RECOGNITION OF HUMAN PROINSULIN LEADER SEQUENCE BY
CLASS I-RESTRICTED T CELLS IN HLA-A*0201 TRANSGENIC MICE
AND IN HUMAN TYPE 1 DIABETES

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ABSTRACT

Objective—A restricted region of proinsulin located in the B chain and adjacent region of C-peptide has been shown to contain numerous candidate epitopes recognized by CD8+ T cells. Our objective is to characterize HLA class I-restricted epitopes located within the preproinsulin leader sequence.

Research Design and Methods—Seven 8- to 11-mer preproinsulin peptides carrying anchoring residues for HLA-A1, -A2, -A24 and -B8 were selected from databases. HLA-A2-restricted peptides were tested for immunogenicity in transgenic mice expressing a chimeric HLA-A*0201/beta2-microglobulin molecule. The peptides were studied for binding to purified HLA class I molecules, selected for carrying C-terminal residues generated by proteasome digestion in vitro and tested for recognition by human lymphocytes using an ex vivo interferon gamma ELISpot assay.

Results—Five HLA-A2-restricted peptides were immunogenic in transgenic mice. Murine T cell clones specific for these peptides were cytotoxic against cells transfected with the preproinsulin gene. They were recognized by PBMCs from 17/21 HLA-A2 type 1 diabetic patients. PBMCs from 25/38 HLA-A1, -A2, -A24 or -B8 patients produced interferon gamma in response to six preproinsulin peptides covering residues 2 to 25 within the preproinsulin region. In most patients, the response was against several class I-restricted peptides. T cells recognizing preproinsulin peptide were characterized as CD8+ T cells by staining with peptide/HLA-A2 tetramers.

Conclusions—We defined class I-restricted epitopes located within the leader sequence of human preproinsulin, through in vivo (transgenic mice) and ex vivo (diabetic patients) assays, illustrating the possible role of preproinsulin-specific CD8+ T cells in human type 1 diabetes.
Type 1 diabetes involves the activation of lymphocytes against β cell autoantigens. In animals, the predominant role of T lymphocytes is supported by experiments in which diabetes is transferred by diabetogenic T cells, is prevented by antibodies that interfere with T lymphocyte activation or fails to develop in diabetes-prone mice in which key genes in T lymphocyte differentiation or activation are deficient. In human, T lymphocytes are predominant within insulitis at early stages of diabetes. Moreover, type 1 diabetes has been reported in an immunodeficient patient deprived of B lymphocytes (1).

MHC class II-restricted CD4+ T cells are central in the diabetes process but CD8+ T cells play a pivotal role in its initiation in NOD mice (2). In human, CD8+ T cells are predominant and a high percentage of IFN-γ-positive cells is detected within insulitis in recent-onset diabetes in most observations (3-6). Recurrent diabetes in recipients of isografts from a discordant twin is accompanied by predominant CD8+ T cell infiltration (7).

Among β cell autoantigens, proinsulin has been ascribed a key role in diabetes. In man, insulin and proinsulin are common targets of autoantibodies (8,9) and T cells (10-17) in diabetic and prediabetic individuals. Anti-insulin antibodies (IAA) are the first to be detected in children at risk for diabetes and carry a high positive predictive value for diabetes (9). In NOD mice, injection of insulin-specific T cell clones accelerate diabetes (18). Protection from diabetes is obtained by injecting insulin in prediabetic mice (19). In addition, proinsulin 1- or 2−/− NOD mice show delayed or accelerated diabetes, respectively (20,21).

Several new β cell HLA class I-restricted epitopes have been reported recently (22-27). We and others have shown that a restricted region of human proinsulin located in the B chain and adjacent C-peptide clusters proteasome cleavage sites generating correct C-termini of putative MHC class I peptides and many epitopes that are recognized by diabetic CD8+ T cells (22,23). Recognition of epitopes that are located within the C peptide and C peptide-B chain junction including residues that are excised during the secretion process makes a strong case for proinsulin as an autoantigen in diabetes. Despite strong evidence that leader sequence peptides are presented by class I HLA molecules, especially HLA-A2.1 (28), only two HLA A2.1 preproinsulin leader sequence peptides have been identified (26,27).

To characterize class I-restricted epitopes within the preproinsulin leader sequence, we selected 8- to 11-mer peptides carrying anchoring residues for class I molecules. These peptides were studied for immunogenicity in HLA-A*0201 transgenic mice (29). Mouse CD8+ T cell clones specific to HLA-A*0201-restricted peptides were tested for cytotoxicity against HLA-A2 target cells transfected with the preproinsulin gene. In human, peptides were studied for binding to common class I molecules, for carrying C-terminal residues generated by proteasome digestion and for recognition by PBMCs from diabetic patients.

**RESEARCH DESIGN AND METHODS**

**Mice.** HHD mice express the chimeric HLA-A*0201 HHD monochain containing the HLA-A*0201 α1/α2 domains and the H-2Db α3 domain linked at its N-terminus to the human β2m C-terminus by a 15 residue linker peptide (29).

**Cytotoxicity assays.** Cytotoxicity assays were performed using RMA-S cells transfected with the HHD monochain or P815-HTR (high efficiency transfection recipient, DBA/2, H-2d) mouse mastocytoma cells expressing HLA-A2.1 and human preproinsulin as targets. P815-HTR cells were
electroporated with respectively 20 μg of HLA-A2.1 plasmid (blue script) and 2 μg of construct plasmid preproinsulin human linearized DNA (PcDNA3.1+, Invitrogen) at 250 V using an Easyject gene pulser. After 24 h, cells were transferred in selective medium containing 500 μg/ml G418 (Gibco BRL, Paislay, U.K.) and cloned by limiting dilution. Neomycine-resistant clones expressing the HLA-A2.1 monochain were analyzed by flow cytometry using anti-Hβ2m FITC-conjugated antibody. Transfected cells expressing high preproinsulin RNA levels were selected by RT-PCR using β-actin RNA as internal standard.

Peptide immunizations were performed by injecting HHD mice with 100 µg peptide and 140 µg of I-Aβ-restricted helper peptide from the hepatitis B core protein (TPPAYRPPNAPIL) in incomplete Freund’s adjuvant (IFA) s.c. at the base of the tail. At day 11, spleen cells were restimulated in vitro for 6 days in the presence of irradiated (3500 rad) HHD mouse spleen cells preactivated for 3 days by 10 μg/ml lipopolysaccharide 055:B5 (Sigma), then pulsed with peptides (5 μg/ml). Spleen cells were recovered and tested for cytotoxicity against HHD-transfected RMA-S cells pulsed with tested or control peptide using chromium release assay.

CTL lines and clones were obtained by culturing cells showing cytolytic activity in RPMI supplemented with 10% FCS and 0.1 mmol/l 2-mercaptoethanol and restimulated weekly with peptide (1 μg/ml down to 0.05 μg/ml)-pulsed HHD cells.

Patients. Type 1 diabetic patients had anti-GAD, -insulin, -islet antigen 2 (IA2) or islet cell antibodies (ICA) at diagnosis. Recent-onset patients (n = 26, 12 females, 14 males) were studied within 3 months of diagnosis. Long-standing patients (n =12, 4 females, 8 males) had been insulin-treated for 5 to 40 years at study. Controls included 12 healthy blood donors (4 females, 8 males) and 6 type 2 diabetic patients (2 females, 4 males), 3 of whom were insulin-treated (Table 1). Informed consent was obtained from all patients. PBMCs were isolated by Ficoll Paque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) and analyzed immediately. HLA class I and class II alleles were determined by serological typing and genotyping, respectively, and class I alleles were confirmed by genotyping.

Peptides. Seven 8- to 11-mer preproinsulin peptides carrying putative binding motifs for HLA-A1, -A2, -A24 and -B8 alleles were selected as predicted from known viral peptide sequences and databases (http://www-bimass.cit.nih.gov; http://www.syfpeithi.de). Selection was based on bimass and syfpeithi scores ≥ 40 and 14, respectively, or known viral peptide sequences (own data, J.C.). Six HLA-A2-restricted peptides (one of them carrying binding motifs for HLA-A24 and -B8) and one peptide containing binding motifs for HLA-A1 and -A24 were selected (Table 2). Peptides were synthesized and purified (23). Peptide nomenclature refers to N- and C-terminal positions along the human preproinsulin sequence.

Peptide-binding assay. HLA heavy (H) chains prepared as reported (30) were incubated with 2µg/ml of exogenous β2m (Sigma, Steinheim, Germany) and with 10⁻⁴, 10⁻⁶ and 10⁻⁸ M exogenous peptides in Eppendorf microtubes (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) for 1 hour at room temperature and then for 24 h at 4°C. Reassembled HLA molecules were further incubated for 90 minutes at 37°C in 96-well microtiter plates coated with anti-HLA monoclonal antibodies (BB7.2 for HLA-A2, B1.23.2 for HLA-B molecules, PA2.6 for HLA-A and -B molecules). Correctly folded HLA complexes were revealed with anti-β2m antibodies coupled to alkaline phosphatase, and 4-methyl-umbelliferyl phosphate (M-8883; Sigma) as substrate. Fluorescence generated was measured at 360/460 nm in a Microfluor reader (Victor 1420; Wallac,
Turku, Finland). Percentage of binding was defined as the binding of tested peptide over the binding of the reference viral peptide x 100. Reference peptides were influenzae virus matrix M.58-66 (GILGFVFTL) for HLA-A2, influenzae virus PB1 peptide 591-599 (VSDGGPNLY) for HLA-A1, EBV LMP2 419-427 (TYGPFMSL) for A-24 and HIV Nef 90-97 (FLKEKGGL) for HLA-B8.

**Proteasome digestion.** Preproinsulin peptide 1-28, which covers leader sequence and adjacent B chain residues (MALWMRLPLLALLALWGPDPAAAANFQ) was digested by a proteasome-enriched extract obtained from T1 lymphoblastoid cells (23). Cleavage products were separated by RP-HPLC (Perkin-Elmer, Norwalk, CT) on a C18 Nucleosil column (10 µm, 250 x 4.0 mm; Macherey-Nagel, Hoerdt, France). Mass analyses were performed on a MALDI-Tof spectrometer in a reflectron delayed extraction ion source over a mass range of 500-3200 Dalton and recorded with a Voyager-DE-Pro mass spectrometer (PerSeptive Biosystems, Framingham, MA). Monoisotopic masses were calculated and peptides corresponding to computed masses identified (23).

**Enzyme-linked immunospot (ELISpot) assay.** IFNγ ELISpot was performed as previously described (23). Background IFNγ response was evaluated in 3-6 wells containing 3x10^5 cells/well incubated without peptide. Responses were considered positive when the number of spots in the presence of peptide was above background + 3 SD. Positive controls consisted of 3 wells containing 3x10^4 cells/well stimulated with 1µg/ml phytohemagglutinin and 3 wells containing 3x10^5 cells/well stimulated with 10 µg/ml of viral peptides (peptide M.58-66 from influenzae virus matrix for HLA-A2, nucleoprotein NP44-52 for HLA-A1, NP380-388 for HLA-B8). Negative controls were HIV Nef 83-91 peptide for HLA-A2 and Nef 182-189 peptide for HLA-B8. A stimulation score (SS) was calculated to take into account interassay variability (SS = mean number of spots in response to peptide – mean number of spots in absence of peptide).

**Expansion of human proinsulin-specific T cells.** PBMCs (3x10^6 cells/ml) were incubated in presence of 10 µg/ml peptide for 2 h at 37°C, washed, seeded in a 6-well plate, maintained for 14 days and fed on days 4 and 7 by replacing half supernatant with fresh medium containing 10U/ml IL-2, and 5 µg/ml and 2 µg/ml proinsulin peptide, respectively.

**MHC class I tetramers and flow cytometry.** HLA-A2 tetramers were produced as previously described (31). Tetramers were titered individually by staining a relevant peptide-specific CD8 T cell line and used at 5-10 µg/ml. For staining, 10^6 PBMC were incubated at 37°C for 30 min with 5-10 µg/ml PE-labeled tetramer, then with anti-CD8-APC and anti-CD3-FITC labeled antibodies (BD/Pharmingen, San Jose, CA) for 15 minutes at 4°C. Small lymphocytes were gated according to forward/side scatter profiles. CD8+ cells were selected among CD3+ cells. Staining with 7AAD (Pharmingen) was used to exclude dead cells. Data were collected on a FACSCalibur™ flow cytometer, and analyzed using Cell Quest software (Becton-Dickinson).

**Statistics.** Comparison of SS values of patients and controls used non-parametric Mann-Whitney test. Student t test was used for 6-14 peptide/HLA-A2 tetramer assessment.

**RESULTS**

**Immunogenicity of HLA-A2-binding peptides.** Six preproinsulin peptides (peptides 1-8, 2-11, 6-14, 6-16, 14-23 and 15-24) selected for predicted binding to HLA-A2 were tested for immunogenicity in HLA-A2 transgenic mice. As shown in Figure 1, spleen cells from mice immunized against five out of six preproinsulin peptides (2-11, 6-14, 6-16, 14-23, 15-24) showed significant cytotoxic
response to HHD-transfected RMA-S cells pulsed with the immunizing peptide. Immunogenic peptides induced cytotoxic T cells, which displayed increasing lytic activity after each of four in vitro restimulations with 1, 0.33, 0.1 and 0.05 μg/ml peptide, respectively (data not shown).

**Murine preproinsulin leader sequence-specific T cell clones.** As four peptides covered overlapping residues, murine T cell clones were generated against preproinsulin peptides 6-14, 6-16, 14-23 and 15-24. All 6-14 and 6-16-specific clones were cytotoxic against 6-14 and 6-16-pulsed HHD transfected RMA-S cells, indicating that cytotoxic T cells recognize a unique epitope. As a rule, clones generated against peptides 6-14 or 6-16 showed higher cytotoxicity to 6-14-pulsed than 6-16-pulsed HHD transfected RMA-S cells. By contrast, 14-23 and 15-24 peptide-specific T cells showed low cross-reactivity with peptides 15-24 and 14-23, respectively (not shown).

To evaluate processing of peptides 2-11, 6-14, 14-23 and 15-24 from the source protein, we tested whether peptide-specific CD8+ T cell clones were cytotoxic against P815 cells co-transfected with the HHD-encoding and human proinsulin genes (Figure 2). T cell clones specific for all four preproinsulin peptides were cytotoxic, indicating that corresponding epitopes were generated from full length preproinsulin through the endogenous processing pathway.

**Binding of selected peptides to HLA-A1, -A2, -A24 and -B8.** Among the six peptides selected for possible binding to HLA-A2, one peptide (2-11) contained binding motifs for HLA-A24 and -B8 in addition to HLA-A2. Another peptide (15-25) contained binding motifs for HLA-A1 and -A24. These peptides were studied for binding to purified HLA molecules. Peptides that yielded >50%, 20-50% and <20% binding at 10^-6 M were considered high, intermediate and low binders, respectively. Four out of 6 peptides were high binders to HLA-A2 (2-11, 6-14, 6-16, and 15-24) while peptide 14-23 showed intermediate binding and peptide 1-8 no binding at 10^-6 M and low binding at 10^-4 M. Peptide 2-11 was high binder to HLA-A24 and intermediate binder to HLA-B8. Peptide 15-25 was intermediate binder to HLA-A1 and high binder to HLA-A24. Overall, six peptides showed binding to HLA class I alleles. Two peptides (2-11 and 15-25) showed high or intermediate binding to different HLA class I molecules (Table 2). Noticeably, binding of preproinsulin peptides to class I molecules was weaker than that of the control viral peptides with the exception of peptides 2-11 and 15-25, which showed a binding equal to the reference viral peptide (100% binding) to HLA-A24 at 10^-6 M (not shown). Peptide 14-23 that elicited low cytotoxicity against 14-23 pulsed HHD transfected RMA-S cells but significant binding to HLA-A2 was included in further experiments. Peptide 1-8, showing no specific cytotoxicity and no significant binding was excluded.

**Processing of preproinsulin 1-28 by proteasome.** We determined whether C-terminal residues of preselected peptides were generated by proteasome digestion. Database (http://www.paproc2.de.paproc2/cgi-bin) predicts proteasome cleavage in preproinsulin leader sequence in positions 10 to 14, 16, and 22 to 24, pointing to peptides 2-11, 6-14, 6-16, 14-23 and 15-24 as possible candidates. We analyzed peptides resulting from digestion of preproinsulin peptide 1-28 by a proteasome-enriched T1 cell extract (23). Several digestions were performed that yielded identical HPLC profiles. A 20h-incubation was retained for fraction collection. Multiple peaks appeared between 18 and 74 min of HPLC column retention time and a late peak corresponding to the entire preproinsulin 1-28 peptide was evidenced. Mass spectrometry analysis of total proteasome digests defined cleavage...
sites that clustered in positions 17 and 22 to 25. HPLC fractions yielded the same cleavage sites as total digests and additional cleavage sites in 9 to 16, 18 to 21, 26 and 27. While numerous cleavage sites predicted by current databases were confirmed in positions 10 to 24, cleavage sites that were not predicted were characterized (in positions 9, 15, 18 to 21, 26, 27). The C-terminal residues of the six preselected peptides that showed a significant binding to HLA class I alleles (peptides 2-11, 6-14, 6-16, 14-23, 15-24, 15-25) were thus generated by proteasome.

Recognition of leader sequence preproinsulin peptides by human T cells. In our study, the most frequent class I alleles were HLA-A2 (21 patients, 12 controls), HLA-B8 (17 patients, 8 controls), HLA-A24 (14 patients, 1 control) and HLA-A1 (11 patients, 7 controls) (Table 1). Peptide binding studies and, in case of HLA-A2-restricted peptides, immunogenicity in HHD transgenic mice defined six possible HLA-A1, -A2, -A24 or -B8-restricted epitopes that carry C-terminal residues generated by proteasome cleavage in vitro. Preproinsulin and control viral peptides were tested for recognition by T cells from control and type 1 diabetic individuals using an IFNγ ELISpot assay.

Viral peptides. To evaluate IFNγ responses to known CD8+ T cell epitopes, responses of control and patient PBMCs to viral peptides was studied. Eighteen out of 20 HLA-A2 patients and 8 out of 11 HLA-A2 controls showed a positive response to influenzae M.58-66 that ranged between 3 and 20 SD above background. Six out of 9 HLA-A1 individuals (3/6 patients, 3/3 controls) tested against influenzae NP44-52 and 9 out of 20 HLA-B8 individuals (7/13 patients, 2/7 controls) tested against influenzae NP380-388 showed a positive response that ranged within 3 and 6 SD above background. None HLA-A2 and -B8 individuals showed a response to HIV peptides. No difference in the response to viral peptides used as positive and negative controls was observed between diabetic and control individuals (Table 3).

Preproinsulin peptides. All preproinsulin peptides tested were recognized by PBMCs of diabetic patients (Tables 4 and 5). When pooling control and patient responses to preproinsulin peptides, 0% and 65%, respectively, showed numbers of spots in the presence of peptide that was 3 SD above background. Setting the threshold for positive responses at background + 3 SD (23), a positive IFNγ response was observed against at least one preproinsulin leader sequence peptide with the same frequency in recent-onset (17/26) and long-standing (8/12) type 1 diabetic patients. No IFNγ response to preproinsulin peptides was detected in 18 control individuals, including 6 type 2 diabetic patients. Some peptides were recognized with a comparable frequency in recent-onset and long-standing diabetic patients, i.e. peptides 6-14 and 6-16 for HLA-A2. Others peptides were selectively recognized in recent onset patients (2-11, 14-23, 15-24 for HLA-A2 and 2-11, 15-25 for HLA-A24). When considering peptides tested with PBMCs of more than 6 patients carrying a defined class I allele, four peptides (2-11 for HLA-B8, 14-23 and 15-24 for HLA-A2, and 15-25 for HLA-A24) were recognized in more than 50% of recent-onset patients. Two peptides (6-16 for HLA-A2, 2-11 for HLA-A2 and -A24) were recognized in at least 30% of recent-onset patients (Table 5).

Characterization of 6-14-specific T cells as CD8+ T cells. In order to set up the staining of CD8+ T cells with 6-14 peptide/HLA-A2 tetramers, cells from patients L4 and R26 were expanded for 14 days with peptide 6-14 and studied for recognition of preproinsulin 6-14/HLA-A2 and HIV gag/HLA-A2 control tetramers. Staining with 6-14/HLA-A2 tetramer was at 1.2 and 0.8% CD8+ T cells from patients L4 and R26, respectively, while the staining with HIVgag/HLA-A2 tetramer
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was at 0.08 and 0.04%, respectively (Figure 3A). In subsequent experiments, some patients who showed a positive ELISpot response to peptide 6-14 were studied for 6-14 peptide/HLA-A2 tetramer binding. In 4 ELISpot-positive patients, 0.13, 0.14, 0.17 and 0.30% 6-14 peptide/HLA-A2 positive cells were detected in the CD8+ T cell fraction, while in ELISpot-negative patients the average of 6-14 peptide/HLA-A2 tetramer binding was 0.05 ± 0.01% (range 0.04-0.08%, n=7, p<0.02), bringing evidence that 6-14 peptide-reactive T cells were clustered in the CD8+ T cell subset as shown in an illustrative experiment (Figure 3B). For comparison, mean HIV gag/HLA-A2 tetramer detection in the eleven patients was 0.04 ± 0.05.

DISCUSSION

Defining epitopes derived from β cell autoantigens is expected to have wide-range implications in development of T cell assays and peptide-based immunotherapy in type 1 diabetes. While numerous HLA class II-restricted CD4+ T cell have been defined in human type 1 diabetes (14) and transgenic mice expressing human class II alleles (32-34), epitopes presented by class I alleles to CD8+ T cells remain largely unknown. An HLA-A2-restricted GAD epitope (GAD 114-123) has been defined as the target of cytotoxic T cells in two subjects with preclinical and recent-onset type 1 diabetes (35). An HLA-A2 epitope derived from islet amyloid polypeptide (IAPP) has been characterized using an IFNγ ELISpot assay (36). More recently, new β cell HLA class I-restricted epitopes have been reported. The proinsulin A chain, B chain, C-peptide and leader sequence contain epitopes recognized by CD8+ T cells (17,22,23,37-41). Recognition of epitopes encompassing residues excised during insulin processing makes a strong case for proinsulin as an autoantigen in diabetes. However, there has been no systematic study of leader sequence epitopes.

Leader sequence peptides are commonly presented to CD8+, especially HLA-A2-restricted, T cells (42). Using a systematic approach to define class I epitopes derived from the proproinsulin leader sequence, we bring evidence that PBMCs from diabetic patients secrete IFNγ in response to several epitopes. Five proproinsulin leader sequence peptides were recognized in 80% HLA-A2 patients. We did not restrict our study to epitopes showing the highest binding affinity to purified class I molecules since rules governing the spectrum of epitopes presented in autoimmunity remain far from clear. All 5 peptides that showed significant binding to purified HLA-A2 molecules in vitro were highly immunogenic in HHD transgenic mice. One peptide (proinsulin 2-11) was recognized in 57% HLA-B8 and 30% HLA-A24 recent-onset diabetic patients. Two peptides covering the 6-16 proproinsulin region correspond to the same epitope since 6-14 and 6-16-specific T cell clones recognize indistinctly both peptides. By contrast, the 14-25 region contains two epitopes that are distinctly recognized by 14-23 and 15-24-specific peptides. Using 6-14/HLA-A2 tetramers, we bring direct evidence that proproinsulin peptide 6-14 is recognized by human CD8+ T cells. By contrast, the small number of patients studied using tetramers preclude firm conclusions as for the concordance observed between IFNγ production and tetramer staining.

In most patients, the response to proproinsulin leader sequence was multiepitopic. The long preclinical phase characterizing diabetes development does not preclude, however, that a more restricted set of peptides is dominantly recognized at initiation of the autoimmune process, as reported in case of a CD4+ T cell-restricted epitope in the NOD mouse (43). A2-restricted GAD, IAPP and proproinsulin peptide 2-10
were predominantly recognized in recent onset diabetic patients (35,36, 27). This was the case in our study for peptides 2-11 and 14-23 although not for HLA A2-restricted peptide 6-14. It should be acknowledged that our data do not directly correlate recognition of preproinsulin peptides by CD8+ T cells and the absence of residual C-peptide in long-standing diabetic patients studied. However, a comparable observation was reported for other proinsulin epitopes (23) and may reconcile with previous observations in human type 1 diabetes (7). This may indicate that long term memory class I-restricted T cells persist in patients who are likely to have been deprived of remnant \( \beta \) cells for years while on exogenous insulin therapy. Finally, the evidence that murine T cell clones that were specific for leader sequence preproinsulin peptides were cytotoxic to P815 target cells expressing the human preproinsulin gene indicate that corresponding CD8+ T cells may point to useful disease markers.

Using a strategy based on peptide library-mediated \textit{in vitro} assembly of class I molecules, preproinsulin peptides have been defined on the basis of their association with HLA-B8, -A2 and -B15. Several epitopes were shown to harbor anchor residues that were weakly predicted by commonly used algorithms or did not contain canonical allele-specific binding motifs (44). In our study, some of these peptides were recognized in more than 40% of patients, such as preproinsulin 2-11 in HLA-B8, 6-16, 14-23 and 15-24 in HLA-A2, or 15-25 in both HLA-A1 and -A24 patients.

Proteasome-mediated proteolysis is a major, although not exclusive, system generating COOH-termini of class I epitopes. Intracellular mechanisms that define the sequence of peptides presented to CD8+ T cells include, in addition, peptide translocation in the endoplasmic reticulum by transporter-associated with antigen processing (TAP) and binding of peptides into the MHC class I groove. As reported in case of viral epitopes (45), we used 20S proteasome-enriched preparations extracted from the T1 lymphoblastoid cell, which contains IFN\( \gamma \)-inducible LMP-2 and LMP-7 proteasome subunits. Among candidate peptides, six carried C-terminal flanking residues that were identified following proteasome processing \textit{in vitro}. One peptide (1-8) that carried a leucine in position 8 was not identified as a proteasome cleavage site. This is not unexpected since many peptides from signal sequences have been characterized as presented by class I alleles in a proteasome-independent and TAP-independent manner (46,47). However, this peptide showed no detectable binding to HLA-A2 nor immunogenicity in HHD transgenic mice. By contrast, peptide 2-11 showed significant binding to HLA-A2, significant immunogenicity in HHD transgenic mice and recognition by PBMCs from 29% HLA-A2 and 57% HLA-B8 diabetic patients.

Considering previous reports (23,26) and the present data, at least nine HLA-A2-restricted epitopes have been identified as recognized by CD8+ T cells from type 1 diabetes patients within preproinsulin. Similar evidence that point to the high diversity of epitopes recognized by CD8+ T cells within restricted regions of myelin autoantigens has been obtained in multiple sclerosis, in contrast with the limited number of immunodominant epitopes usually reported on viral antigens (48). Furthermore, epitope diversity was not restricted to HLA-A2 in multiple sclerosis (49). In the same line, there was no clear correlation between the prevalence of positive responses to peptides within the restricted preproinsulin region studied and affinity levels of peptide binding to HLA class I molecules. \textit{In vivo} priming in HHD mice and study of peptide binding \textit{in vitro} possibly biased our peptide selection procedure toward peptides with significant affinity, possibly
underestimating the number of peptides recognized along diabetes development. However, frequent responses to intermediate affinity peptides were observed, as proinsulin 2-11 in HLA-B8 and 14-23 in HLA-A2 patients. Since autoimmune reactions develop against self proteins, the autoantigen-specific T cell repertoire is expected to be purged from high-avidity T cells specific for dominant T cell epitopes, opening the likelihood of autoreactive T cells that are specific for subdominant or cryptic epitopes.

Our data bring evidence that type 1 diabetic patients shows class I-restricted responses to 8- to 11-mer peptides within the preproinsulin leader sequence. The absence of recognition of preproinsulin peptides by PBMCs from control individuals may point of leader sequence preproinsulin-specific CD8+ T cells as useful disease markers.

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REFERENCES

1. Martin S, Wolf-Eichbaum D, Duinkerken G, Scherbaum WA, Kolb H, Noordzij JG, Roep BO: Development of type 1 diabetes despite severe hereditary B-lymphocyte deficiency. *N Engl J Med* 345:1036-1040, 2001
2. Verdaguer J, Schmidt D, Amrani A, Anderson B, Averill N, Santamaria P: Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. *J Exp Med* 186:1663-1676, 1997
3. Itoh N, Hanafusa T, Miyazaki A, Miyagawa J, Yamagata K, Yamamoto K, Waguri M, Imagawa A, Tamura S, Inada M, Kawata S, Tarui S, Kono N, Matsuzawa Y: Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. *J Clin Invest* 92:2313-2322, 1993
4. Somoza N, Vargas F, Roura-Mir C, Vives-Pi M, Fernandez-Figuera MT, Ariza A, Gomis R, Bragado R, Marti M, Jaraquemada D: Pancreas in recent onset insulin-dependent diabetes mellitus. Changes in HLA, adhesion molecules and autoantigens, restricted T cell receptor V. *J Immunol* 153:1360-1377, 1994
5. Conrad B, Weidmann E, Trucco G, Rudert WA, Behboo R, Ricordi C, Rodriquez-Rilo H, Finegold D, Trucco M: Evidence for superantigen involvement in insulin-dependent diabetes mellitus aetiology. *Nature* 371:351-355, 1994
6. Foulis AK, McGill M, Farquharson MA: Insulitis in type 1 (insulin-dependent) diabetes mellitus in man--macrophages, lymphocytes, and interferon-gamma containing cells. *J Pathol* 165:97-103, 1991
7. Sibley RK, Sutherland DE, Goetz F, Michael AF: Recurrent diabetes mellitus in the pancreas iso- and allograft. A light and electron microscopic and immunohistochemical analysis of four cases. *Lab Invest* 53:132-144, 1985
8. Yu L, Robles DT, Abiru N, Kaur P, Rewers M, Kelemen K, Eisenbarth GS: Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc Natl Acad Sci U S A* 97:1701-1706, 2000
9. Ziegler AG, Hummel M, Schenker M, Bonifacio E: Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study. *Diabetes* 48:460-468, 1999.
10. Keller RJ: Cellular immunity to human insulin in individuals at high risk for the development of type I diabetes mellitus. *J Autoimmun* 3:321-327, 1990
11. Schloot NC, Roep BO, Wegmann D, Yu L, Chase HP, Wang T, Eisenbarth GS: Altered immune response to insulin in newly diagnosed compared to insulin-treated diabetic patients and healthy control subjects. *Diabetologia* 40:564-572, 1997
12. Rudy G, Stone N, Harrison LC, Colman PG, McNair P, Brusic V, French MB, Honeyman MC, Tait B, Lew AM: Similar peptides from two beta cell autoantigens, proinsulin and glutamic acid decarboxylase, stimulate T cells of individuals at risk for insulin-dependent diabetes. *Mol Med* 1:625-633, 1995
13. Dubois-LaForgue D, Carel JC, Bougneres PF, Guillet JG, Boitard C: T-cell response to proinsulin and insulin in type 1 and pretype 1 diabetes. *J Clin Immunol* 19:127-134, 1999
14. Alleva DG, Crowe PD, Jin L, Kwok WW, Ling N, Gottschalk M, Conlon PJ, Gottlieb PA, Putnam AL, Gaur A: A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin. *J Clin Invest* 107:173-180, 2001
15. Petersen LD, Duinkerken G, Bruining GJ, van Lier RA, de Vries RR, Roep BO: Increased numbers of in vivo activated T cells in patients with recent onset insulin-dependent diabetes mellitus. *J Autoimmun* 9:731-737, 1996

16. Durinovic-Bello I, Schlosser M, Riedl M, Maisel N, Rosinger S, Kalbacher H, Deeg M, Ziegler M, Elliott J, Roep BO, Karges W, Boehm BO: Pro- and anti-inflammatory cytokine production by autoimmune T cells against preproinsulin in HLA-DRB1*04, DQ8 Type 1 diabetes. *Diabetologia* 47:439-450, 2004

17. Naquet P, Ellis J, Tibensky D, Keshole A, Singh B, Hodges R, Delovitch TL: T cell autoreactivity to insulin in diabetic and related non-diabetic individuals. *J Immunol* 140:2569-2578, 1988

18. Daniel D, Gill RG, Schloot N, Wegmann D: Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. *Eur J Immunol* 25:1056-1062, 1995

19. Daniel D, Wegmann DR: Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23). *Proc Natl Acad Sci U S A* 93:956-960, 1996

20. Thebault-Baumont K, Dubois-Laforgue D, Krief P, Briand JP, Halbout P, Vallon-Geoffroy K, Morin J, Laloux V, Lehuen A, Carel JC, Jami J, Muller S, Boitard C: Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice. *J Clin Invest* 111:851-857, 2003

21. Moriyama H, Abiru N, Paronen J, Sikora K, Liu E, Miao D, Devendra D, Beilke J, Gianani R, Gill RG, Eisenbarth GS: Evidence for a primary islet autoantigen (preproinsulin 1) for insulitis and diabetes in the nonobese diabetic mouse. *Proc Natl Acad Sci U S A* 100:10376-10381, 2003

22. Hassainya Y, Garcia-Pons F, Kratzer R, Lindo V, Greer F, Lemonnier FA, Niedermann G, van Endert PM: Identification of naturally processed HLA-A2-restricted proinsulin epitopes by reverse immunology. *Diabetes* 54:2053-2059, 2005

23. Toma A, Haddouk S, Briand JP, Camoin L, Gahery H, Connnan F, Dubois-Laforgue D, Caillat-Zucman S, Guillet JG, Carel JC, Muller S, Choppin J, Boitard C: Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients. *Proc Natl Acad Sci U S A* 102:10581-10586, 2005

24. Jarchum I, Nichol L, Trucco M, Santamaria P, DiLorenzo TP: Identification of novel IGRP epitopes targeted in type 1 diabetes patients. *Clin Immunol* 127(3):359-365, 2008

25. Baker C, Petrich de Marquesini LG, Bishop AJ, Hedges AJ, Dayan CM, Wong FS: Human CD8 responses to a complete epitope set from preproinsulin: implications for approaches to epitope discovery. *J Clin Immunol* 28(4):350-360, 2008

26. Mallone R, Martinuzzi E, Blancou P, Novelli G, Afonso G, Dolz M, Bruno G, Chaillous L, Chatenoud L, Bach JM, van Endert P: CD8+ T-cell responses identify beta-cell autoimmunity in human type 1 diabetes. *Diabetes* 56(3):613-621, 2007

27. Martinuzzi E, Novelli G, Scotto M, Blancou P, Bach JM, Chaillous L, Bruno G, Chatenoud L, van Endert P, Mallone R: The frequency and immunodominance of islet-specific CD8+ T-cell responses change after type 1 diabetes diagnosis and treatment. *Diabetes* 57(5):1312-1320, 2008

28. Henderson RA, Michel H, Sakaguchi K, Shabanowitz J, Appella E, Hunt DF, Engelhard VH: HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science* 255:1264-1266, 1992

29. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA, Perarnau B: HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m)
Class I-Restricted Insulin-Specific T Cells

- HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. *J Exp Med* 185:2043-2051, 1997

- Choppin J, Cohen W, Bianco A, Briand JP, Connan F, Dalod M, Guillet JG: Characteristics of HIV-1 Nef regions containing multiple CD8+ T cell epitopes: wealth of HLA-binding motifs and sensitivity to proteasome degradation. *J Immunol* 166:6164-6169, 2001

- Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM: Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94-96, 1996

- Raju R, Munn SR, David CS: T cell recognition of human pre-proinsulin peptides depends on the polymorphism at HLA DQ locus: a study using HLA DQ8 and DQ6 transgenic mice. *Hum Immunol* 58:21-29, 1997

- Endl J, Otto H, Jung G, Dreisbusch B, Donie F, Stahl P, Elbracht R, Schmitz G, Meinl E, Hummel M, Ziegler AG, Wank R, Schendel DJ: Identification of naturally processed T cell epitopes from glutamic acid decarboxylase presented in the context of HLA-DR alleles by T lymphocytes of recent onset IDDM patients. *J Clin Invest* 99:2405-2415, 1997

- Herman AE, Tisch RM, Patel SD, Parry SL, Olson J, Noble JA, Cope AP, Cox B, Congia M, McDevitt HO: Determination of glutamic acid decarboxylase 65 peptides presented by the type 1 diabetes-associated HLA-DQ8 class II molecule identifies an immunogenic peptide motif. *J Immunol* 163:6275-6282, 1999

- Panina-Bordignon P, Lang R, van Endert PM, Benazzi E, Felix AM, Pastore RM, Spinas GA, Sinigaglia F: Cytotoxic T cells specific for glutamic acid decarboxylase in autoimmune diabetes. *J Exp Med* 181:1923-1927, 1995

- Panagiotopoulos C, Qin H, Tan R, Verchere CB: Identification of a beta-cell-specific HLA class I restricted epitope in type 1 diabetes. *Diabetes* 52:2647-2651, 2003

- Ouyang Q, Standifer NE, Qin H, Gottlieb P, Verchere CB, Nepom GT, Tan R, Panagiotopoulos C: Recognition of HLA class I-restricted beta-cell epitopes in type 1 diabetes. *Diabetes* 55(11):3068-3074, 2006

- Standifer NE, Ouyang Q, Panagiotopoulos C, Verchere CB, Tan R, Greenbaum CJ, Pihoker C, Nepom GT: Identification of novel HLA-A*0201-restricted epitopes in recent-onset type 1 diabetic subjects and antibody-positive relatives. *Diabetes* 55(11):3061-3067, 2006

- Pinkse GG, Boitard C, Tree TI, Peakman M, Roep BO: HLA class I epitope discovery in type 1 diabetes: independent and reproducible identification of proinsulin epitopes of CD8 T cells—report of the IDS T Cell Workshop Committee. *Ann N Y Acad Sci* 1079:19-23, 2006

- van Endert P, Hassainya Y, Lindo V, Bach JM, Blancou P, Lemonnier F, Mallone R: HLA class I epitope discovery in type 1 diabetes. *Ann N Y Acad Sci* 1079:190-197, 2006

- Pinkse GG, Tysma OH, Bergen CA, Kester MG, Ossendorp F, van Veelen PA, Keymeulen B, Pipeleers D, Drijfhout JW, Roep BO: Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. *Proc Natl Acad Sci U S A* 102:18425-18430, 2005

- Gueguen M, Biddison WE, Long EO: T cell recognition of an HLA-A2-restricted epitope derived from a cleaved signal sequence. *J Exp Med* 180:1989-1994, 1994
45. Kessler JH, Beekman NJ, Bres-Vloemans SA, Verdijk P, van Veelen PA, Kloosterman-Joosten AM, Vissers DC, ten Bosch GJ, Kester MG, Sijts A, Drijfhout JW, Ossendorp F, Offringa R, Melief CJ: Efficient identification of novel HLA-A(*0201)-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis. *J Exp Med* 193:73-88, 2001

46. Kloetzel PM: Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII. *Nat Immunol* 5:661-669, 2004

47. Engelhard VH, Brickner AG, Zarling AL: Insights into antigen processing gained by direct analysis of the naturally processed class I MHC associated peptide repertoire. *Mol Immunol* 39:127-137, 2002

48. Yewdell JW: Confronting complexity: real-world immunodominance in antiviral CD8+ T cell responses. *Immunity* 25(4):533-543, 2006

49. Berthelot L, Laplaud DA, Petrê S, Ballet C, Michel L, Hillian S, Braudeau C, Connan F, Lefrère F, Wiertlewski S, Guillet JG, Brouard S, Choppin J, Soulillou JP: Blood CD8(+) T cell responses against myelin determinants in multiple sclerosis and healthy individuals. *Eur J Immunol* 38(7):1889-1899, 2008
### Table 1: Patients and control individuals.

|   | P       | -DRB1* | -A, -B* |   | P       | -DRB1* | -A, -B* | C       | -DRB1* | -A, -B* |
|---|---------|---------|---------|---|---------|---------|---------|---------|---------|---------|
| R1| 03/04   | A2-3, B18-38 |         | R20| 03/04   | A1-24, B8- | A1   | nd      | A2-29, B7-44 |
| R2| 04/13   | A2-24, B27- |         | R21| 03/04   | A24-25, B18-56 | A2   | nd      | A2-3, B38-51 |
| R3| 03/11   | A2-30, B16-18 |       | R22| 03/03   | A1-24, B16-39 | C2   |         | A24-28, B7-8 |
| R4| 03/16   | A2-, B45-18 |         | R23| 04/08   | A3-24, B39-49 | C4   |         | A1-30, B8-13 |
| R5| 01/03   | A2-, B8-   |         | R24| 04/08   | A24-80, B44-51 | C5   |         | A1-32, B8-13 |
| R6| 03/16   | A2-11, B7-55 |       | R25| 03/03   | A9-10, B8-14 | C6   |         | A2-31, B35-60 |
| R7| 03/04   | A2-, B62-52 |         | R26| 03/04   | A1-2, B8-15  | C7   |         | A2-, B8- |
| R8| 03/04   | A2-, B8-44 |         | L1 | 03/04   | A2-, B35-62 | C8   |         | A2-, B7-12 |
| R9| 03/11   | A2-, B8-51 |         | L2 | 03/04   | A2-29, B7-35 | C9   |         | A2-, B27-44 |
| R10| 04/04  | A2-3, B14-44 |       | L3 | 01/01   | A2-24, B7-35 | C10  |         | A1-2, B8-50 |
| R11| 04/13  | A2-3, B15-50 |       | L4 | 04/04   | A2-, B39-62 | C11  |         | A2-, B7-50 |
| R12| 01/04  | A2-3, B15-35 |       | L5 | 03/03   | A1-24, B8-45 | C12  |         | A1-3, B8-27 |
| R13| 03/04  | A2-24, B49- |         | L6 | 03/04   | A1-24, B8-49 | D1   |         | A2-10, B12-44 |
| R14| 07/13  | A1-2, B12-44 |       | L7 | 04/13   | A11-24, B50-62 | D2   |         | A2-, B35-44 |
| R15| 03/13  | A2-, B8-   |         | L8 | 03/04   | A1-3, B8-49  | D3   |         | A1-11, B8-55 |
| R16| 03/04  | A2-3, B8-18 |         | L9 | 03/03   | A3-31, B8-51 | D4†  |         | A2-, B38-45 |
| R17| 04/16  | A1-24, B8-44 |       | L10| 03/04   | A1-9, B8-21  | D5†  |         | A1-2, B7- |

*Note: C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12 are control individuals.*

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**Class I-Restricted Insulin-Specific T Cells**
|   |   |   |   |   |
|---|---|---|---|---|
| R18 | 03/08 | A3-24, B8-58 | L11 | 03/04 | A1-, B8-38 | D6† | 03/07 | A1-19, B8-44 |
| R19 | 01/04 | A3-24, B35-47 | L12 | 03/04 | A1-, B8-44 |

P, patients; R, recent-onset diabetes; L, long-standing diabetes; C, healthy control subjects; D, type 2 diabetic control subjects; *, HLA alleles; †, insulin-treated; nd, not done.
Table 2: Binding of preproinsulin peptides to selected HLA class I alleles.

| Peptide*  | Sequence   | HLA I | HLA motif† | Anchoring | % of binding§ | Type of binder¶ |
|-----------|------------|-------|------------|-----------|---------------|-----------------|
|           |            |       |            |           | 10-4  | 10-6 |        |
| 1-8       | MALWMRLL   | A2    | MALWMRLL   | *         | 46%   | 0%   | L |
| 2-11      | ALWMRLLPLL | A2    | ALWMRLLPLL | **        | 55%   | 53%  | H |
|           |            |       |            |           | 55%   | 53%  | H |
|           |            |       |            |           | 53%   | 53%  | H |
| 6-14      | RLLPLLALL  | A2    | RLLPLLALL  | **        | 36%   | 65%  | H |
| 6-16      | RLLPLLALLAL| A2    | RLLPLLALLAL| **        | 96%   | 71%  | H |
| 14-23     | LALWGPDPAA | A2    | LALWGPDPAA | *         | 44%   | 36%  | I |
| 15-24     | ALWGPDPAAA | A2    | ALWGPDPAAA | *         | 96%   | 94%  | H |
| 15-25     | ALWGPDPAAAF| A1    | ALWGPDPAAAF| *         | 18%   | 41%  | I |
|           | ALWGPDPAAAF| A24   | ALWGPDPAAAF| **        | 82%   | 100% | H |

* N- and C- terminal positions in preproinsulin;
† Anchoring positions are in bold characters;
‡ HLA anchoring motifs: ** = strict, * = tolerated;
§ % of binding = binding of tested peptide/binding of viral reference peptide;
¶ L, low binder (binding <20% at 10⁻⁶ M and binding >20% at 10⁻⁴ M); I, intermediate binder (binding between 20% and 50% at 10⁻⁶ M); H, high binder (binding >50% at 10⁻⁶ M).
Table 3: Recognition of viral peptides by PBMCs of patients and of controls individuals.

| V* | Epitope | Sequence | HLA† | Frequencies of recognition ‡ | Median (mean) responses§ |
|-----|---------|----------|------|-------------------------------|-------------------------|
|     |         |          |      | Patients | Controls | Patients | Controls | Comparision (p) |
| IV  | M.58-66 | GILGFVFTL | A2   | 18/20 | 8/11 | 89 (0-676) | 49 (0-246) | 0.36 |
| IV  | NP 44-52| CTEKLSDY | A1   | 3/6   | 3/3 | 39 (7-40) | 14 (10-20) | 0.43 |
| IV  | NP 380-388| ELRSRYWAI | B8   | 7/13 | 2/7 | 8 (0-119) | 5 (2-131) | 0.30 |
| HIV | Nef 83-91| AAVDLSHFL | A2   | 0/12 | 0/4 | 3 (0-20) | 0 (0-5) | 0.06 |
| HIV | Nef 182-189| EWRFDRLA | B8   | 0/8 | 0/3 | 1 (0-35) | 0 (0-0) | 0.07 |

* V, virus; IV, influenzae virus;
† HLA, presenting HLA molecule;
‡ ELISpot responses, number of responders/number of individuals tested;
§ median (range) of stimulation score (SS); SS = mean number of spots in response to peptide – mean number of spots in absence of peptide; p, p values comparing the median responses in patients versus controls; non parametric Mann-Whitney test.

P, patients; C, controls.
Table 4: Relative recognition frequencies of preproinsulin peptides in diabetic versus control subjects.

| P* | HLA | Frequencies of recognition† | Comparison |
|-----|-----|-----------------------------|------------|
|     |     | L | R | C | D | Patients | Controls | p‡ |
| 2-11 | A2 | 0/4 | 5/17 | 0/7 | 0/4 | 5/21 | 0/11 | 0.01 |
|      | A24 | 0/4 | 3/10 | 0/1 | - | 3/14 | 0/1 | - |
|      | B8  | 4/10 | 4/7 | 0/6 | 0/2 | 8/17 | 0/8 | 0.0008 |
| 6-16 | A2  | 2/4 | 7/16 | 0/8 | 0/4 | 9/20 | 0/12 | 0.0004 |
| 6-14 | A2  | 1/4 | 4/14 | 0/4 | 0/2 | 5/18 | 0/6 | 0.01 |
| 14-23 | A2 | 0/4 | 9/14 | 0/4 | 0/2 | 9/18 | 0/6 | 0.0003 |
| 15-24 | A2 | 1/4 | 7/13 | 0/4 | 0/2 | 8/17 | 0/6 | 0.0008 |
| 15-25 | A1 | 2/3 | 1/3 | 0/1 | - | 3/6 | 0/1 | - |
|      | A24 | 0/3 | 10/10 | 0/1 | - | 10/13 | 0/1 | - |

P, peptide; R, recent-onset diabetes; L, long-standing diabetes; C, healthy control subjects; D, type 2 diabetic control subjects;

* N- and C- terminal amino acids in preproinsulin.

† ELISpot responses, number of responders / number of individuals tested;

‡ p values comparing the relative epitope recognition frequencies in patients (L+R) versus controls (C+D); non parametric Mann-Whitney test.
Table 5: Recognition of preproinsulin peptides by PBMCs of diabetic patients: median values of stimulation score.

| P   | HLA | Median (range) responses† | Comparison‡ |
|-----|-----|---------------------------|-------------|
|     | L   | R  | C  | D  | Patients | Controls | p      |
| 2-11| A2  | 3.3 (0-12.2) | 10.0 (0-41.8) | 0.56 (0-8.3) | 0 (0-0) | 7.8 (0-41.8) | 0 (0-8.3) | 0.01 |
|     | A24 | 5.2 (2.2-12.2) | 0 (0-41.8) | - | - | 2.8 (0-41.8) | - | - |
|     | B8  | 25 (0-105) | 8.2 (0-37.2) | 0 (0-1.1) | 0 (0-5.6) | 15.6 (0-105) | 0 (0-5.6) | 0.01 |
| 6-16| A2  | 12.2 (10-54.5) | 5 (0-111.7) | 0 (0-9.5) | 0 (0-0) | 7.3 (0-111.7) | 0 (0-9.5) | 0.004 |
|     | A2  | 28.9 (21.1-144) | 30.6 (7.8-144) | 4.7 (3.9-7.2) | - | 30.6 (7.8-144) | 4.7 (3.9-7.2) | 0.01 |
|     | A2  | 11.1 (7.8-12.2) | 7.7 (54-0) | 1.4 (0-5.6) | - | 8.9 (54-0) | 1.4 (0-5.6) | 0.04 |
| 15-24| A2  | 12.8 (2.4-32.2) | 8.7 (0-52.8) | 0 (0-0) | - | 12.2 (0-52.8) | 0 (0-0) | 0.002 |
| 15-25| A1  | 13.6 (6.1-25.6) | 1.7 (0-14.4) | - | - | 9.2 (0-25.6) | - | - |
|     | A24 | 11.1 (5.8-25.6) | 0 (0-14.4) | - | - | 0 (0-25.6) | - | - |

P, peptide; R, recent-onset diabetes; L, long-standing diabetes; C, healthy control subjects; D, type 2 diabetic control subjects;

* N- and C-terminal amino acids in preproinsulin.

† ELISpot responses; median (range) of stimulation score (SS); SS = mean number of spots in response to peptide – mean number of spots in absence of peptide

‡ p values comparing the median responses in patients versus controls; non parametric Mann-Whitney test.
Figure 1: Cytotoxic responses of peptide-specific HLA-A2.1-restricted murine T cells against preproinsulin peptides.

Five to six HHD mice were immunized in vivo for each preproinsulin peptide (peptides 6-14, 6-16, 2-11, 14-23, 15-24, 1-8). Spleen cells were re-stimulated in vitro eleven days later using irradiated peptide-pulsed HHD lymphoblasts. Spleen cells were then tested for cytolytic activity against HHD-transfected RMA-S targets loaded with relevant preproinsulin peptide (white circles) or irrelevant negative control peptide (dark circles), using chromium release assay. The figure represents the % of specific lysis (vertical axis) obtained for 1/1, 2/1, 3/1 and 4/1 effector/target (E/T) ratio (horizontal axis).
Figure 2: Cytotoxicity of anti-2-11, anti-6-14, anti-14-23 and anti-15-24 CD8$^+$ T cell clones against P815 cells transfected with the HHD-encoding and the human preproinsulin genes. Individual CD8$^+$ T cell clones were tested for cytolytic activity against HHD- and preproinsulin-transfected P815 targets loaded with relevant preproinsulin peptide (circles for 2-11, rectangles for 6-14, triangles for 14-23, squares for 15-24; open symbols) or without peptide (dark symbols, respectively), using chromium release assay. The figure represent the % of specific lysis (vertical axis) obtained for 1/1, 2/1 and 3/1 effector/target (E/T) ratio (horizontal axis).
Figure 3: Peptide/HLA-A2 tetramers staining of CD8⁺ T cells from diabetic patients.

3A: Staining of expanded CD8⁺ T cells from two diabetic patients.
PBMC from one long standing (L4) and one recent onset (R26) diabetic patients were cultured in presence of peptides (peptide 6-14 or HIV gag peptide), then stained by 6-14 peptide/HLA-A2 and HIV gag peptide/HLA-A2 tetramers. Cultures were harvested for tetramer staining on day 14. HIV gag peptide/HLA-A2 tetramer was used as a negative control. Detection of tetramer-stained cells was performed by gating out 7 AAD⁺ dead cells and selecting CD8⁺ T cells. The figure shows the staining with 6-14 peptide/HLA-A2 tetramer (left panels) and with HIV gag peptide/HLA-A2 tetramer (right panels) at day 14 of cell expansion. Dot plots show tetramer (vertical axis) versus CD8⁺ cells (horizontal axis) staining. The numbers displayed in each dot plot indicate the percentages of cells stained by tetramers.

3B: Ex vivo tetramer staining of PBMCs from 6-14 peptide ELISpot-positive and ELISpot-negative diabetic patients.
PBMC from 6-14 peptide ELISpot-positive diabetic patients were stained ex vivo with 6-14 peptide/HLA-A2 and HIV gag peptide/HLA-A2 tetramers. The figure shows the ex vivo staining with 6-14 peptide/HLA-A2 tetramer (left panels) and with HIV gag peptide/HLA-A2 tetramer (right panels) for one 6-14 peptide ELISpot-positive patient (R9). As a control, a 6-14 peptide ELISpot-negative patient (R10).