Prediction of HLA class I-restricted T-cell epitopes of islet autoantigen combined with binding and dissociation assays

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Abstract
Identification of cognate peptides recognized by human leucocyte antigen (HLA)/T cell receptor (TCR) complex provides insight into the pathogenic process of type 1 diabetes (T1D). We hypothesize that HLA-binding assays alone are inadequate metrics for the affinity of peptides. Zinc transporter-8 (ZnT8) has emerged in recent years as a novel, major, human autoantigen. Therefore, we aim to identify the HLA-A2-restricted ZnT8 epitopes using both binding and dissociation assays. HLA class I peptide affinity algorithms were used to predict candidate ZnT8 peptides that bind to HLA-A2. We analyzed 15 reported epitopes of seven β-cell candidate autoantigens and eight predicted candidate ZnT8 peptides using binding and dissociation assays. Using IFN-γ ELISpot assay, we tested peripheral blood mononuclear cells (PBMCs) from recent-onset T1D patients and healthy controls for reactivity to seven reported epitopes and eight candidate ZnT8 peptides directly ex vivo. We found five of seven recently reported epitopes in Chinese T1D patients. Of the eight predicted ZnT8 peptides, ZnT8153–161 had a strong binding affinity and the lowest dissociation rate to HLA-A*0201. We identified it as a novel HLA-A*0201-restricted T-cell epitope in three of eight T1D patients. We conclude that ZnT8153–161 is a novel HLA-A*0201-restricted T-cell epitope. We did not observe a significant correlation (P = 0.3, R = –0.5) between cytotoxic T cell (CTL) response and peptide/HLA*0201 complex stability. However, selection of peptides based on affinity and their dissociation rate may be helpful for the identification of candidate CTL epitopes. Thus, we can minimize the number of experiments for the identification of T-cell epitopes from interesting antigens.

Keywords: T-cell epitope, type 1 diabetes, ZnT8, autoantigen, autoimmunity

Introduction
Type 1 diabetes (T1D) in both humans and nonobese diabetic mice is a chronic autoimmune disease that results from inflammation of pancreatic islets and destruction of pancreatic β cells by T cells targeting numerous cell autoantigens [1,2]. CD8+ CTLs, recognizing β-cell-specific peptides presented by HLA class I molecules, have the pivotal role in selective cell death [3]. Self-reactive CTLs recognize short peptides displayed on the β-cell surface in the form of HLA class I molecules, and upon T-cell receptor activation they induce β-cell apoptosis.

Early investigation of T-cell responses to islet autoantigens in T1D primarily focused on CD4+ T cells [1]. However, recently, much attention has been directed toward the CD8+ T-cell subset [4–6]. Epitopes have now been identified from multiple islet antigens including proinsulin [6–9], glutamic acid decarboxylase-65 (GAD65) [6,10,11], proproislet amyloid polypeptide protein [12–14], glial fibrillary acidic protein [13], insulinoma-associated antigen-2 (IA-2) [14], and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) [6,13,14]. It is clear that T cells from both patients newly diagnosed with T1D and from patients with
long-standing disease recognize a variety of epitopes from different autoantigens. The identification of target autoantigens for CD8^+ T cells is particularly important because manipulation of immune responses to these antigens provides hope for removing the specific cells that damage β cells.

Zinc transporter-8 (ZnT8), belonging to the ZnT protein family, localizes in insulin secretory granules. It plays an important role in replenishing zinc following exocytosis [15]. Recently, ZnT8 has been identified as a novel autoantigen from microarray expression profiling of human and rodent pancreas and islet cells [16,17]. Anti-ZnT8 autoantibodies are detected in 60–80% of new-onset T1D patients [16]. Some studies suggested that ZnT8 expression could affect β-cell physiology [18,19]. Moreover, a single nucleotide polymorphism (SNP R325W) in the ZnT8 gene (SLC30A8) was shown to increase the risk of developing type 2 diabetes [20]. These findings confirm association either between a ZnT8 gene and diabetes or between its potential biomarker of islet cell mass and function. However, no report shows its potential epitopes for evaluating T-cell-based autoimmunity.

Investigations have selected CD8^+ T-cell epitopes using a number of different methods. In the current study, different from previously published work, we screened HLA class I-restricted T-cell epitopes of ZnT8 combined with binding and dissociation assays. In addition, we also tested 15 candidate peptides of the β-cell proteins and candidate autoantigens in T1D. We investigated CD8^+ T-cell responses to these peptides, using the sensitive CD8^+ -specific IFN-γ ELISpot assay in patients newly diagnosed with T1D. Here, we found that ZnT8 shows such epitope could be a novel human CD8^+ T-cell epitope, and we showed that these peptides when combined with binding and dissociation methods may help or provide more information in screening T-cell epitopes.

Methods

Peripheral blood samples were collected from patients (n = 8, mean age 29.8 ± 8.1, range 20–41 years) with recent-onset T1D (within 1 year of diagnosis) as well as from healthy HLA-A*0201 control subjects (n = 5, mean age 24.8 ± 1.0, range 24–26 years) with no family history of T1D. PBMCs were isolated from fresh peripheral blood (within 4 h of taking the sample) over LymphoprepTM (Nycomed, Pharma AS, Oslo, Norway) gradients according to the manufacturer’s protocol. PBMCs were resuspended in complete Roswell Park Memorial Institute (RPMI) medium (RPMI 1640 plus Glutamax plus 25 mM HEPES with 1% antibiotic/antimycotic, supplemented with 10% FCS) at a concentration of 2–4 × 10^6/ml. All subjects gave informed consent, and the study was approved by the relevant ethics committees.

HLA analysis and measurement of autoantibodies

HLA analysis was performed on deoxyribonucleic acid extracted from peripheral blood by polymerase chain reaction—Sequence-Specific Oligonucleotide Probe in the HLA Laboratory (The First Affiliated Hospital of Nanjing Medical University, Nanjing, China). Autoantibodies to GAD, IA-2, and ICA (gift from Prof. John C. Hutton, Barbara Davis Center for Childhood Diabetes, University of Colorado at Denver and Health Sciences Center, CO, USA) were measured in serum by radioimmunoassay by Prof. George S. Eisenbarth and Liping Yu as previously described [21].

Peptides

Putative HLA-A*0201 CD8^+ T-cell epitopes were predicted using the BIMAS (Section of Bio-Informatics and Molecular Analysis, National Institutes of Health, Bethesda, MD, USA) [22] and SYFPEITHI (Institute for Immunology, University of Tübingen, Tübingen, Germany) [23]. A viral peptide mix of Flu MP 48–66; Epstein-Barr virus (EBV) BMLF 280–288; and cytomegalovirus (CMV) pp65 495–503 was used as positive controls. HIV gag 77–85 was used as a negative control. Predicted β-cell peptides and control peptides were synthesized by Nanjing Jinsite Biology and Technology Company (Nanjing, China) and purified by reverse-phase high-performance liquid chromatography. Mass spectrometry was performed for quality control purpose.

Peptide-binding assay

The ability of peptides to bind HLA-A*0201 was confirmed by cell membrane stabilization of the HLA-A2 molecule in TAP (transporter associated with antigen processing)-deficient 174 × CEM.T2 cells [24]. Briefly, T2 cells were loaded with 100 µg/ml peptide during an overnight incubation at room temperature in the presence of 3 µg/ml β2m (Sigma-Aldrich, Oakville, ON, Canada) in serum-free medium (12440, Invitrogen) and then washed and stained with FITC-conjugated anti-HLA-A2 monoclonal antibody (BB7.2; BD, Pharmingen, San diego, CA). The HLA-A2 surface expression was measured by flow cytometry (FACSCalibur), and the mean fluorescence intensity was recorded. The high-affinity immunodominant HLA-A2 CMV peptide [25] was used as a positive control. Results of β-cell peptide binding to HLA-A*0201 are expressed as the percentage relative binding of the CMV peptide to HLA-A*0201 = 100 × ([MFI with given peptide–MFI without peptide] / [MFI with control peptide–MFI without control peptide]) × 100%.
without peptide)/(MFI with CMV/A2 peptide–MFI without peptide)], where MFI is the mean fluorescence intensity.

**HLA–peptide complex stability**

The temporal stability of peptide/HLA-A*0201 complexes was assessed as previously described [14]. Briefly, T2 cells were cultured with synthetic peptides overnight at room temperature as performed for peptide-binding assays. The following day, after removing peptide and adding emetine (10^{-4}\text{ mol/l}; Sigma-Aldrich) to block protein synthesis, cells were incubated at 37°C for the indicated time periods. At each time point, an aliquot of cells was washed and stained with FITC-conjugated anti-HLA-A2 monoclonal antibody (BB7.2). The HLA-A2 surface expression was assessed by flow cytometry (FACSCalibur), and the mean fluorescence intensity was recorded. The CMV peptide forms highly stable complexes with HLA-A*0201 and was used as a positive control. Results are expressed as the relative complex stability \( = 100 \times \frac{(\text{MFI with given peptide–MFI without peptide})}{(\text{MFI with CMV peptide–MFI without peptide})} \), where MFI is the mean fluorescence intensity.

**ELISpot assay**

Ninety-six-well PVDF plates (Millipore, Saint-Quentin-en-Yvelines, France) were coated overnight with an anti-IFN-\( \gamma \) antibody (U-CyTech, Utrecht, The Netherlands). Plates were subsequently blocked with RPMI plus 10\% human serum (PAA Laboratories GmbH, Pasching, Austria) and peptides were added (20\( \mu \text{M} \) final concentration) in triplicate wells as previously described [6]. PBMCs were seeded at 2–4\times10^5\text{ cells/well} and cultured for 20–24h. Following PBMC removal, IFN-\( \gamma \) secretion was visualized with a biotin-conjugated anti-IFN-\( \gamma \) antibody (U-CyTech), alkaline phosphatase-conjugated ExtrAvidin, and Sigmafast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (both from Sigma).

Spots were counted using an AID reader (AID-GmbH, Strassberg, Germany), and means of triplicate wells were calculated. All ELISpot readouts are expressed as spot-forming cells (SFCs)/10^6 PBMCs. The cutoff for a positive response was set at three SDs above the average basal reactivity (i.e. reactivity against HIV gag_{77–85} and DMSO diluent alone). This was chosen as the cutoff allowing for the best diagnostic sensitivity (i.e. highest number of positive responses to \( \beta \)-cell epitopes in T1D patients) and specificity (i.e. lowest number of positive responses in healthy controls), as determined by the receiver-operating characteristics analysis [6].

**Statistical analysis**

GraphPad PRISM 4.0 Software was used for statistical analysis. Values are expressed as means ± SD or medians (range) according to their distribution. Comparisons of means between HLA–peptide complex stability and CTL responses to \( \beta \)-cell peptides in ELISpot assays were carried out with paired Student’s \( t \)-test for normal distributed variances. \( P \leq 0.05 \) was considered to be of statistical significance.

**Results**

**Prediction of epitopic peptides**

The SLC30A8 sequence was scanned for HLA-A*0201-binding peptides using the prediction softwares BIMAS and SYFPEITHI. A threshold of 60 (BIMAS) or 20 (SYFPEITHI) resulted in a short list of the top 2\% of peptides generated by both algorithms. The top 2\% of peptides produced by the SYFPEITHI algorithm is thought to generate an 80\% probability in identifying a naturally presented epitope

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Position</th>
<th>Sequence</th>
<th>SYFPEITHI</th>
<th>BIMAS</th>
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<tr>
<td>IGRP152–160</td>
<td>FLWSVFWLI</td>
<td>20</td>
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<td>IGRP215–223</td>
<td>FLFAVGFYL</td>
<td>22</td>
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<td>ALWMRILLPL</td>
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Table II. ZnT8 peptides predicted to bind HLA-A*0201.

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<th>BIMAS</th>
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<td>ZnT8</td>
<td>153–161</td>
<td>VVTGVLYL</td>
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<td>CLGHNHKEV</td>
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<td>245–254</td>
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<td>ZnT8</td>
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<td>LAVDGVLVS</td>
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<td>15</td>
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<td>ZnT8</td>
<td>314–322</td>
<td>ILSAHVATA</td>
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<td>19</td>
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</table>

β-Cell peptide binding to HLA-A*0201

To determine whether the candidate peptides were able to bind HLA-A*0201 in vitro, the level of HLA-A*0201 surface expression on T2 cells after the addition of exogenous peptides was measured [24]. Given that the greatest stability of HLA-A2, correlating with the highest affinity peptide binding was observed using the CMV peptide, this binding level was set at 100% with all other peptides expressed relative to this level. As shown in Figure 1A, IGRP (152–160), IGRP (215–223), IGRP (228–236), PPI (10–18), PPI (2–10), IA (215–223), and the control peptide FluMP stabilized HLA-A2 expression at levels >80%. Peptides GFAP (214–222) and PPI (10–18) resulted in intermediate (40–80%) expression of HLA-A2, whereas other peptides and the negative control peptide HIV gag bound poorly to HLA-A*0201 (<40%). IGRP (265–273) stabilized HLA-A2 expression at the lowest level, but it was predicted to be strong binders by both algorithms (Figure 1A).

Of the eight ZnT8 peptides predicted to bind HLA-A2, ZnT8 (153–161), ZnT8 (245–254), ZnT8 (273–282), and ZnT8 (314–322) were able to stabilize HLA-A2 expression at levels >80%. ZnT8 (200–208) and ZnT8 (292–300) were predicted to be weak binders at levels (40–80%). ZnT8 (177–186) and ZnT8 (281–290) were predicted to be strong binders by both algorithms, yet it stabilized HLA-A2 expression at the lowest level of all predicted binders (Figure 1B). We found that the relative binding affinities of β-cell peptides to HLA-A*0201 did not correlate with the binding affinities predicted by SYFPEITHI ($r = 0.217$) and BIMAS ($r = 0.092$; data not shown). We only used those peptides that stabilized HLA-A2 expression at >80% of the maximal level in subsequent experiments.

Dissociation rate of β-cell peptides from HLA-A*0201

Previous studies have shown that a stable peptide/major histocompatibility complex (MHC) complex could facilitate the formation of synapses between T cells and antigen-presenting cells, and the stability of peptide/MHC complex was the key factor for CTL activation [27]. Therefore, we assessed the stability of complexes formed with the candidate epitopes and HLA-A2 on T2 cells over a 4-h period at 37°C. The stability of the various peptide/HLA-A2 complexes was then normalized relative to that observed for CMV/HLA-A2 complexes that were set at 100%.

As shown in seven high-binding epitopes of β-cell proteins and candidate autoantigens, peptide/HLA-A2 complexes formed with IGRP (215–223) and PPI (10–18) were unstable, completely dissociating within 4 h of incubation at 37°C. Complexes of relatively low stability were observed for IGRP (228–236) and IA (2172–180) (~20% dissociated in 4 h). Whereas the remaining peptides tested—PPI (2–10) and GFAP (143–151)—produced HLA complexes that were stable over a 4-h period (~5% dissociation in 4 h; Figure 2A). Of the four high-affinity ZnT8 peptides, ZnT8 (245–254) completely dissociated within 4 h. ZnT8 (153–161) produced HLA complex that was more stable over a 4-h period (~20% dissociated in 4 h; Figure 2B).

Recognition of β-cell peptides by PBMCs from patients with recent-onset T1D

To test the ability to induce CD8+ T-cell response for each putative epitope, PBMCs obtained from eight HLA-A*0201 patients with recent-onset T1D and five HLA-A*0201 nondiabetic control subjects were assayed for peptide recognition by IFN-γ ELISpot assays. Reactivities were ranked as absent (<3 SD above the mean basal reactivity), low (>3 and <4 SD), intermediate (>4 and <5 SD), and high (>5 SD). The previously reported epitopes of seven β-cell candidate autoantigens were also studied. On comparing our results with those previously published, we have confirmed some of the existing epitopes. PPI (10–18) was recognized by ~75% (6/8) of T1D patients. It showed stronger CD8+ T-cell
Figure 1. A and B. Binding of β-cell peptides to HLA-A*0201. T2 cells lacking stable HLA-A*0201 surface expression were incubated with synthetic β-cell peptides, or equimolar amounts of control peptide known to bind to HLA-A*0201 with high affinity (CMVpp65), or peptide did not bind to HLA-A*0201 (HIV gag). Summary of the relative affinity of β-cell peptides (derived from IAPP, IGRP, PI, PPI, IA-2, GAD65, GFAP, and ZnT8) for HLA-A*0201. The error bars refer to the SE from three independent experiments. The relative binding affinity for each peptide is expressed as a percentage of maximal (CMV) binding as described in the Research design and methods section. For details of peptide origin and amino acid sequence, refer to Tables I and II.
subjects but none of the control subjects secreted a uniform stable over the 4-h period. IGRP, ZnT8, and PPI have been normalized relative to that observed for the CMV/HLA-A*0201 complex stability. Of the predicted MHC class I peptides, ZnT8 was found to inhibit protein synthesis and incubated with peptides at 37°C. Of HIV gag and the HLA-A2 expression. Peptide/HLA-A*0201 stability in the presence of PI for identifying a pool of candidate epitopes in known combination of binding and dissociation assays, we first demonstrated that ZnT8 autoantigen involved in T1D patients. Our findings confirm previous literature of some demonstrated epitopes of several β-cell candidate autoantigens in Chinese T1D patients. Using a combination of binding and dissociation assays, the rate of dissociation of MHC/peptide complexes and the self-reactive T-cell response in patients with T1D.

Correlations between the stability of the peptide/HLA-A2 complex and the self-reactive T-cell response in patients with T1D

Of 15 β-cell peptides screened by IFN-γ ELISPOT assays from eight patients with recent onset T1D, CD8⁺ T-cell responses were detected against six peptides. All these peptides had high-binding affinity. We analyzed the correlation between the specific CTL reactivity to each peptide in T1D patients and the dissociation rate. We did not observe a significant correlation (P = 0.339, R = −0.554) within the small number of patients analyzed. However, we found that PI₁₂₀–₁₈₀, IA-2₁₇₂–₁₈₀, and ZnT₈₁₅₃–₁₆₁ which had low dissociation rate showed a high CD8⁺ T-cell response (Table III).

Discussion and conclusions

β-Cell destruction and subsequent insulin deficiency are responsible for the clinical symptoms associated with T1D. Our findings confirm previous literature of some demonstrated epitopes of several β-cell candidate autoantigens in Chinese T1D patients. Using a combination of binding and dissociation assays, we first demonstrated that ZnT8 autoantigen involved autoactive CD8⁺ T-cell response in T1D. The immunogenicity of a peptide is related to its binding affinity for class I molecules [28–31]. Our data were consistent with previous literature in the mouse model [32], in that the predicted binding affinity of β-cell peptides to HLA-A*0201 did not correlate well with the actual binding affinity. Thus, the actual affinity of peptides IGRP₁₅₂–₁₆₀, IGRP₂₁₅–₂₂₃, and IGRP₂₂₈–₂₃₆ was higher than that of IAPP₅–₁₃, IA-2₄₈₂–₄₉₀, and GFAP₂₁₄–₂₂₂, despite the lower predicted binding scores. It was also found in ZnT₈₁₇₇–₁₈₆ and ZnT₈₂₈₁–₂₉₀ which showed high binding scores, and that the actual affinity of these two peptides was low. Actual binding affinities of candidate β-cell epitopes are likely attributable to the 3D structure of the peptide–HLA interaction, such as hydrophobic interaction, hydrogen bonds, and salt bonds. Therefore, it cannot be accurately determined by existing algorithms. Nonetheless, computer-based prediction algorithms are helpful for identifying a pool of candidate epitopes in known target proteins that require confirmation of binding in vitro [33].

The rate of dissociation of MHC/peptide complexes appears to be an important binding parameter, with highly immunogenic peptides displaying slower off-rates [34, 35]. It may have an effect on CTL recognition of (self) β-cell peptides in the periphery.
Rapid peptide dissociation from HLA-A*0201 may lead to a paucity of specific peptide/HLA-A*0201 complexes at the surface of the cell, insufficient for recognition and triggering the circulating T cells [14]. In previously reported epitope, we assessed the stability of complexes used which HLA-A2 expressed at 80% of the maximum level. We found that IGRP215–223 and PPI2–10 were completely dissociated in 4 h, although they had high-binding affinity. We found that PI210–18 and ZnT8153–161 had high-binding affinities and the lowest dissociation rate. Also, they both showed high immunogenicity.

Figure 3. Summary of HLA-A2 + T1D patients ($n = 8$), HLA-A2 + healthy control subjects ($n = 5$) assayed for $\beta$-cell reactivities. All values, including basal $+ n$ SD cutoffs, are expressed as SFC/106 PBMCs and are basal subtracted. Unsubtracted basal values (reactivities to DMSO and gag 77–85) are shown in the last row of each column. Reactivities are ranked as low (between 3 and 4 SD, in yellow), intermediate (between 4 and 5 SD, in orange), and high (more than 5 SD, in red). ++ + + +, off-scale ELISpot reading.

Rapid peptide dissociation from HLA-A*0201 may lead to a paucity of specific peptide/HLA-A*0201 complexes at the surface of the cell, insufficient for recognition and triggering the circulating T cells [14]. In previously reported epitope, we assessed the stability of complexes used which HLA-A2 expressed at >80% of the maximum level. We found that IGRP215–223 and PPI2–10 were completely dissociated in 4 h, although they had high-binding affinity. We found that PI180–18 and ZnT8153–161 had high-binding affinities and the lowest dissociation rate. Also, they both showed high immunogenicity,
and were recognized by ~75% (6/8) or ~37.5% (3/8) of recent-onset T1D patients in our study. These data may suggest a useful role of combining HLA-binding affinity and dissociation assays in determining both immunogenicity and recognition of naturally processed antigen production with respect to significant enhanced killing by CTLs. Collecting a larger number of HLA-A*0201 patients from T1D is difficult in a short period of time. We did not have a large number of patients to detect their CD8^+ T-cell response in this study. Thus, we could not observe a significant correlation between the dissociation rate and strong self-reactive T-cell response in T1D patients. However, in our data, most of the epitopes which had low dissociation rate showed high CD8^+ T-cell response.

Recently, the pattern of expression of IGRP splice variants in human tissues was investigated; the only transcripts detected in human thymus were lacking exon 4 [37]. This would result in a frameshift mutation, preventing expression not only of exon 4, but also of the last and largest exon (exon 5), and a failure to establish tolerance to IGRP epitopes found in this region of the protein. Interestingly, all reported epitopes here lied within exons 4 and 5. We also found that IGRP_{152–160} and IGRP_{215–223} could induce CD8^+ T-cell response in T1D patients. Thus, epitopes 152–160 and 215–223 may represent a “hot spot” for T-cell reactivity.

Insulin is clearly an important autoepitope in T1D [38], and proinsulin-derived epitopes are the targets of T cells in both diabetic and prediabetic subjects [39,40]. We observed that in addition to recent-onset patients, the nondiabetic control subjects also exhibited CD8^+ T-cell responses to the peptides PI_{B10–18} and PPI_{2–10}. This finding is similar to other reports of CTL recognition of insulin 2 and PI_{B10–18} [6,14] present in healthy individuals. The importance of this observation is unclear.

ZnT8 is a member of a family of 10 zinc transporters (ZnT1–10; encoded by SLC30A1–10) thought to catalyze the extrusion of Zn^{2+} from the cell cytosol into the extracellular space or intracellular organelles. ZnT8 has been shown to be a major autoantigen in T1D [16]. Moreover, recent genome-wide studies [41] have shown that a nonsynonymous SNP rs13266634 in the SLC30A8 gene, resulting in the replacement of tryptophan-325 with arginine, increases the risk of type 2 diabetes (OR 1.58) possibly

<table>
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<th>Peptide</th>
<th>IFN-γ producing spots/106 PBMC</th>
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<tr>
<td>PI_{B10–18}</td>
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<tr>
<td>IA-2_{172–180}</td>
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<tr>
<td>IGRP_{215–223}</td>
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<tr>
<td>ZnT8_{153–161}</td>
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<td>PPI_{2–10}</td>
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</table>

It appeared that the dissociation studies may be useful for CTL response against those peptides. We only observed this phenomenon within a small number of patients. A bigger pool of patients and more advanced research will be done to further demonstrate our study. In our data, it seemed to form an exception that IGRP_{215–223} was unstable over a 4-h period and recognized by PBMC from four out of eight T1D patients. It is possible for T cells to react to unstable MHC–peptide complexes in such a way that they recognize the endogenously processed peptide when this particular epitope is expressed at high levels [36].

Recently, the pattern of expression of IGRP splice variants in human tissues was investigated; the only transcripts detected in human thymus were lacking exon 4 [37]. This would result in a frameshift mutation, preventing expression not only of exon 4, but also of the last and largest exon (exon 5), and a failure to establish tolerance to IGRP epitopes found in this region of the protein. Interestingly, all reported epitopes here lied within exons 4 and 5. We also found that IGRP_{152–160} and IGRP_{215–223} could induce CD8^+ T-cell response in T1D patients. Thus, epitopes 152–160 and 215–223 may represent a “hot spot” for T-cell reactivity.
by decreasing insulin secretion and/or proinsulin processing. These findings represent the association between a zinc transporter gene and diabetes.

However, little information on the role of ZnT8-specific CD8+ T-cell responses in human T1D is available. Our report here first demonstrates that ZnT8 [53–161] is a novel CD8+ T-cell epitope in human T1D. Besides, ZnT8[153–161], ZnT8[245–254], and ZnT8[314–322] also have high-binding affinity and low dissociation rate. The requirement to test such a broad range of peptides in the current study inevitably leads to limitations. First, we do not have a large sample size.

We were only able to study adult subjects (because of the large volume of blood required), and these responses may be different in children with newly diagnosed disease. Second, because of a large number of peptides to be tested, all these peptides that we now select are of high-binding affinity. Third, recently, Ouyang et al. [14] showed an inverse relationship between MHC/peptide binding and T-cell response measured by IFN-γ production. In our study, these peptides that bind with a high affinity may not be the most immunogenic peptides in the T-cell assays.

In this study, we first demonstrated that ZnT8 autoantigen involved autoreactive CD8+ T-cell response in T1D. Although for this correlation between MHC–peptide complexes dissociation and T-cell response, we did not observe a significant correlation within the small numbers of patients analyzed. However, we suggested that it may be helpful for the identification of T-cell epitopes from interesting antigens using HLA binding and dissociation assays.

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