Islet Amyloid Polypeptide is not a Target Antigen for CD8+ T-Cells in Type 2 Diabetes

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ABSTRACT

Background: Type 2 diabetes (T2D) is a chronic metabolic disorder in which beta-cells are destroyed. The islet amyloid polypeptide (IAPP) produced by beta-cells has been reported to influence beta-cell destruction. Objective: To evaluate if IAPP can act as an autoantigen and therefore, to see if CD8+ T-cells specific for this protein might be present in T2D patients. Methods: Peripheral blood mononuclear cells (PBMC) were obtained from human leukocyte antigen (HLA)-A2+ T2D patients and non-diabetic healthy subjects. Cells were then screened for peptide recognition using ELISPOT assay for the presence of IFN-γ producing CD8+ T-cells against two HLA Class I-restricted epitopes derived from IAPP (IAPP5-13 and IAPP9-17) and common viral antigenic minimal epitopes Flu MP 58-66, CMV 495–503, EBV 280–288 and HIV 77–85 as controls. Results: A total of 36.4% of patients and 56.2% of healthy subjects showed a response against IAPP5-13 peptide. No significant difference in response against this peptide was noted between the patients and the healthy donors. With respect to peptide IAPP9-17, although healthy subjects showed a higher mean number of spot forming cells than the patients, the difference was not significant; 36.4% of patients and 37.5% of controls responded to this peptide. The response of healthy subjects to the common viral peptides was stronger than that of the patients, though the result was not significant. Conclusions: It is unlikely that IAPP would be a target for CD8+ T-cells in diabetic patients; however, the trend observed toward a lower response of T2D patients against IAPP and common viral peptides may imply a decreased immune response in these patients.


Keywords: CD8-Positive T-Lymphocytes, ELISPOT, Islet Amyloid Polypeptide, Type 2 Diabetes

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INTRODUCTION

Type 2 diabetes (T2D) is the most common metabolic disorder in the world (1). Diabetes has multiple serious negative impacts on people’s health, including nephropathy, retinopathy, neuropathy, and cardiovascular diseases (2). T2D is caused by the failure of β-cells to compensate for insulin resistance (3). The failure could be mainly a result of decreased β-cell mass and defective insulin secretion in the body (4). A few mechanisms have been suggested to explain this failure, including endoplasmic reticulum stress, oxidative stress, lipotoxicity, glucotoxicity, and amyloid deposition (5). Inflammatory and immune factors have also been implicated in both insulin resistance and beta-cell dysfunction. For example, in a study by Vozarova and colleagues, a positive correlation between elevated numbers of white blood cells (WBC) and increases in insulin resistance (that eventually resulted in T2D) was shown (6). One hypothesis for this correlation was that the production of interleukin (IL)-6 by the WBC could induce inflammation that, in turn, results in decreased insulin sensitivity.

T2D is also characterized by increases in amyloid deposits in pancreatic islets; this is often a hallmark of end-stage chronic inflammation (5). Islet amyloid poly-peptide (IAPP, or amylin) is a 37 amino acid protein produced by β-cells and is involved in the regulation of glucose homeostasis (7). IAPP, first isolated from extracts of amyloid from the pancreas of T2D patients with insulinomas (8), has been implicated in macrophage infiltration into islets (9). Glucose-induced production of IL-1β by beta-cells has been linked to β-cell destruction by apoptosis (10). Moreover, IAPP could induce macrophage processing of IL-1β by activating nuclear-localization receptor protein 3 inflammasome (11-12). Thus a strong association between amyloid deposition in T2D subjects and presence of beta-cell apoptosis might suggest a role for IAPP in beta-cell death.

In T2D patients, adipose tissue and pancreatic islets are infiltrated by cytokine-producing macrophages that affect insulin action and beta-cell function and viability (13). Nishimura et al. showed that CD8+ T-cells are responsible for macrophage activation and recruitment into adipose tissue of obese mice; these authors proposed a key role for these cells in obese adipose tissue inflammation (14). Several studies have reported the infiltration of macrophages into the islets of T2D patients, but the role of CD8+ T-cells in this process has not been fully elucidated. If these cells are responsible for macrophage infiltration into the T2D patient islets, as was observed in obese mice, this raises the question as to if there are also CD8+ T-cells potentially in the islets. One hypothesis has suggested involvement of an endogenous factor like an unknown autoantigen(s) in recruiting CD8+ T-cells into the adipose tissue and islets of T2D subjects. This would not be unprecedented; in Type 1 diabetes, several antigens have been reported to act as autoantigens that are subsequently targeted by CD8+ T-cells. One of these autoantigens is IAPP that may be involved in islet cell inflammation.

Based on this information, the current study sought to investigate if IAPP antigen-specific T-cells were present in T2D patients. For this, two HLA-A2-restricted epitopes of IAPP, each capable of stimulating specific CD8+ T-cells to produce IFN-γ in Type 1 diabetes patients (15,16), were employed to treat blood cells collected from patients and control subjects and the release of IFN-γ was then measured by an enzyme-linked immunospot (ELISPOT) assay.
MATERIALS AND METHODS

Patients and Controls. Based on the previous studies (15,16), twenty-eight T2D patients with disease duration not longer than 1 year (followed at the Diabetes Clinic of Shiraz University of Medical Sciences) were screened. Of these patients, 11 (39.3%) were HLA-A2+. Forty healthy donors were also enrolled in the study, of which 16 (40.0%) were HLA-A2+. Among patients there were 7 female and 4 male participants in comparison to 10 female and 6 male from controls. T2D patients had mean age 41.2 ± 2.2 years compared to controls with mean age of 36.6 ± 1.05 years. Mean BMI of patients was 24.8 ± 2.1 kg/m² compared to controls 22.8 ± 1.9 kg/m². Patients were diagnosed as having T2D according to the American Diabetes Association criteria (17). They had no sign of other autoimmune disease(s). Control subjects were healthy individuals with no diseases; all had no history of diabetes and had normal glucose level. None was receiving any medication. Informed consent was obtained from all subjects. The relevant human and research ethics committees of the Shiraz University of Medical Sciences approved all aspects of the study.

Peptides. The peptides used in this study (all used at 10 μM) were >80% pure (China Peptides, Shanghai, China) (Table 1).

<table>
<thead>
<tr>
<th>Sequences</th>
<th>SYFPEITHI score</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAPP 5-13</td>
<td>KLQVFLIVL</td>
<td>26</td>
</tr>
<tr>
<td>IAPP 9-17</td>
<td>FLIVLSVAL</td>
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<td>Flu 58-66</td>
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<td>EBV 280-288</td>
<td>GLCTLVAML</td>
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<td>CMV 495-503</td>
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</tr>
<tr>
<td>HIV 77-85</td>
<td>SLYNTVATL</td>
<td>31</td>
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IAPP, Islet amyloid polypeptide; Flu, Influenza; EBV, Epstein–Barr virus; CMV Cytomegalovirus; HIV, human immunodeficiency virus; and SYFPEITHI (database for MHC ligands and peptide motifs).

These peptides ranked the highest among all IAPP sequences in the SYFPEITHI algorithm that predicts sequences likely to bind to HLA-A2 (18). Two HLA-A2-restricted peptides, including IAPP 5-13 and IAPP 9-17 derived from the leader sequence of IAPP, were used here. The viral peptides of Influenza (Flu) MP (matrix protein) 58-66, Epstein-Barr virus (EBV 280-288) and Cytomegalovirus pp65 (CMV 495-503), were used as positive controls. This selection was based on the fact that most of the people are exposed to these viruses at least once in their life. Phytohemagglutinin (PHA) (1 mg/ml; Sigma, Lyon, France) as a polyclonal activator was used as an additional control.
treatment for evaluating the validity of the test. A human immunodeficiency virus peptide (HIV\textsuperscript{77-85}) was used as negative control because all subjects in both groups were HIV negative.

**Sampling and Peripheral Blood Mononuclear Cells (PBMC) Isolation.** Single blood samples from patients and controls (5 ml) were collected into heparin tubes and kept at room temperature (RT) until processing. Mononuclear cells were isolated using lymphocyte separation medium (Lymphoprep\textsuperscript{TM}, Stem Cell Technologies, Vancouver, Canada). All samplings took place at 7 AM and were fasting samples.

**HLA-A2 Typing by Flow Cytometry.** Blood samples were screened for HLA-A2 by flow cytometry. In brief, 5 ml of lysis buffer was added to 300 µl of blood and incubated for 5 min at RT. The suspension was washed 5-times with phosphate-buffered saline (PBS, pH 7.4) and then centrifuged at 1600 rpm for 5 min. The cells were then resuspended in 200-µl fluorescence-activated cell sorting (FACS) buffer (BD Biosciences, San Jose, CA) and stained with 1 µl anti HLA-A2 monoclonal antibody (BB7.2, IgG2b; a kind gift from Dr. R. Mallone, INSERM U1016, Paris, France). After 30 min incubation at 4°C, the cells were washed twice with PBS and then 10 µl fluorescent isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (IgG\textsubscript{2a}; Abcam, Cambridge, UK) was added. The mixture was incubated 20 min at 4°C before the cells were washed one final time and then immediately analyzed on a FACS-Calibur flow cytometer (BD Sciences). Cells stained only with the secondary antibody were used as negative control and cells stained with W6/32 anti HLA- Class I antibody (IgG\textsubscript{2a}, Abcam) used as the positive control. All analyses were performed using FlowJo (version 7) software (TreeStar, Ashland, OR). A minimum of 5000 events per sample was acquired in each assay.

**ELISPOT Assay.** A highly sensitive ELISPOT assay (19) was used to detect IFN-γ producing cells. Addition of both human serum-free AIM-V (Life Technologies, Carlsbad, CA) which does not require human serum for optimal growth and activation of differentiated lymphoid cells and a low dose of IL-7 has been previously shown to increase assay sensitivity. In brief, 96-well polyvinylidene plates (Millipore, Saint-Quentin-en-Yvelines, France) were coated overnight with anti-IFN-γ mouse antibody (U-CyTech, Utrecht, the Netherlands). The plates were subsequently blocked with RPMI medium containing 10% human serum, and then peptides were added at a final concentration of 10 µM (in triplicate wells) along with recombinant human IL-7 (0.5 U/ml; R&D Systems, Germany). PBMC isolated from the test subjects/controls were then seeded at 3 × 10\textsuperscript{5} cells/well and cultured for 24 hr at 37°C under 5% CO\textsubscript{2}. The cells were subsequently removed and the plates washed five times with PBS (at RT). Thereafter, biotin-conjugated anti-IFN-γ mouse antibody (U-CyTech) and alkaline phosphatase-conjugated ExtrAvidin (Sigma, St. Louis, MO) were added to each well and the plates were incubated 1 hr at RT. After five washes of each plate, a SigmaFast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablet was dissolved in 10 ml distilled water and then 100 µl of the solution was added to each well. The plate was then left to air-dry for 10 min in the dark.

Resultant spots were counted using an ELISPOT reader (AID ELISPOT Reader ELR04; AID GmbH, Strasberg, Germany), and the mean number of spots (in triplicate wells) was calculated. All results were expressed as spot-forming cells/10\textsuperscript{6} PBMC. The threshold for determining a positive response was defined as a value three standard deviations (SD) above the mean spot-forming cell counts of control (basal) wells (cells
without peptides) for each epitope. The results were ranked as low (≥3 and <4 SD), intermediate (≥4 and <5 SD), and high (≥5 SD).

**Statistical Analysis.** The statistical significance of the ELISPOT results was analyzed using a Mann-Whitney U test (SPSS, SPSS Inc, Chicago, IL). Statistical significance was accepted at p-values < 0.05.

**RESULTS**

**Selection of HLA-A2⁺ Patients and Controls.** All patients and controls were investigated for positivity for the HLA-A2 Class I allele by flow cytometry. Eleven T2D patients and 16 healthy controls positive for HLA-A2 were enrolled in this study and their PMBC were analyzed for inducible IFN-γ release when exposed to the study peptides. An example of a flow cytometry typing result is shown in Figure 1.

**Positive Control**

**Test**

![Flow cytometry histograms for HLA-A2 typing. PBMCs were obtained from Type 2 diabetes patients and healthy donors and then analyzed for HLA-A2 positivity. Cells were stained with BB.7.2 anti-HLA-A2 antibody followed by FITC-conjugated anti-human globulin (test). Controls were unstained cells (without antibody), cells stained only with secondary antibody, or cells stained with W6/32 anti-HLA-Class I antibody (positive control).](image-url)
Table 2. ELISPOT detection of specific response of HLA-A2+ T2D patients (n=11) and healthy controls (n=16) to IAPP<sub>5-13</sub>, IAPP<sub>9-17</sub>, and viral peptides.

<table>
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<tr>
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<th>M01</th>
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<td>5.6</td>
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<td>7.8</td>
<td>86.4</td>
<td>326.4</td>
<td>120.0</td>
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<td>90.0</td>
<td>383.1</td>
<td>2.1</td>
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<td>185.5</td>
<td>20.5</td>
<td>89.5</td>
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<td>33.3</td>
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PBMC were stimulated with the peptides and the frequency of IFN-γ releasing T-cells was then detected by ELISPOT. Values shown are the number of spot-forming cells (SFC)/10<sup>6</sup> PBMC and are basal subtracted. Basal values (cells cultured without peptide) are shown in last row of each column. Reactivity is ranked as 3, 4, and 5 SD of basal value; low (3 - 4 SD, in bright grey), intermediate (4 - 5 SD, in dark grey), and high (>5 SD, in black). All spot counts were determined from means of replicate assay wells. IAPP, Islet amyloid polypeptide; Flu MP, Influenza matrix protein; EBV, Epstein-Barr virus; CMV, Cytomegalovirus; HIV, human immunodeficiency virus. P = T2D patient; H = healthy donor.
Identification of IFN-γ Producing Cells. An ELISPOT assay was used to quantify IFN-γ secretion by PBMCs from patients and healthy donors in the response of each peptide. The results of peptide stimulation of PBMCs from T2D patients and healthy subjects treated are shown in Table 2. The threshold for determining a positive response was defined as 3 SD above the mean spot-forming cell value in the control wells for each epitope. The results were ranked as low (>3 and <4 SD), intermediate (>4 and <5 SD), and high (>5 SD) according to a previous study (20). Four out of eleven patients (36.4%) were positive for IAPP5-13. Patients P04 and P09 showed a high response, while P08 and P11 were moderate responders to IAPP5-13 against the peptide. The healthy subjects had also a marked number of PBMC responding to IAPP5-13 (56.25%). Subjects H01, H02, H03, H09, H11, H12, and H13 yielded high responses while subjects H06 and H16 had low responses against this peptide. There were no significant differences in the outcomes against IAPP5-13 between the diabetic (68.33 ± 26.78) patients and healthy donors (121.86 ± 45.40, p=0.71) (Figure 2A).

Figure 2. IFN-γ ELISPOT responses of PBMC from patients and control subjects against putative peptides from (A) IAPP and (B) different viral peptides. The frequency of IFN-γ releasing T cells presented as spot-forming cells (SFC)/10^6 PBMC. All spot counts were determined from the means of replicate assay wells. Horizontal bars represent number of spot-forming cells and error bars SEM. No significant differences between the two groups were found for the peptides.

With respect to IAPP9-17 peptide, the frequency of positive T2D patients (36.4%) was not significantly different from that among the controls (37.5%, Figure 2A). Against IAPP9-17, high responses were detected in healthy subjects H02, H08, H09, H12, and
H13, and a low response was obtained in subject H06. The T2D patients P04, P07, and P08 that were responsive to IAPP\textsubscript{9-17} peptide showed moderate responses, whereas patient P09 had a strong IFN-\textgreek{y} production. In healthy donors, five of 16 were positive for both IAPP\textsubscript{5-13} and IAPP\textsubscript{9-17}. Among the T2D patients, three out of 11 were positive for both peptides. Overall, the mean number of spots for both IAPP epitopes was lower in patients than among healthy donors, but the difference was not significant (Table 3).

### Table 3. Reactivity of PBMC from T2D patients and controls against different peptides.

<table>
<thead>
<tr>
<th>Peptides</th>
<th>T2D (n =11)</th>
<th>Healthy Donors (n= 16)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flu MP \textsubscript{58-66}</td>
<td>85.91 ± 39.26</td>
<td>170.00 ± 54.07</td>
<td>0.17</td>
</tr>
<tr>
<td>CMV \textsubscript{295-303}</td>
<td>151.28 ± 66.49</td>
<td>164.99 ± 39.83</td>
<td>0.88</td>
</tr>
<tr>
<td>EBV \textsubscript{280-288}</td>
<td>69.50 ± 22.44</td>
<td>160.16 ± 50.76</td>
<td>0.12</td>
</tr>
<tr>
<td>HIV \textsubscript{77-85}</td>
<td>37.36 ± 21.42</td>
<td>12.59 ± 10.64</td>
<td>0.82</td>
</tr>
<tr>
<td>IAPP\textsubscript{5-13}</td>
<td>68.33 ± 26.78</td>
<td>121.86 ± 45.40</td>
<td>0.71</td>
</tr>
<tr>
<td>IAPP\textsubscript{9-17}</td>
<td>35.71 ± 17.24</td>
<td>48.58 ± 22.91</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Values expressed as mean spot forming cells (SFC) \textsubscript{10\textsuperscript{6}} PBMC (± SEM).

IAPP, Islet amyloid polypeptide; Flu MP, Influenza matrix protein; EBV, Epstein–Barr virus; CMV, Cytomegalovirus; HIV, human immunodeficiency virus.

Nine of 11 patients (81.8\%) were positive for at least one viral peptide. One patient only showed a low response for HIV, possibly due to cross-reactivity. Fourteen out of 16 (87.5\%) of the healthy donors were positive for viral peptides and all were negative for HIV. For the viral peptides, the mean numbers of spot forming cells in PBMC from all T2D patients were lower than those for the healthy donors (e.g., Flu MP \textsubscript{58-66}; 85.91 ± 39.26 vs 170 ± 54.07) though the difference was not significant (Table 3, Figure 2B). An example of an ELISPOT assay result with PBMC from a T2D patient and a healthy donor is shown in Figure 3.

### DISCUSSION

T2D is a complex metabolic disorder. Macrophage infiltration of islets is one of the main models have been proposed for disease development (21). In several studies, the role of macrophage recruitment into islet cell regions has been investigated. In a study in obese mice an accumulation of macrophages in these areas in the early stages of disease has been shown (13).
In another study the dominance of M1 macrophages in the islets of T2D patients and also obese mice has been shown (22). These macrophages often appeared to reflect an inflammatory condition and they produced IL-1 that, in turn, could contribute to islet cell damage. Nishimura et al. studied the interaction between adipose tissue and CD8⁺ T-cells in obese mice and showed this interaction was crucial for distal macrophage differentiation, migration, and activation (14). Several chemokines secreted by CD8⁺ T-cells were suggested to be involved in this macrophage migration, including interferon-inducible protein-10, monocyte chemoattractant protein (MCP)-1 and MCP-3.

The association of T2D with a low-grade inflammation has made some researchers to believe that an antigen could be involved in triggering of inflammation (9,23). However, due to the chronic nature of T2D, no antigen has yet been identified as the autoantigen trigger for this disease (9,10). There is some evidence suggesting a possible role of antigens derived from stressed cells, such as heat shock proteins, as danger signals in chronic inflammation (24,25), however the role of such antigens in the etiology of T2D is unclear. In human Type 1 diabetes, IAPP has recently attracted attention as a possible autoantigen; some studies have demonstrated an existence of autoreactive CD8⁺ T-cells against two peptides of this specific protein (26).

In our study, most of the peptides used were recently characterized as immunogenic by investigators (15,16,19). The peptide fragments that were chosen for use in this study...
are the same as those derived from IAPP and are restricted to the HLA-A2, and therefore can stimulate CD8\(^+\) cells (15,16). In addition, in the present study, PBMC were treated with IL-7 that has been shown to boost memory/effector T-cell responses (19, 27). As results of this study showed, there was no significant difference between the response to IAPP\(_{5-13}\) and IAPP\(_{9-17}\) by cells from T2D patients and healthy controls, indicating these specific peptide pieces may not act as an autoantigen to stimulate CD8\(^+\) cells. However, a trend was seen toward a weaker response to both peptides in the cells from patients compared to that by those of the healthy subjects; this could suggest a generalized decreased immune response among the diabetics. This data seemed to be in line with results of some other studies on the quality of immune responses in T2D patients. In a study by Andreasen and his colleagues, an impaired response against in vivo administration of LPS has been reported. They showed attenuated LPS-induced increases in TNF-\(\alpha\) and several adhesion molecules in T2D patients compared to healthy controls (28). Of note, as it was expected, the subjects responded differently to the viral peptides in ELISPOT assay. This difference could be due to the influence of genetic backgrounds and the inheritance of different MHC alleles that affect the immune responses. However, a generalized decreased immune response among the diabetics would also help to explain the comparative responses of the patients and controls to the viral peptides. Here the cells from the T2D subjects trended toward lower levels of response. A suppressed immune response against viral infections in diabetics has been shown by several investigators (29,30). Diabetic patients infected with H1N1 influenza virus often present with a more severe disease state than do healthy control subjects (29). Breton and colleagues also suggested that diabetic adults are at greater risk for infection-related mortality (30). It is thus not surprising that our subjects had lower responses to the test peptide pieces.

In this study for the first time we examined the presence of specific CD8\(^+\) T-cells against IAPP as an autoantigen in T2D patients compared to the healthy individuals. Although, the results did not show significant differences between the two groups, it is possible that study of other IAPP-derived peptides clarify the role of this molecule and of specific CD8\(^+\) T-cells in autoimmune processes in the T2D patients. A limitation of the study was the relatively small number of evaluated patients which can be investigated in further studies.

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