Hybrid Insulin Peptides Are Autoantigens in Type 1 Diabetes

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We recently established that hybrid insulin peptides (HIPs) are present in human islets and that T cells reactive to HIPs are found in the residual islets of organ donors with type 1 diabetes (T1D). Here, we investigate whether HIP-reactive T cells are indicative of ongoing autoimmunity in patients with T1D. We used interferon-γ enzyme-linked immune absorbent spot analyses on peripheral blood mononuclear cells (PBMCs) to determine whether patients with new-onset T1D or control subjects displayed T-cell reactivity to a panel of 16 HIPs. We observed that nearly one-half of the patients responded to one or more HIPs. Responses to four HIPs were significantly elevated in patients with T1D but not in control subjects. To characterize the T cells reactive to HIPs, we used a carboxyfluorescein succinimidyl ester–based assay to clone T cells from PBMCs. We isolated six nonredundant, antigen-specific T-cell clones, most of which reacting to their target HIPs in the low nanomolar range. One T-cell clone was isolated from the same patient on two different blood draws, indicating persistence of this T-cell clone in the peripheral blood. This work suggests that HIPs are important target antigens in human subjects with T1D and may play a critical role in disease.

Type 1 diabetes (T1D) is caused by the T-cell–mediated destruction of insulin-producing β-cells in the islets of Langerhans. We previously reported that diabetes-triggering T cells, isolated from the NOD mouse model of autoimmune diabetes, respond to hybrid insulin peptides (HIPs). These peptides represent a novel form of posttranslational modification involving the covalent linkage of insulin fragments to other protein fragments obtained from separate parent molecules through a peptide bond (1). Several diabetogenic T-cell clones isolated from NOD mice target two distinct HIPs. BDC-2.5 and four additional T-cell clones from the BDC panel target the 2.5HIP, a peptide formed by fusion of an insulin C-peptide fragment ($ins2C_{77–82}$) on the N-terminal side (left peptide) to WE14, a natural cleavage product from chromogranin A ($ChgA_{358–371}$) on the COOH-terminal side (right peptide) (1,2). BDC-6.9 and two additional T-cell clones from the BDC panel of clones target the 6.9HIP, a peptide formed between the same C-peptide fragment and IAPP2, a natural cleavage product from pro-islet amyloid polypeptide ($IAPP_{74–80}$) (1,3). Recent mass spectrometric data confirm the presence of HIPs in murine islets as well as in islets of organ donors without diabetes (4). T cells recognizing these HIPs not only are present in large numbers in the islets (3,5) but also can be detected in the peripheral blood of NOD mice, displaying a memory phenotype and increasing in frequency as the mice progress toward diabetes (2). We also established that several CD4 T-cell clones, isolated from the residual islets of deceased donors with T1D, recognize HIPs (1,6). These T-cell clones reacted to human HIP sequences containing a fragment of insulin C-peptide ($insC_{64–71}$) as the left peptide linked to the N termini of natural cleavage products on the right side of the insulin A chain ($insA_{90–96}$), neuropeptide Y ($NPY_{68–74}$), or IAPP2 ($IAPP_{74–80}$).

Our primary goal in this study was to determine whether HIP-reactive T cells could be observed in the peripheral blood of patients with new-onset T1D. Peripheral

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blood mononuclear cells (PBMCs) from living patients are much more readily obtained than T cells from the residual islets of organ donors with T1D, and therefore, the presence of HIP-reactive T cells with an inflammatory phenotype in the peripheral blood of patients at different stages of disease could serve as a key biomarker of T1D. We used a panel of 16 different HIPs to determine by interferon-γ (IFN-γ) enzyme-linked immune absorbent spot (ELISPOT) analysis whether T-cell responses to these HIPs could be detected in patients with T1D but not in age- and HLA-DQ-DR–matched control subjects.

Table 1—Subject demographics

Demographic	Patients with new-onset T1D (*n* = 35)	Control subjects without diabetes (*n* = 19)
Age (years)		
Mean (SD)	12.2 (4)	13.4 (8)
Median	11.6	11.3
Range	5–19	7–35
Sex, *n* (%)		
Male	17 (49)	9 (47)
Female	18 (51)	10 (53)
Diabetes duration (days)		
Mean (SD)	20.6	NA
Median	12	NA
Range	0–163	NA
Islet autoantibody-positive (%)	97	0
HLA-DQ genotype, *n* (%)		
HLA-DQ2/8	9 (26)	1 (5)
HLA-DQ2/X	11 (31)	9 (47)
HLA-DQ8/X	14 (40)	8 (42)
ND	1	1

NA, not applicable; ND, not determined.

RESEARCH DESIGN AND METHODS

Flow Cytometry

Antibodies used for staining of T cells were CD4 BV711 (SK3; BD Biosciences), CD25 BV421 (M-A251; BD Biosciences), and CD8 APC-H7 (SK1; BD Biosciences). 7AAD or fixable viability dye eFluor 780 was used to discriminate live cells. Gating strategies are indicated in each figure; the lymphocyte gate was based on forward scatter (FSC)/side scatter properties, and the singlets gate was based on the FSC-A/FSC-H. For carboxyfluorescein succinimidyl ester (CFSE) assays, unfractionated PBMCs were washed two times with PBS, resuspended in a 1 μmol/L solution of CFSE (10^7 cells/mL), and incubated at 37°C. After 10 min, cells were washed two times with AIM V media (Thermo Fisher Scientific) containing 2% normal human serum (AB serum; Gemini Bio-Products) and then resuspended in AIM V containing 2% AB serum. Cells (at 1–8 × 10^5 cells/well) were then plated in a flat-bottom 96-well plate and cultured for 7 days at 37°C. Samples were run on an LSRFortessa X-20 (BD Biosciences) flow cytometer or a CytoFLEX (Beckman Coulter) flow cytometer, and data were analyzed using FlowJo v10 software (Tree Star, Ashland, OR).

Peptides

The peptides used in this study were obtained from CHI Scientific (Maynard, MA) at a purity >95% and are listed in Table 2. The insulin B-chain amino acids 9–23 (insB:9–23) and the insB:9–23 R22E mimotope peptide were provided by Dr. Aaron Michels (purity >95%; Genemed Synthesis) and were described previously (7).

ELISPOT

Indirect ELISPOT analyses were conducted as described previously (8) using the human IFN-γ ELISPOT kit (U-CyTech biosciences, Utrecht, the Netherlands). Briefly, PBMCs from subjects were incubated with peptide or antigens for 72 h and subsequently transferred in triplicate wells to plates coated with the anti–IFN-γ capture antibody for overnight incubation. Cells were removed, and wells were washed. Spots were then formed by sequential incubations with the biotinylated secondary site anti–IFN-γ, gold-labeled goat anti-biotin, and a precipitating silver substrate; spots were enumerated with a Bioreader 4000 Pro-X (BIO-SYS, Karben, Germany). For each antigen tested, the total spots from three wells from the 96-well plates were added, reflecting spot-forming cells per 1 × 10^6 total PBMCs. In this study, a number of spots per 1 × 10^6 PBMCs >20 was considered significant (mean number of spots for the DMSO negative control + 3 SDs). All subjects were tested with the panel of 16 HIPs and control peptides (tetanus toxoid or Pediarix). Because of the limited cell numbers obtained, some subjects were not tested with all native peptides.

T-Cell Cloning

PBMCs were labeled with CFSE and cultured for 7–8 days in AIM V containing individual peptides and normal human AB serum. $CFSE^{dim}$, $CD25^{hi}$, and $7AAD^{neg}$ cells were index sorted into a round-bottom 96-well plate using a FACSAria cell sorter (BD Biosciences) at one cell per well. Wells were supplemented with recombinant human IL-2 (20 units/mL), IL-4 (5 ng/mL), anti-CD3 (30 ng/mL), irradiated mixed-matched PBMCs from two different donors (1 × 10^6/mL), and irradiated PRIESS cells (1 × 10^6/mL). Wells were regularly scored for growth by visual inspection, and cells were expanded using IL-2 and IL-4. After 28 days, T-cell clones were challenged with peptides using autologous irradiated PBMCs, and supernatants were tested for IFN-γ by ELISA. Wells that gave a positive response with little background were selected for further expansion and T-cell receptor (TCR) sequencing.

Antigen Assay

Early passaged CD4 T-cell clones were thawed and restimulated with peptide and irradiated autologous Epstein-Barr

Table 2—Peptides used in the study

HIPs	Peptide Sequence*	B-Chain	Peptide Sequence
HIP1	GQVELGG-WSKMDQLA	insB:9-23	SHLVEALYLVCGER
HIP2	GQVELGG-LEGQEEEE	insB:9-23R22E	SHLVEALYLVCGEE
HIP3	GQVELGGG-EAEDLQV		
HIP4	GQVELGGG-GIVEQCC	**Left control peptides**	**Peptide Sequence****
HIP5	GQVELGGG-TPIESHQ	$ins_{64–79}$	GQVELGGGPGAGSLQP
HIP6	GQVELGGG-NAVEVLK	$ins_{75–90}$	GSLQPLALEGSLQKRG
HIP7	GQVELGGG-FLGEGHH		
HIP8	GQVELGGG-SSPETLI	**Right control peptides**	**Peptide Sequence*****
HIP9	SLQPLAL-WSKMDQL	$ChgA_{334–349}$	KEWEDSKRWSKMDQLA
HIP10	SLQPLAL-LEGQEEE	$ChgA_{350–365}$	KELTAEKRLEGQEEEE
HIP11	SLQPLAL-EAEDLQV	$Ins_{49–64}$	FYTPKTRREAEDLQVG
HIP12	SLQPLAL-GIVEQCC	$Ins_{82–97}$	LEGSLQKRGIVEQCCT
HIP13	SLQPLAL-TPIESHQ	$IAPP_{15–30}$	VALNHLKATPIESHQV
HIP14	SLQPLAL-NAVEVLK	$IAPP_{66–81}$	GSNTYGKRNAVEVLKR
HIP15	SLQPLAL-FLGEGHH	$ScG1_{432–447}$	SDTREEKRFLGEGHHR
HIP16	SLQPLAL-SSPETLI	$NP\text{-}Y_{60–75}$	TRQRYGKRSSPETLIS

*Hyphenation indicates hybrid peptide junction. **Highlighted sequences represent C-peptide components of HIPs. ***Highlighted sequences represent N-terminal sequences of natural cleavage products.

virus (EBV)–transformed B-cell line. Twenty-four hours later, IL-2 and IL-4 were added to the T-cell cultures for T-cell expansion. T cells were maintained in culture for 21–28 days before functional assays were performed. For T-cell assays, CD4 T-cell clones ($0.5–5 \times 10^5$) were incubated with irradiated autologous EBV-transformed B-cell line ($0.5–5 \times 10^5$) in the presence or absence of antigen at concentrations indicated in the figure legends. For IFN-γ ELISA, supernatants were collected 24–48 h after culture and analyzed using a kit from eBiosciences according to the manufacturer's protocol. For the CD25 upregulation assay, cells were harvested and stained for CD4, CD25, and a viability dye before analysis by flow cytometry. To determine the HLA restriction of HIP responses, the antigen assay was performed in the presence and absence of anti-DR (L243) or anti-DQ (SPV-L3) antibodies. The EBV-transformed cell lines were first pulsed with peptide and washed twice with AIM V. Antibodies were added at a final concentration of 1 μg/mL.

TCR Sequencing

TCR α- and β-chain sequences were determined using a modified method of our previous report (7). Briefly, RNA was extracted from expanded CD4 T-cell cultures using RNAeasy Mini Kit (QIAGEN) followed by RT-PCR to synthesize single-strand cDNA ligated to the universal oligonucleotide sequence at the 5′ end. Two-step PCR was performed to amplify TCR α- and β-chain genes using primers containing identifier oligonucleotides and the Illumina adaptor sequences. PCR products were purified using Pippin Prep (Sage Science) and were subject to sequencing on MiSeq (Illumina) using paired-end 250-cycle V2 chemistry (Illumina). All sequences were separated by the identifier oligonucleotides and analyzed by the IMGT/HighV-QUEST algorithm to identify variable gene, joining gene, and junction sequences for individual T-cell cultures.

Statistical Analysis

The total number of spots per 10^6 PBMCs per analyte was analyzed with a two-tailed paired *t* test by comparing the no antigen condition to the antigen condition. All statistical analyses were performed with the GraphPad Prism version 6 software. $P < 0.05$ was considered significant.

Peripheral Blood Processing, Islet Autoantibodies, and HLA Typing

Peripheral blood was obtained for T-cell assays, HLA genotyping, and monitoring of islet autoantibodies. Islet autoantibodies present in serum to insulin, GAD65, IA-2, and ZnT8 were measured by radioimmunoassay as previously described (9,10). HLA-DRB, -DQA, and -DQB genotyping was performed as previously described (11) by the HLA service core at the Barbara Davis Center for Diabetes.

Study Approval

Participants were recruited from patients, relatives, and volunteers attending the Barbara Davis Center for Diabetes. Written informed consent was obtained in accordance with protocols approved by the Colorado Multiple Institutional Review Board.

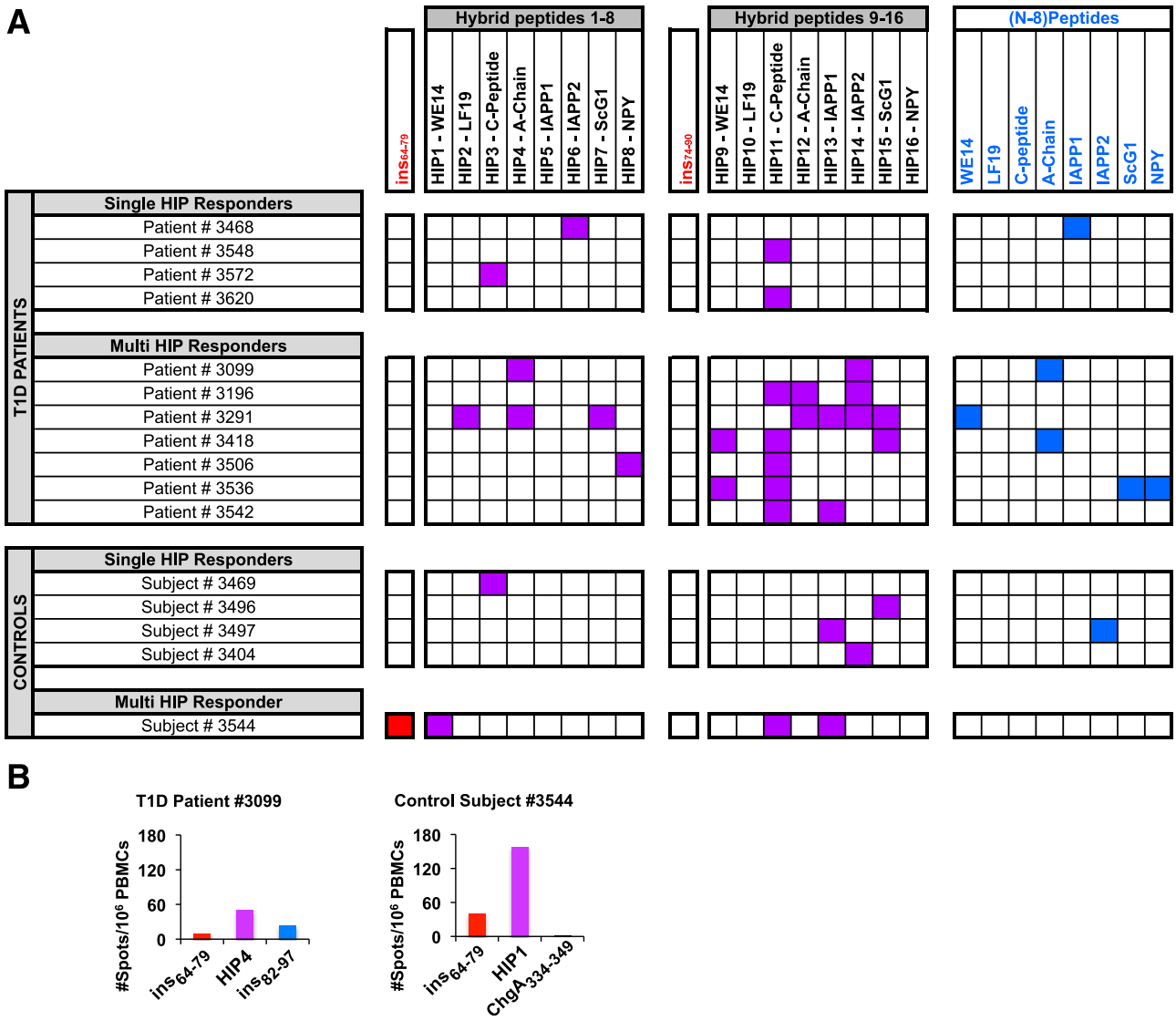

Figure 1—HIP responders in T1D and nondiabetic control cohorts. Freshly isolated PBMCs were cultured in the presence or absence of antigen for 96 h, washed, and transferred to an IFN-γ–coated plate for overnight culture. After 18 h, plates were developed according to the manufacturer's protocol, and spots were enumerated. *A*: To test the biological significance of HIP responses, HIP responders (total number of spots/10^6 PBMCs >20) were reported (purple squares) as well as responses to left peptides (red squares) and right peptides (blue squares). Cumulative data are shown for 35 patients with diabetes and 19 control subjects. *B*: Responses to HIPs and corresponding control peptides for subjects #3099 and #3544, showing an increased response to HIPs compared with the native control peptides. Data are from one experiment per subject.

RESULTS

Study Subjects

Subject demographics are summarized in Table 1 and described in detail in Supplementary Table 1. A total of 35 patients with new-onset T1D (<6 months from the date of diagnosis) and 19 control subjects (age and HLA matched, nondiabetic, islet autoantibody negative) were enrolled. A majority (84%) of the control subjects were first-degree relatives of patients with T1D, and these control subjects without diabetes were unrelated to the patients enrolled in this study, except for one control subject (#3293) who was a sibling of a patient in this study (#3291) (Supplementary Table 1). The study protocol was approved by the institutional review board, and written informed consent was obtained from all participants. HLA genotyping was performed on all subjects and because all patients with new-onset T1D in the study were HLA-DQ2, -DQ8, or -DQ2/8, only control subjects with matching genotypes were included.

Study Design

We used an IFN-γ ELISPOT assay to test T-cell reactivity to a panel of HIPs and native secretory granule peptide sequences. For determination of reactivity to various peptides, the assay readout for the ELISPOT analyses was the total number of spots per million PBMCs. A total of 16 HIPs and

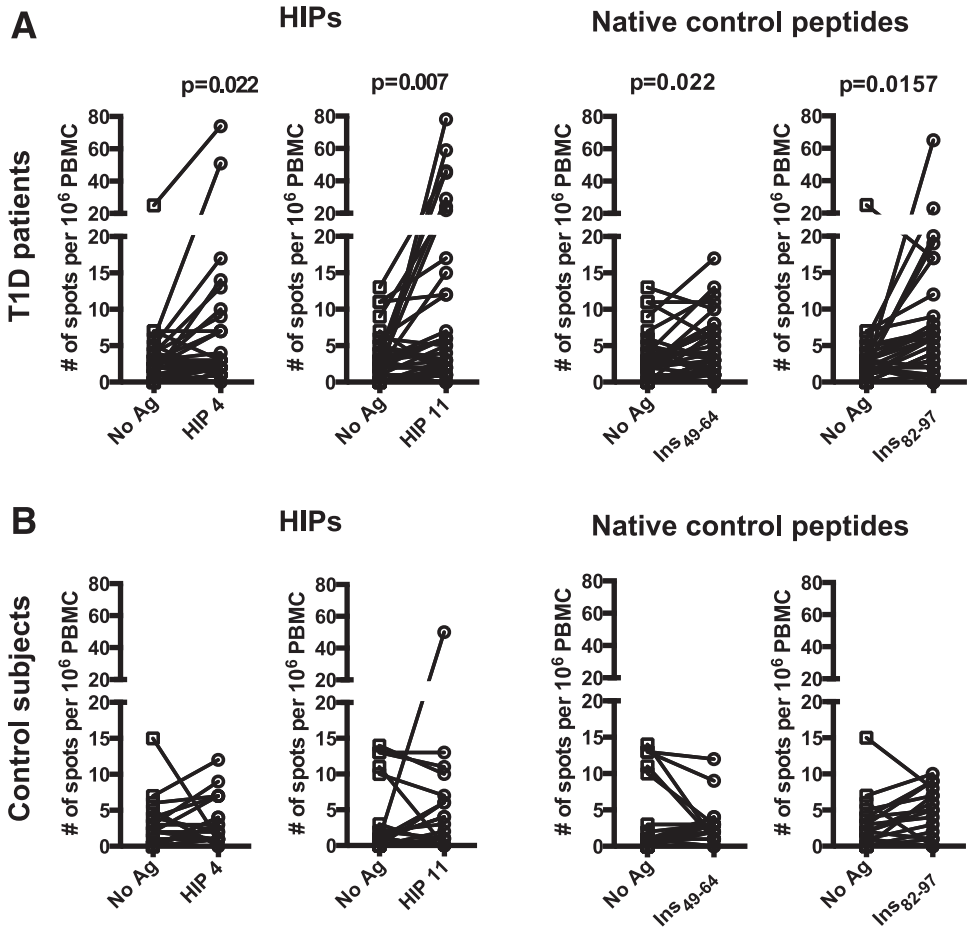

Figure 2—Identification of disease-relevant HIPs. Freshly isolated PBMCs were cultured in the presence or absence of antigen for 96 h, washed, and transferred to an IFN-γ–coated plate for overnight culture. After 18 h, plates were developed according to the manufacturer's protocol, and spots were enumerated. Cumulative data are shown for 35 patients with diabetes and 19 control subjects. *A*: Responses to HIP4, HIP11, $ins_{49–64}$, or $ins_{82–97}$ in patients with T1D. *B*: Responses to HIP4, HIP11, $ins_{49–64}$, or $ins_{82–97}$ in control subjects without diabetes. The total number of spots per 10^6 PBMCs is indicated for each condition. *P* values, when significant ($P < 0.05$), are reported and were obtained using a two-tailed *t* test. Ag, antigen.

10 native secretory granule peptide sequences were used as epitopes for this study. Each HIP contained a fragment of insulin C-peptide on the N-terminal (left) side and a natural β-cell peptide on the COOH-terminal (right) side. For HIPs 1–8, the C-peptide fragments were selected to contain amino acid residues that bind with high affinity to positions 1 and 4 of DQ8 (12). In contrast, HIPs 9–16 contain the human equivalent C-peptide sequence that makes up the left side of the murine 2.5 and 6.9HIPs. The secretory granule peptides, comprising the right side of HIPs 1–8, were chosen to contain negatively charged anchor residues that, in combination with the C-peptide fragments, are predicted to bind with high affinity to p9 of DQ2 and/or DQ8. The same list of right-side peptides was used for the design of HIPs 9–16, and the resulting HIPs include the human equivalents of the 2.5HIP (HIP9) and 6.9HIP (HIP14). Secretory granule peptides (right peptides) were derived from natural cleavage products of proinsulin, NPY, ChgA, IAPP, and secretogranin 1 (Scg1), all highly abundant in β-cell secretory granules.

We previously demonstrated that 4 of the 16 HIPs could elicit responses from T cells isolated from residual islets of organ donors with T1D (1,6). One of the HIPs (HIP9) is the human equivalent of the murine 2.5HIP, targeted by the NOD-derived diabetogenic T-cell clone BDC-2.5. As control peptides, we included the native peptide components of the 16 HIPs wherein the left peptides were COOH-terminally extended by seven or eight residues of their germline-encoded peptide sequences and the right peptides were N-terminally extended by eight amino acid residues. In an example shown in Supplementary Fig. 1, the control peptides for HIP9, a fusion peptide between $ins_{75–82}$ and $ChgA_{342–349}$, are $ins_{75–90}$ and $ChgA_{334–349}$. $ins_{75–90}$ contains the C-peptide component (left side) of HIP9 extended at the COOH terminus by the natural C-peptide sequence, and $ChgA_{334–349}$ is the WE14 sequence extended by N-terminal residues of the natural ChgA sequence. In addition, we included insB:9–23 and a mimotope of insB:9–23 (R22E) because T cells reactive to these peptides were also found in

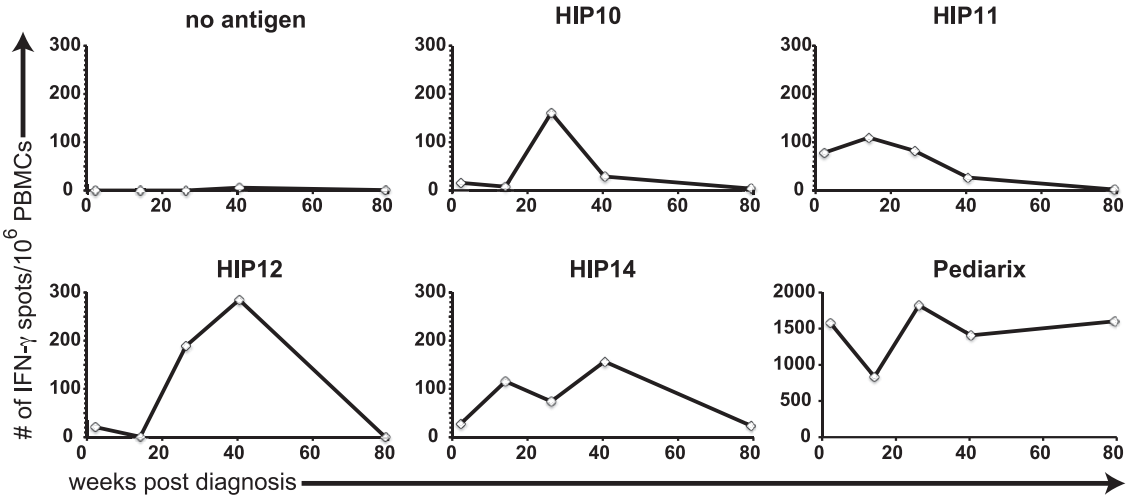

Figure 3— Longitudinal analysis of responses to Pediarix or HIPs in one patient with T1D. Freshly isolated PBMCs from patient #3196 were collected 2 weeks, 14 weeks, 26 weeks, 40 weeks, or 79 weeks after diagnosis and cultured in the presence or absence of antigen for 72 h, washed, and transferred to an IFN-γ–coated plate for overnight culture. After 18 h, plates were developed according to the manufacturer's protocol, and spots were enumerated. The total number of spots per 10^6 PBMCs is indicated for each condition. Data are cumulative of five independent experiments.

residual islets of donors with T1D (13) and peripheral blood of patients with new-onset T1D (7). Table 2 provides a detailed list of all the HIPs and control peptides used in this study.

T Cells From Patients With T1D Produce IFN-γ in Response to HIPs

As a positive control, we included tetanus toxoid or Pediarix, a vaccine containing five different immunogens. Our results indicate that both patients with new-onset T1D and control subjects responded significantly to Pediarix and tetanus toxoid (Supplementary Fig. 2). As a vehicle control, we used media (AIM V) with or without DMSO. The mean responses to media or media + DMSO were 3.0 (SD 4.3) or 3.3 (3.4) spots per 10^6 PBMCs, respectively.

To analyze the reactivity profile, we graphed the responses from subjects that showed significant responses to at least one HIP (Fig. 1*A*). On the basis of the average number of spots observed in the no antigen condition, significant responses were set at >20 spots/10^6 PBMCs (> mean of no antigen condition + 3 SDs). For the HIP-responding subjects, we also show the responses to the 10 native control peptides. The data from all study participants are shown in Supplementary Figs. 3 and 4.

Of the 19 control subjects tested, 5 showed significant responses to HIPs. Of the 19 subjects only 1 responded to multiple HIPs (subject #3544). Eleven patients with new-onset T1D responded to at least one HIP, with the majority responding to more than one HIP (7 of 35). For example, patient #3291 showed significant responses to seven distinct HIPs: HIP2, HIP4, HIP7, HIP12, HIP13, HIP14, and HIP15. We also observed that various HIPs were recognized by more than one patient. For example, HIP9 and HIP14 (the equivalent human sequences of the murine 2.5HIP and the 6.9HIP, respectively) triggered responses from several patients with T1D. Our data show that HIP11, a C-peptide/C-peptide HIP, triggered responses in seven patients (Fig. 1*A*). This HIP was also recognized in one control subject (#3544, homozygous for HLA-DRB1*0301, HLA-DQA1*0501, and HLA-DQB1*0201), the only control subject without diabetes (who was a first-degree relative) to respond to multiple HIPs (Fig. 1). In two subjects, we observed responses to HIPs and corresponding control peptides. For example, responses from subject #3544 to HIP1 were significantly elevated compared with responses to control peptides ($ins_{64–79}$ and $ChgA_{334–349}$), suggesting that peptide fusion leads to a gain of antigenicity (Fig. 1*B*).

When we compared HIP responses from PBMCs of all patients with T1D with the responses toward the no antigen condition (media ± DMSO) using a pairwise analysis, we found a significant increase in the number of IFN-γ–producing T cells to HIP4 (P = 0.0299), HIP11 (P = 0.0070), HIP12 (P = 0.0489), and HIP16 (P = 0.0005) (Fig. 2 and Supplementary Fig. 3). Although responses to HIP16 were highly significant over background in the paired analysis, the total number of spots/10^6 PBMCs never exceeded 20. These differences were trending toward, but not significant for, HIP14 (P = 0.0513). In contrast, none of the control subjects showed significantly increased responses to any of the 16 HIPs tested (Supplementary Fig. 3). This pairwise analysis demonstrates that there are increased responses to four distinct HIPs compared with the no antigen condition in the new-onset T1D cohort.

To assess reactivity to native, nonhybrid peptide sequences, we tested responses to 10 control peptides (Table 2) of PBMCs from the same individuals with new-onset T1D and without diabetes. We observed that a few

patients with T1D and control subjects responded to several of these native peptides (Fig. 2 and Supplementary Fig. 4). In the pairwise analysis (no antigen vs. native peptide), these differences were significant for $ChgA_{350–365}$, $ins_{82–97}$, $ins_{49–64}$, and $IAPP_{15–30}$ in the T1D group only. Additionally, the two C-peptide fragments that entail the left components of our 16 HIPs ($ins_{64–79}$ and $ins_{74–90}$) also triggered increased responses in patients with T1D but not in control subjects without diabetes (Supplementary Fig. 4).

We also investigated the presence of T cells reactive to insB:9–23 or a mimotope peptide for the insB:9–23 (insB:9–23 R22E) (7). Our data indicate that a subset of subjects in both groups responded to the mimotope but not to the native insulin peptide, with the insB:9–23 R22E triggering significant responses in patients with new-onset T1D (Supplementary Fig. 2).

IFN-γ Responses to HIPs Persist Over Time

One of the patients who responded to multiple HIPs (#3196) was followed for >1 year, which allowed us to study the HIP responses over a prolonged time frame, TCR usage of HIP-reactive T cells, and HLA restriction of HIP responses. This patient was followed for 79 weeks after diagnosis, and the responses to HIPs were screened at various intervals. At the initial visit (2 weeks after diagnosis), significant responses were observed to HIP10, HIP11, HIP12, and HIP14 (total number of spots/10^6 PBMC >20) (Fig. 3). We observed different kinetics of reactivity to these HIPs: significant responses to HIP11 and HIP14 were maintained consecutively until at least 40 weeks postdiagnosis, whereas responses to HIP10 and HIP12 were transient (Fig. 3). A final test was performed 77 weeks after diagnosis at which time point we only observed responses to HIP14 (total number of spots/10^6 PBMC >20). These data indicate that the frequency of HIP-reactive T cells may decrease within ≤1 year after diagnosis. Pediarix was used as a positive control throughout the screening period, and PBMCs showed a robust response to this antigen at all time points, whereas very few spots were observed in response to the no antigen control throughout this study.

Proliferation of PBMCs in Response to HIPs

As a second measure to assess peripheral blood T-cell responses to HIPs, we also carried out T-cell proliferation assays on PBMCs from patient #3196 who was screened in the longitudinal study. PBMCs from this patient, tested 24 and 38 weeks after the initial visit, were labeled with CFSE and cultured in the presence of the 16 HIPs, the insB:9–23 peptide, or the insB:9–23 R22E mimotope peptide. Pediarix was used as a positive control and media only as a negative control. After 6 days of culture, cells were harvested, stained for CD25 (marker of activation) and CD4, and analyzed by flow cytometry (Fig. 4*A*).

Proliferation of CD4 T cells was observed in response to HIP10, HIP11, HIP12, and HIP14 and the insB:9–23 R22E mimotope peptide (Fig. 4*A* and *B*). T-cell proliferation to these antigens was also observed in a second assay performed 40 weeks postdiagnosis (data not shown). The positive control condition (Pediarix) elicited strong T-cell proliferation because 50% of CD4 T cells were $CFSE^{dim}$ $CD25^{hi}$ after 6 days in culture. In contrast, very little proliferation was observed when PBMCs were cultured in the absence of antigen (Fig. 4*A*). IFN-γ production by T cells in response to the same antigens was analyzed in

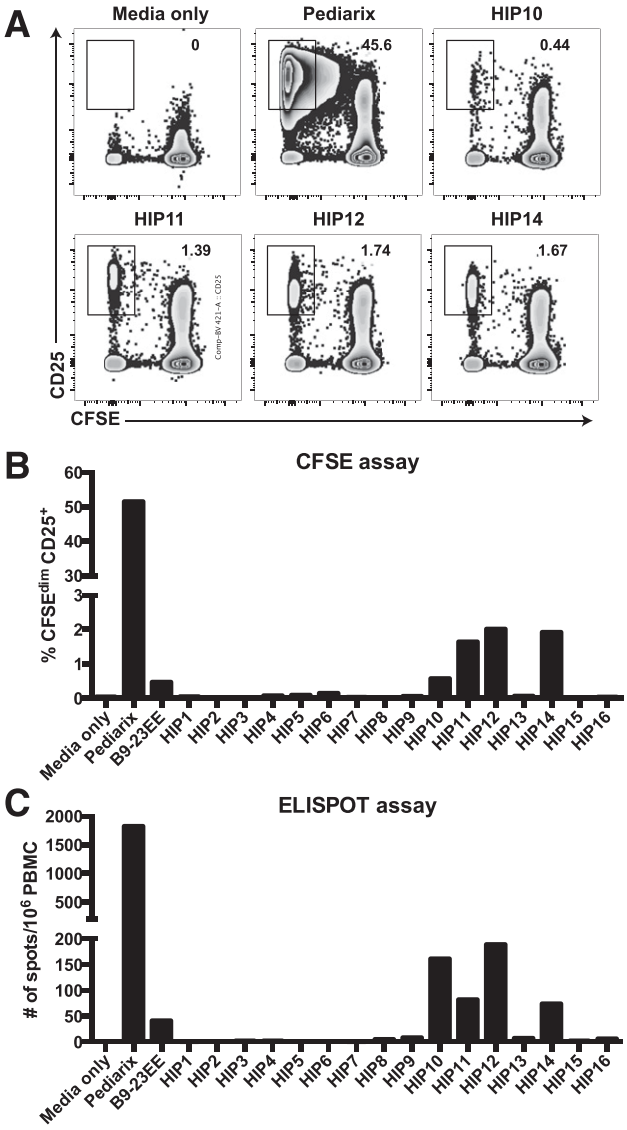

Figure 4—Comparison of proliferation assay and ELISPOT assay. Freshly isolated PBMCs from patient #3196 were isolated 26 weeks after diagnosis. *A* and *B*: Cells were labeled with CFSE and cultured in the presence or absence of antigen. After 6 days of culture, cells were harvested and stained for CD4, CD25, and a fixable viability dye. Gates were set on live, single $CD4^+$ cells, and the percentage of $CFSE^{dim}$ $CD25^+$ cells is reported. *C*: Cells were cultured in the presence or absence of antigen for 72 h, washed, and transferred to an IFN-γ–coated plate for overnight culture. After 18 h, plates were developed according to the manufacturer's protocol, and spots were enumerated. The total number of spots per 10^6 PBMCs is indicated for each condition. Data are representative of two independent experiments.

parallel by ELISPOT to allow for a comparison of the results from both assays. Results from the ELISPOT analysis at 26 weeks are shown in Fig. 4*C* and compare well with T-cell responses to HIPs observed in the proliferation assays (Fig. 4*B*).

Phenotypic Characteristics of HIP-Reactive CD4 T-Cell Clones

To further characterize T cells responsive to HIP10, HIP11, HIP12, and HIP14, $CFSE^{dim}$ $CD25^{hi}$ T cells from patient #3196 were single-cell sorted by FACS and cloned as described in RESEARCH DESIGN AND METHODS. One T-cell clone that responded to HIP14 was successfully isolated (HIP14-G10) from the 26-week blood draw, and we determined that the TCR contains TRVB27 along with two α-chains containing TRAV5 and TRAV16; the same T-cell clone (see Supplementary Table 2) was isolated from the 40-week blood draw (HIP14-D3), indicating that this T-cell clone was present in the peripheral blood of this subject over a time frame of at least 14 weeks. Furthermore, following the 40-week blood draw, we isolated two additional HIP14-reactive T-cell clones (HIP14-G10b and HIP14-C8) with different TCRs (TRBV20-1 and TRBV7-7, respectively). In addition to the HIP14-reactive clones, HIP11 and HIP12-reactive T-cell clones (HIP11-E2, HIP12-B6, and HIP12-F11) were isolated at week 40. These clones all used different TCR Vβ regions (TRBV5-5, TRBV29-1, and TRBV19, respectively). Although we expanded 17 T-cell clones from the HIP10 culture, none of them showed specific responses to HIP10. The TCR Vβs of all HIP-reactive T-cell clones isolated from patient #3196 are summarized in Table 3.

To monitor T-cell activation of these clones, we measured CD25 upregulation in response to HIPs by flow cytometry. As illustrated in Fig. 5*B*, all T-cell clones upregulated CD25 in response to their cognate HIPs. While HIP11- and HIP14-reactive T-cell clones responded to their cognate antigens at very low concentrations (half-maximal effective concentration [EC_{50}] ~10 nmol/L), HIP12-reactive clones required higher concentrations of antigen (EC_{50} ~1 μmol/L). Similar results were observed when IFN-γ secretion was investigated as a measure of T-cell activation (Fig. 5*B*). To determine the HLA restriction of HIP responses in patient #3196, we measured CD25 upregulation in the presence and absence of anti-DR (L243) or anti-DQ (SPV-L3) antibodies. We observed that CD25 upregulation was completely abrogated when HIP12- or HIP14-reactive T-cell clones were activated in the presence of anti-DR antibody, indicating that responses by HIP12- and HIP14-reactive T-cell clones are HLA-DR restricted (Fig. 5*C* and Table 3). In contrast, our antibody blocking studies indicated that the restriction element for the HIP11-reactive T-cell clone was HLA-DQ (Fig. 5*C* and Table 3). Finally, we observed strong upregulation of CD25 in response to HIPs but not to native $ins_{75–90}$ C-peptide (all T-cell clones), $ins_{82–97}$ (clone HIP11-E2), $ins_{49–64}$ (clone HIP12-F11), or $IAPP_{66–81}$ (clone HIP14-G10b), indicating that T-cell responses could be attributed to the hybrid peptides and not to the native, nonhybrid peptides (Fig. 5*D*).

DISCUSSION

We report here for the first time to our knowledge that HIP-reactive T cells can be observed in the peripheral blood of patients with newly diagnosed T1D. Analyses of PBMCs from patients with new-onset T1D and control subjects without diabetes revealed that ~30% of patients with T1D produce a robust IFN-γ response to at least one HIP and that some patients respond to up to seven HIPs. A total of 20% of subjects with new-onset T1D responded to multiple HIPs, whereas only one control subject, a 9-year-old first-degree relative of a patient with T1D, responded to more than one HIP. In addition, we found that four HIPs, (HIP4, HIP11, HIP12, and HIP16) elicited responses by PBMCs from subjects with T1D that were significantly higher than responses to the no antigen condition; no significant

Table 3—TCR usage of HIP-reactive T-cell clones isolated from patient #3196 isolated at two different visits

Weeks after diagnosis	HIP reactivity	Clone name	TRBV	TRBJ	α-1		α-2		DR/DQ restriction
					TRAV	TRAJ	TRAV	TRAJ	
26	HIP14	F5	TRBV20-1	TRBJ2-1	TRAV17	TRAJ54			ND
26	HIP14	G10	TRBV27	TRBJ2-5	TRAV16	TRAJ37	TRAV5	TRAJ40	DR
40	HIP11	E2	TRBV5-4	TRBJ2-1	TRAV6	TRAJ31	TRAV8-2/8-4	TRAJ43	DQ
40	HIP12	B6	TRBV29-1	TRBJ1-2	TRAV38-1	TRAJ43			DR
40	HIP12	F11	TRBV19	TRBJ1-1	TRAV17	TRAJ57			DR
40	HIP12	G5	TRBV19	TRBJ1-1	TRAV17	TRAJ57			ND
40	HIP14	B7	TRBV27	TRBJ2-5	TRAV16	TRAJ37	TRAV5	TRAJ40	DR
40	HIP14	C8	TRBV7-7	TRBJ2-5	TRAV22	TRAJ36			ND
40	HIP14	D3	TRBV27	TRBJ2-5	TRAV16	TRAJ37	TRAV5	TRAJ40	DR
40	HIP14	D8	TRBV27	TRBJ2-5	TRAV16	TRAJ37	TRAV5	TRAJ40	DR
40	HIP14	G10b	TRBV20-1	TRBJ1-2	TRAV13–1	TRAJ58			DR

ND, not determined.

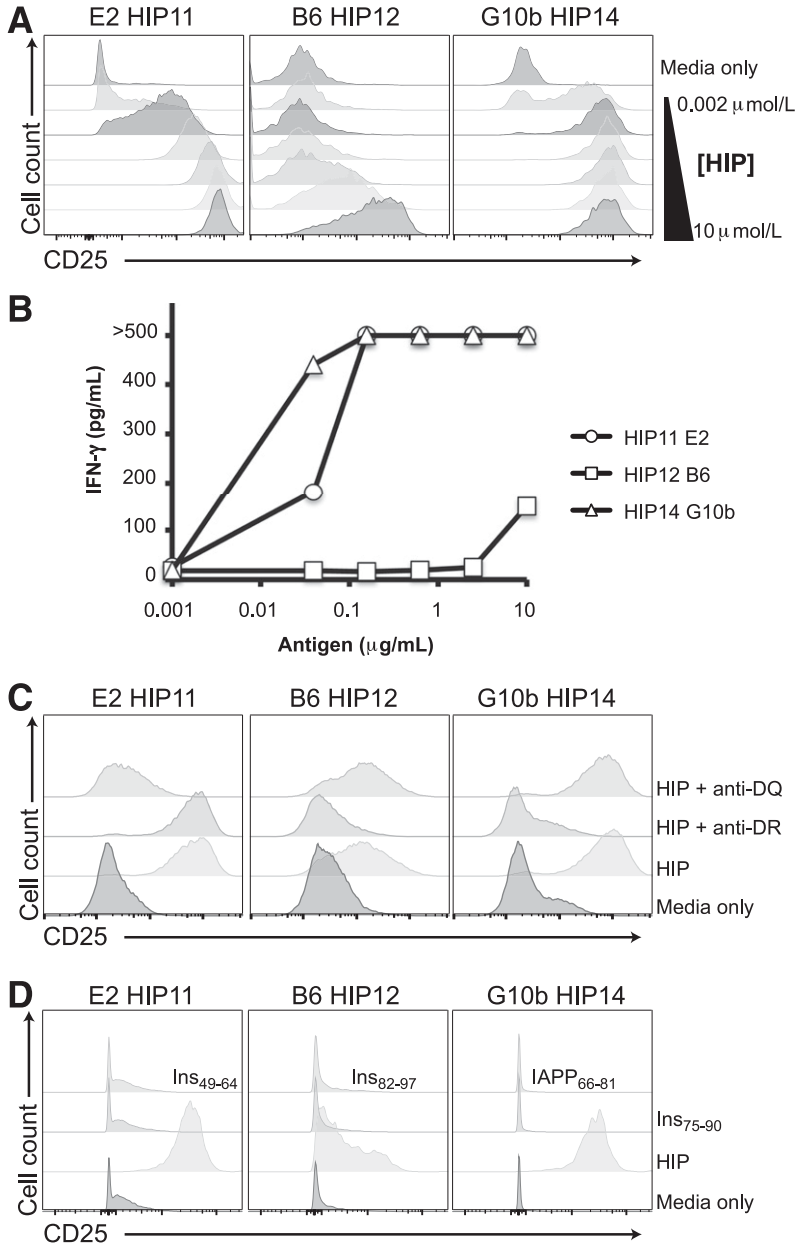

Figure 5—Characterization of HIP14-reactive T-cell clones. Freshly isolated PBMCs from patient #3196 were isolated 40 weeks after diagnosis, labeled with CFSE, and cultured with HIP11, HIP12, or HIP14. After 7 days, $CFSE^{dim}$ $CD25^{+}$ cells were single-cell sorted by FACS and expanded in vitro to generate the HIP-reactive T-cell clones. *A* and *B*: The HIP-reactive T-cell clone E2, B6, or G10b (1×10^5 cells) was challenged with HIP11, HIP12, or HIP14, respectively, at indicated concentrations. After 48 h, cells were harvested and stained with CD4 and CD25 before flow cytometry analysis (*A*), and supernatants from cell cultures were harvested and an IFN-γ ELISA was performed (*B*). *C*: The HIP-reactive T-cell clones (1×10^5 cells) were challenged with indicated HIP in the presence or absence of antigen, an anti-DR blocking antibody, or an anti-DQ blocking antibody. After overnight culture, cells were harvested and stained with CD4 and CD25 before flow cytometry analysis. *D*: The HIP-reactive T-cell clones E2, B6, and G10b (5×10^5 cells) were cultured in the presence of HIPs and indicated left or right peptides. Data are representative of three independent experiments.

differences were observed in the HLA-matched control group. A longitudinal analysis carried out on one patient (#3196) from the T1D cohort showed that HIP-specific responses can persist up to 1 year following diagnosis, after which time these responses were greatly diminished. Using a CFSE-based T-cell proliferation assay, we isolated six different HIP-reactive T-cell clones from a patient with T1D, with specificities toward three distinct HIPs (HIP11, HIP12, and HIP14). These T-cell clones, some of which responded to HIPs in the low nanomolar range, allowed us to confirm that responses were directed toward HIPs and not the germline-encoded amino acid sequences of the native parent proteins. We also tracked one HIP14-reactive T-cell clone present in a patient at two different blood draws spaced 14 weeks apart, which indicated that HIP-reactive T cells are

circulating in the peripheral blood for an extended period following disease onset.

We previously established that HIP-reactive T cells are present in residual islets of organ donors with T1D (1,6), and our current study demonstrates that these reactivities are also present in the peripheral blood in a subset of new-onset patients. We observed T-cell specificities toward four distinct HIPs, HIP4 (C-peptide/A-chain), HIP5 (C-peptide/IAPP1), HIP6 (C-peptide/IAPP2) (6), and HIP8 (C-peptide/NPY) (1), which confirms and extends our previous findings of islet-resident CD4 T cells in organ donors with T1D. Our data in the NOD mouse model, where we found that the phenotype of HIP-reactive T cells is an indicator of disease activity (2), along with the observation that HIP-reactive T cells are present in the islets of NOD mice (1,6) suggest a role for these T cells in the disease process. A recent study by Ito et al. (14) has confirmed and extended these findings by analyzing HIP tetramer-positive (tet+) cells isolated from the islets of NOD mice. Using single-cell RNA sequencing, the authors found that 2.5HIP tet+ cells have an activated phenotype in the islets and that very few of these cells express FoxP3, a marker of regulatory T cells. This was in contrast to insulin tet+ T cells, most of which were FoxP3+. Furthermore, they found that 2.5HIP tet+ cells isolated from the islets had a strong T-helper 1 bias, secreting large amounts of IFN-γ and tumor necrosis factor-α. T-helper 1 CD4 T cells have been strongly implicated in the pathogenesis of T1D (15), and therefore, the inflammatory phenotype of HIP-reactive T cells observed through our ELISPOT analysis on patient PBMCs is a possible indication of their contribution to disease.

The discovery of HIPs as T-cell epitopes and the observation of HIP-reactive T cells in the peripheral blood of patients with new-onset T1D, along with HIP T-cell reactivity in the inflamed islets of organ donors with T1D, provide strong rationale for investigating the relevance of HIP-reactive T cells as biomarkers of disease and for potential therapeutic intervention. Future studies should address several questions. First, how diverse are the HIPs that form in pancreatic β-cells? Comprehensive mass spectrometric analyses of pancreatic islets will allow us to address this question in greater detail. Results from such studies may provide new HIP sequences that can be used to identify additional HIP-reactive T cells that can be used as biomarkers for T1D. Second, how are HIPs formed? The mechanism by which hybrid peptide formation occurs is currently unknown, and elucidating how HIPs form in vivo could potentially identify new therapeutic targets. Third, are different HIP specificities found in the target organ? Further study of islet-infiltrating T cells could lead to identification of other HIP specificities in the pancreas. For example, T cells reactive to HIP4 have been found in the islets of a donor with T1D, and the current study demonstrated significantly elevated responses to this HIP in the peripheral blood of patients with T1D but not in control subjects without diabetes. We have observed increased reactivity to HIP11 (C-peptide/C-peptide) in the peripheral blood of seven patients with T1D, but this HIP has not been tested on T cells isolated from residual islets of organ donors with T1D. Finally, it will be of great interest to determine whether at-risk subjects (those positive for multiple islet autoantibodies) exhibit T-cell reactivity to HIPs before clinical T1D onset.

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Data and Resource Availability. The data sets generated during the current study are available from the corresponding author upon reasonable request. The CD4 T-cell clones generated during the current study are available from the corresponding author upon reasonable request.

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