Original Article

CD8$^+$ T-Cell Responses Identify β-Cell Autoimmunity in Human Type 1 Diabetes

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Despite the understanding that type 1 diabetes pathogenesis is mediated by T-cells, detection of these rare lymphocytes remains largely elusive. Suitable T-cell assays are highly needed, since they could offer preclinical diagnoses and immune surrogate end points for clinical trials. Although CD4$^+$ T-cell assays have met with limited success, CD8$^+$ T-cells are increasingly recognized as key actors in the diabetes of the NOD mouse. CD8$^+$ T-cells are likely to play a role also in humans and may provide new markers of β-cell autoimmunity. Taking advantage of a panel of HLA-A2–restricted β-cell epitopes derived from preproinsulin, GAD, and islet glucose-6-phosphatase catalytic subunit-related protein (IGRP), we have implemented an islet-specific CD8$^+$ T-cell interferon-γ enzyme-linked immunospot (ISL8Spot) assay. The ISL8Spot assay is capable of detecting and quantifying β-cell–reactive CD8$^+$ T-cells directly ex vivo, without any preliminary expansion, using either fresh or frozen samples. Positive ISL8Spot responses separate new-onset diabetic and healthy samples with high accuracy (86% sensitivity, 91% specificity), using as few as five immunodominant epitopes. Moreover, sensitivity reaches 100% when the ISL8Spot assay is complemented by antibody determinations. Combination of CD8$^+$ T-cell measurements with immune intervention strategies may open new avenues toward type 1 diabetes prediction and prevention. *Diabetes* 56:613–621, 2007

T-cells play the central pathogenic role in the diabetes of the NOD mouse. Despite this knowledge, the relevance of T-cell effectors in human type 1 diabetes has been difficult to assess because of their very low frequencies in peripheral blood. Indeed, current etiopathological diagnosis of new-onset diabetes as type 1 disease, as well as type 1 diabetes risk assessment, heavily relies on antibody (Ab) markers. However, Abs are not required for β-cell destruction (1,2), and their titers do not seem to correlate with T-cell responses (3–5). More importantly, the kinetics of their fluctuations make them unsuitable to predict the time to type 1 diabetes onset in at-risk individuals and to reflect tolerance induction during immune modulatory treatment (6). For these reasons, development of suitable T-cell assays remains a primary goal in type 1 diabetes research. First, it is important for deciphering the underlying disease process. Second, surrogate immune markers closely reflecting the pathogenic evolution could be highly valuable to optimally assess eligibility for immune interventions (i.e., which patients and when) and to monitor clinical response.

Given the strong association between type 1 diabetes and the HLA class II locus (7), most studies to date have focused on β-cell–specific autoimmune responses driven by CD4$^+$ T-cells. However, recent work in the NOD mouse has established a prominent role for CD8$^+$ T-cells (8–10). NOD mice devoid of CD8$^+$ lymphocytes do not experience insulitis (11,12), and CD8$^+$ clones from pre-diabetic and diabetic animals can provoke diabetes (13–15). More importantly, CD8$^+$ T-cells specific for insulin $B_{15–23}$ (13,16) and for islet-specific glucose-6-phosphatase catalytic subunit–related protein $(IGRP)_{206–214}$ (17) are early pathogenic actors in the diabetes pathogenesis of NOD mice. Quantification of $IGRP_{206–214}$–specific CD8$^+$ T-cells in the peripheral blood of NOD mice has also been shown to predict subsequent diabetes development (18). Therefore, CD8$^+$ T-cell monitoring holds promise for the "immune staging" of human type 1 diabetes but is hampered by technical difficulties in their measurement.

Development of suitable CD8$^+$ T-cell assays depends on several factors. First, appropriate readouts and measurement techniques must be selected. Interferon (IFN)-γ secretion, as measured by enzyme-linked immunospot (ELISpot), is currently the preferred system in the viral and tumor immunology setting, because of its excellent sensitivity and simple format. Second, assays should incorporate the most relevant HLA class I restrictions. Although no strong association has been proposed between particular HLA class I alleles and type 1 diabetes, HLA-A2 (HLA-A*0201) is the most represented allele among Caucasians (~45% of subjects), and it has been suggested to mediate an additional susceptibility to diabetes in NOD mice (19,20). Third, the relevant epitopes should be identified. We recently reported a systematic HLA-A2–restricted epitope exploration for preproinsulin (PPI), using a reverse immunology approach combining

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Ab, antibody; ELISpot, enzyme-linked immunospot; IFN, interferon; IGRP, islet glucose-6-phosphatase catalytic subunit–related protein; ISL8Spot, islet-specific CD8$^+$ T-cell interferon-γ enzyme-linked immunospot; PBMC, peripheral blood mononuclear cell; PPI, preproinsulin; SFC, spot-forming cells.

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TABLE 1
The HLA-A2–restricted epitope panel used in the ISL8Spot assay

	Sequence	SYFPEITHI score	Reference
$PPI_{2–10}$	ALWMRLLPL	28	Unpublished
$PPI_{6–14}$	RLLPLLALL	31	Unpublished
$PI_{B10–18}$ ($PPI_{34–42}$)	HLVEALYLV	27	Hassainya et al. (21); Toma et al. (23); Pinkse et al. (24)
$PI_{B18–27}$ ($PPI_{42–51}$)	VCGERGFFYT	7	Hassainya et al. (21); Toma et al. (23)
$PI_{C20–28}$ ($PPI_{76–84}$)	SLQPLALEG	18	Hassainya et al. (21)
PI_{C29-A5} ($PPI_{85–94}$)	SLQKRGIVEQ	20	Hassainya et al. (21)
PI_{A1-A10} ($PPI_{90–99}$)	GIVEQCCTSI	21	Hassainya et al. (21)
$PI_{A12–20}$ ($PPI_{101–109}$)	SLYQLENYC	15	Hassainya et al. (21)
$GAD65_{114–123}$	VMNILLQYVV	21	Panina-Bordignon et al. (29)
$IGRP_{228–236}$	LNIDLLWSV	22	Takaki et al. (20)
$IGRP_{265–273}$	VLFGLGFAI	24	Takaki et al. (20)
Flu $MP_{58–66}$	GILGFVFTL	30	Viral mix positive control
EBV $BMLF1_{280–288}$	GLCTLVAML	28	Viral mix positive control
CMV $pp65_{495–503}$	NLVPMVATV	30	Viral mix positive control
HIV $gag_{77–85}$	SLYNTVATL	31	Negative control

For PPI, amino acid (aa) numbering is given both with respect to the PI sequence alone (aa B1-A21) and to the complete PPI sequence (aa 1–110).

proteasome digestions with prediction algorithms and subsequent verification of natural processing and immunogenicity (21,22). This study led to the identification of seven novel PPI candidate epitopes, two of which were subsequently shown to be targeted by $CD8^+$ T-cells from type 1 diabetic patients (23,24). Taking advantage of this epitope information, we here describe an islet-specific $CD8^+$ T-cell interferon-γ enzyme-linked immunospot (ISL8Spot) assay that reliably separates type 1 diabetic subjects from healthy subjects.

RESEARCH DESIGN AND METHODS

Peptides. The epitope peptides used (>80% pure; Schafer-N, Copenhagen, Denmark) are listed in Table 1. A viral peptide mix of Flu $MP_{58–66}$, Epstein-Barr virus $BMLF_{280–288}$, and cytomegalovirus $pp65_{495–503}$ (10 μmol/l each) and phytohemagglutinin (1 μg/ml; Sigma, Lyon, France) were used as positive controls. HIV $gag_{77–85}$ and DMSO diluent were used as negative controls.

Patients. Caucasian new-onset adult (>16 years old) type 1 diabetic patients with acute onset of symptoms requiring permanent insulin treatment from the time of diagnosis (25) were recruited from the Diabetes Registry of the Province of Turin, Italy, and from the GOFEDI Network, France, as detailed in the APPENDIX. All patients had metabolically controlled disease and were free of recent (<2 weeks) infectious or inflammatory conditions at the time of blood draw. Parallel recruitment of Caucasian healthy control subjects took place at the same institutions. Type 2 diabetic patients were defined as Ab^- subjects with normal fasting C-peptide levels (normal values 0.36–1.17 nmol/l; DPC, Los Angeles, CA). All subjects gave informed consent, and the study was approved by the relevant ethics committees.

Blood processing. All blood samples were shipped overnight at room temperature in blinded fashion (typically, each type 1 diabetes sample was paired with a healthy sample). Rapid HLA-A2 screening was performed with the BB7.2 mAb (26), followed by subtyping using the Olerup SSP HLA*02 kit (GenoVision/Qiagen, Vienna, Austria). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using lymphocyte separation medium (PAA, Les Mureaux, France) and immediately used or stored frozen (10% DMSO in pooled human male AB serum).

Islet Abs. Serum Abs were measured by radio-binding assays, using ^{35}S-labeled GAD65 and intracellular insulinoma-associated protein 2 (IA-2) and ^{125}I-labeled insulin, following protocols previously evaluated within the Diabetes Antibody Standardization Program (lab number 137) (27). Sensitivities in the 2005 Diabetes Antibody Standardization Program were 84% for anti-GAD, 76% for anti–IA-2, and 28% for insulin autoantibodies, where the specificity was set at 95%. Islet cell Abs were assayed by indirect immunofluorescence on frozen sections of human blood group 0 pancreas.

ISL8Spot assay. Ninety-six–well PVDF plates (Millipore, Saint-Quentin-en-Yvelines, France) were coated overnight with an anti–IFN-γ Ab (U-CyTech, Utrecht, the Netherlands). Plates were subsequently blocked with RPMI + 10% human serum (PAA), and peptides were added (10 μmol/l final concentration) in triplicate wells along with recombinant human IL-2 (0.5 units/ml; R&D Systems, Lille, France). PBMCs were seeded at 3×10^5 cells/well and cultured for 20–24 h. In selected experiments, PBMCs preincubated for 10 min at room temperature with 25 μg/ml anti-CD8 (OKT8) or with 50 μg/ml anti–HLA-A2 (BB7.2) mAb, or the $CD8^-$ fraction negatively selected by anti-CD8 microbeads (Miltenyi Biotech, Paris, France), were seeded in parallel. After PBMC removal, IFN-γ secretion was visualized with a biotin-conjugated anti–IFN-γ Ab (U-CyTech), alkaline phosphatase-conjugated Extr-Avidin, and Sigmafast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (both from Sigma). Spots were counted using an AID reader (Strassberg, Germany), and means of triplicate wells were calculated. All ISL8Spot readouts are expressed as spot-forming cells (SFC)/10^6 PBMCs.

Statistical analysis. Values are expressed as means ± SD or median (range), according to their distribution. All graphs are displayed as means ± SE. Comparisons between proportions were made with the χ^2 test and Fisher's exact test, when appropriate. Comparisons of means between two groups were carried out with the Student's t test for normal distributed variables or with the Mann-Whitney U test for non-normal variables. Analysis of data was done using Stata 7.0 (Stata, College Station, TX). $P < 0.05$ was considered to be of statistical significance.

RESULTS

Selection of HLA-A2–restricted β-cell epitopes. The peptides used in the ISL8Spot assay are listed in Table 1. Most of these epitopes are derived from PPI and were recently characterized by us and others as naturally processed and immunogenic (21,23,24). Two $PPI_{2–10}$ and $PPI_{6–14}$ leader sequence peptides rating the highest in the SYFPEITHI algorithm (28) were also included, as this region is associated exclusively with β-cells and may contain additional epitopes. This 24–amino acid region was not included in our previous PPI epitope search (21), since it was found to be resistant to proteasome digestion, presumably because of its hydrophobicity (P.v.E., unpublished data). The $GAD_{114–123}$ epitope originally described 10 years ago by Panina-Bordignon et al. (29) and two IGRP epitopes recently proposed by Takaki et al. (20) were also considered to cover additional β-cell antigens.

ISL8Spot validation. We aimed to implement an assay format amenable to standardization for routine clinical use, with minimal sample handling. For this reason, our IFN-γ ELISpot system was set up to work with unfractionated PBMCs, without preliminary expansions, and with both freshly prepared and frozen samples. This format was first validated by studying PBMC responses against mixed viral epitopes. Suboptimal epitope concentrations (30

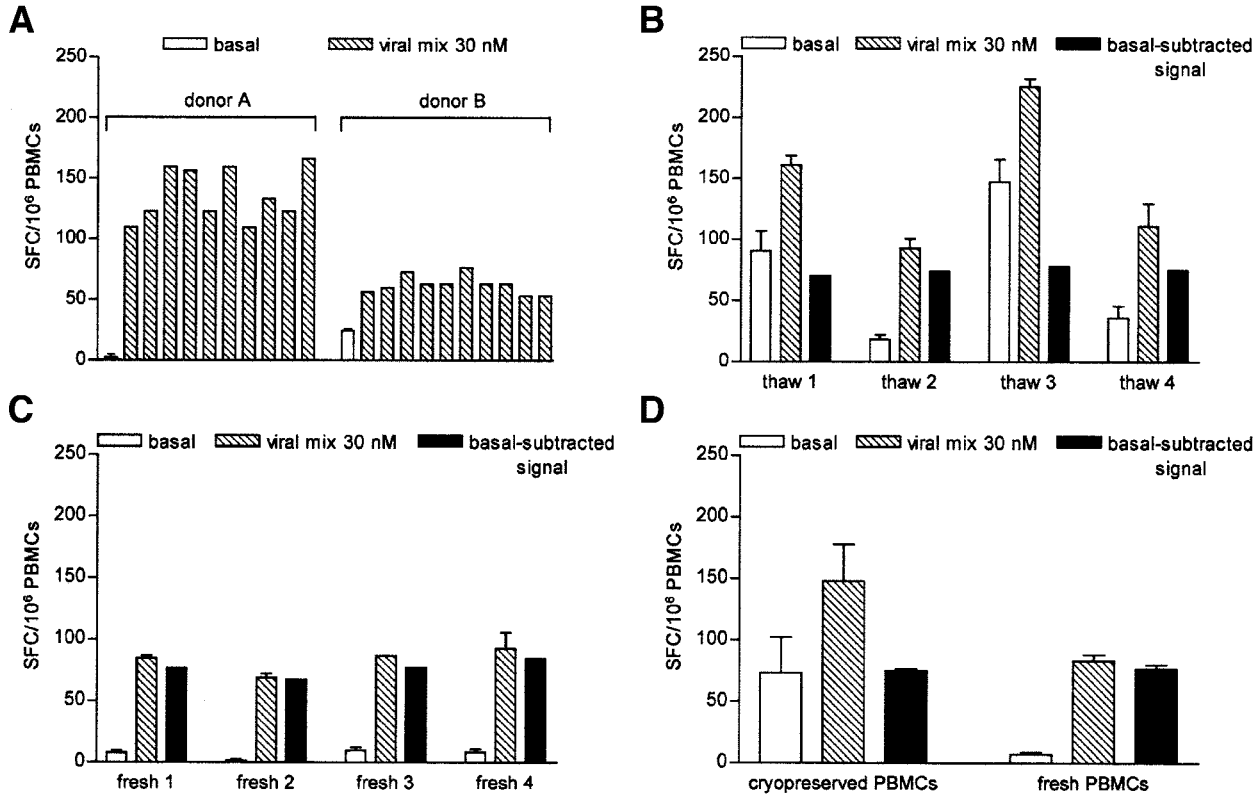

FIG. 1. ***A*: ISL8Spot intra-assay variability. PBMCs from two HLA-A2$^+$ donors were assayed against gag$_{77–85}$ control peptide and DMSO diluent alone ("basal," □; mean ± SE of 10 wells is shown) or with 30 nmol/l viral peptide mix (▨; single well counts are shown). CVs are 16.1 and 12.2% for donor A and B, respectively. *B*: Interassay variability, analytical level. An HLA-A2$^+$ donor was venesected on a single occasion, PBMCs were frozen down, and single aliquots were subsequently thawed and tested in four separate experiments as above. Basal and viral mix–specific readouts are shown (□ and ▨, respectively) along with final basal-subtracted values (■; CV 4.2%). *C*: Interassay variability, analytical and preanalytical level. Blood was drawn from the same donor on four different occasions and at the same time of the day. The following day, PBMCs were prepared and assayed as above; CV 9.2%. *D*: Interassay variability, frozen versus fresh samples. The graph shows means ± SE for SFC/10^6 values of fresh PBMCs prepared and tested on four separate occasions and of cryopreserved PBMCs frozen on the same occasion and tested in four separate experiments.**

nmol/l) were used in order to have responses of a magnitude similar to that found against β-cell epitopes (basal-subtracted median positive responses of 44.0 SFC/10^6 PBMCs, range 6.0–832.7; Fig. 3). Within this range of sensitivity, intra-assay variability, as measured in single wells from single experiments, was typically 12.2–16.1% (Fig. 1*A*). Despite variable basal reactivities against the gag$_{77–85}$ control peptide and the DMSO diluent alone, the interassay analytical variability, as determined by multiple assays on different PBMC aliquots frozen on the same occasion, was 4.2% (Fig. 1*B*). Because interassay variability in the clinical setting can also be determined by changes at the pre-analytical level, we determined the coefficients of variation (CVs) for multiple assays performed on different blood draws from the same subjects, finding a value of 9.2% (Fig. 1*C*). Finally, we compared the difference in sensitivity between fresh and frozen samples by testing PBMCs from the same subject either fresh or upon cryopreservation and thawing (Fig. 1*D*). Despite a higher average background reactivity in frozen PBMCs, the basal-subtracted signal was equivalent in both types of samples (74.8 vs. 76.3 SFC/10^6 PBMCs for cryopreserved vs. fresh PBMCs). This validation also supports the use of basal-subtracted values to compare different experiments.

A receiver-operator characteristic analysis was performed to select the best cutoff for assigning positive responses (Fig. 2*A*). The mean + 3 SD of basal readouts (DMSO and gag$_{77–85}$) was thus selected as the cutoff attaining the best sensitivity and specificity.

Representative examples of ISL8Spot readouts are shown in Fig. 2*B*. As expected based on the 9– to 10–amino acid length of the peptides used, the visualized reactivities originated from HLA-A2–restricted CD8$^+$ T-cells, as the signal was abolished by blocking anti–HLA-A2 or anti-CD8 mAbs (91.4 and 95.0% inhibition, respectively; Fig. 2*C*) or by depletion of the CD8$^+$ fraction (not shown).

Detection of β-cell–specific CD8$^+$ T-cells by ISL8Spot. Characteristics of the study subjects are summarized in Table 2. For the HLA-A2$^+$ type 1 diabetic patients, 86.4% (19/22) were islet Ab$^+$ (1 of 17 healthy control subjects, 5.9%; $P < 0.0001$), 84.2% (16 of 19) were HLA-DR3$^+$ and/or -DR4$^+$ (5 of 18 healthy control subjects, 27.8%; $P = 0.0006$), and the median type 1 diabetes duration was 12 days (range 3–180). The type 1 diabetic and healthy cohorts were age matched (29.2 ± 8.5 vs. 32.7 ± 10.9 years; $P = 0.55$).

Results are summarized in Fig. 3. All data were obtained with blood samples shipped overnight and freshly prepared PBMCs, except for patients P03, P04, P12, P15, and P22, for whom blood samples were shipped overnight, processed, frozen, and used upon thawing. Reactivities were ranked as low (>3 and <4 SD), intermediate (>4 and <5 SD), and high (>5 SD).

Of 22 HLA-A2$^+$ type 1 diabetic patients, 19 were positive for at least one epitope (86.4% sensitivity); reactivity frequently targeted multiple epitopes (median 2.0, range 0–5). Epitope-specific CD8$^+$ T-cells were detected also in most (80.0%; 4 of 5) frozen PBMC samples. Of the 22

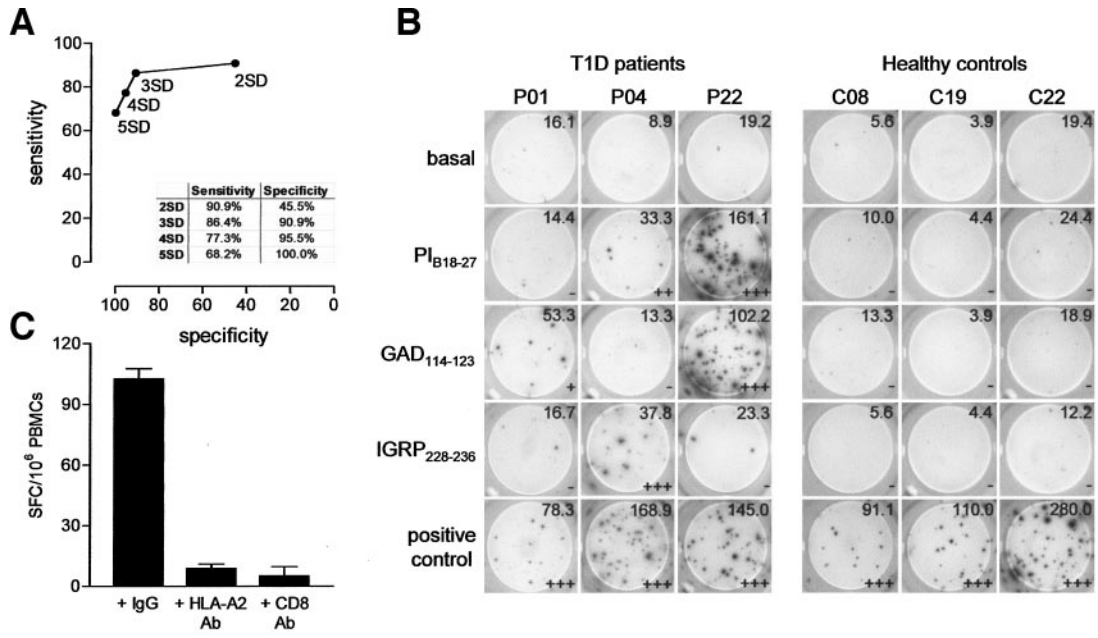

FIG. 2. ***A*: Receiver-operator characteristic plot analysis was used to select the cutoff value attaining the best assay accuracy. Sensitivity (i.e., number of type 1 diabetic [T1D] patients testing positive to at least one of the 11 β-cell epitopes listed in Table 1; $n = 22$) and specificity (i.e., number of healthy control subjects testing negative to all β-cell epitopes; $n = 22$) are compared for each possible cutoff value, and the value providing the best sensitivity and specificity is selected. *B*: Representative ISL8Spot assays against selected β-cell epitopes for three HLA-A2$^+$ type 1 diabetic patients and three healthy control subjects. Numbers in the top right corner display the SFC/10^6 PBMCs (without basal subtraction). Each peptide is assayed in triplicate, while basal wells (DMSO diluent alone or irrelevant peptide) are assayed in sextuplicate. The symbol in the bottom right corner refers to whether reactivity was scored as negative (−), weakly positive (+; between 3 and 4 SD), intermediate positive (++; between 4 and 5 SD), or strongly positive (+++; >5 SD). *C*: ISL8Spot reactivity is mediated by HLA-A2–restricted CD8$^+$ T-cells. A representative analysis from a type 1 diabetic patient positive for the $GAD_{114–123}$ epitope is shown. PBMCs preincubated with an irrelevant IgG, anti–HLA-A2, or anti-CD8 mAb were subjected to the ISL8Spot assay in parallel wells. Means ± SE of basal-subtracted values are shown, where the basal was 2.3 SFC/10^6.**

age-matched HLA-A2$^+$ healthy control subjects tested, 20 were negative for all epitopes (90.9% specificity; $P < 0.0001$ for the type 1 diabetes vs. healthy cohort comparison). Interestingly, subject C12 was positive for T-cell responses and also harbored risk factors for type 1 diabetes, being HLA-DR3$^+$ and weakly positive for insulin autoantibodies. Testing of HLA-A2$^+$ type 2 diabetic patients ($n = 5$) and of age-matched new-onset HLA-A2$^-$ type 1 diabetic patients ($n = 4$) did not reveal any reactivity (Fig. 3).

Five epitopes ($PPI_{2–10}$, $PI_{B18–27}$, $PI_{A12–20}$, $GAD_{114–123}$, and $IGRP_{228–236}$) were recognized by >25% of type 1 diabetic patients. Importantly, when the analysis is restricted to these five immunodominant epitopes, the same type 1 diabetic patients are correctly identified without any loss in sensitivity and specificity.

There was no correlation between number or quality of epitopes recognized and type 1 diabetes duration, age of onset, or sex. Although unlikely, a modifying effect of DR3/DR4 haplotypes on CD8$^+$ T-cell responses was excluded, since the frequency of positive responses remained significantly higher in type 1 diabetic patients than in control subjects when the analysis was restricted to DR3$^+$ and/or DR4$^+$ subgroups (87.5% [14 of 16] vs. 14.3% [1 of 7] for type 1 diabetic vs. healthy and type 2 diabetic donors, $P = 0.0006$; $P = 0.003$ for type 1 diabetic vs. healthy subjects alone). Similarly, no correlation emerged between Ab and T-cell responses, considering single antigens separately (e.g., insulin autoantibodies vs. PPI T-cell responses) or together (i.e., any Ab vs. any T-cell response). However, the three patients negative by ISL8Spot were all Ab$^+$, whereas the three Ab$^-$ patients (P04, P18, and P22) tested positive by ISL8Spot. Thus, a combination of ISL8Spot and Ab testing achieved 100% sensitivity in identifying type 1 diabetic patients.

DISCUSSION

Several immune intervention strategies for type 1 diabetes are currently under scrutiny (6,30–33). These interventions could achieve better results if implemented at a preclinical stage, when a larger β-cell mass can still be rescued from autoimmune destruction (34). However, this requires both accurate prediction of type 1 diabetes development and the possibility to monitor clinical efficacy through immune surrogate markers reflecting restoration of tolerance. It has emerged that anti-islet Abs are not sufficient to fulfill this task (6), and great hopes have therefore been invested in markers of T-cell activation against β-cell epitopes (35).

Because the association between DR3/DR4 alleles and type 1 diabetes skewed the interest of researchers, a decade of studies investigated whether CD4$^+$ T-cell responses could differentiate between type 1 diabetes and health. Most of these studies were unsuccessful, since β-cell–reactive CD4$^+$ T-cells were difficult to visualize, usually requiring in vitro pre-expansion (36,37). When detected, β-cell–specific CD4$^+$ T-cells were also identified in healthy individuals (38,39), although possibly characterized by different functional phenotypes (37,40). The emergence of CD8$^+$ T-cells as crucial actors in the type 1 diabetes pathogenesis of the NOD mouse (8–10) has brought up the possibility to study this lymphocyte subpopulation to the same end. However, only anecdotal analyses, mostly oriented toward epitope identification, have been reported for human type 1 diabetes (23,24,29,41,42).

TABLE 2
Characteristics of study subjects

Case number	Age (years)	Sex	Diabetes duration (days)	HLA DRB1	GAD Ab* (arbitrary units)	IA-2 Ab† (arbitrary units)	Insulin autoantibodies (arbitrary units)‡	Islet cell Abs (Juvenile Diabetes Foundation units)§	
HLA-A2$^+$ type 1 diabetic patients (n = 22)									
P01	17	M	122	—	**1,939**	**1,529**	**3.77**	Negative	
P02	38	M	180	04–16	**2,817**	13	**2.54**	**640**	
P03	37	M	60	07–08	**2,898**	29.9	0.24	—	
P04	43	M	5	11–15	60	11	0.30	Negative	
P05	36	F	6	03–08	**3,848**	19	**1.04**	—	
P06	21	F	24	03–16	**2,894**	**598**	0.40	—	
P07	33	M	8	01–03	95	13	0.27	**20**	
P08	23	M	16	04–13	58	18	0.31	**5**	
P09	32	M	29	—	**2,792**	13	0.28	Negative	
P10	33	M	19	—	**2,114**	**505**	0.44	—	
P11	16	F	6	01–01	**1,441**	**578**	0.29	**20**	
P12	19	M	20	03–03	**283**	15	0.34	Negative	
P13	25	M	3	01–03	**2,970**	—	—	—	
P14	28	M	4	03–04	**3,125**	**2,439**	**0.86**	—	
P15	18	M	13	03–15	**883**	11	0.35	—	
P16	29	M	26	03–04	**2,548**	**1,570**	0.35	**20**	
P17	27	M	70	03–13	**3,292**	30	**0.77**	Negative	
P18	36	M	5	04–13	58	15	0.35	Negative	
P19	46	F	11	01–03	**3,192**	**133**	0.27	—	
P20	24	F	4	03–13	**1,602**	**2,876**	0.33	—	
P21	36	F	4	04–04	126	**2,039**	**1.00**	—	
P22	26	M	3	03–10	28	21	0.30	Negative	
HLA-A2$^+$ healthy subjects (n = 22)									
C01	25	F	—	13–14	40	19	0.23	Negative	
C02	26	F	—	09–11	37	11	0.20	Negative	
C03	30	F	—	01–16	41	15	0.37	Negative	
C04	32	M	—	11–16	60	10	0.22	Negative	
C05	55	M	—	03–13	—	—	—	—	
C06	26	F	—	07–07	48	16	0.40	Negative	
C07	29	M	—	09–13	63	5	0.21	—	
C08	33	F	—	04–14	63	9	0.23	Negative	
C09	27	M	—	07–15	25	16	0.26	Negative	
C10	26	F	—	08–11	—	—	—	—	
C11	25	F	—	11–15	48	14	0.36	Negative	
C12	23	F	—	03–11	41	26	**0.76**	Negative	
C13	58	F	—	07–14	—	—	—	—	
C14	29	F	—	04–11	46	13	0.19	Negative	
C15	27	M	—	01–11	66	28	0.20	Negative	
C16	56	F	—	—	—	—	—	—	
C17	26	F	—	01–07	43	27	0.28	Negative	
C18	26	F	—	13–15	70	14	0.15	—	
C19	25	M	—	—	76	20	0.28	Negative	
C20	48	F	—	04–11	70	13	0.13	—	
C21	35	M	—	—	—	—	—	—	
C22	33	M	—	—	60	14	0.49	—	
HLA-A2$^+$ type 2 diabetic patients (n = 5)									Therapy
Q01	26	M	7	04–09	48	25	—	—	Insulin
Q02	31	M	5	11–12	46	6	—	Negative	Insulin
Q03	46	M	162	11–16	15	9	—	Negative	OHA
Q04	56	M	192	04–16	17	12	—	Negative	OHA
Q05	41	M	360	11–11	66	13	—	Negative	OHA
HLA-A2$^-$ type 1 diabetic patients (n = 4)									
N01	42	F	29	—	**3,784**	**2,688**	0.30	—	
N02	41	M	18	—	**1,113**	**1,405**	0.22	—	
N03	15	F	6	04–04	**594**	19	0.42	—	
N04	19	F	16	03–04	**1,175**	**295**	**3.66**	**80**	

The 97.5th percentile cutoff values for Ab titers are as follows: *GAD Abs: 180 arbitrary units. †IA-2 Abs: 80 arbitrary units. ‡Insulin autoantibodies: 0.7 arbitrary units. §Islet cell Abs: 5 Juvenile Diabetes Foundation units. Positive Ab titers are displayed in bold. —, Not done; OHA, oral hypoglycemic agent.

		T1D PATIENTS (n=22)																					
	% positive	P01	P02	P03	P04	P05	P06	P07	P08	P09	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22
PPI_{2-10}	31.8 (7/22)	0	27.2	0	5.5	1.5	4.5	0	0	44.6	0	16.7	48.0	0	26.7	16.8	7.1	697.1	260.0	15.6	19.4	2.2	47.5
PPI_{5-14}	6.7 (1/15)	0	22.5	5.0	0	1.3	0	10.0	0	18.0	0	27.8	41.4	0		0	2.6						
PI_{B10-18} (PPI_{24-42})	19.0 (4/21)	0.6	18.3	43.4	12.2	0	2.2	0	0	0	0	35.5	12.5	6.7	63.3	0	0	26.1	190.0	7.8	0	0	
PI_{B18-27} (PPI_{42-51})	40.0 (8/20)	0	29.3	0	24.4	3.2	0	0	0	58.0	2.8	4.4	39.2	36.1		0		832.7	372.7	0.0	7.8	0	141.9
PI_{C20-28} (PPI_{78-84})	13.3 (2/15)	0	13.3	36.7	7.8	2.4	2.2	0	0	18.0	0	5.5	8.1	10.0		0	6.0						
PI_{C29-A5} (PPI_{85-94})	13.3 (2/15)	0.6	47.8	0	1.1	92.4	1.1	0	0	0	0	0	0	4.4		20.2	0						
PI_{A1-A10} (PPI_{90-99})	0 (0/12)	1.7	24.4	0	0	0	3.3	2.3	0	0	0	14.4					3.7						
PI_{A12-20} ($PPI_{101-109}$)	33.3 (7/21)	0	78.3	7.3	7.8	0	10.0	5.6	0	44.6	2.6	20.0	106.9	2.2	108.3	21.3	0		336.8	4.5	0	0	4.1
$GAD65_{114-123}$	50.0 (11/22)	37.2	34.4	35.0	4.4	86.4	10.0	0	11.1	0	0	0	22.5	2.2	8.9	9.1	20.4	820.5	281.1	8.9	7.8	82.2	83.0
$IGRP_{228-236}$	25.0 (3/12)	0.6	81.7	0	28.9	3.5	1.1	1.2	36.1		0.4		0			12.4							4.1
$IGRP_{265-273}$	20.0 (2/10)	0	11.1	6.1		2.4	3.3	2.3	41.1		0					13.5							148.6
Viral mix control	95.4 (21/22)	62.2	53.3	532.8	160.0	841.2	343.3	4.5	223.3	310.2	837.0	287.8	253.6	197.7	++++	160.2	458.2	1053.8	++++	45.6	32.2	422.2	125.8
PHA control	100 (22/22)	519.4	++++	++++	++++	++++	712.2	752.2	638.8	729.0	++++	211.1	++++	++++	++++	++++	1557.8	++++	++++	684.3	733.4	++++	++++
Basal+3SD		31.2	26.5	19.1	16.3	28.1	5.2	12.1	25.8	23.6	15.1	16.3	53.8	20.8	28.3	55.0	4.4	66.2	159.5	15.3	5.8	3.0	47.9
Basal+4SD		41.7	35.3	25.5	21.8	37.5	6.9	16.1	34.4	31.5	20.1	21.8	71.7	27.8	37.7	73.4	5.9	88.3	212.6	20.4	7.7	4.0	63.8
Basal+5SD		52.1	44.0	32.0	27.3	46.8	8.6	20.2	43.0	39.4	25.3	27.3	89.6	34.8	47.1	91.7	7.3	110.5	265.8	25.5	9.5	5.0	79.8
Basal		16.1	20.0	18.3	8.9	14.3	1.1	4.4	42.2	68.7	8.5	8.9	37.5	8.9	90	68.7	0.7	85.0	152.2	14.4	2.2	3.3	19.2
positive epitopes	2.0 (0-5)	1	5	3	2	2	2	0	2	3	0	4	1	1	2	0	3	3	5	1	3	1	3

		HEALTHY CONTROLS (n=22)																					
	% positive	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22
PPI_{2-10}	0 (0/22)	0	0	19.5	0	0	0	0	0	8.9	0	7.7	5.6	10.4	4.4	0	0	2.7	0	0	1.2	26.1	0
PPI_{5-14}	4.5 (1/22)	0	0	30.6	21.5	0	0	0.5	1.1	3.4	0	17.7	34.4	0	0	0	10.0	3.8	0.6	0	10.0	38.4	2.3
PI_{B10-18} (PPI_{24-42})	0 (0/22)	0	0	0	5.9	0	0	0.5	2.2	7.8	0	0	12.2	12.7	0	0	0	2.7	0	0.5	0	32.8	0
PI_{B18-27} (PPI_{42-51})	0 (0/21)	0	0	0	4.8	0	11.1	1.6	4.4	5.6	0.4	0		10.4	2.2	0	0	0	0	0.5	2.3	0	5.0
PI_{C20-28} (PPI_{78-84})	0 (0/21)	0	0	0	17.1	0	24.5	0	4.4	8.9	0		17.8	6.0	0	0	0	1.6	0	1.7	15.6	0	0
PI_{C29-A5} (PPI_{85-94})	0 (0/22)	0	0	0	13.7	0	0	0	2.2	4.5	0	0	15.6	21.6	0	0	0	1.6	0	1.7	15.6	17.3	0
PI_{A1-A10} (PPI_{90-99})	0 (0/22)	0	0	0	33.7	0	0	0	0	7.8	0	0	4.4	12.7	6.7	0	0	3.8	0	0	20.0	8.4	0
PI_{A12-20} ($PPI_{101-109}$)	4.5 (1/22)	0	0	0	0	0	0	2.7	2.2	1.1	0	10.0	31.1	24.9	21.1	18.3	0	1.6	2.8	0.5	31.1	6.2	0.6
$GAD65_{114-123}$	0 (0/21)	1.1	0	4.0	11.5	0	0	5.0	7.7	6.7	0		12.2	29.3	4.4	20.6	0	2.7	12.8	0	37.8	49.5	0
$IGRP_{228-236}$	4.8 (1/21)	0	0	0	0	0.6	60.0	1.6	0	4.5	42.7	117.7		0	0	0	6.7	2.7	0	0.5	6.7	0	0
$IGRP_{265-273}$	0 (0/20)	3.3	34.0	0	20.4	2.8	0	0	0	7.8	0			1.6	0	0	0	2.7	0	0	52.3	0	0.6
Viral mix control	86.4 (19/22)	13.3	466.2	806.1	238.1	347.2	201.1	458.3	85.5	364.4	3.8	326.6	202.2	618.2	308.9	345.0	308.9	502.7	353.9	106.1	534.4	30.0	260.6
PHA control	100 (22/22)	677.7	++++	++++	++++	++++	++++	612.7	724.3	806.6	++++	++++	1340.0	577.1	++++	++++	++++	674.9	819.4	397.2	++++	++++	407.2
Basal+3SD		127.9	44.8	32.7	57.1	9.8	164.0	5.5	8.2	26.3	44.4	114.7	19.0	51.2	24.5	102.4	104.1	4.1	13.3	9.8	73.1	64.9	40.7
Basal+4SD		170.5	59.8	43.6	76.1	13.1	218.7	7.3	10.9	35.1	59.2	152.9	22.7	68.2	32.7	136.6	138.7	5.4	17.7	13.1	97.5	86.6	54.3
Basal+5SD		213.3	74.8	54.5	95.3	16.5	273.3	9.1	13.6	43.9	74.0	191.3	38.0	85.3	40.8	170.7	173.5	6.8	22.2	16.4	121.9	108.2	67.8
Basal		36.7	30.4	32.7	59.6	6.1	165.5	1.7	5.6	3.3	34.0	66.7	7.8	24	10.0	57.2	50.0	0.6	6.1	3.9	54.4	49.4	19.4
positive epitopes	0 (0-2)	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0

		T2D PATIENTS (n=5)					HLA-A2- T1D PATIENTS (n=4)			
	% positive	Q01	Q02	Q03	Q04	Q05	N01	N02	N03	N04
PPI_{2-10}	0 (0/5)	0.4	1.1	0	0	1.1	0	2.9	32.0	2.3
PPI_{5-14}	0 (0/5)	0	1.1	0	1.6	0	1.1	15.1		0
PI_{B10-18} (PPI_{24-42})	0 (0/5)	0	1.1	0	0	1.1	0	0.7	0	3.4
PI_{B18-27} (PPI_{42-51})	0 (0/5)	0	0	0	0.5	3.4	2.2	0	32.0	3.4
PI_{C20-28} (PPI_{78-84})	0 (0/5)	0	10.0	0	0.5	0		0		2.3
PI_{C29-A5} (PPI_{85-94})	0 (0/5)	0	0	0	2.8	2.2		4.0	29.7	5.6
PI_{A1-A10} (PPI_{90-99})	0 (0/5)	0	2.2	0	1.6	1.1		0		13.4
PI_{A12-20} ($PPI_{101-109}$)	0 (0/5)	5.6	0	0	5.0	1.1	2.2	7.3	24.2	10.0
$GAD65_{114-123}$	0 (0/5)	0.4	1.1	0	1.6	4.5	0	0	0	1.1
$IGRP_{228-236}$	0 (0/5)	1.1	3.3	0	0	1.1	0	0	36.4	
$IGRP_{265-273}$	0 (0/5)	0	3.3	0	0	0		0		
Viral mix control	100 (5/5)	832.1	405.5	167.7	409.4	156.7	0	8.4	22.0	187.8
PHA control	100 (5/5)	++++	663.2	584.3	494.9	200.0	296.6	++++	++++	727.7
Basal+3SD		16.6	20.7	8.2	9.8	8.2	15.8	31.1	70.9	15.5
Basal+4SD		22.1	27.5	10.9	13.1	10.9	21.1	41.5	94.6	20.7
Basal+5SD		27.8	34.5	13.6	16.4	13.6	26.3	51.9	118.2	25.8
Basal		8.9	7.8	5.6	2.8	2.2	16.7	36.0	94.7	3.3
positive epitopes	0	0	0	0	0	0	0	0	0	0

FIG. 3. Summary of HLA-A2+ type 1 diabetic (T1D) patients (n = 22), HLA-A2+ healthy control subjects (n = 22), HLA-A2+ type 2 diabetic (T2D) patients (n = 5), and HLA-A2− type 1 diabetic patients (n = 4) assayed for β-cell reactivities. All values, including basal + n SD cutoffs, are expressed as SFC/10^6 PBMCs and are basal subtracted. Unsubtracted basal values (reactivities to DMSO and gag_{77-85}) are shown in the penultimate row of each column. Subjects positive to at least one epitope are highlighted in green in the second row. Reactivities are ranked as low (between 3 and 4 SD, in yellow), intermediate (between 4 and 5 SD, in orange), and high (more than 5 SD, in red). The five epitopes testing positive in more than 25% of type 1 diabetic patients are highlighted in blue. The number of positive epitopes in each subject is indicated in the last row. ++++, off-scale ELISpot reading.

We here propose the first validation of CD8+ T-cell measurements to differentiate between type 1 diabetes and health, using a portfolio of well-defined islet epitopes applied to an IFN-γ ELISpot format (ISL8Spot). Several possibilities may explain the accuracy we achieved by CD8+ T-cell measures compared with previous attempts with CD4+ T-cells (37,39,43). The simplest speculation is that β-cell–reactive CD8+ T-cells may be more numerous in the bloodstream than their CD4+ counterparts, at least at type 1 diabetes onset. This could relate in part to a different recirculation of these two subsets from target tissues to peripheral blood. Alternatively, β-cell–reactive CD8+ T-cells may preferentially undergo avidity maturation (17) or may display a more homogeneous IFN-γ–producing phenotype. Either feature would facilitate their detection, as well as their power to discriminate between type 1 diabetes and health by a single cytokine measure. Besides the biological differences between CD4+ and CD8+ T-cells, some specifics of our study probably contributed to these results. These may include the recruitment of type 1 diabetic patients with disease of very recent onset, the preliminary selection of a robust candidate epitope panel based on both immunogenicity and natural processing, and addition of low-dose IL-2 to increase the epitope-specific signal (R.M., unpublished data). Moreover, the short median type 1 diabetes duration of our study cohort (12 days) and the lack of correlation between T-cell responses and type 1 diabetes duration minimize the

possibility of a bias on PPI-specific responses introduced by the daily insulin injections. Selection of patients based on permanent insulin requirement from the time of diagnosis also limited the potential inclusion of latent autoimmune diabetes in adults (LADA) patients (44).

Importantly (35,45,46), detection of β-cell–specific $CD8^+$ T-cells was accomplished on both fresh and frozen PBMCs and directly ex vivo. This rules out any bias introduced by in vitro manipulation, while encouraging the transfer to clinical application. Furthermore, these measurements could be performed with as little as 10 ml of blood, since a panel of five immunodominant epitopes is sufficient for optimal assay accuracy. T-cell testing could therefore be offered also to children. We hypothesize that higher T-cell responses may be found in type 1 diabetic children, as their faster decline in β-cell function could subtend a more aggressive autoimmunity (47,48).

A recent study conducted by the Immune Tolerance Network recapitulated the state-of-the-art for T-cell assays in type 1 diabetes (35). Different assays were compared for their ability to distinguish type 1 diabetes from healthy donors, using 23 frozen samples from new-onset patients. While HLA class II tetramer (36) and IFN-γ ELISpot assays (49) could not be evaluated because of poor performance on frozen samples (45,46), a cellular immunoblot assay reached 91% sensitivity and 83% specificity (50,51). A T-cell proliferation assay (52) showed a lower sensitivity (58%) but higher specificity (94%). Both of these assays rely on [^{3}H]thymidine incorporation as the final readout after a 5- to 7-day culture, using eluted proteins from whole islet cell lysates or different self-antigens/epitopes as stimuli. Given the nature of these stimuli (whole proteins and/or 13- to 15-mer peptides), the responding cells are likely to be $CD4^+$ T-cells. The higher sensitivity of cellular immunoblotting compared with ISL8Spot is of note and may be due to the coverage of all potential T-cell targets ensured by the islet cell lysates. However, besides achieving a higher specificity, the ISL8Spot assay offers some distinct advantages. First, the ISL8Spot T-cell targets are molecularly defined epitopes that can be chemically synthesized. This allows for better consistency and makes the technique easier to implement. Second, direct ex vivo testing avoids any potential bias brought in by in vitro expansions. Third, the ISL8Spot assay requires smaller blood volumes, making it more suitable for younger patients and for repeated testing at different time points.

The 86% sensitivity of our prototype ISL8Spot is in the same range of that of GAD Ab radioimmunoassays, which have reached 80–85% sensitivity and 95–98% specificity through several years of improvements (27). The lower specificity (91%) of our T-cell measurements versus the Ab determinations may reflect detection of autoreactive T-cells without pathological significance, or identification of subclinical β-cell autoimmunity. Indeed, the observation that one of the two healthy subjects positive for T-cell responses was also a carrier of type 1 diabetes risk factors is intriguing. Although the predictive power of T-cell measures for subsequent type 1 diabetes development remains to be established, combined T-cell and Ab measures (i.e., positivity to either one) were able to correctly identify all type 1 diabetic patients, thus reaching 100% sensitivity. Larger multicentric studies will further elucidate whether T-cell assays can complement islet Ab determinations for an etiology-based diagnosis of type 1 diabetes, as currently recommended (25). Moreover, the recent description of additional T-cell epitopes derived from IGRP, IA-2, glial fibrillary acidic protein (GFAP), and islet amyloid polypeptide (IAPP) extends the panel available for testing (41,42).

This proof-of-concept study demonstrates that IFN-γ $CD8^+$ T-cell responses, as measured by ISL8Spot, can propose themselves as new autoimmune markers of type 1 diabetes. After epitope identification, this strategy can be expanded to other HLA class I restrictions to cover the majority of the population. Characterization of the $CD8^+$ T-cells recognizing the identified epitopes may provide new insights into type 1 diabetes pathogenesis. Further validation of the ISL8Spot assay in (pre)-diabetic subjects and patients enrolled in immune prevention trials will clarify their potential for risk assessment, etiology-based diagnosis, and therapeutic monitoring of type 1 diabetes.

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APPENDIX

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