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Characterization of immune response to novel HLA-A2-restricted epitopes from zinc transporter 8 in type 1 diabetes

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ABSTRACT

Objective: ZnT8-specific CD8+ T cells in human type 1 diabetes (T1D) have been reported recently, although the results from different laboratories are inconsistent. We aimed to characterize these ZnT8 specific CD8+ T cells and validate assays to screen peptide libraries.
Methods: We screened HLA-A2-restricted T cell candidate peptides of ZnT8 with different methods including computer algorithms, MHC-peptide binding and dissociation assays in T2 cell line, identification in HLA-A2 transgenic (Tg) mice and *in vivo* CTL assays. Then ELISpot assay was used to measure peptide-reactive T cell responses in 49 HLA-A2-restricted T1D patients.
Results: We demonstrated that $ZnT8_{107–116(115)}$, $ZnT8_{110–118}$, and $ZnT8_{177–186}$ were novel HLA-A*0201-restricted CTL epitopes in T1D patients. $ZnT8_{107–116(115)}$, $ZnT8_{115–123}$, $ZnT8_{153–161}$, $ZnT8_{177–186}$ and $ZnT8_{291–300}$ represent potentially major biomarkers for T1D. T cell responses against these epitopes showed different distributions between recently diagnosed and long-standing patients. Furthermore, they displayed discriminating performance among different ethnicities. We also compared the performance of the epitope identification strategies used herein. The epitopes which exhibited strong immunogenicity in HLA-A2 Tg mice were also well recognized by T1D patients.
Conclusions: The differences in autoimmune T cell responses among T1D individuals may open new avenues toward T1D prediction and prevention. It also provides efficient strategies for immune intervention.

1. Introduction

CD8+ T cells play a central role in the development [1] and probably the final effector phase of autoimmune beta-cell destruction in type 1 diabetes [2,3]. Rapid diabetes recurrence is seen in recipients of an isograft from a discordant, nondiabetic twin and is accompanied by an almost exclusive $CD8^+$ T cell islet infiltration [4]. Thus, it seems that beta-cell-specific T lymphocytes maintain immune memory for years after disease onset. However, differentiation patterns of autoreactive T lymphocytes, once diabetes is diagnosed remain largely unknown. Therefore, discovery and characterization of the antigens recognized by such T cells is important [5]. A variety of functional and biochemical approaches have been developed

Abbreviations: Tg, transgenic; ZnT8, zinc transporter-8; CTL, cytotoxic lymphocyte; GAD65, glutamic acid decarboxylase; IA-2, tyrosine phosphatase-related islet antigen 2; DMSO, dimethyl sulphoxide; CMV, cytomegalovirus; PBMCs, peripheral blood mononuclear cells; CFSE, carboxyfluorescein diacetate succinimidyl ester; HLA, human leukocyte antigen.

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to identify peptide epitopes [6–8]. Reliable approaches to predict optimal CD8+ T cell epitopes are still needed.

Zinc transporter-8 (ZnT8) has emerged in recent years as a key player in regulation of both insulin secretion and beta-cell mass, with the highest expression in human pancreatic beta-cells [9]. ZnT8 is further implicated as an autoantigen targeted by disease-associated autoreactive T cells in humans, with 60–80% of recent-onset type 1 diabetic patients presenting with autoantibodies to ZnT8 [10–14]. Previous studies have shown that T cells epitopes derived from other autoantigens such as proinsulin, GAD65, and IA-2 can be detected in patients at the onset of clinical disease [7,8,15–17]. However, little information regarding ZnT8-associated CTL specific-peptides is available. Recently, as a result of the efforts of several groups, including our own, several HLA-A2-restricted-CD8+ T cell epitopes derived from ZnT8 have been identified [18–21]. A variety of methods have been used to predict which peptides are likely T cell epitopes, such as computational algorithms, cell lines and Tg mice. However, diabetic CD8+ T cells exhibited different responses to the same epitopes in different studies, such as $ZnT8_{153–161}$ [18,20]. Some peptides selected as potential epitopes with different screening methods were not confirmed as recognized once verified in diabetic patients [18,20]. This discrepancy among the results from different laboratories raises doubt about the exact immunodominant epitopes recognized by T cells in patients [8,15–17].

To further characterize these ZnT8 specific CD8+ T cells, we identified novel candidate epitopes from ZnT8 and analyzed their cognate T cell responses in patients with different disease duration. We characterized known ZnT8 specific CD8+ T cell responses between Caucasian and Chinese patients. Comparing the methods which are used to predict candidate T cell epitopes in parallel, we tried to validate suitable assays to screen peptide libraries in humans.

2. Methods and methods

2.1. Peptides

Predicted ZnT8 peptides and control peptides were synthesized by Nanjing Jinsite Biology and Technology Company (Nanjing, China). Individual peptides were synthesized and purified to 98% purity.

2.2. Blood processing and serum Abs

Rapid HLA-A2 screening was performed with the FITC mouse anti-human HLA-A2 mAb (BB7.2, BD Pharmingen™), followed by subtyping using the Morgan™ HLA SSP Typing kit (Texas Bio-Gene Inc.). Serum Abs were measured by radio-binding assays, using ^{35}S-labeled glutamic acid decarboxylase-$_{65}$ (GAD_{65}), protein-tyrosine-phosphatase-2 (IA-2), zinc transporter-8 (ZnT8) [11]. The cut-off values for positivity for GADA, IA2A and ZnT8A were values above 0.015, 0.048, and 0.018 respectively, based on the 99th percentile of healthy control subjects. PBMCs were isolated by density gradient centrifugation using Lymphoprep™ lymphocyte separation medium (Fresenius Kabi Norge AS, Norway) and immediately used.

2.3. Patients and control subjects

Type 1 diabetic patients (n = 49; 27 male, 22 female; median age 36 years [range 9–65] were recruited. Healthy controls (n = 30; 14 men, 16 women; median age 28 years [range 18–51]) were selected. Data are shown in Supplementary Fig. 1. Patients with acute onset of symptoms requiring permanent insulin treatment from the time of diagnosis were diagnosed with type 1 diabetes [22]. All participants were HLA-A2+ (HLA-A*02:01) by genotyping and gave written informed consent. The protocol was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University and was carried out in agreement with the Declaration of Helsinki as revised in 2008.

2.4. Human ELISpot assay

Ninety-six-well PVDF plates pre-coated with an anti-IFN-γ monoclonal antibody (Dakewe Biotech Co™ Human IFN-γ pre-coated ELISpot kit) were blocked with RPMI-1640 plus 10% human serum (PAA Laboratories GmbH, Pasching, Austria). Peripheral blood mononuclear cells (PBMCs) were prepared as described previously [19]. Fresh PBMCs were added to triplicate wells ($2–3 \times 10^5$ cells per well) and incubated for 20–24 h in the absence or presence of peptides (20 μM final concentration) or phytohemagglutin (Sigma L8902 PHA lectin from phaseolus vulgaris). After PBMC removal, IFN-γ secretion was detected with a secondary biotin-conjugated anti-IFN-γ monoclonal antibody. Subsequent processing of the plates was identical to the mouse ELISpot protocol described above. All data shown are means of triplicate wells and expressed as spot-forming cells per 10^6 lymphocytes.

2.5. Peptide binding assay

Briefly, T2 cells were loaded with 100 μg/ml peptide during an overnight incubation at room temperature (RT) in the presence of 3 μg/ml β2-microglobulin ($\beta2^m$) (Sigma–Aldrich, Oakville, ON, Canada) in Iscove's modified Dulbecco's medium serum-free Iscove's modified Dulbecco's medium (IMDM, Invitrogen) and then washed and stained with FITC-conjugated anti-HLA-A2 monoclonal antibody (BB7.2; BD Pharmingen, San Diego, CA). The surface HLA-A2 expression was measured by flow cytometry (FACSCalibur), and the mean fluorescence intensity was recorded. The high-affinity immunodominant HLA-A2 CMV peptide [23] was used as a positive control. Results of ZnT8 peptide binding to HLA-A*0201 are expressed as: percentage relative binding of the CMV peptide to HLA-A*0201 = 100 × [(MFI with given peptide – MFI without peptide)/(MFI with CMV/A2 peptide – MFI without peptide)], where MFI is the mean fluorescence intensity.

2.6. HLA-peptide complex stability

Briefly, T2 cells were cultured with synthetic peptides overnight at RT as was performed for peptide binding assay. The following day, after removing peptide and adding emetine (10^{-4} M; 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). Surface HLA-A2 expression was assessed by flow cytometry (FACSCalibur), and 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: relative complex stability = 100 × [(MFI with given peptide – MFI without peptide)/(MFI with CMV peptide – MFI without peptide)], where MFI is the mean fluorescence intensity[8].

2.7. Mice

Female or male HHD II mice [24] were 12–14 week old and were maintained in pathogen-free conditions in the model animal research center of Nanjing University. Animal experiments were