HLA-B7–Restricted Islet Epitopes Are Differentially Recognized in Type 1 Diabetic Children and Adults and Form Weak Peptide-HLA Complexes

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The cartography of β-cell epitopes targeted by $CD8^+$ T cells in type 1 diabetic (T1D) patients remains largely confined to the common HLA-A2 restriction. We aimed to identify β-cell epitopes restricted by the HLA-B7 (B*07:02) molecule, which is associated with mild T1D protection. Using DNA immunization on HLA-B7–transgenic mice and prediction algorithms, we identified GAD and preproinsulin candidate epitopes. Interferon-γ (IFN-γ) enzyme-linked immunospot assays on peripheral blood mononuclear cells showed that most candidates were recognized by new-onset T1D patients, but not by type 2 diabetic and healthy subjects. Some epitopes were highly immunodominant and specific to either T1D children ($GAD_{530–538}$; 44% T cell–positive patients) or adults ($GAD_{311–320}$; 38%). All epitopes displayed weak binding affinity and stability for HLA-B7 compared with HLA-A2–restricted ones, a general feature of HLA-B7. Single-cell PCR analysis on β-cell–specific (HLA-B7 tetramer–positive) T cells revealed uniform IFN-γ and transforming growth factor-β (TGF-β) mRNA expression, different from HLA-A2–restricted T cells. We conclude that HLA-B7–restricted islet epitopes display weak HLA-binding profiles, are different in T1D children and adults, and are recognized by $IFN\text{-}\gamma^+TGF\text{-}\beta^+CD8^+$ T cells. These features may explain the T1D-protective effect of HLA-B7. The novel epitopes identified should find valuable applications for immune staging of $HLA\text{-}B7^+$ individuals. ***Diabetes*** **61:2546–2555, 2012**

Identification of the epitope targets igniting β-cell autoimmunity provides molecular probes to detect their cognate T cells. Detection of these autoreactive T cells offers useful biomarkers to monitor autoimmune progression along the course of disease (1,2) and regression after immunotherapeutic interventions (3). Second, epitopes can be used as therapeutic agents to neutralize pathogenic T cells after tolerogenic administration (4).

A wealth of information has been harvested in recent years through identification of different epitopes targeted by autoreactive $CD8^+$ T cells. This information is highly relevant to the understanding of type 1 diabetes (T1D) pathogenesis, as $CD8^+$ T cells play a central role in the development (5) and probably the final effector phase of autoimmune β-cell destruction (6). Despite this steady progress, this catalog of β-cell epitopes remains incomplete. With few exceptions (7,8), epitope discovery has been limited to peptides restricted for the most common HLA-A2 allele. This allele allows coverage of ~40% of the T1D population, thus leaving another 60% not amenable to be monitored for T-cell responses. Another point of interest in achieving additional epitope coverage is that some HLA class I alleles have recently been recognized as additional T1D-susceptible or -protective alleles independently of the long-known HLA class II haplotypes (9,10). While HLA-B*39:06 is the strongest T1D-predisposing HLA class I allele (odds ratio [OR] 10.31), HLA-B*57:01 is the one conferring the highest protection (OR 0.19) (10). Thus, it is relevant to address the immune recognition mechanisms underlining these protective effects. However, HLA-B*57:01 is relatively infrequent in the general Caucasian population (~3.5%) and exceedingly rare in matched T1D patients (~0.5%) (10), thus making these studies difficult to perform and yielding marginal population coverage.

HLA-B7 (B*07:02, accounting for ~98% of Caucasian $HLA\text{-}B7^+$ subjects) (10,11) has also been associated with T1D protection (10). This association persists once adjusted for linkage disequilibrium with class II alleles (OR 0.58 [95% CI 0.47–0.70]) (10). HLA-B7 is expressed in ~15% of the healthy population and in ~7% of T1D patients of Caucasian descent (10). Identification of HLA-B7–restricted β-cell epitopes would thus provide coverage of a significant subgroup of patients. Moreover, the relative T1D protection of $HLA\text{-}B7^+$ patients may allow using epitope identification to generate hypotheses for this protective effect. We therefore set forth to identify HLA-B7–restricted epitopes derived from the two β-cell antigens (Ags) GAD and preproinsulin (PPI).

RESEARCH DESIGN AND METHODS

Peptides. A library of 37 9–10-mer GAD peptides selected using SYFPEITHI (score ≥14) (Table 1) was synthesized (>70% purity; GL Biochem). Candidate epitopes identified by DNA immunization were resynthesized at >85% purity for further studies.

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TABLE 1
List of GAD candidate epitopes (n = 37) tested in DNA immunization experiments

Epitope	Sequence	SYFPEITHI	% Mice responding (n = 9)
$GAD_{3–11}$	**S P G S G F W S F**	**18**	**11.1**
$GAD_{36–44}$	F T G G I G N K L	14	0
$GAD_{62–71}$	Q P P R A A A R K A	16	0
$GAD_{63–72}$	P P R A A A R K A A	21	0
$GAD_{78–87}$	K P C S C S K V D V	19	0
$GAD_{84–92}$	K V D V N Y A F L	14	0
$GAD_{98–107}$	L P A C D G E R P T	18	0
$GAD_{100–108}$	**A C D G E R P T L**	**17**	**44.4**
$GAD_{103–111}$	G E R P T L A F L	15	0
$GAD_{105–114}$	R P T L A F L Q D V	17	0
$GAD_{152–160}$	Q P Q N L E E I L	20	0
$GAD_{175–184}$	**H P R Y F N Q L S T**	**20**	**66.7**
$GAD_{182–191}$	L S T G L D M V G L	14	0
$GAD_{207–215}$	Y E I A P V F V L	16	0
$GAD_{208–216}$	E I A P V F V L L	16	0
$GAD_{210–219}$	A P V F V L L E Y V	18	0
$GAD_{230–238}$	W P G G S G D G I	20	0
$GAD_{240–248}$	S P G G A I S N M	19	0
$GAD_{259–267}$	F P E V K E K G M	16	0
$GAD_{265–273}$	K G M A A L P R L	14	0
$GAD_{270–279}$	L P R L I A F T S E	14	0
$GAD_{292–301}$	L G I G T D S V I L	14	0
$GAD_{311–320}$	**I P S D L E R R I L**	**24**	**11.1**
$GAD_{322–331}$	A K Q K G F V P F L	16	0
$GAD_{323–331}$	K Q K G F V P F L	16	0
$GAD_{328–336}$	V P F L V S A T A	17	0
$GAD_{339–347}$	T V Y G A F D P L	14	0
$GAD_{345–353}$	D P L L A V A D I	18	0
$GAD_{393–402}$	N P H K M M G V P L	24	0
$GAD_{400–408}$	V P L Q C S A L L	21	0
$GAD_{469–477}$	A H V D K C L E L	14	0
$GAD_{498–506}$	**K P Q H T N V C F**	**19**	**22.2**
$GAD_{509–518}$	I P P S L R T L E D	14	0
$GAD_{510–518}$	P P S L R T L E D	15	0
$GAD_{530–538}$	**A P V I K A R M M**	**18**	**11.1**
$GAD_{548–556}$	Q P L G D K V N F	20	0
$GAD_{563–571}$	N P A A T H Q D I	18	0

The candidate epitopes selected are shown in boldface.

DNA immunization of HLA-B7–transgenic mice. HLA-B7 (B*07:02)–transgenic *H-2K*^{b}/*D*$^{b-/-}$ mice (12) were pretreated by injecting cardiotoxin (100 μL, 10 μmol/L) in each tibialis anterioris muscle, followed 5 and 19 days later by 50 μg of GAD or noncoding plasmid (13). After 2 weeks, splenocytes were collected and plated (5×10^5 per well) on enzyme-linked immunospot (ELISpot) polyvinylidene fluoride plates (Millipore) coated with anti–interferon-γ (IFN-γ) antibodies (Abs) (U-CyTech). Peptides (10 μmol/L) or DMSO diluent alone were added along with recombinant human interleukin-2 (IL-2) (0.5 units/mL; R&D Systems) (14), and plates were incubated for 20–24 h. Reactions were revealed with a biotinylated anti–IFN-γ Ab (U-CyTech), alkaline phosphatase–conjugated streptavidin, and NBT-BCIP substrate (both from Sigma-Aldrich). Spots were counted on a Bioreader 5000 Pro-SF (BIO-SYS), and means of triplicate wells were calculated. Candidate epitopes were selected based on a positive IFN-γ response in one or more mice out of nine that were immunized, with frequencies of >15 spot-forming cells (SFCs) per 10^6 splenocytes.

Study subjects. Patients (Table 2) were recruited in Paris and Turin along with healthy controls (n = 18: 8 males and 10 females; median age = 28 years, range 20–56). New-onset, auto-Ab$^+$, insulin-dependent T1D adults (n = 8: 5 males and 3 females; median age = 29 years, 18–75; diabetes duration 5 days, 0–90), new-onset T1D children (n = 11: 8 males and 3 females; median age = 10 years, 3–15; diabetes duration 1 day, 0–106), and type 2 diabetes (T2D) patients (n = 13: 4 males and 9 females; median age 59 years, 11–73; diabetes duration 13 years, 0–30) were diagnosed according to the American Diabetes Association criteria (15). All study subjects were HLA-B7$^+$ (HLA-B*07:02) by genotyping using Ambisolv HLA-B*07:02–specific primers (Invitrogen). A group of HLA-B27$^+$, new-onset T1D patients (n = 4: 3 males and 1 female; median age 14 years, 10–32; diabetes duration 4 days, 1–26) and HLA-B7$^+$, long-standing T1D patients (n = 6: 5 males and 1 female; median age 41 years, 32–68; diabetes duration 7 years, 3–28) were further included as controls. Written informed consent was obtained from all participants, and the relevant ethics committees approved the study. HLA-A2$^+$, new-onset T1D patients have been previously characterized (16).

Human IFN-γ ELISpot assay. Peripheral blood mononuclear cells (PBMCs) were prepared as previously described (17,18) and used either fresh or frozen with similar results (1,2). PBMCs (3×10^5 per well) were seeded in anti-IFN-γ–coated ELISpot polyvinylidene fluoride plates in AIM-V medium (Invitrogen) containing 0.5 ng/mL IL-7 and 10 μmol/L peptide (14). A viral peptide mix of HLA-B7–restricted Flu $NP_{418–426}$ (LPFDKTTVM), Epstein-Barr virus $EBNA\text{-}3A_{247–255}$ (RPPIFIRRL), and cytomegalovirus (CMV) $pp65_{417–426}$ (TPRVTGGGAM) (10 μmol/L for each) and phytohemagglutinin (PHA) (1 μg/mL; Sigma-Aldrich) were the positive controls. A naturally processed and presented epitope derived from the HLA-A2 signal sequence (APRTLVLLL) (19) and DMSO diluent were negative controls. After a 20–24-h culture, plates were developed and counted as above. ELISpot readouts are expressed as SFCs/10^6 PBMCs after subtraction of background responses in the presence of control and no peptide (which were identical in all cases). The cutoff for a positive response was set at 3 SDs above the average background of each individual, as determined by receiver-operator characteristics analysis (1). Positive responses were further ranked as low (≥3 SDs above background), medium (≥4 SDs), and high (≥5 SDs). Intra- and interassay reproducibility is 14 and 9%, respectively (1).

HLA-B7 tetramers. Tetramers (TMrs) were generated using the one-pot, mix-and-read technology (20). PBMCs were TMr stained (~150 nmol/L monomers complexed 4:1 with phycoerythrin-labeled streptavidin) for 20 min at room temperature in the presence of lymphocyte-specific protein tyrosine kinase inhibitor II (50 nmol/L; Merck) (17), after which anti-CD8 and anti-CD14/CD19 Abs were added for 20 min at room temperature. After washing and addition of 7-aminoactinomycin D, ≥500,000 events were acquired on a BD Fortessa cytometer. Small and large, viable, CD14/CD19-negative lymphocytes were gated before plotting CD8 and TMr labels.

Single-cell sorting and reverse-transcriptase PCR. PBMCs (10^6) were TMr stained and acquired as above. TMr$^+$CD8$^+$ T cells were single-cell sorted on a BD FACSAria II into 96-well PCR plates containing 5 μL PBS treated with diethyl pyrocarbonate and immediately frozen at −80°C. After thawing, 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 as previously described (16). 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 (Eurogentech) and AmpliTAQ Gold Polymerase (Applied Biosystems) by touch-down PCR. PCR products were resolved on a 2.5% (weight/volume) agarose gel. *IFNG*, *TNFA*, *TGFB* (transforming growth factor-β1 [TGF-β1]), *MIP1A*, *MIP1B*, *IL7R* (IL-7 receptor α), *IL10R* (IL-10 receptor α), *PD1*, and *CD3E* mRNAs were analyzed.

HLA-B7 peptide binding affinity and stability assays. The HLA-A2–restricted epitopes used for comparison were $PPI_{2–10}$ (1), $PPI_{6–14}$ (1,16), $PPI_{15–24}$ (6), $PI_{B10–18}$ (1,7), $PI_{B18–27}$ (1,7), $PPI_{A12–20}$ (1), $GAD_{114–122}$ (1,21), and $GAD_{536–545}$ (13) and the viral epitopes Flu $MP_{58–66}$, CMV $pp65_{495–503}$, and Epstein-Barr virus $BMLF1_{280–288}$. Additional HLA-B7– and HLA-A2–restricted viral, bacterial, parasitic, and tumor epitopes known to be recognized by CD8$^+$ T cells were selected using the Immune Epitope Database (www.iedb.org) (Supplementary Table 1).

Recombinant HLA-A2.1 (0.7 nmol/L) or HLA-B7.2 (2 nmol/L) was mixed in 96-well polypropylene plates (Nunc) with β2-microglobulin (25–50 nmol/L) and peptide (fivefold titrations from 33 μmol/L to 0.01 nmol/L) in PBS supplemented with 0.1% Lutrol F-68 and allowed to form complexes at 18°C for 48 h. Amounts of peptide-HLA complex were determined in an Alphascreen assay (PerkinElmer) with streptavidin-conjugated donor beads and anti-HLA class I Ab W6/32–conjugated acceptor beads (22). Peptide-HLA complexes (10 μL) were transferred to 384-well Optiplates (PerkinElmer) in duplicates, mixed with 30 μL of streptavidin donor beads and W6/32 acceptor beads, and incubated in the dark for 6–8 h at room temperature. Plates were read in an Envision reader (PerkinElmer), and affinities were calculated by nonlinear regression fit using the GraphPad Prism 5 software.

Peptide-HLA stability assays were conducted as previously described (23). In brief, biotinylated, recombinant HLA-A2.1 (30 nmol/L) or HLA-B7.2 (100 nmol/L) was mixed with peptides (10 μmol/L) and ^{125}I-labeled β2-microglobulin (25,000 counts per minute/well), transferred to 96-well Flashplate Plus (PerkinElmer), and allowed to form peptide-HLA complexes overnight at 18°C. Dissociation was initiated by adding unlabeled β2-microglobulin to a final concentration of 360 nmol/L and by consecutively reading the

TABLE 2
Characteristics of diabetic study subjects

Subject number	Age (years)	Sex	Diabetes duration	Therapy	GAD Abs	IA2 Abs	IAA	ZnT8 Abs
HLA-B7$^+$, new-onset T1D adults (n = 8)								
A01	42	F	78 days	Insulin	+	neg	—	—
A02	32	M	0 days	Insulin	+	+	—	—
A03	59	F	90 days	Insulin	+	+	—	—
A04	25	F	4 days	Insulin	+	+	neg	—
A05	18	M	0 days	Insulin	+	+	—	—
A06	19	M	7 days	Insulin	+	+	neg	—
A07	75	M	5 days	Insulin + OHA	neg	neg	—	+
A08	21	M	4 days	Insulin	+	neg	—	neg
HLA-B7$^+$, new-onset T1D children (n = 11)								
C01	10	M	5 days	Insulin	+	—	—	—
C02	3	M	1 day	Insulin	+	+	+	—
C03	11	F	0 days	Insulin	neg	+	neg	—
C04	10	M	1 day	Insulin	+	+	+	—
C05	9	M	2 days	Insulin	+	+	+	—
C06	15	M	1 day	Insulin	+	neg	+	—
C07	5	M	2 days	Insulin	neg	neg	+	—
C08	5	F	1 day	Insulin	+	+	neg	—
C09	14	M	1 day	Insulin	+	+	neg	—
C10	15	M	5 days	Insulin	+	neg	neg	—
^{11}C	4	F	106 days	Insulin	neg	+	+	—
HLA-B7$^+$, long-standing T1D adults (n = 6)								
L01	32	M	28 years	Insulin	—	—	—	—
L02	46	F	3 years	Insulin	+	neg	—	—
L03	33	M	3 years	Insulin	+	neg	—	—
L04	51	M	7 years	Insulin	—	—	—	—
L05	35	M	9 years	Insulin	—	—	—	—
L06	68	M	8 years	Insulin	—	—	—	—
HLA-B7$^+$, T2D subjects (n = 13)								
D01	63	F	20 years	Insulin	neg	neg	—	—
D02	54	F	6 years	OHA	neg	neg	—	—
D03	73	M	9 years	Insulin + OHA	neg	neg	—	—
D04	60	F	21 years	Insulin	neg	neg	—	—
D05	65	F	14 years	Insulin	neg	neg	—	—
D06	53	F	13 years	OHA	neg	neg	—	neg
D07	61	F	9 years	OHA	neg	neg	neg	—
D08	66	F	15 years	OHA	neg	neg	—	—
D09	61	M	30 years	Insulin	neg	neg	—	—
D10	59	M	8 years	Insulin + OHA	neg	neg	—	—
D11	54	F	26 years	Insulin	—	—	—	—
D12	11	F	29 days	OHA	neg	neg	neg	—
D13	28	M	6 days	Insulin	neg	neg	—	neg
HLA-B27$^+$, new-onset T1D subjects (n = 4)								
B01	32	M	4 days	Insulin	neg	neg	—	+
B02	13	M	4 days	Insulin	neg	neg	neg	+
B03	15	M	1 day	Insulin	+	neg	+	—
B04	10	F	26 days	Insulin	+	+	+	—

All Abs were tested at the time of blood draw for T-cell assays. F, female; IA2, insulinoma-associated protein 2; IAA, insulin auto-Abs; M, male; OHA, oral hypoglycemic agents; ZnT8, zinc transporter 8; —, not done or not applicable; neg, negative.

microplates in a TopCount NTX scintillation counter (PerkinElmer) at 37°C for 12 h. Half-lives were calculated as above.

Statistical analysis. Values are expressed as means ± SD or median (range), according to distribution. Comparisons between proportions were made with the Fisher exact test. Comparisons of means between two groups were performed with the Mann-Whitney U test.

RESULTS

Selection of HLA-B7–restricted candidate β-cell epitopes. A GAD peptide library of 37 potential CD8$^+$ T-cell epitopes was selected using the SYFPEITHI algorithm (score ≥14) (Table 1). We used our previously described DNA immunization technique (13,24) on HLA-B7–transgenic mice to select candidate epitopes. Six candidates were selected, being recognized by murine splenocytes after in vitro recall (Fig. 1). No positive responses were observed in control-immunized mice (data not shown). A candidate epitope derived from PPI ($PPI_{8–16}$; LPLLALLAL) ranking highest in SYFPEITHI (score = 23) was further selected.

Recognition of candidate epitopes in T1D patients. PBMCs from new-onset T1D patients were subsequently assayed for recognition of these candidate epitopes, using a modified IFN-γ ELISpot format (1,2). As previously reported (9,10), the proportion of HLA-B7$^+$ subjects was lower in our local T1D population (28/304, 9.2%) compared

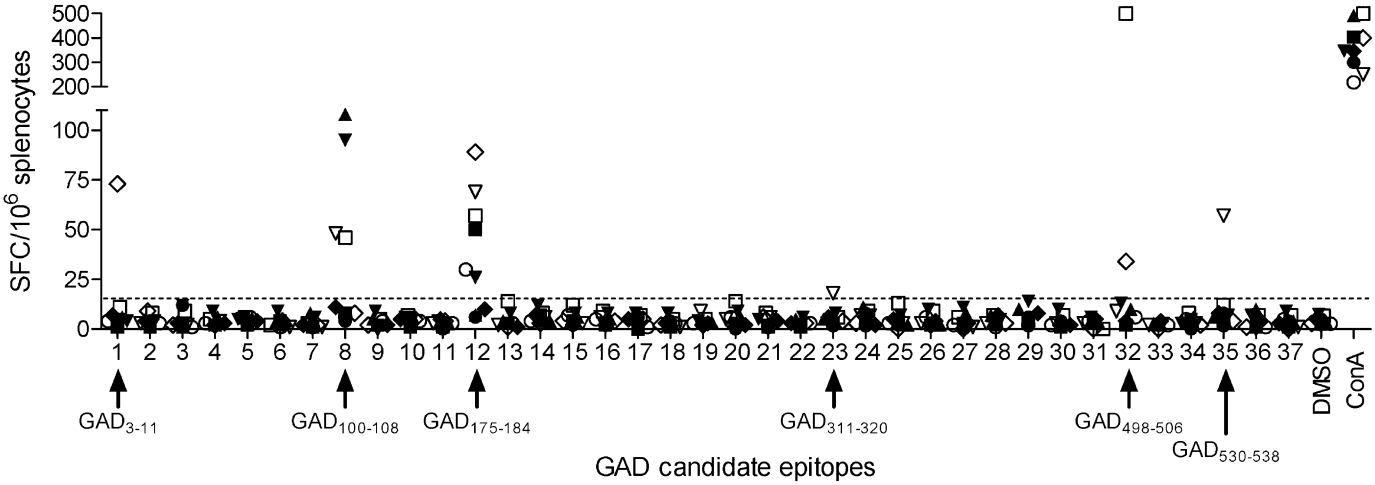

FIG. 1. HLA-B7–restricted GAD candidate epitopes were identified. HLA-B7–transgenic mice (n = 9) were immunized with plasmid DNA encoding full-length human GAD. Their splenocytes were subsequently recalled with the 37 GAD peptides listed in Table 1 in IFN-γ ELISpot plates. Each symbol represents an individual mouse, and the number of IFN-γ SFCs/10^6 splenocytes is given for each peptide. The dotted line represents the positive cutoff (15 SFCs/10^6 splenocytes). Peptides recognized in at least one mouse were selected and are indicated by arrows. The DMSO-negative control and concanavalin A (ConA)–positive control stimulations are also shown.

with healthy controls (35/163, 21.5%; $P < 0.001$), with no significant difference between T1D adults and children.

Raw ELISpot counts are shown in Supplementary Figs. 1 and 2. As summarized in Fig. 2*A*, all candidates were recognized in at least one of the new-onset adult and pediatric patients studied. Three epitopes ($PPI_{8–16}$, $GAD_{311–320}$, and $GAD_{530–538}$) were immunodominant, as they were recognized by 33, 21, and 23% of the patients studied, respectively. Moreover, most (13/19, 68%) responses were of high magnitude (>5 SDs above background) (Supplementary Fig. 1) and in the range of 10–100 IFN-γ SFCs/10^6 PBMCs. This corresponds to median precursor frequencies of 0.003% (range 0.001–0.1) out of total PBMCs (Fig. 2*B*), in the same range of what was previously reported for HLA-A2–restricted epitopes (median frequencies = 0.004%) (1,24). Responses to a viral mix control were positive in 58% of patients, whereas all patients responded to the PHA polyclonal stimulus.

T-cell reactivities to these epitopes were further validated for their T1D specificity in T2D and healthy controls (Fig. 3*A*). Although most (5/7, 71%) epitopes ($GAD_{3–11}$, $GAD_{100–108}$, $GAD_{311–320}$, $GAD_{498–506}$, and $GAD_{530–538}$) were only recognized by new-onset T1D patients, $PPI_{8–16}$ and $GAD_{175–184}$ were less T1D specific, being recognized by 11 and 6% of healthy subjects, respectively. Moreover, $PPI_{8–16}$ was also recognized by a significant fraction (4/12, 33%) of T2D patients. Further controls included four new-onset T1D patients (one adult and three children) who were positive for the B7-related HLA-B27 restriction element. None of these subjects tested positive to any of the epitopes considered (data not shown).

Different epitopes are targeted in T1D children and adults. For those epitopes that were T1D specific, different profiles of T-cell reactivity were observed in T1D children and adults (Fig. 3*A*). Although some epitopes ($GAD_{3–11}$ and $GAD_{100–108}$) were recognized in both patient groups with

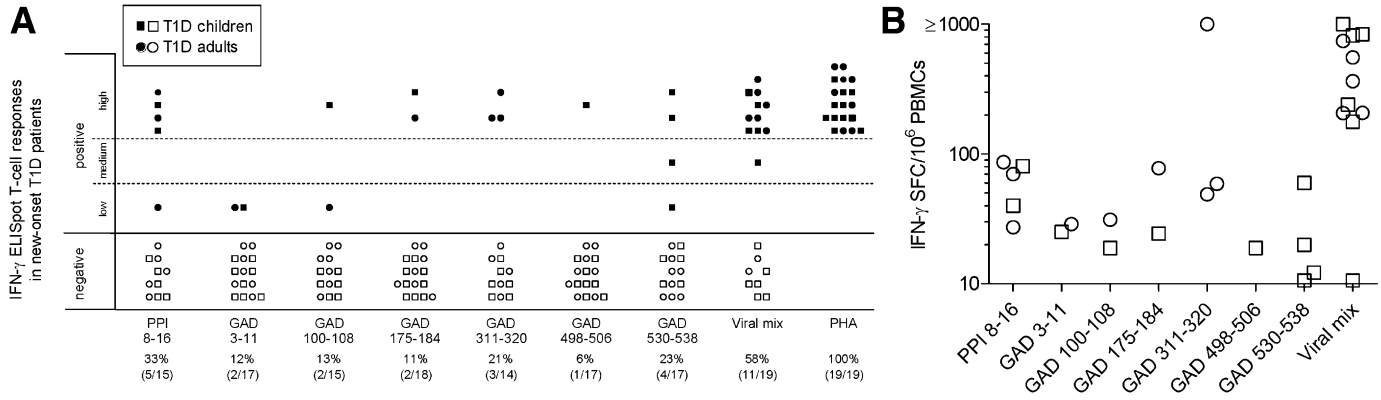

FIG. 2. Validation of HLA-B7–restricted candidate GAD and PPI epitopes in HLA-B7$^+$, new-onset T1D children and adults. Whole PBMCs were tested using an IFN-γ ELISpot format, as detailed in RESEARCH DESIGN AND METHODS. *A*: Number of T1D children (square symbols) and adults (round symbols) testing positive to each peptide. Reactivities are ranked as negative (<3 SDs above basal; white symbols) or positive (≥3 SDs; black symbols). When positive, responses are further ranked as low (3–4 SDs), medium (4–5 SDs), and high (≥5 SDs). The percentage of patients positive to each epitope are indicated. Responses to a viral epitope mix and to PHA are included as positive controls. *B*: Frequencies (IFN-γ SFCs/10^6 PBMCs) of T cells reactive to each individual epitope. Each symbol represents an individual patient.

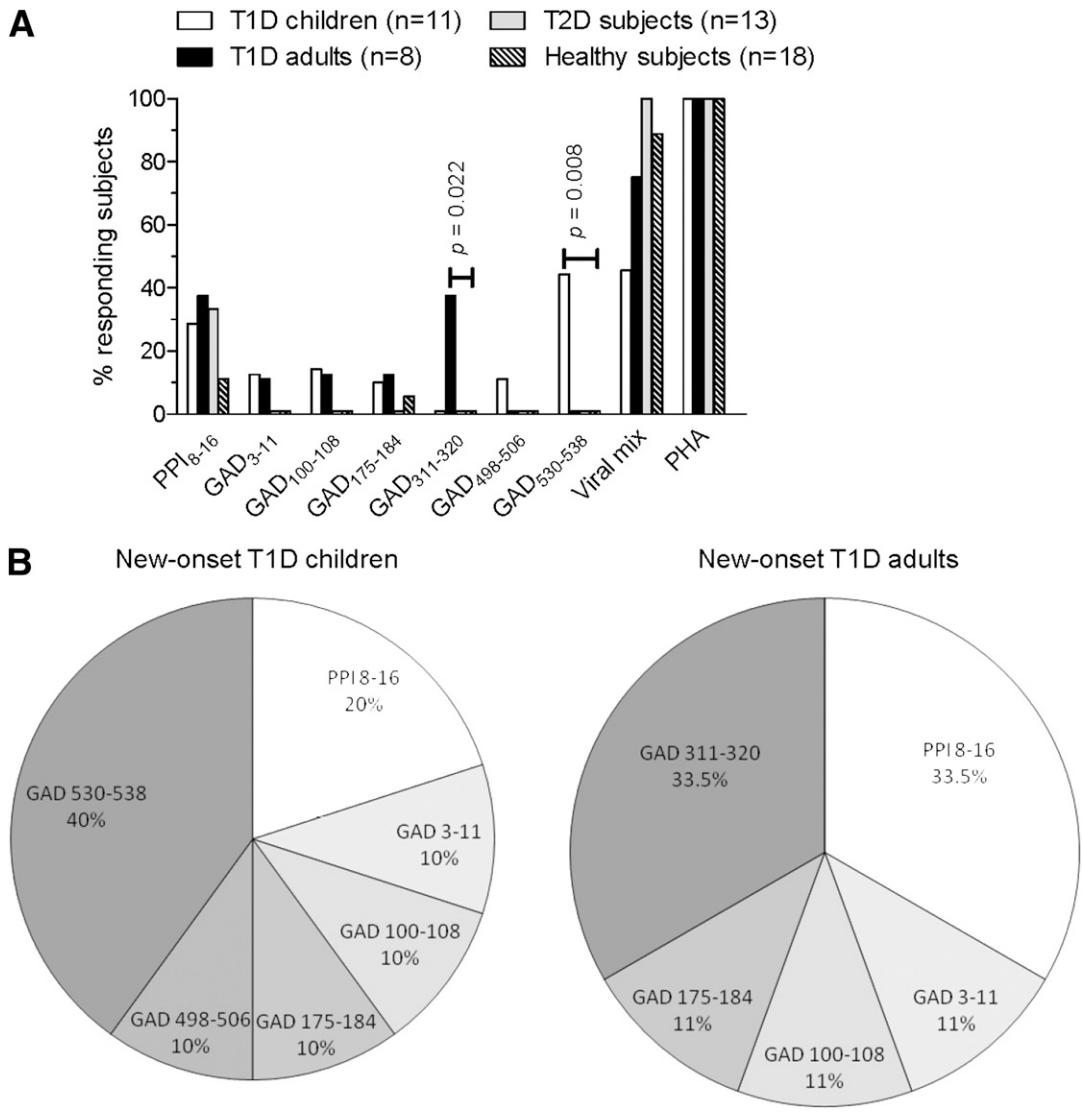

FIG. 3. Recognition of HLA-B7–restricted islet epitopes in different study subjects. *A*: Percentage of new-onset T1D children (white bars), new-onset T1D adults (back bars), T2D subjects (gray bars), and healthy subjects (hatched bars) responding to each individual epitope. *B*: Relative distribution of epitope specificities among total β-cell epitope-reactive CD8$^+$ T cells. The percent prevalence of each epitope out of all epitopes recognized among new-onset T1D children (n = 10) (*left*) and adults (n = 9) (*right*) is shown.

similar frequencies (11–14%), others were specific for either T1D children ($GAD_{498–506}$ and $GAD_{530–538}$) or adults ($GAD_{311–320}$). This was evident not only when analyzing the prevalence of T-cell responses but also when considering the relative immunodominance of these epitopes among the total β-cell epitope-reactive T cells detected (Fig. 3*B*). Indeed, $GAD_{530–538}$ was highly immunodominant in T1D children, as it was targeted by 40% of the total autoreactive T-cell responses and recognized by 44% of pediatric patients (P = 0.008), but in none of the T1D adults. Conversely, $GAD_{311–320}$ was the major T1D-specific epitope in T1D adults, as it accounted for 34% of the total T-cell responses and was recognized in 38% of T1D adults (P = 0.022), but in none of the T1D children.

Most CD8$^+$ T-cell IFN-γ responses rapidly wane after T1D onset. We previously reported that IFN-γ–producing CD8$^+$ T-cell responses against different HLA-A2–restricted β-cell epitopes rapidly wane after diagnosis (2). This was the case also for HLA-B7–restricted epitopes. Testing a subgroup of five patients (one adult and four children) near diagnosis and 7 days to 14 months thereafter (Fig. 4), most (6/10, 60%) responses that were positive at diagnosis became undetectable. There was only one instance of a $GAD_{175–184}$–specific response that was absent at diagnosis and became detectable 14 days after (patient A03). As an internal control, T-cell responses against a viral epitope pool remained stable in four of the five patients studied and disappeared in one child (C05), perhaps witnessing the resolution of an acute viral infection. Moreover, the same T-cell responses tested in six long-standing HLA-B7$^+$ T1D adults were infrequent, with only two patients (33%) scoring positive for a single epitope ($PPI_{8–16}$, which was also targeted in T2D patients, and $GAD_{311–320}$) (Supplementary Fig. 3) compared with 63% (5/8) of new-onset T1D adults testing positive, most frequently for two different epitopes (Supplementary Fig. 1).

HLA-B7–restricted epitopes show lower HLA binding affinity and stability than HLA-A2–restricted ones. Next, we analyzed the HLA binding affinity and stability of the identified HLA-B7–restricted epitopes and compared

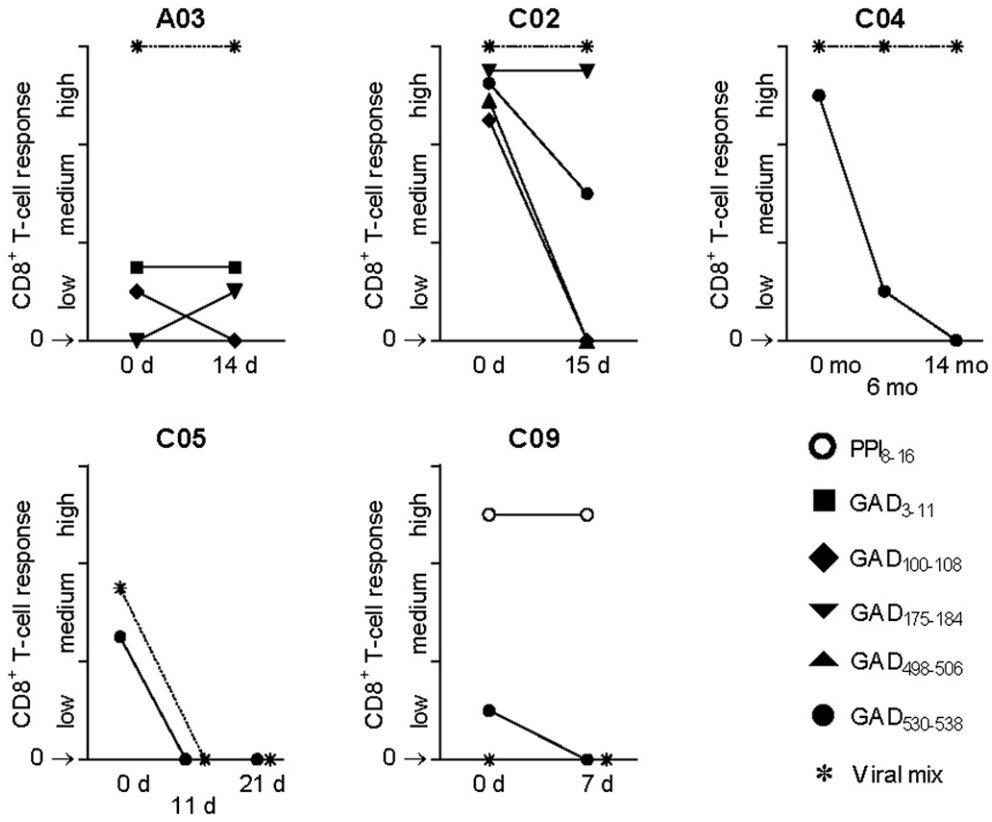

FIG. 4. Longitudinal follow-up of IFN-γ ELISpot responses in T1D patients. Patients previously tested by ELISpot close to diagnosis (t = 0) (Supplementary Fig. 1) were reassayed under identical conditions after 7 days to 14 months of follow-up, as indicated. Reactivities testing positive at either time point are depicted and ranked as absent (<3 SDs above basal), low (3–4 SDs), medium (4–5 SDs), and high (≥5 SDs) as in Fig. 2*A*. Responses against a pool of viral epitopes are included as positive controls.

them with those of HLA-A2–restricted epitopes previously identified (Fig. 5; see Supplementary Table 1 for details). The binding affinity of HLA-B7–restricted islet epitopes (Fig. 5*A*) was one log lower than for HLA-A2–restricted ones (median Kd = 95 nmol/L, range 23–1088, vs. 12 nmol/L, 3–1837; P = 0.012). This difference was also present for HLA-B7– and HLA-A2–restricted nonautoimmune T-cell epitopes derived from infectious and tumor Ags (median Kd = 35 nmol/L, 7–356, vs. 2 nmol/L, 0.2–163, respectively; P = 0.001). The same was true when analyzing the stability of HLA-B7 and -A2 molecules complexed with the corresponding epitopes (Fig. 5*B*). Whereas islet epitopes formed unstable complexes with HLA-B7 (median half-life = 2.4 h, 0.6–7.7), HLA-A2 complexes were more stable (median half-life = 7.1 h, 4.2–21.0; P = 0.003). Also in this case, this difference reflected the general binding properties of HLA-B7 compared with HLA-A2, as it was also observed for nonautoimmune epitopes (median half-life = 2.4 h, 1.0–5.7,

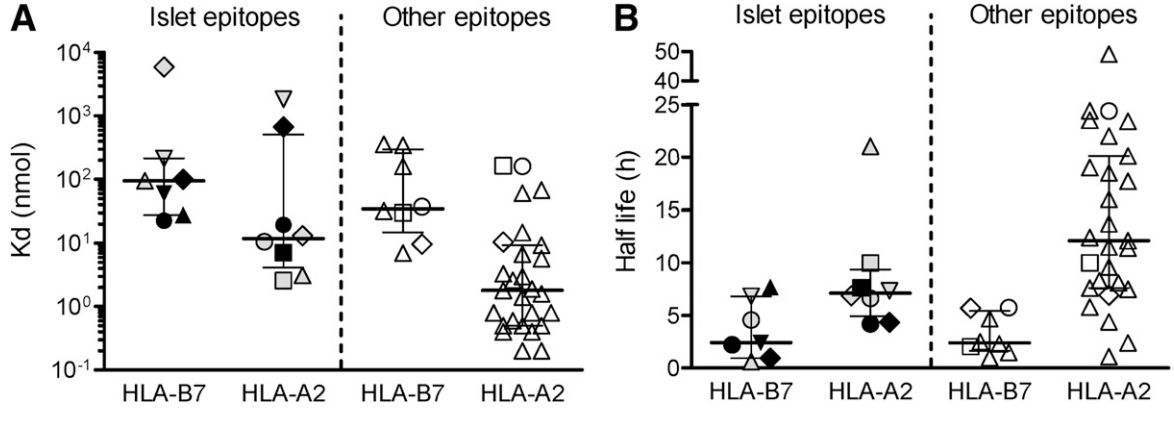

FIG. 5. HLA affinity and stability measurements. *A*: HLA-B7 and -A2 Kd binding affinity for T-cell epitopes derived from islet Ags or from infectious and tumor Ags identified here and in previous reports, as detailed in RESEARCH DESIGN AND METHODS and Supplementary Table 1. *B*: HLA-B7 and -A2 binding stability (half-life) measured for the same epitopes. Each symbol represents an individual epitope, and bars show median and interquartile range values for each distribution. Each symbol depicts the mean value of at least two separate measurements.

vs. 12.1 h, 1.1–49.1; $P = 0.0002$). Moreover, the lower HLA binding affinity and stability frequently reported for autoimmune epitopes (25) compared with nonautoimmune ones was observed for HLA-A2 (median affinity = 12 vs. 2 nmol/L, $P = 0.001$; stability = 7.1 vs. 12.1 h, $P = 0.043$), but not for HLA-B7 (median affinity = 95 vs. 35 nmol/L, $P = 0.78$; stability = 2.4 vs. 2.4 h, $P = 1$), further suggesting a general feature of weak binding for HLA-B7.

HLA-B7–restricted autoreactive CD8$^+$ T cells show higher *IFNG* and *TGFB* expression than HLA-A2–restricted ones. The lower binding affinity and stability imposed by HLA-B7 compared with HLA-A2 would lead to more transient availability of epitope/HLA-B7 versus epitope/HLA-A2 complexes, which may deliver weaker signals to T cells. To gain insight into the functional consequences of this weak epitope binding to HLA-B7, we used single-cell PCR to functionally characterize epitope-specific CD8$^+$ T cells. PBMCs from the T1D patient C09, who tested positive for $PPI_{8–16}$ and $GAD_{530–538}$, and A07, who tested positive for $GAD_{311–320}$ (Supplementary Fig. 1), were further assayed using HLA-B7 TMrs loaded with the corresponding peptides. Results were concordant with those of IFN-γ ELISpot, yielding significant fractions of TMr$^+$CD8$^+$ cells compared with samples stained with TMrs loaded with a control peptide (Fig. 6*A*). As expected, this concordance was observed relative to the absence or presence of epitope-specific T cells and not to their frequency, given the different readout of the two assays (functional vs. structural) and the lower threshold needed for T-cell activation compared with stable TMr binding (26), especially in the presence of weak peptide/HLA-B7 complexes.

These $PPI_{8–16}$, $GAD_{530–538}$, and $GAD_{311–320}$ epitopes are weak HLA-B7 binders (Kd = 1088, 61, and 100 nmol/L and half-life = 4.6, 2.4, and 0.9 h, respectively), falling within the typical range of HLA-B7–restricted islet epitopes (median Kd = 95 nmol/L; half-life = 2.4 h). Twenty TMr$^+$ cells recognizing these three epitopes were single-cell sorted from these positive PBMC samples, analyzed for their mRNA expression, and compared with the expression profiles of HLA-A2–restricted CD8$^+$ T cells specific for $PPI_{6–14}$ from new-onset T1D patients as previously described (16) (Fig. 6*B*). $PPI_{6–14}$ displays strong HLA-A2–binding characteristics (Kd = 13 nmol/L; half-life = 6.9 h), within the range of HLA-A2–restricted islet epitopes (median Kd = 12 nmol/L; half-life = 7.1 h). With the exception of *IL-10R* mRNA, the three HLA-B7–restricted β-cell epitope-specific populations sorted (black symbols) were remarkably homogeneous for their expression of *IFNG*, *TNFA*, *TGFB*, *MIP1A*, *MIP1B*, *IL7R*, and *PD1*. This profile was also similar to that of HLA-A2–restricted $PPI_{6–14}$–specific CD8$^+$ T cells (white square symbols), with the exception of *IFNG* and *TGFB*, which were more and uniformly expressed by HLA-B7–restricted cells (95–100% and 100%, respectively) compared with HLA-A2–restricted ones (median expression = 60 and 13%, $P < 0.001$ for both). CMV-specific CD8$^+$ T cells sorted from HLA-B7$^+$ (gray round symbol) and HLA-A2$^+$ (gray square symbols) subjects were also different for their expression of *TGFB* (100 vs. 74%, $P = 0.008$), but not of *IFNG* (21 vs. 28%, $P = 0.435$); and they coexpressed both *IFNG* and *TGFB* in only ~20% of cases (not shown) (16). Thus, circulating HLA-B7–restricted autoreactive T cells recognizing weak HLA-binding epitopes uniformly coexpress *IFNG* and *TGFB*, a molecular signature different from that of HLA-A2–restricted counterparts that recognize stronger HLA-binding epitopes.

DISCUSSION

Identification of β-cell epitopes allows tracking of autoreactive T cells during disease progression and after immunotherapeutic interventions. Although T-cell assays bypassing this need for HLA-specific epitope identification have been previously described (3,27), it remains important to draw a comprehensive epitope cartography not only for the most prevalent HLA-A2 allele but also for other common restriction elements. HLA-B7 fits these requirements, being expressed by ~15% of the Caucasian general population and ~7% of T1D patients (10). This difference in frequency has suggested a relative protection conferred by this allele, which was confirmed in genetic studies correcting for linkage disequilibrium (10). This feature of HLA-B7 makes it additionally relevant to explore immune mechanisms underlying this protection.

We elected to identify HLA-B7–restricted epitopes derived from GAD and PPI, as these two Ags yielded most of the immunodominant epitopes described for HLA-A2 (1,13). The epitopes thus obtained should prove useful for immune staging of HLA-B7$^+$ individuals. One lesson learned is that epitope panels need to be tailored for the T1D population under study, i.e., pediatric versus adult. It has long been postulated that T1D children harbor more aggressive β-cell autoimmunity, which may explain their earlier age at onset compared with adults. The immunological bases for this difference are unclear, and previous T-cell studies have not compared these two groups. We show that the β-cell epitopes targeted are only partially shared. Indeed, some epitopes were exclusively targeted in T1D children (i.e., $GAD_{530–538}$), whereas others were typical of T1D adults (i.e., $GAD_{311–320}$). Although the size of our study groups was relatively small due to the rarity of HLA-B7$^+$ patients, this age-related epitope difference proved significant. Furthermore, age-specific epitopes were highly immunodominant in the corresponding population. Why these epitopes are differentially selected and whether they are more immunogenic or give rise to more aggressive cytotoxic T lymphocytes in children remains to be elucidated. There was also one epitope ($PPI_{8–16}$) that was found to not be T1D specific, being similarly recognized in T2D patients and healthy controls. Similar observations have been made for some HLA-A2–restricted β-cell epitopes (8,28) and may reflect a high number of naïve precursors that succeed in activating upon epitope-driven stimulation. As previously observed in HLA-A2$^+$ T1D patients (1,2) and even in inbred NOD mice (29,30), there was considerable heterogeneity in the epitopes targeted in different individuals. These repeated findings may explain the disappointing results of vaccination trials using one single islet Ag such as insulin or GAD (4).

The current study also gives further validation of our DNA immunization technique on HLA-transgenic mice for epitope identification (13). We were not able to apply the same strategy to PPI due to inefficient in vivo expression of the corresponding plasmid. All the GAD epitopes preselected in immunized mice proved to be recognized by patient T cells, as was previously the case for HLA-A2–restricted islet epitopes (13,28). Although the reactivities detected in mice are induced by exogenous immunization, they prove relevant once transferred to human T1D. The major advantage of this approach is its rapidity and ease of use, as panels of candidate epitopes can be generated within weeks with minimal hands-on work. Moreover, candidates are selected based not only on immunogenicity but also on natural processing, as DNA immunization

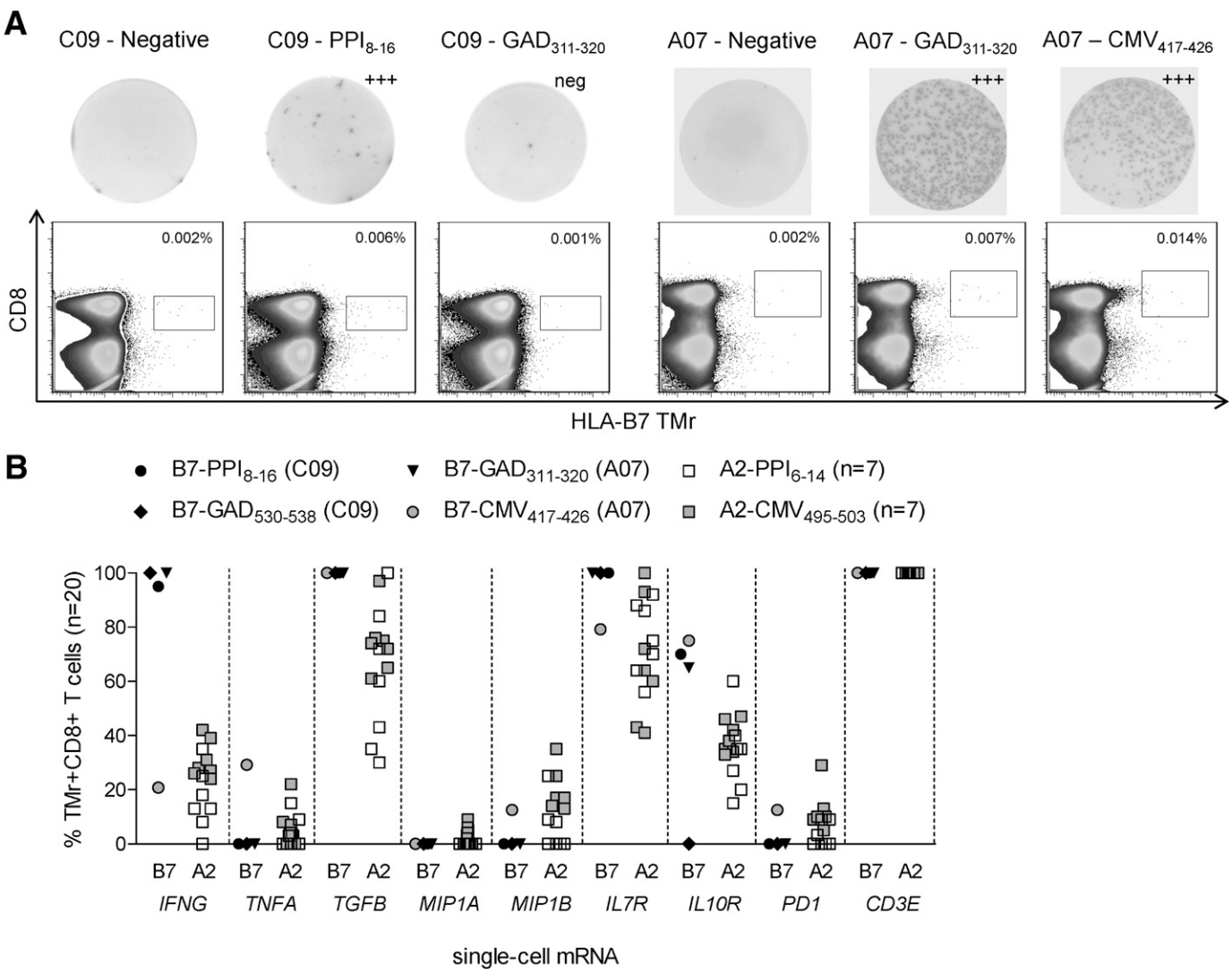

FIG. 6. mRNA expression profiles of epitope-specific HLA-B7– and HLA-A2–restricted TMr$^+$CD8$^+$ T cells. *A*: Comparison of IFN-γ ELISpot and HLA-B7 TMr readouts for different epitopes in patients C09 and A07, as indicated at the top of each column. Top row displays representative ELISpot wells out of triplicate measurements. Bottom row shows the corresponding fraction of TMr$^+$CD8$^+$ cells detected in the same individual. *B*: TMr$^+$CD8$^+$ cells were single-cell sorted, analyzed by reverse-transcriptase PCR, and compared with similar measurements previously performed on HLA-A2$^+$, new-onset T1D patients (16). Percent numbers of HLA-B7–restricted PPI$_{8–16}$- (black round symbols), GAD$_{530–538}$- (black diamond symbols), GAD$_{311–320}$- (back triangle symbols), and CMV$_{417–426}$–specific (gray round symbols) T cells (n = 20/each) expressing each mRNA are depicted. White and gray squares show frequencies of gene-expressing single HLA-A2–restricted PPI$_{6–14}$- and CMV$_{495–503}$–specific T cells sorted from 7 HLA-A2$^+$, new-onset T1D patients. neg, negative; +++, positive response ≥5 SDs above background.

ensures in vivo Ag translation and processing (24). Selection of the initial peptides to test based on a wide range of predicted HLA-binding affinities also yields weak binding epitopes, which are particularly relevant in autoimmunity. Indeed, most of the epitopes identified here were weak and unstable HLA-B7 binders, and would thus be missed if initially selected based on strong binding. HLA-B7–restricted epitopes were uniformly weak binders irrespective of their Ag source (islet or nonautoimmune), pointing to a general feature of HLA-B7, as recently described (31). This feature is not shared by all HLA-B alleles, as it is not observed for HLA-B*35:01 (31). Apart from the special case of HLA-B7, the HLA-A2–restricted β-cell epitopes previously characterized displayed significantly weaker HLA binding than epitopes derived from nonautoimmune Ags. Thus, autoreactive epitope-HLA complexes are not only characterized by weaker recognition (T-cell receptor affinity) from cognate T cells (32), but also by weaker peptide-HLA interactions, probably leading to decreased epitope presentation. These two features may explain failure of thymic negative selection.

The study of HLA-B7$^+$ T1D patients also allows a comparison with patients carrying the T1D-indifferent allele HLA-A2 (9,10). We show that, despite the mild T1D protection conferred, HLA-B7 can function as a restriction element for β-cell–reactive CD8$^+$ T cells. These cells are present in the blood of T1D patients at frequencies (0.003% of PBMCs) similar to those of HLA-A2–restricted T cells (1,24). However, their functional profile is different, being characterized by higher and uniform IFN-γ and TGF-β mRNA expression. These cells might represent a regulatory subset, which may explain the HLA-B7 protective effect. Indeed, Weiner and colleagues (33) described a regulatory population of murine CD8$^+$ T cells that are LAP$^+$ (latency-associated peptide) and produce high amounts of IFN-γ and TGF-β. These cells are regulatory both in vitro and in vivo

and suppress experimental autoimmune encephalomyelitis in an IFN-γ– and TGF-β–dependent fashion (33). An Ag-specific $CD8^+$ T-regulatory subset with similar characteristics has been reported after peptide immunization (34). The observed IFN-γ^+TGF-β^+ HLA-B7–restricted T cells may be generated after low-density epitope-HLA-B7 stimulation, either in the thymus or in the periphery. Nonetheless, the subjects analyzed here developed T1D, suggesting that HLA-B7–mediated protection is fallible, as is the case for that conferred by HLA class II alleles (35).

HLA-B7–restricted $CD8^+$ T-cell responses producing IFN-γ rapidly waned after disease onset, as previously reported for HLA-A2 (2). This rapid disappearance may be due to loss of residual β-cells, which limits Ag availability, or to the effects of insulin therapy, which may induce tolerance to insulin-derived epitopes and relieve metabolic stress from β-cells, possibly making them less immunogenic (36). This decrease in autoreactive T cells, however, seems specific to IFN-γ–producing cells, as it is not observed in studies using TMrs, including our own (16). The fact that TMr^+CD8^+ T cells in long-standing patients are predominantly central memory (16), i.e., they secrete little IFN-γ (37), may explain these findings. TMrs may thus allow the use of the identified epitopes for immune staging of both the prediabetic phase, to stratify risk, and after T1D onset, to monitor response to immunotherapies.

In conclusion, the novel epitopes identified in this study demonstrate that the T1D-protective HLA-B7 molecule is capable of restricting the response of β-cell–reactive T cells. These responses target different epitopes in T1D children and adults and are imprinted by the weak peptide-binding features of HLA-B7, possibly conditioning their phenotype. Further studies are warranted to verify whether an opposite phenomenon takes place for HLA class I alleles associated with T1D susceptibility.

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M.S., G.A., and T.Ø. designed and performed experiments and participated in data analysis. E.L. designed experiments, participated in data analysis, and provided blood samples and reagents. S.L. designed experiments. C.R., G.N., and F.A.L. participated in data analysis and provided blood samples and reagents. G.B., C.G.-L., O.L., and J.-C.C. provided blood samples and reagents. S.B. designed experiments and participated in data analysis. C.B. designed experiments and provided blood samples and reagents. R.M. designed experiments, participated in data analysis, provided blood samples and reagents, and wrote the manuscript. All authors reviewed and edited the manuscript. R.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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