. Staining was performed for the indicated tetramer (positive cells are marked with red crosses in whole-slide images), glucagon (white staining), and DAPI (blue staining). Consecutive sections are shown to illustrate the staining and quantification of antigen-specific cells reactive against PPI, CMV, EBV, FLU, EV, and a negative control without tetramer (no tetramer). Scale bars, 1,000 μm in whole-slide images. Magnified images show staining for tetramer (red) and CD8 (green). Yellow arrows indicate tetramer-positive cells. Scale bars, 25 μm for PPI; 20 μm for CMV.

reflecting the presence or absence of islets with insulinitis (Fig. 1C). Then, cells positive for CD8 and tetramer signal were identified, counted, and the percentage of tetramer-positive CD8⁺ cells was calculated, as illustrated in Fig. 2 and Supplementary Fig. 2. First, the frequency of CD8⁺ T cells reactive against PPI (PPI₁₅₋₂₄), a highly relevant antigen in T1D, was analyzed. The mean percentage of PPI⁺ CD8⁺ T cells was 10.4% (patient 1 in the DiViD study [DiViD-1] = 9.5% vs. DiViD-2 = 12.8% vs. DiViD-4 =

10.8% vs. DiViD-5 = 9.5%) (Fig. 3). Positive cells were observed mainly in the exocrine compartment, but they could also be found near or infiltrating the islets (Supplementary Figs. 1 and 2). To assess the relative abundance of PPI⁺ CD8⁺ T cells, their frequency was compared with that of relevant viral specificities. The mean percentage of CD8⁺ cells reactive against CMV (CMV₄₉₅₋₅₀₃) was 5.1% (DiViD-1 = 4.5% vs. DiViD-2 = 5.9% vs. DiViD-4 = 1.6% vs. DiViD-5 = 6.7%), and was lower than the

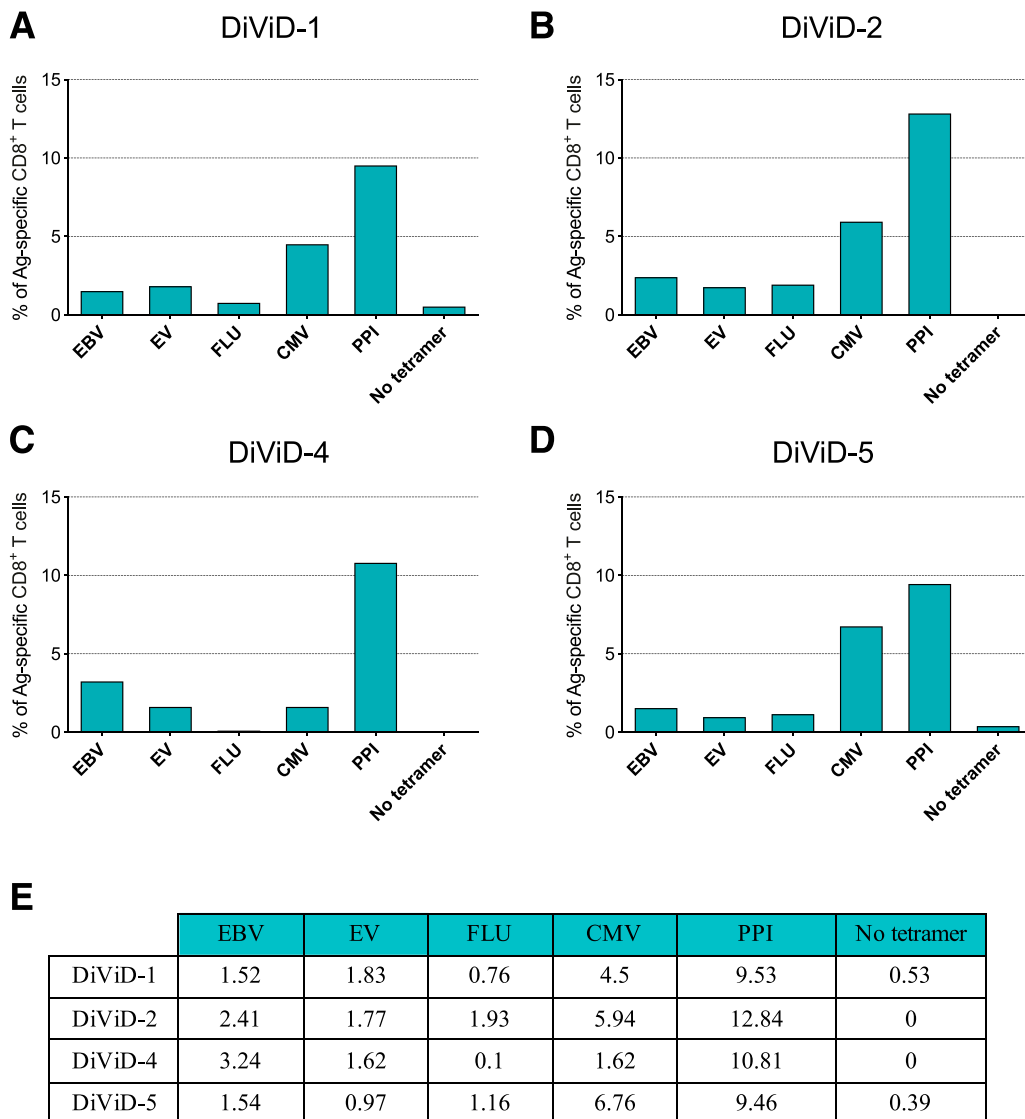


Figure 3—Quantification of tetramer-positive cells in the entire tissue section. *A–D*: Bar graphs show the percentage of antigen (Ag)-specific CD8⁺ T cells for each tissue section and individual (DiViD-1, 2, 4, and 5). PPI, CMV, EBV, FLU, EV, and negative control without tetramer (no tetramer). *E*: The summary table shows the percentage of Ag-specific CD8⁺ cells for each antigen and patient.

frequency of PPI⁺ CD8⁺ cells (Fig. 3). As with PPI⁺ cells, CMV⁺ cells were found predominantly in the exocrine tissue. Conversely, their presence around or inside the islets was rare (≤ 4 CMV⁺ CD8⁺ cells) (Supplementary Fig. 2). Last, the frequency of cells reactive against EBV (EBV_{426–434}; mean percentage EBV⁺, 1.8), FLU (FLU_{58–66}; mean percentage FLU⁺, 1.1), and the EV (EV_{1,587–1,596}; mean percentage EV⁺, 1.6) was low in the islets and exocrine tissue for all the individuals compared with CMV- or PPI-specific CD8⁺ T cells (Fig. 3 and Supplementary Fig. 1).

DISCUSSION

Previous *in situ* studies have shown the presence of PPI-specific cells in the pancreas of donors with T1D, but the frequency of these cells, as well as relative abundance

compared with other known viral specificities, has not been reported to date. Here, we show the presence of a high frequency of PPI-reactive CD8⁺ cells in the pancreas of living individuals whose tissue samples were collected shortly after diagnosis. PPI_{15–24} has been identified as one of the major known epitopes in T1D on the basis of well-defined criteria (14): 1) it is naturally processed and presented by β -cells; 2) reactivity against this epitope has been validated in human pancreatic lymph nodes or islet-infiltrating T cells, and 3) a confirmed recognition of this epitope exists through isolated T clones and lines. Furthermore, PPI-specific CD8⁺ T cells exhibit cytotoxic activity when exposed to human islets (12,15). Among the studied specificities, PPI-specific CD8⁺ T cells could be detected more frequently in islets but, as shown in Fig. 1, the heterogeneity in the number of islets and in the

number of CD8⁺ T cells per islet limited our ability to form strong conclusions regarding islet infiltration. Conversely, quantification of the total number of cells in the entire tissue section provided robust data. The frequency of PPI-specific cells was close to 10% for most of the four patients. Even though this percentage could seem low, it has been shown in transgenic mouse models and virus-induced diabetes that only 1–2% of antigen-specific cells are sufficient to induce diabetes (16,17), thus highlighting the efficiency of these cells in killing their target.

The significance of PPI-specific CD8 T cells in the pancreas in the context of autoimmunity highlights the importance of PPI as a major antigen in T1D. β -cells have an extraordinary capacity to increase PPI translation in response to glucose stimulation, increasing its potential exposure to the immune system under conditions of high insulin demand and cellular stress. As mentioned, PPI is naturally processed and presented by β -cells (5,14), and it is a major source of epitopes for HLA-A and HLA-B alleles (9). Recently, no differences in the frequency of β -cell-reactive CD8 T cells against multiple antigens were reported in the blood of individuals with and without T1D. The notable exception was the epitope studied here, PPI_{15–24}, whose frequency was higher in individuals with T1D (8). In agreement with the data presented here, an increased presence of PPI_{15–24} CD8 T cells has been reported recently in the pancreas of individuals with T1D up to 8 years postdiagnosis (13). In addition, multiple studies have shown the capacity of PPI-specific CD8 T cells to kill β -cells in vitro (12,15), highlighting their potential implication in disease pathogenesis. Taken together, data reported by us and others point to a scenario in which, in the context of autoimmunity, increased β -cell stress in response to insulin demand could have a strong impact on epitope generation, antigen presentation, and immune activation, making β -cells an attractive target for PPI-specific cells that could be probing for antigen in the pancreas.

A strength, and simultaneously a limitation, of our study is the uniqueness of the samples, which were collected from living individuals by pancreatic-tail resection. This precluded us from including samples from nondiabetic donors, which would have been collected and preserved in very different conditions and have been already studied elsewhere elsewhere (13). Instead, our objective was to compare the frequency of PPI-specific cells to the frequency of well-known viral reactivities within individuals with diabetes. Our study was not designed to target all the epitopes spanning multiple antigens but rather to offer an overview of what could be the tip of the autoreactive iceberg. Therefore, it is possible the data presented here underestimate the number of reactive cells against a given antigen, whether viral or from β -cells. This could be the case for EV-specific cells, which were not found in the islets despite the reported presence of a low-grade EV infection in these individuals (18). It is important, therefore, to foster epitope discovery efforts to sample a wider antigenic repertoire and to extract conclusions regarding antigen recognition in T1D.

Our study increases the amount of data from the relatively limited number of pancreas samples in which antigen-specific T cells have been studied in situ in donors with recent-onset T1D. Given the scientific value of these tissues and the current clinical relevance of immune interventions targeting T cells (19) and antigen-specific immunotherapies like oral insulin (20), our findings, together with previous reports, could inform future clinical trials. This study contributes to the current existing knowledge about PPI and indicates that antigen-specific therapies directed toward PPI could have substantial clinical impact.

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Author Contributions. T.R.-C. participated in project design, performed experiments, analyzed and interpreted data, and wrote the manuscript. N.A. performed experiments. L.K. and K.D.-J. provided samples from the DiViD study and participated in writing the manuscript. M.v.H. supervised the project, interpreted data, and participated in writing the manuscript. M.v.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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