Single Insulin-Specific CD8$^+$ T Cells Show Characteristic Gene Expression Profiles in Human Type 1 Diabetes

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OBJECTIVE—Both the early steps and the high recurrence of autoimmunity once the disease is established are unexplained in human type 1 diabetes. Because CD8$^+$ T cells are central and insulin is a key autoantigen in the disease process, our objective was to characterize HLA class I–restricted autoreactive CD8$^+$ T cells specific for preproinsulin (PPI) in recent-onset and long-standing type 1 diabetic patients and healthy control subjects.

RESEARCH DESIGN AND METHODS—We used HLA-A*02:01 tetramers complexed to PPI peptides to enumerate circulating PPI-specific CD8$^+$ T cells in patients and characterize them using membrane markers and single-cell PCR.

RESULTS—Most autoreactive CD8$^+$ T cells detected in recent-onset type 1 diabetic patients are specific for leader sequence peptides, notably $PPI_{6–14}$, whereas CD8$^+$ T cells in long-standing patients recognize the B-chain peptide $PPI_{33–42}$ ($B_{9–18}$). Both CD8$^+$ T-cell specificities are predominantly naïve, central, and effector memory cells, and their gene expression profile differs from cytomegalovirus-specific CD8$^+$ T cells. $PPI_{6–14}$–specific CD8$^+$ T cells detected in one healthy control displayed *Il-10* mRNA expression, which was not observed in diabetic patients.

CONCLUSIONS—PPI-specific CD8$^+$ T cells in type 1 diabetic patients include central memory and target different epitopes in new-onset versus long-standing disease. Our data support the hypothesis that insulin therapy may contribute to the expansion of autoreactive CD8$^+$ T cells in the long term. ***Diabetes*** **60:3289–3299, 2011**

Type 1 diabetes is an autoimmune disease driven by the activation of lymphocytes against pancreatic β-cells. Although the successive steps that control the activation of autoreactive lymphocytes have been extensively studied in animal models, the disease process remains ill defined in human type 1 diabetes (1). In mice, type 1 diabetes is transferred into naïve recipients by T cells, is prevented by antibodies that target T-cell differentiation, and fails to develop when key genes in T-cell differentiation or activation are nonfunctional. In humans, T cells—especially CD8$^+$ T cells—are predominant within insulitis in most observations (2–5). Occurrence of type 1 diabetes in a patient deprived of B lymphocytes further underscores the role of T cells (6). An unexpected feature is the high recurrence level of autoimmunity in long-standing type 1 diabetic patients. Rapid diabetes recurrence is seen in type 1 diabetic recipients of isograft from a nondiabetic twin, accompanied by an almost exclusive islet CD8$^+$ T cell infiltration (7). β-Cell–specific T cells thus seem to maintain immune memory for years after type 1 diabetes onset. However, the differentiation patterns of autoreactive T cells once diabetes is diagnosed remain largely unknown.

Insulin has been ascribed a key antigenic role in type 1 diabetes. Altered diabetes development in proinsulin $1^{-/-}$ or $2^{-/-}$ NOD mice makes a strong case for the primary role of insulin in the NOD mouse (8–10). In humans, insulin and proinsulin are common targets of autoantibodies and T cells in prediabetic and diabetic individuals. Insulin autoantibodies are the first to be detected in children at risk for type 1 diabetes and carry a high positive predictive value for diabetes in siblings of patients (11). A restricted region of proinsulin located in the B chain and adjacent C-peptide clusters has epitopes that are recognized by CD8$^+$ T cells (12–16). Other epitopes are located within the A chain, the C-peptide and C-peptide–B chain junction (14), and preproinsulin (PPI) leader sequence (13,17,18), including residues excised during insulin processing. PPI-reactive CD8$^+$ T cells have mostly been observed in recent-onset type 1 diabetic patients (12,13). However, some peptides, in particular peptides located in the insulin B chain, allow the detection of interferon-γ (IFN-γ)–producing CD8$^+$ T cells in long-standing patients who have been treated with insulin for decades (12).

Most studies have used IFN-γ enzyme-linked immunospot (ELISpot) assays to characterize autoreactive CD8$^+$ T cells (12–15). More recently, HLA class I tetramers (TMrs) have identified CD8$^+$ T cells expressing a T-cell receptor specific for HLA class I–restricted peptides, independently of cytokine production (13,18,19). Developing T-cell assays is a major challenge in human type 1 diabetes. It is therefore expected to help in identifying epitopes recognized by T cells and to pave the way toward immunotherapy to restore tolerance to β-cells. New T-cell technologies are expected to allow defining autoreactive T-cell differentiation programs and characterizing autoimmune responses in comparison with physiologically appropriate immune responses.

Here, we used TMrs to detect insulin-reactive CD8$^+$ T cells in type 1 diabetic patients and characterize their functional profile in comparison with cytomegalovirus (CMV)-specific CD8$^+$ T cells. Remarkably, CD8$^+$ T cells detected in recent-onset type 1 diabetic patients are specific for leader sequence peptides, especially $PPI_{6–14}$. The detection of CD8$^+$ T cells against an insulin B-chain peptide in patients with established disease raises the hypothesis that insulin therapy may also contribute to expansion of autoreactive CD8$^+$ T cells.

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RESEARCH DESIGN AND METHODS

Patients. Type 1 diabetic patients (n = 49) were HLA-A*0201$^+$ and positive for anti-GAD, anti-insulin, or anti-IA2 autoantibodies at diagnosis (Table 1). Recent-onset patients (14 males, 11 females; aged 38.1 ± 11.1 years) were studied within 3 months from diagnosis (median 3 days; range 2–70 days). Long-standing type 1 diabetic patients (13 males, 11 females; aged 41.4 ± 11.6 years) had a median type 1 diabetes duration of 4 years (range 2–40). Control subjects were healthy blood donors (12 males, 9 females; aged 35.6 ± 11.8 years), type 2 diabetic patients under oral treatment (3 males, 4 females; mean age 56.6 ± 9.0 years) or insulin treatment (3 males, 4 females; mean age 55.4 ± 7.7 years) selected as expressing HLA-A*0201. Informed consent was obtained and the study was approved by the local ethics committees. HLA class I alleles were determined by Ambisolv genotyping (Dynal/Invitrogen) on DNA with Gentra Puregene Blood kit A (Qiagen). C-peptide was measured by time-resolved fluoroimmunoassay using Wallac Delfia reagents (Perkin-Elmer SAS, Courtaboeuf, France). Lower detection limit was 3.3 pmol/L. The intra- and interassay coefficients of variation at the level 1.1 ng/mL (364 pmol/L) were 3.9 and 5.2%, respectively, and at the level 6 ng/mL (1986 pmol/L) 3.1 and 3.4%, respectively. Intact insulin did not cross-react with C-peptide in this assay.

TMrs. PPI peptides were selected from PPI sequence, synthetized, and purified as previously described (20) (Table 2) and were controlled by MALDI-TOF (matrix-assisted laser desorption/ionization−top of flight) on a Bruker Protein TOF mass spectrometer (12). Peptide nomenclature refers to N and C termini along the PPI protein sequence. Peptide binding to HLA-A*0201 was determined by major histocompatibility complex stabilization assays using TAP2-deficient HLA-A*0201–transfected T2 cells (21,22). Percent mean fluorescence intensity (MFI) increase corresponds to (MFI with a given peptide – MFI without peptide)/(MFI without peptide). For TMr assembly, HLA-A*0201 α-chain and human β2-microglobulin were produced in *E. coli*. HLA-A*0201/peptide monomers were refolded in vitro using 12 mg of peptide, purified by gel filtration (Superdex 200HR 10/30, Pharmacia), and biotinylated using the biotin-protein ligase BirA (Avidity) (23). Purified biotinylated HLA-A*0201/peptide monomers were tetramerized with Extravidin-R-Phycoerythrin conjugate (Sigma) (21,24).

TMr assays. Peripheral blood mononuclear cells (PBMCs) were prepared from fresh blood using Lymphoprep (Axis Shield) separation in Leucosep tubes. TMr staining was performed by incubating 10^6 PBMCs with 5–10 μg/mL phycoerythrin (PE)-labeled TMrs at 37°C for 30 min. Anti–CD45RA-FITC, anti–CCR7-PE-Cy7, anti–CD8-PerCP (SK1 clone), and anti–CD3-APC-Cy7 antibodies (BD Pharmingen, eBioscience) were then added for 15 min at 4°C. After washing, cells were acquired on a BD FACSAria and analyzed using FlowJo (Tree Star) (25,26). The minimal number of TMr$^+$ events analyzed in phenotyping studies was 5.10^3.

Cell sorting. CD8$^+$ PBMCs were negatively selected (Dynal/Invitrogen) by depleting CD4-, CD14-, CD16-, CD19-, CD36-, CD56-, CDw123-, and CD235a-expressing cells. Staining was performed on 10^6 CD8-enriched cells by adding 10–20 μg/mL of nonpooled PE-labeled TMr at 37°C for 30 min. Anti–CD8-PerCP–labeled antibody was then added for 15 min at 4°C. Small lymphocytes were gated according to forward/side scatter profiles. CD8$^+$/TMr$^+$ T cells were sorted on a FACSAria at 1 cell/well into 96-well PCR plates containing 5 μl PBS treated with diethyl pyrocarbonate (Sigma) and immediately frozen at −80°C.

Single-cell reverse transcriptase PCR. RNA was extracted from sorted cells by direct cellular lysis for 2 min at 65°C. Procedures and primers for coamplification of multiple genes in single cells were designed as described (27,28). Reverse transcription was carried out with murine leukemia virus reverse transcriptase (Applied Biosystems) for 60 min at 37°C. Seminested PCR was then performed with gene-specific primers (Eurogentec) and AmpliTAQ Gold Polymerase (Applied Biosystems) by touch-down PCR. PCR products were resolved on a 2.5% (w/v) agarose gel. Perforin (*Prf1*), granzyme A (*Gzma*), granzyme B (*Gzmb*), Fas ligand (*Fasl*), IFN-γ (*Ifng*), TGF-β1 (*Tgfb1*), TNF-α (*Tnfa*), IL-2 (*Il2*), IL-10 (*Il10*), IL-10Rα (*Il10ra*), IL-7R (*Il7r*), MIP-1α (*Mip1a*), MIP-1β (*Mip1b*), PD1 (*Pd1*), RANTES (*Ccl5*), KLRG1 (*Klrg1*), CCR7 (*Ccr7*), and CD3ε (*Cd3e*) mRNAs were analyzed.

Statistical methods. Comparison of distribution scores between patients and control subjects and TMr assessment used the nonparametric Kruskal-Wallis test or Fisher exact test. To study assay reliability, we used the graphical Altman and Bland method, which focuses on means and variability of differences between repeated measurement pairs (29). C-peptide levels were compared using the Mann-Whitney U test.

RESULTS

Insulin-specific CD8$^+$ T cells are detected in type 1 diabetic patients. PPI peptides are recognized by CD8$^+$ T cells from type 1 diabetic patients using IFN-γ ELISpot (12–15), which, methodologically, restricts recognition to

TABLE 1
Characteristics of patients and control subjects

Patient	Sex	Age (years)	Diabetes duration	Patient	Sex	Age (years)	Diabetes duration	Control subject	Sex	Age (years)	T2D	Sex	Age (years)
R1	F	54	8 weeks	L1	F	34	3 years	H1	M	49	T2D1	M	78
R2	M	66	72 h	L2	M	52	18 years	H2	F	26	T2D2	F	66
R3	F	49	72 h	L3	F	44	31 years	H3	M	57	T2D3	F	54
R4	M	42	72 h	L4	F	48	39 years	H4	F	29	T2D4	M	61
R5	M	33	48 h	L5	M	34	29 years	H5	F	32	T2D5	F	51
R6	F	39	2 weeks	L6	M	48	4 years	H6	M	29	T2D6	M	64
R7	M	35	10 weeks	L7	F	63	4 years	H7	F	50	T2D7	F	57
R8	F	35	9 weeks	L8	F	47	3 years	H8	M	28	T2D8	F	63
R9	M	30	72 h	L9	M	29	3.5 years	H9	M	21	T2D9	M	53
R10	F	28	72 h	L10	M	42	4 years	H10	M	32	T2D10	M	57
R11	M	37	72 h	L11	M	24	4 years	H11	M	21	T2D11	F	47
R12	M	34	72 h	L12	F	58	3.5 years	H12	F	32	T2D12	F	51
R13	M	23	72 h	L13	M	36	2 years	H13	F	32	T2D13	M	68
R14	F	28	72 h	L14	F	49	4 years	H14	M	28	T2D14	F	49
R15	M	33	72 h	L15	M	29	4 years	H15	M	35			
R16	F	52	72 h	L16	M	64	40 years	H16	M	66			
R17	F	26	72 h	L17	M	35	2 years	H17	F	28			
R18	F	38	72 h	L18	M	28	3 years	H18	M	48			
R19	M	38	72 h	L19	M	29	4 years	H19	F	35			
R20	M	38	72 h	L20	F	24	14 years	H20	F	32			
R21	M	41	72 h	L21	M	44	13 years	H21	M	37			
R22	M	63	72 h	L22	F	45	25 years						
R23	F	26	72 h	L23	F	37	31 years						
R24	M	36	72 h	L24	F	51	9 years						
R25	F	28	72 h										

R, recent-onset type 1 diabetic patients; L, long-standing type 1 diabetic patients; H, healthy control subjects; T2D, type 2 diabetic control subjects.

TABLE 2
HLA-A*0201/peptide TMrs

PPI peptides	Sequence	SYFPEITHI score	BIMAS score	T2/A2*0201 MFI	TMr production	Positive cutoff (%)	TMr frequencies: Recent-onset T1D	TMr frequencies: Long-standing T1D	Ref
2–11	ALWMRLLPLL	28	400	0.575	+	0.023	7/25	4/24	12,13
6–14	RLLPLLALL	31	180	0.9	+	0.051	8/25	9/24	12,13
6–15	RLLPLLALLA	0	0	0.45	Unstable				na
7–16	LLPLLALLAL	28	0	0.063	—				na
14–23	LALWGPDPAA	14	<1	0.075	—				12,13
15–24	ALWGPDPAAA	22	40	1.18	+	0.029	8/25	7/24	12,13
30–39 (B6–15)	LCGSHLVEAL	17	0	0.507	+	0.077	1/25	1/24	na
33–42 (B9–18)	SHLVEALYLV	19	0	0.79	+	0.046	7/25	12/24	18
34–42 (B10–18)	HLVEALYLV	27	22	1.16	+	0.041	5/25	5/24	12,14,18
41–51 (B17–27)	LVCGERGFFYT	0	0	0.105	—				na
42–51 (B18–27)	VCGERGFFYT	7	20	0.11	+	0.03	3/25	4/24	12
60–68 (C3–11)	DLQVGQVEL	25	>2	0.095	—				na
76–84 (C20–28)	SLQPLALEG	18	0	0.215	—				14
81–89 (C25-KR1)	ALEGSLQKR	19	0	0.19	—				na
85–94 (C29-A5)	SLQKRGIVEQ	20	0	0.135	—				14
97–105 (A8–16)	TSICSLYQL	17	0	0.22	—				na
101–109 (A12–20)	SLYQLENYC	15	87	0.54	+	0.026	2/25	4/24	14
PDHase (208–216)	TLLEIENAV	26	1,055	na	+	0.0165			na
CMV (pp65 495–503)	NLVPMVATV	30	160	1.5	+	1.433			46

MFI, mean fluorescent intensities; na, not available; T1D, type 1 diabetes.

a functional subset of T cells, precluding characterization of their differentiation stage. To characterize PPI-specific $CD8^+$ T cells independently of their function, we prepared HLA-A*0201 PE-labeled TMrs complexed with previously defined PPI peptides (A2/PPI) (Table 2) (12–15). Stable TMrs were obtained with peptides that showed a percent MFI >0.5 in HLA-A2 stabilization assays (Table 2). HLA-A2 TMrs complexed with CMV peptide $pp65_{495–503}$ (A2/CMV TMrs) and $PDHase_{208–216}$ (A2/PDHase TMrs) were used as positive and negative controls, respectively. A2/PPI $TMrs^+$ $CD8^+$ T cells were detected (Fig. 1*A–E*) in 35/49 type 1 diabetic patients (71.43%). Detection thresholds for each individual TMr^+ were set at the 95th percentile of values obtained in controls. Percentages of TMr^+ $CD8^+$ T cells above 95th percentile of controls were considered positive. (Table 2). No $CD8^+$ T cells were stained with A2/PDHase TMrs (Fig. 1*F*) or in absence of TMrs (Fig. 1*G*). As expected, more cells were often stained with HLA-A2/CMV TMrs than with A2/PPI TMrs (Fig. 1*H*).

Significant differences were observed among recent-onset, long-standing type 1 diabetic patients, and control subjects in the frequencies of TMr^+ $CD8^+$ T cells specific for PPI leader sequence peptides $PPI_{6–14}$, $PPI_{15–24}$, and B-chain peptide $PPI_{33–42}$ ($B_{9–18;}$ Fig. 2*A*). Percentages of $CD8^+$ T cells specific for leader sequence peptide $PPI_{2–11}$, B chain peptides $PPI_{30–39}$, $PPI_{34–42}$, and $PPI_{42–51}$, and A chain peptide $PPI_{101–109}$ did not differ significantly among groups, but few individual responses were seen against these peptides (Fig. 2*A* and Supplementary Table 1). There was no significant difference in PPI-specific $CD8^+$ T cells between healthy controls and type 2 diabetic patients, including with insulin-treated Type 2 diabetic patients (data not shown), and in CMV-specific $CD8^+$ T cells between type 1 diabetic patients and control subjects (Fig. 2*A*). The number of individuals whose TMr^+ frequencies were ≥95th percentile of control subjects was different between recent-onset patients and control subjects for $PPI_{6–14}$ TMr^+ cells (8/25, 32.0%; $P < 0.03$) and $PPI_{15–24}$ (8/25, 32.0%; $P < 0.03$) but not for $PPI_{33–42}$ (7/25, 28.0%; $P = 0.06$) and between long-standing type 1 diabetic patients and control subjects for $PPI_{33–42}$ (12/24, 50.0%; $P < 0.002$) but not for $PPI_{6–14}$ (9/24, 37.5%; $P = 0.35$) and $PPI_{15–24}$ (7/24, 29.2%; $P = 0.055$). In a healthy control subject, a high (0.14%) frequency of $PPI_{6–14}$–specific $CD8^+$ T cells was observed (Fig. 2*A*).

Assay reliability was calculated from values obtained from two assays performed in two different samples collected at median intervals of 3 days following diagnosis (range 2–90 days) in the same individuals using $PPI_{6–14}$, $PPI_{15–24}$, $PPI_{33–42}$, and CMV $pp65_{495–503}$ TMrs. Differences of up to ±0.038, 0.042, 0.060, and 0.186% were observed for $PPI_{6–14}$, $PPI_{15–24}$, $PPI_{33–42}$, and CMV $pp65_{495–503}$ TMrs, respectively. Mean differences represent interassay mean errors and intervals acceptable errors. There is good interassay agreement for $PPI_{6–14}$, $PPI_{15–24}$, and $PPI_{33–42}$ peptides, each value being included in the error interval, which is ≤0.05 (Fig. 2*B*). Overall, 68% of recent-onset and 83.3% of long-standing type 1 diabetic patients had detectable PPI-specific $CD8^+$ T cells above the 95th centile of control subjects. Responses were observed against at least two peptides in 44.9% of patients (22/49), including 32.6% (16/49) who showed a response to either $PPI_{6–14}$, $PPI_{15–24}$, $PPI_{33–42}$.

In long-standing patients who stained positive for A2/PPI TMrs, mean diabetes duration was 6.5 years (range: 3–25) for $PPI_{6–14}$ peptide, 14.4 years (3–39) for $PPI_{15–24}$ peptide, and 10.7 (3–39) for $PPI_{33–42}$ peptide. Basal C-peptide values were available in 11/19 patients in whom increased A2/$PPI_{33–42}$ TMr^+ $CD8^+$ T cells (TMr^+ patients) were detected and 7/30 patients in whom A2/$PPI_{33–42}$ TMr^+ $CD8^+$ T cells were in the basal range (TMr^- patients). Although differences between both groups were not significant, lower C-peptide values were observed in TMr^+ patients (median 0.07 ± 0.09 nmol/L; range 0.01–0.27) than in TMr^- ones (median 0.22 ± 0.19 nmol/L; range 0.01–0.59; $P = 0.07$) (Fig. 3*A*). Moreover, an inverse correlation was observed between percentages of A2/$PPI_{33–42}$ TMr^+ $CD8^+$ T cells detected and fasting C-peptide values ($r = -0.63$; $P = 0.05$) (Fig. 3*B*).

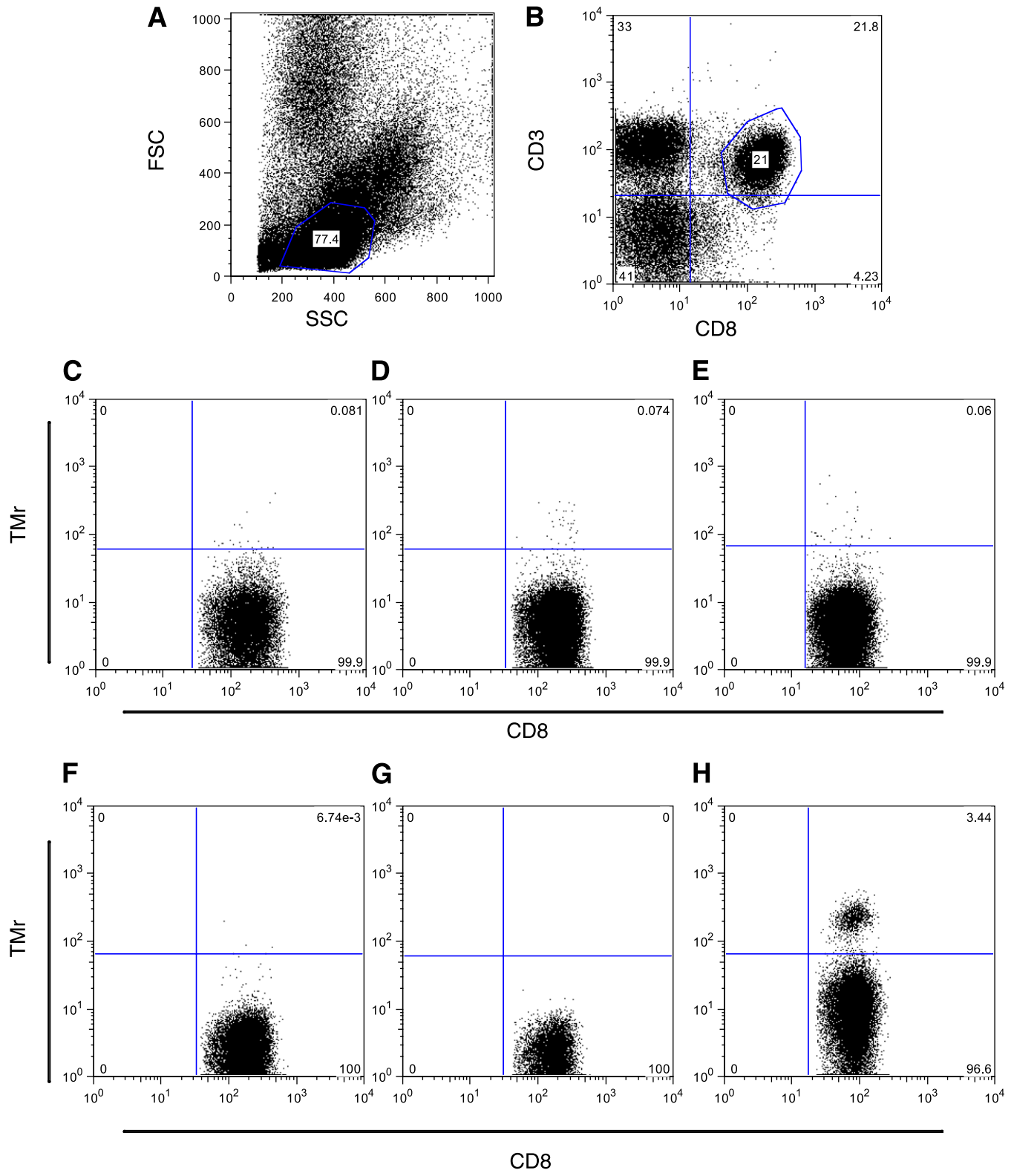

FIG. 1. TMr detection of CD8+ T cells specific for A2.1-restricted PPI peptides. *A*: Lymphocytes were gated according to forward/side scatter profiles. *B*: CD3$^+$CD8$^+$ T cells were gated to evaluate percent of TMr$^+$ cells. *C–E*: CD8$^+$ T cells stained with A2/PPI peptide TMr complexed with $PPI_{6–14}$ (*C*), $PPI_{15–24}$ (*D*), and $PPI_{33–42}$ (*E*) peptides were detected in L24, R2, and L11 type 1 diabetic patients, respectively. *F* and *G*: For each staining, compensation settings were verified with the HLA-A2/$PDHase_{208–216}$ negative control TMrs (*F*) and with a staining without TMr (*G*), here shown on the R2 patient. *H*: HLA-A2/CMV $pp65_{495–503}$ TMrs were used as the virus-specific positive control (R2 patient). (A high-quality color representation of this figure is available in the online issue.)

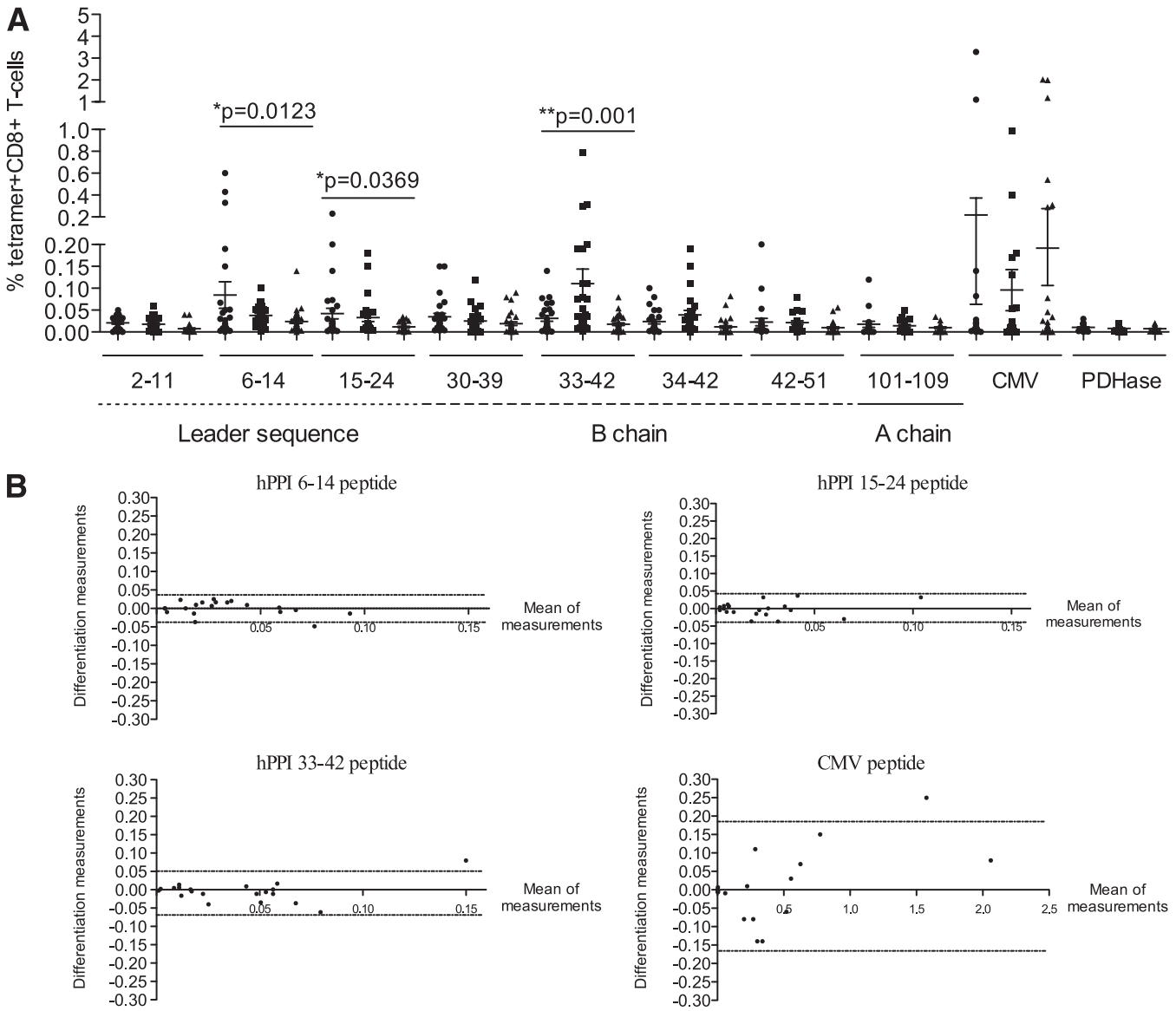

FIG. 2. Distribution of CD8+ T cells specific for A2.1-restricted PPI peptides in type 1 diabetic patients and control subjects. *A*: Each symbol represents the percentage of CD8$^+$ T cells specific for A2.1-restricted PPI and control peptides detected in individual recent-onset type 1 diabetic (●), long-standing type 1 diabetic (■), and healthy and type 2 diabetic control (▲) subjects. A significantly higher percentage of CD8$^+$ T cells specific for PPI$_{6–14}$ (P = 0.0123), PPI$_{15–24}$ (P = 0.0369), and PPI$_{33–42}$ (P = 0.001) was observed in recent and long-standing type 1 diabetic patients compared with control subjects (Kruskal-Wallis test). Differences in detection of A2/PPI TMr$^+$ were significant for leader sequence PPI$_{6–14}$ (recent-onset: median 0.085% [range 0.009–0.6]; long-standing: 0.042% [0.003–0.1]; control: 0.014% [0.007–0.14]; P = 0.0123), PPI$_{15–24}$ (recent-onset: 0.042% [0.002–0.2]; long-standing: 0.034% [0.004–0.18]; control: 0.012% [0.002–0.035]; P = 0.0369), and B chain PPI$_{33–42}$ (recent-onset: 0.031% [0.001–0.14]; long-standing: 0.115% [0.007–0.79]; control: 0.018% [0.001–0.07]; P = 0.001). *B*: Plots for pairs of measurement scores of HLA-A2/peptide TMrs. Differences of ±0.000–0.005 were observed for PPI$_{6–14}$, PPI$_{15–24}$, and PPI$_{33–42}$ TMrs (mean difference ± SD: 0.000 ± 0.019, 0.001 ± 0.021, and 0.005 ± 0.028, respectively) and 0.000–0.008 for CMV pp65$_{495–503}$ TMrs (0.008 ± 0.09). The scatter plot of the difference between the measurements against their mean allows detecting no lack of individual reliability, which may be hidden by the use of global reliability statistics for PPI$_{6–14}$, PPI$_{15–24}$, PPI$_{33–42}$, and CMV pp65$_{495–503}$ TMr levels (24).

PPI-specific CD8$^+$ T cells express central memory cell markers. Upon antigen encounter, CD8$^+$ T cells express differentiation programs that imprint their homing, survival, activation, and functions. Detection of circulating TMr$^+$ CD8$^+$ T cells allows characterizing membrane markers, especially homing and signaling markers that discriminate effector and memory cells from naïve cells. PPI-specific CD8$^+$ T cells were gated to evaluate percent TMr$^+$ cells and define (Fig. 4) (30) CD45RA$^+$CCR7$^+$ naïve T cells (TN), CD45RA$^-$ CCR7$^+$ central memory T cells (TCM), CCR7$^-$CD45RA$^-$ effector memory T cells (TEM), and CCR7$^-$ CD45RA$^+$ effector memory T cells (TEMRA) (Fig. 4*A* and *B*). Significant differences were seen in the distribution of CMV-specific and PPI-specific CD8$^+$ naïve and memory T cells between type 1 diabetic patients and control subjects using the Kruskal-Wallis test, but not between recent-onset and long-standing type 1 diabetic patients (Fig. 4*C*). In recent-onset and long-standing type 1 diabetic patients, 31.5 and 32.0% TN PPI-specific CD8$^+$ cells were detected, respectively, whereas these cells were almost undetectable among CMV-specific T cells. Similarly, PPI-specific TCMs represented 25.4 and 29.8% of CD8$^+$ T cells in the two respective type 1 diabetic populations, whereas they represented only 15.6% of CMV-specific CD8$^+$ T cells (P = 0.0071).

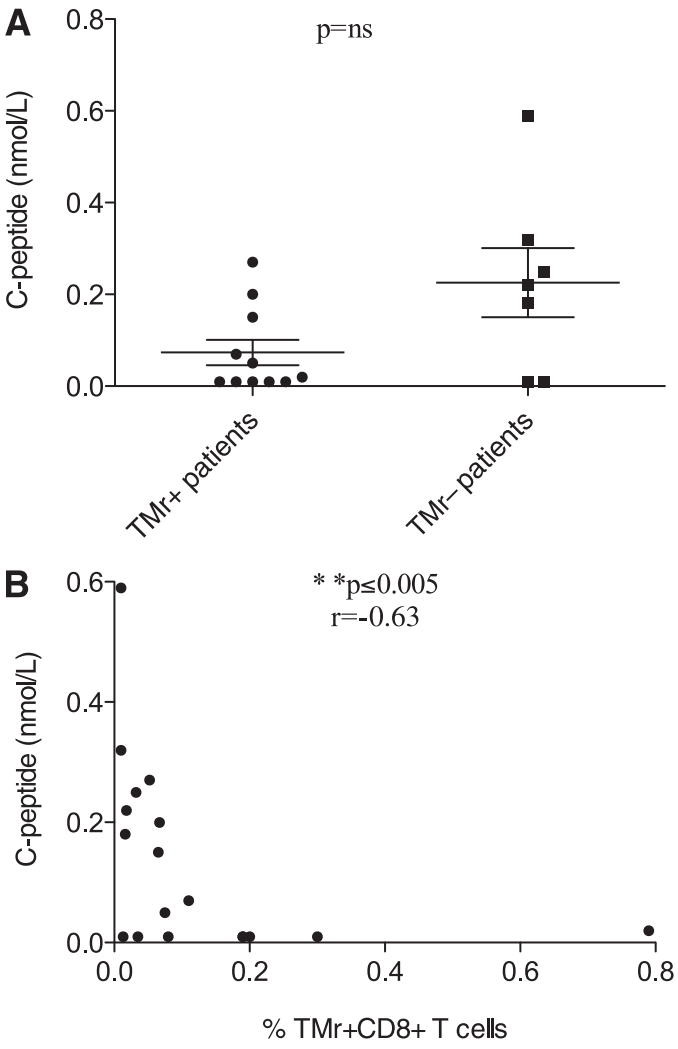

FIG. 3. Correlation between C-peptide level and the presence of $PPI_{33–42}$–specific CD8+ T cells in type 1 diabetic patients. *A*: Each symbol represents the fasting C-peptide level detected in type 1 diabetic patients depending on whether A2.1/$PPI_{33–42}$ TMr^+ $CD8^+$ T cells were above (●) or below (■) detection threshold (TMr^+ and TMr^- patients, respectively; threshold = 0.046%). *P* = 0.07 by Mann-Whitney *U* test. *B*: Each symbol represents the correlation between the percentage of $PPI_{33–42}$–specific $CD8^+$ T cells and basal C-peptide levels detected in type 1 diabetic patients in whom increased A2.1/$PPI_{33–42}$ TMr^+ $CD8^+$ T cells were detected. A negative correlation ($P \leq 0.005$, Spearman $r = -0.63$) was observed.

In contrast, PPI-specific TEM and TEMRA were underrepresented in recent-onset (8.19%) and long-standing (12.1%) patients, as compared with CMV-specific TEM and TEMRA $CD8^+$ T cells (50.47 and 29.97%, respectively). There was no difference in distributions of $PPI_{6–14}$–, $PPI_{15–24}$–, $PPI_{33–42}$–specific $CD8^+$ T cells. Altogether, these data show that PPI-specific $CD8^+$ memory T cells are predominantly TN, TCM, and TEM cells, whereas the majority of CMV-specific $CD8^+$ T cells are TEM and TEMRA cells. We observed no correlation between diabetes duration and percentage of PPI-specific $CD8^+$ TN or TCM T cells on long-standing type 1 diabetic patients (data not shown).

PPI-specific $CD8^+$ T cells show characteristic $CD8^+$ T cells gene expression programs. Cell surface phenotypes often fail to ascribe functional attributes to memory $CD8^+$ T cells (25,31,32). To address how differentiation programs are established at clinical onset and in long-standing type 1 diabetes, we studied 18 genes coding for inflammatory chemokines, cytokines, cytotoxic molecules, and receptors involved in $CD8^+$ T-cell responses. Gene expression was evaluated in TMr^+ $CD8^+$ T cells and nonpurified TMr^+ $CD8^+$-naïve and central memory cells. Cells were obtained by purifying $CD8^+$ T cells, then sorting TMr^+ cells from patients in whom a minimum of 0.05% TMr^+ $CD8^+$ T cells were detected. In these patients, the percentage of cells expressing the *CD3e* gene was 82.06 ± 14.35%, in contrast with patients who did not reach this threshold (16.50 ± 8.96%). Gene expression profiles were heterogeneous between single cells carrying the same peptide specificity, but a significant percentage of single cells coexpressed genes involved in the same functional program (Fig. 5). Gene expression in PPI-specific (Fig. 5*A*) and CMV-specific (Fig. 5*B*) $CD8^+$ T cells was different. Figure 5*A* illustrates a representative subject (R18) displaying 0.19% A2/$PPI_{6–14}$ TMr^+ $CD8^+$ T cells of which only 4.3% coexpressed *Pfr* and *Gzma* or *Gzmb*, none coexpressed *Ccl5* (coding for RANTES) and *Mip1b*, but 40% expressed *Ccr7*. Opposite profiles were seen in A2/CMV-specific $CD8^+$ T cells. In patient L9 (Fig. 5*B*), 2.0% A2/CMV TMr^+ $CD8^+$ T cells were detected of which 36% coexpressed *Pfr* and *Gzma* or *Gzmb* but only 4% expressed *Ccr7*, while 32% coexpressed *Ccl5* and *Mip1b*.

Gene expressions were strikingly comparable in A2/$PPI_{6–14}$, $PPI_{15–24}$, and $PPI_{33–42}$ TMr^+ $CD8^+$ T cells (data not shown). Considering the whole type 1 diabetic population, the mean percentage of PPI-specific $CD8^+$ T cells that expressed *Pfr* and *Gzma* and *Gzmb* was 16.1, 11.0, and 6.9%, respectively, as compared with 51.5, 40.7, and 21.4% for CMV-specific $CD8^+$ T cells (P = 0.0003, P = 0.0092, P = 0.0125, respectively; Fig. 5*D*). Differences in chemokine gene expression were also seen, such as *Ccl5* and *Mip1b*, which drive immune cells to inflammatory sites. Indeed, a higher number of CMV-specific $CD8^+$ T cells expressed *Ccl5* compared with PPI-specific $CD8^+$ T cells (81.9 vs. 27.8%; P=0.0026), and the same result was observed for *Mip1b* (20.6 vs. 6.0%; P = 0.01). By contrast, *Ccr7*, which controls recirculation through lymph nodes, was more expressed by PPI-specific $CD8^+$ T cells (30.9%) than CMV-specific $CD8^+$ cells (3.4%; $P < 0.001$). These data suggest that PPI-specific $CD8^+$ T cells display gene expression programs that largely overlap TCM rather than TN phenotypes, based on expression of *IFN-γ* and *Rantes* genes (33), whereas CMV-specific $CD8^+$ T cells are predominantly TEM cells. Expression of *Fasl*, *Tnfa*, *Il2*, *Mip1a*, and *Il10* genes was remarkably rare. Expression of the *Il7ra* gene was above 70% in both A2/CMV and A2/PPI peptide TMr^+ $CD8^+$ T cells. In contrast with previous reports (34), the expression of *Klrg1* by CMV-specific $CD8^+$ T cells was heterogeneous, whereas it was low in most PPI-specific $CD8^+$ T cells. A significant difference was further observed in expression of *Pd1* between PPI- and CMV-specific $CD8^+$ T cells (3.0 vs. 11.1%; P = 0.0117). There was no significant difference in gene expression levels by PPI-specific $CD8^+$ T cells between recent-onset and long-standing type 1 diabetic patients (data not shown). In the only control individual who showed detectable $PPI_{6–14}$–specific $CD8^+$ T cells (Fig. 2*A*), a unique gene expression program was observed, with 34% $CD8^+$ T cells expressing *Il10*, as compared with 2.2 ± 3.3% of $PPI_{6–14}$–specific $CD8^+$ T cells in type 1 diabetic patients.

DISCUSSION

HLA class I TMrs may prove instrumental in characterizing T cells in autoimmunity. Following previous identification of

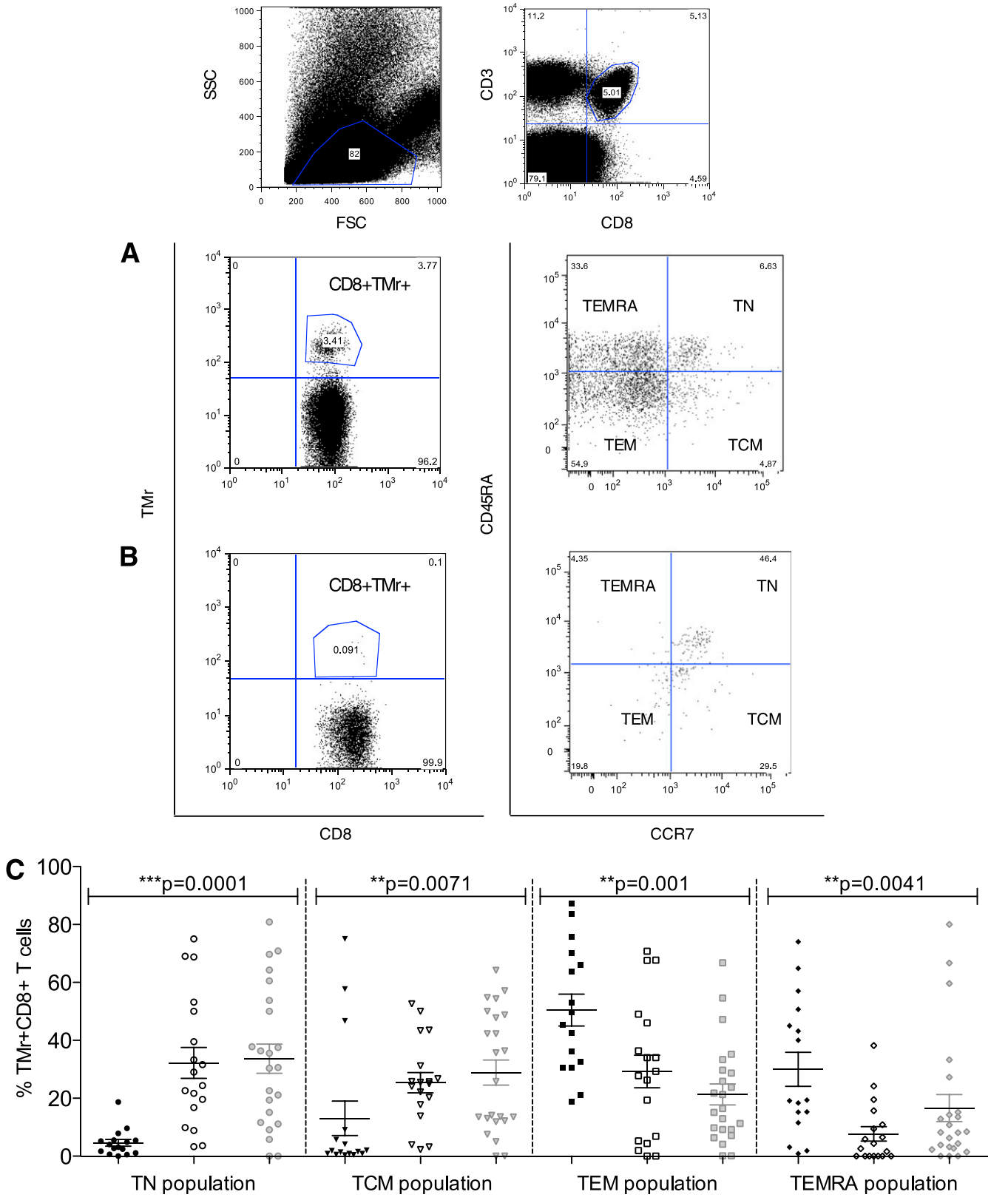

FIG. 4. Phenotype of PPI- and CMV-specific CD8+ T cells. Lymphocytes were gated according to forward/side scatter profiles and then on $CD3^+CD8^+$ T cells to evaluate percent of TMr^+ cells on the R2 patient. *A* and *B*: Among CMV-specific (*A*) and $PPI_{6–14}$–specific (*B*) $CD3^+CD8^+$ T cells, TN, TCM, TEM, and TEMRA were characterized. *C*: Distribution within each subset compartment of CMV-specific (black symbols) and PPI-specific $CD3^+CD8^+$ T cells in recent-onset (white symbols) and long-standing (gray symbols) type 1 diabetic patients. *P* values were calculated using the Kruskal-Wallis test. PPI-specific $CD8^+$ TN cells represent 32.2% (range 3.3–75) and 33.6% (0–80.9), PPI-specific $CD8^+$ TCM cells represent 25.4% (2.3–52) and 28.9% (0–6.2), and PPI-specific $CD8^+$ TEM/TEMRA cells represent 18.5% (0–78.8) and 18.9% (0–80) in recent-onset and long-standing type 1 diabetic patients, respectively; CMV-specific TN cells 4.6% (0–18.7), TCM 13.1% (0.5–75), and TEM/TEMRA 40.2% (0.9–87.4). (A high-quality color representation of this figure is available in the online issue.)

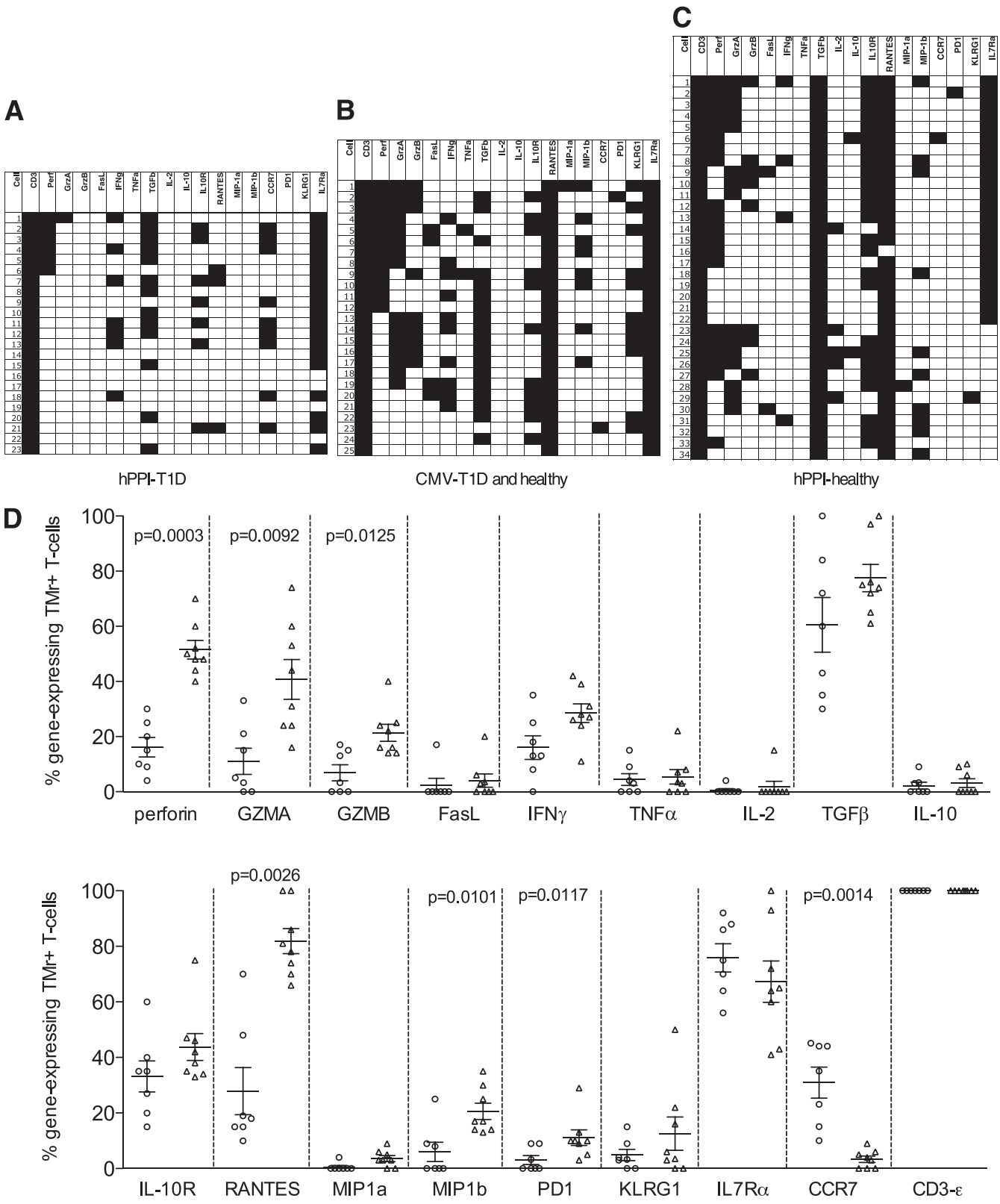

FIG. 5. Gene expression profiles and distribution of PPI- and CMV-specific CD8+ single T cells. *A–C*: Examples of single-cell PCR of CD8+ T cells specific for $PPI_{6–14}$ (*A*; patient R18), $pp65_{495–503}$ CMV peptide (*B*; patient L9), and $PPI_{6–14}$ (*C*; control subject H20). Genes tested are indicated in the upper row of each diagram. Each single cell tested is numbered in the first column on the left. Black squares indicate expression of the corresponding mRNA, and white squares indicate lack thereof. Gene expression is reported for cells expressing *CD3ε* mRNA. *D*: Each point represents, for a single individual, the mean percent of cells expressing the indicated gene out of the 35–40 single CD8+ T cells specific for PPI (○) or CMV $pp65_{495–503}$ (△) $CD8^+$ T cells studied. Results shown for PPI-specific $CD8^+$ T cells correspond to pooled $PPI_{6–14}$, $PPI_{15–24}$, and $PPI_{33–42}$ TMr^+ cells in recent and long-standing type 1 diabetic patients. Only individuals in whom PPI or $pp65_{495–503}$ CMV-specific $CD8^+$ T cells were detected were further studied and are here represented. *P* values indicate statistically significant difference (Mann-Whitney *U* test) in the expression of *Prf*, *Gzma*, *Gzmb*, *Ccl5*, *Mip1b*, and *Ccr7* between PPI- and CMV-specific $CD8^+$ T cells. No significant difference was observed in the expression of the other genes tested.

PPI-specific $CD8^+$ T cells in type 1 diabetes using IFN-γ ELISpot (12), we here report the presence of circulating PPI-specific $CD8^+$ T cells in type 1 diabetes. PPI-specific $CD8^+$ T cells were detected against three major peptides, two within the PPI leader sequence ($PPI_{6–14}$ and $PPI_{15–24}$) and one within the insulin B chain ($PPI_{33–42}$; also known as $B_{9–18}$). $CD8^+$ T cells that were specific for other PPI peptides were only seen in few patients. Overall, $CD8^+$ T cells specific for at least one PPI peptide were detected in 68% of recent-onset and 83% of long-standing type 1 diabetic patients. These figures are likely underestimated, since stable TMrs were only obtained with peptides that showed sufficient binding affinity to HLA-A*0201 (Table 2). However, we cannot rule out that PPI-specific T cells not tested here are activated in type 1 diabetes and that different peptides are recognized at different stages of type 1 diabetes because we only tested individuals with clinical diabetes.

HLA-A*0201–restricted leader sequence peptides are commonly presented to $CD8^+$ T cells, as previously reported for $PPI_{6–14}$ and $PPI_{15–24}$ (12,15). $PPI_{6–14}$– and $PPI_{15–24}$–specific $CD8^+$ T cells have previously been shown to be cytotoxic against human islet T cells and HLA-A*0201–transfected P815 target cells, respectively (13,17). Recognition of $PPI_{15–24}$ has been reported to increase when human islets were incubated in the presence of high glucose concentrations (17), adding further insight into its role in the natural history of human type 1 diabetes.

The differentiation of memory T cells in relation to antigen exposure has been extensively studied in immune responses to viruses but remains poorly understood in autoimmunity. Clinical diabetes occurs after a preclinical phase that spans over years in many patients. However, the β-cell mass in prediabetes and the level of autoantigen exposure once diabetes is diagnosed remain unknown (35,36). The persistence of CMV infection with time makes it a relevant control, since T cells are kept exposed to low levels of CMV antigens, as is the case for autoimmune T cells exposed to residual β-cells (36). We limited our study of membrane markers of PPI-specific $CD8^+$ T cells to CCR7 and CD45RA, which discriminate central memory from effector memory $CD8^+$ T cells. However, CD45RA does not fully correlate with effector memory functions. Markers such as CD27 and CD28 further discriminate at least three and four different $CD8^+$ subsets within $CCR7^-$ $CD45RA^-$ and $CCR7^-CD45RA^+$ CMV-specific cells, respectively (25). These markers were, however, less relevant to study PPI-specific $CD8^+$ T cells as these cells were mostly within the $CD45RA^-$ $CCR7^+$ central memory compartment. $CD45RA^+CD45RO^+$ double-positive T cells have been identified as possibly representing a transition population from naïve $CD45RA^+$ to memory $CD45RO^+$ T cells. This population has been shown to be increased in recent-onset type 1 diabetic patients but not in long-standing patients, possibly reflecting an imbalance between the two CD45 isoforms in autoimmunity (37). Because we did not exclude $CD45RA^+CD45RO^+$ T cells from the analysis of memory cells, we cannot exclude that we lost these transition cells that are likely to be included in naïve cells in our analysis. However, we saw the same increase in memory PPI-specific $CD8^+$ T cells in long-standing type 1 diabetic patients in whom the double-positive $CD45RA^+CD45RO^+$ T cell is not increased. A recent work has reported the predominant detection of TN and TEMRA $CD8^+$ T cells, but they were specific for different peptides, i.e., $PPI_{2–10}$ and $PPI_{34–42}$, with higher percentages of detection and in subjects who had possibly been treated with anti-CD3 antibody (38).

Analysis of postactivation $CD8^+$ T cells actually illustrates much greater diversity in survival, recall potentials as well as tissue specificity than is defined by current phenotypic markers (32). We turned to single cell PCR to study gene expression programs in $CD8^+$ T cells. In contrast to CMV-specific $CD8^+$ T cells that showed effector/effector memory phenotypes, PPI-specific $CD8^+$ T cells showed profiles that overlap with those of central memory cells. Gene expression profiles of $PPI_{6–14}$– and $PPI_{15–24}$–specific $CD8^+$ T cells in recent-onset type 1 diabetic patients as well as $PPI_{33–42}$– and $PPI_{15–24}$–specific $CD8^+$ T cells in long-standing type 1 diabetic patients were identical. Similarities in gene expression patterns of $CD8^+$ T cells specific for PPI leader sequence peptides and B chain $PPI_{33–42}$ are remarkable because exposition to B chain epitopes persists in insulin-treated, long-standing, type 1 diabetic patients independently of the persistence of residual β-cells. However, this may not be an exclusive mechanism, since $PPI_{15–24}$–specific $CD8^+$ T cells were detected in a subset of patients with long-standing diabetes, and thus independently of insulin therapy because insulin preparations are devoid of PPI leader sequence. No correlation was observed between detection of $PPI_{15–24}$–specific $CD8^+$ T cells and type 1 diabetes duration. Of further note, a significant fraction of PPI-specific T cells displayed a naïve status that was virtually absent in the CMV-specific repertoire, suggesting that there is still an unexpressed potential for further autoimmune activation in most patients. This unexpressed potential may explain the arousal of $PPI_{33–42}$–specific $CD8^+$ T cells upon insulin treatment in long-standing patients. Indeed, these cells were not detected in type 2 diabetic patients treated with insulin, suggesting that insulin treatment may effectively expand them only in the presence of preexisting autoimmunity. The detection of naïve PPI-specific $CD8^+$ T cells may indicate their preexistence and maintenance in the repertoire of type 1 diabetic patients in relation with the slow chronic process that characterizes type 1 diabetes. Adding further value to the extensive functional characterization carried out by single-cell PCR, one single healthy subject harbored $PPI_{6–14}$–specific $CD8^+$ T cells expressing the regulatory cytokine *Il10*. Thus, the mere presence of autoreactive $CD8^+$ T cells may not be considered pathogenic, as previously suggested for islet-reactive $CD4^+$ T cells (39).

We observed a differentiated CMV-specific $CD8^+$ T cell phenotype, as previously reported (25), with low expression of *Ccr7* and high expression of cytolytic effector molecules and significant diversity among individuals. By contrast, PPI-specific $CD8^+$ T cells expressed higher levels of *Ccr7*—presumably allowing them to repeatedly circulate to lymph nodes rather than directly homing to the islets—and lower levels of cytolytic effector molecules. An important factor in driving distinct memory T-cell phenotypes is the strength of T-cell activation in relationship with local factors in secondary lymphoid organs in which the initial T-cell activation takes place and at the inflammatory site (32,40). Expression of homing receptors for lymph nodes have usually been associated with situations in which low strength of activation or a low antigen load is seen (41). Although in most instances antigen persistence is not required for maintenance of memory T cells, recent data in type 1 diabetes suggest that long-standing type 1 diabetic patients may still harbor residual β-cells (42,43).

The detection of B chain–specific $CD8^+$ T cells in a significant fraction of long-standing type 1 diabetic patients indicates that memory $CD8^+$ T cells persist in the long-term range despite the likelihood that autoimmunity to β-cells is

progressively downregulated, since residual β-cells disappear. This persistence of detectable central memory $CD8^+$ T cells in the long term raises several nonexclusive hypotheses. They may persist in the absence of residual β-cells or in the presence of a minimal residual β-cell mass, either resisting autoimmune destruction or regenerating as destruction proceeds, as proposed in the NOD model (44). The persistent detection of $PPI_{6–14}$– and, to a larger extent, $PPI_{15–24}$–specific $CD8^+$ T cells in few long-standing patients is compatible with either hypothesis. Alternatively, long-term treatment with exogenous insulin may contribute to activation of T cells against B-chain peptides. This is relevant in the light of the clinical observation that type 1 diabetes is a highly recurrent disease in patients who have been treated with exogenous insulin for years. The negative correlation observed between the percentage of $A2/PPI_{33–42}$ TMr^+ $CD8^+$ T cells detected in type 1 diabetic patients and residual basal C-peptide values is compatible with this hypothesis.

In conclusion, a majority of type 1 diabetic patients harbor PPI-specific $CD8^+$ central memory T cells. The absence of these cells in most control individuals makes a strong case for the role of PPI-specific $CD8^+$ T cells in type 1 diabetes. Although type 1 diabetes is characterized by active autoimmunity many years after disease onset, our observation that exogenous insulin may contribute to expansion of autoreactive $CD8^+$ T cells in the long term brings a new light to the natural history of the disease.

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S.L. performed experiments and contributed to writing the manuscript. J.-P.B., S.M., N.L., and B.R. performed experiments and contributed to discussions. J.C. performed statistical evaluations and reviewed and edited the manuscript. E.L. was responsible for patient recruitment and follow-up. F.L. and R.M. were involved in discussion and manuscript editing. C.B. designed experiments, chaired discussions, and wrote the manuscript.

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