Human Type 1 Diabetes Is Associated with T Cell Autoimmunity to Zinc Transporter 8

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Human Type 1 Diabetes Is Associated with T Cell Autoimmunity to Zinc Transporter 8

MyLinh Dang,1 Jennifer Rockell,1 Rebecca Wagner,1 Janet M. Wenzlau, Liping Yu, John C. Hutton, Peter A. Gottlieb, and Howard W. Davidson

Recently we demonstrated that zinc transporter 8 (ZnT8) is a major target of autoantibodies in human type 1 diabetes (T1D). Because the molecules recognized by TID autoantibodies are typically also targets of autoreactive T cells, we reasoned that this would likely be the case for ZnT8. To test this hypothesis, IFN-γ–producing T cells specific for ZnT8 in the peripheral blood of 35 patients with T1D (<6 mo after onset at blood draw) and 41 age-matched controls were assayed by ELISPOT using a library of 23 overlapping dipeptide pools covering the entire 369 aa primary sequence. Consistent with our hypothesis, patients showed significantly higher T cell reactivity than the matched controls, manifest in terms of the breadth of the overall response and the magnitude of responses to individual pools. Therefore, the median number of pools giving positive responses (stimulation index > 3) in the control group was 1.0 (range, 0–7) compared with 6.0 (range, 1–20; \( p < 0.0001 \)) for the patients. Similarly, the median stimulation index of positive responses in controls was 3.1 versus 5.0 in the patients (\( p < 0.0001 \)). Individually, 7 of 23 pools showed significant disease association (\( p < 0.001 \)), with several of the component peptides binding the disease associated HLA-DR3 (0301) and -DR4 (0401) molecules in vitro. We conclude that ZnT8 is also a major target of disease-associated autoreactive T cells in human TID, and we suggest that reagents that target ZnT8-specific T cells could have therapeutic potential in preventing or arresting the progression of this disease. The Journal of Immunology, 2011, 186: 6056–6063.

Although autoantibodies are currently the best biomarkers of type 1 diabetes (T1D) in humans, it is generally accepted that the destruction of pancreatic β-cells is principally the result of the activation and expansion of autoreactive T cells specific for β-cell Ags (1). Consequently, considerable efforts have been made over the past 25 y to identify the molecular targets of potentially diabetogenic T cells and the immunodominant epitopes within them (2). Such information has contributed significantly to our understanding of the pathophysiology of T1D, for example by revealing possible molecular mimicry between viral and islet Ags (3). It also underpins the development of peptide-based, Ag-specific therapeutic strategies, and it is responsible for the identification of novel biomarkers of preclinical disease (4).

Zinc transporter 8 (ZnT8) is primarily restricted to the islets of Langerhans, with the highest expression being in pancreatic β cells

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The online version of this article contains supplemental material.

Abbreviations used in this article: GAD65, glutamic acid decarboxylase; GADA, autoantibodies to GAD65; IA-2, insulinoma Ag 2; IAA, insulin autoantibodies; ICA512, autoantibodies to IA-2; SI, stimulation index; T1D, type 1 diabetes; ZnT8, zinc transporter 8; ZnT8A, autoantibodies to ZnT8.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/$16.00 (5). Recently we demonstrated that, depending on the age of onset, autoantibodies to ZnT8 (ZnT8A) are present in 60–80% of newly diabetic subjects attending the Barbara Davis Center clinic (6) (J.M. Wenzlau, H.W. Davidson, and J.C. Hutton, unpublished observations). This result has since been replicated by ourselves and others in different populations (7, 8); it is now evident that ZnT8A occur with a similar prevalence to those against the “gold standard” TID autoantigens proinsulin, the 65-kDa form of glutamic acid decarboxylase (GAD65), and insulinoma Ag 2 (IA-2). In addition, ZnT8A overlap with, but are independent of, these other biomarkers. Consequently, protocols that measure ZnT8A in addition to Abs against the other three major targets have a significantly enhanced detection rate of diabetes-related autoimmunity (6, 7, 9, 10).

Although humoral and cellular responses to diabetes autoantigens are believed to be activated independently (11), previous studies have shown that T cells recognizing epitopes from proinsulin, GAD65, and IA-2 can be detected in the peripheral blood of patients at the onset of clinical disease (2). Given this precedent, we reasoned that ZnT8 is also likely to be a target of autoreactive T cells in human T1D. To investigate this hypothesis, we analyzed the frequency of proinflammatory IFN-γ secreting cells in PBMCs from 35 patients with recently diagnosed diabetes (<6 mo after diagnosis) and 41 age-matched controls, using a library of overlapping peptides spanning the entire 369 aa primary sequence of ZnT8. The results of this initial study suggest that ZnT8 is indeed a significant T cell target in T1D, and identify 6 regions of the molecule that likely contain key disease associated epitopes.

Materials and Methods

Study participants

Participants of either gender aged 6–30y (Table I) were recruited from patients, relatives, and volunteers attending the Barbara Davis Center for Childhood Diabetes in accordance with protocols approved by the Colorado Multiple Institute Review Board. Subjects with clinical disease of >6 mo duration at the time of blood draw were excluded from the study, as
were control subjects who tested positive for one or more diabetes auto-
antibodies (insulin autoantibody [IAA], autoantibody to GAD65 [GADA], 
ICA512, or ZnT8). PBMCs were prepared from heparinized blood using 
Ficoll-paque Plus (GE Healthcare, Piscataway, NJ), and aliquots were 
immediately used for either ELISPOT analysis or DNA preparation.

**Peptides**

Control peptides P1 (Proinsulin C19-A3; GSLQLPLEAGEQLQKRGI), 
Insulin B9-23 (SHLYEALYYVCCGERG), GAD3 (GAD65 335-352; 
TAGITTVYAGFDPPLAVAD), GAD4 (GAD65 554-573; VNFRMYVIS- 
NHGRNQIDEDFL), R2 (1A 2 853-872; SFYLKVNOQGTRILTOEFH), 
and R5 (IA2 709-736; LAKEWLQCALYQAEPNCTATAGCENIK) (12, 
13) were synthesized at >95% purity (University of Colorado Cancer 
Center Proteomics Core) and added from stocks of 100 mM in DMSO. A 
PESPscreen library of overlapping 20mers spanning the entire 369 residue 
primary sequence of ZnT8 was obtained from Sigma Genosys (St. Louis, 
MO). The library was designed to contain 51 20mers, with consecutive 
peptide pools spanning by 3 residues. Based on this design, this provided 
an actual library of 46 peptides. Individual peptides were resus-

In vitro binding assay

Binding of ZnT8 peptides to recombinant HLA-DR3 (0301) and -DR4 
(0401) was performed by Proimmune (Oxford, UK) using their Class II 
REVEAL binding assay.

**Statistical analyses**

Statistical analyses were conducted using Prism 5 software (GraphPad 
Software, La Jolla, CA). Group comparisons used the Mann-Whitney 
U-test, and categorical variables used Fisher’s exact test. In each case, 
$p < 0.05$ was considered significant.

**Results**

Overall responses to ZnT8 in patients and controls

ZnT8 is a recently described humoral autoantigen in T1D (6). However, at present little is known regarding its relevance as a target of potentially diabetogenic T cells. The goal of this study was to determine whether ZnT8 is a significant target of proin-
flammatory T cells in patients with recently diagnosed T1D. In 
contrast to proinsulin, GAD65, and IA-2, ZnT8 is a polytopic 
integral membrane protein with six predicted transmembrane he-
lies (5), and we are currently unable to express the intact re-
combinant molecule in a form suitable for conducting T cell 
assays. Thus, as a surrogate for the intact protein, we used a 
library of overlapping peptides encompassing the entire 369 aa 
primary sequence (Fig. 1A). Given the relatively low frequency of 
islet Ag-specific autoreactive T cells in the peripheral blood of 
most patients with T1D, and the complexity of the human MHC, 
which exhibits codominant expression of alleles thereby creating 
the possibility for both cis and trans-pairing of the polymorphic 
HLA-DP and -DQ α and β-chains (for example, Ref. 19) and 
potential expression of up to 12 distinct class II molecules by 
a single individual, each with its own unique binding motif, it is 
unsurprising that previous studies have suggested that T1D asso-
ciation is most evident when multiple epitopes are considered 
together, rather than on the basis of responses to a single antigenic 
peptide (12, 14, 20, 21). Accordingly, we first examined the total 
number of ZnT8-specific T cells detected in the samples from 
each donor (Fig. 1B). A highly significant disease association was observed. Although there was no statistically significant difference 
between the background signals in the two groups (median 0 spots 
[range, 0–16] for controls and 1 spot [range, 0–24] for patients; 
p = 0.11), the median number of spots above background in the 
summed incubations from the control group was 13 (range, 0– 
300) compared with 81 (range, 18–689; p < 0.0001) in PBMCs 
from the subjects with a recent diagnosis of diabetes. Using a cut-
off defined as the upper 99% confidence limit of the control 
subjects (50.2 spots) 24 in 35 (68.6%) of the patients but only 3 in 
41 (7.3%) of controls, showed significant ZnT8-specific responses. 
To estimate the breadth of the autoresponse in each individual, 
we next calculated the total number of peptide pools giving a 
positive response (SI ≥ 3) (14) in the two groups (Fig. 1C). 
Again, a highly significant expansion in the patient group was 
observed. In the control group the median number of positive 
peptide pools was 1.0 (range, 0–7), compared with 6.0 (range, 1– 
20; p < 0.0001) in PBMCs from the patients. By this criterion 
of the subjects with T1D, but only 29/41 (70.7%) of the control 
subjects, responded to at least one ZnT8 peptide pool, although all 
control subjects responded to the positive control that contained 
a mixture of pediatric recall Ags (data not shown). The significant 
association between this measure of ZnT8 autoreactivity and T1D 
was unchanged if either less stringent (SI ≥ 2.1) or more stringent 
(SI ≥ 5) cut-offs were applied (1.0 versus 9.0 pools, p < 0.0001; 
and 0 versus 3.0 pools, p < 0.0001, respectively; Fig. 1D, 1E). 
However, using the more stringent cut-off, only 29 in 35 (82.9%)
matched controls were analyzed by IFN-γ ELISPOT as described in Materials and Methods using 23 ZnT8 dipeptide pools. We therefore repeated our analysis disregarding the second of sequential positive responses in which any duplication could occur (i.e., if positive responses were observed in pools 1–4, only the nonoverlapping pools 1 and 3 were counted; Fig. 1F). As expected, this correction selectively reduced the value for the median number of positive pools (SI ≥ 3) in the patients (controls, 1.0 versus 1.0; patients, 5.0 versus 6.0), but did not alter the level of statistically significant difference between them.

There was also a significant difference in the magnitude of the positive responses between the two groups. Overall the median SI in control individuals who responded (SI ≥ 3) to at least one peptide pool was 3.1 (29 individuals, 84 positive responses, range 3.0–30) compared with 5.0 in the patient group (35 individuals; 258 positive responses; range, 3.0–222; p < 0.0001). Similarly, on an individual basis the median SIs of responders were also significantly different in the two groups (controls [29 individuals, median SI, 3.2; range 3.0–9.0] versus patients [35 individuals, median SI, 4.5; range, 3.2–18.0; p < 0.0001]; Fig. 1G). However there was no correlation between the number of peptide pools to which any individual responded and the median SI of the positive responses in that individual (controls, p = 0.73; patients, p = 0.63; Spearman’s test).

We also analyzed responses in our subjects to a series of six previously validated control peptides from proinsulin, IA-2, and GAD65 (12, 13). Of these only GAD65335–352 showed statistically significant disease association when considered alone (p = 0.0047; Supplemental Fig. 1B). In contrast, as has been reported previously (14), when the combined responses to the control peptides were examined a highly significant association was revealed (Supplemental Fig. 1B).

**HLA-association of ZnT8 T cell autoreactivity**

T1D shows a strong association with the expression of particular MHC class II alleles, with the HLA-DRB1, -DQA1, and -DQB1 loci on chromosome 6p21 responsible for ∼50% of familial aggregation of the disease (22). In particular there is a strong positive association with the DRB1*0301-DQA1*0501-DQB1*0201, DRB1*0401-DQA1*0301-DQB1*0302, and DRB1*0405-DQA1*0301-DQB1*0302 (DR3/DR2 and DR4/DR8) haplotypes. Consistent with this association, 30 in 35 of the patient group, but only 28 in 41 of the control group, expressed at least one HLA-DQB1*0201 or -DQB1*0302 allele (Table I, Supplemental Table I). Preliminary bioinformatics analyses predicted that both low- and high-risk molecules would likely bind similar numbers of
Individuals were stratified on the basis of their expression of selected HLA molecules, and the total responses (spots above background) in each included subject is shown. A, Subjects expressing HLA-DR3/DQ2 and/or HLA-DR4/DQ8. B, Controls were stratified on the basis of HLA-DQB1*0602 or HLA-DRB1*0403 expression. C, The control group was restricted to subjects who did not express either HLA-DQB1*0602 or HLA-DRB1*0403. D, The control group was restricted to subjects who expressed either HLA-DQB1*0602 and/or HLA-DRB1*0403. The median and interquartile ranges of each group are indicated.

Table II. Summary of overall responses to ZnT8 assessed by the total number of positive pools (SI ≥ 3.0)

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>T1D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Positive Pools (range)</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>1.0 (0–7)</td>
</tr>
<tr>
<td>HLA-DR3 and/or DR4(8)</td>
<td>28</td>
<td>1.0 (0–7)</td>
</tr>
<tr>
<td>HLA-DR3 / non DR4(8)</td>
<td>12</td>
<td>1.5 (0–7)</td>
</tr>
<tr>
<td>HLA-DR4(8) / non -DR3</td>
<td>14</td>
<td>1.0 (0–7)</td>
</tr>
</tbody>
</table>

Data are median and range. Statistical comparisons were conducted with the Mann–Whitney U test.
likely, we examined whether this gender discrepancy contributed to the significant differences in ZnT8 T cell responses that we observed. As expected, no significant correlation with gender was observed in either the patient or control groups (Supplemental Fig. 1C, 1D).

The generation of high-affinity class-switched Abs, including diabetes autoantibodies, is a T-dependent process. Nevertheless, previous studies that investigated potential relationships between humoral and cellular autoimmunity to proinsulin, IA-2, and GAD65 did not reveal any significant association (11, 26), likely because of the dependence of B cell help and tissue inflammation on separate effector T cell populations (27). Consistent with the results obtained for the other diabetes autoantigens, no correlation between the ZnT8 index and the magnitude of the T cell response was observed (Supplemental Fig. 1E). Likewise, there was also no association between T cell reactivity to ZnT8 and humoral autoimmunity to insulin, GAD65, or IA-2 (data not shown).

**Multiple peptide pools from ZnT8 bind to HLA-DR4 in vitro**

Patients with recently diagnosed diabetes who express at least one copy of the risk-conferring HLA-DR4/DQ8 and/or -DR3/DQ2 haplotypes exhibit a significantly higher frequency of proinflammatory ZnT8-specific T cells in their peripheral blood than do age- and HLA-matched control subjects (Fig. 2A). Preliminary bioinformatic analyses using several public domain MHC class II prediction servers suggested that multiple peptides from ZnT8 would be expected to bind to the HLA molecules expressed by these subjects (data not shown). However, there was considerable divergence between the various algorithms, precluding a definitive conclusion regarding the behavior of individual peptides from being made. To begin to address this issue empirically, and to corroborate our ex vivo data, we used a commercial in vitro binding assay (Reveal and Prove; ProImmune) to directly examine binding peptides were present in 2 of 4 pools that showed statistically significant disease association in vitro, of which eight had assay values more than twice the threshold for positivity. Comparison between the ex vivo and in vitro libraries indicated that 16 of the 23 pools used for the T cell assays contained at least one peptide capable of binding to HLA-DR4 (0401), including 5 of 6 pools that showed substantially significant disease association in subjects expressing HLA-DR4 but not -DR3 (Fig. 3B). In contrast, only 4 in 52 (7.7%) peptides showed significant binding to recombinant HLA-DR3 (0301), with only one giving a value more than 200% of the threshold (Fig. 3A). Nevertheless, HLA-DR3 binding peptides were present in 2 of 4 pools that showed statistically significant disease association in subjects expressing HLA-DR3 but not -DR4.

A limited number of individual peptide pools from ZnT8 show T1D association

The magnitude and diversity of the overall response to ZnT8 (Fig. 1) suggests that T cells recognizing multiple epitopes within this autoantigen are expanded in the peripheral blood of subjects with recent onset T1D. Although the data shown in Fig. 3 indicate that multiple peptides from ZnT8 can bind both HLA-DR3 and -DR4, they do not determine which are naturally processed and presented. For the other major autoantigens, CD4+ T cell responses restricted to a particular MHC molecule are typically focused on a limited subset of immunodominant peptides (28). We therefore analyzed the expression of positive responses to individual ZnT8 peptide pools in the diabetic and control groups (Table III). Perhaps surprisingly, all the peptide pools elicited a response in at least 4 in 35 (11%) of the newly diabetic subjects, with T cells reactive to four pools (7, 9, 17, 21) being present in >40% of the members of this group. In contrast, only 13 in 23 pools elicited a positive response in more than 2 in 41 (4.9%) of the control subjects, with reactivity to two pools (3, 18) being undetectable in these individuals. Pair-wise analysis (Fischer’s exact test) revealed that 13 in 23 pools showed a statistically significant disease as-

![FIGURE 3. Binding of ZnT8 peptides to recombinant HLA-DR3 and -DR4 in vitro. A library of 52 15mers encompassing the entire 369 aa sequence of ZnT8 was tested for binding to recombinant HLA-DR3(0301) (A) and HLA-DR4(0401) (B), using the Class II REVEAL binding assay (ProImmune). The boxes above each profile indicate the peptide pools used in the ex vivo assays and are filled to indicate statistical association with T1D in relevant groups. *p < 0.05, **p < 0.01 (Supplemental Table III). The solid lines show the cut-off for positivity of the assay.](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Pool</th>
<th>Control (%)</th>
<th>T1D (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4</td>
<td>40.0</td>
<td>&lt;0.0001 ***</td>
</tr>
<tr>
<td>2</td>
<td>2.4</td>
<td>25.7</td>
<td>0.0044 **</td>
</tr>
<tr>
<td>3</td>
<td>0.0</td>
<td>14.3</td>
<td>0.0176 *</td>
</tr>
<tr>
<td>4</td>
<td>9.8</td>
<td>20.0</td>
<td>0.3272 NS</td>
</tr>
<tr>
<td>5</td>
<td>4.9</td>
<td>37.1</td>
<td>0.0005 ***</td>
</tr>
<tr>
<td>6</td>
<td>14.6</td>
<td>28.6</td>
<td>0.1655 NS</td>
</tr>
<tr>
<td>7</td>
<td>17.1</td>
<td>40.0</td>
<td>0.0389 *</td>
</tr>
<tr>
<td>8</td>
<td>4.9</td>
<td>28.6</td>
<td>0.0093 **</td>
</tr>
<tr>
<td>9</td>
<td>9.8</td>
<td>45.7</td>
<td>0.0005 ***</td>
</tr>
<tr>
<td>10</td>
<td>14.6</td>
<td>42.9</td>
<td>0.0095 **</td>
</tr>
<tr>
<td>11</td>
<td>24.4</td>
<td>31.4</td>
<td>0.6086 NS</td>
</tr>
<tr>
<td>12</td>
<td>4.9</td>
<td>11.4</td>
<td>0.4052 NS</td>
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<tr>
<td>13</td>
<td>14.6</td>
<td>22.9</td>
<td>0.3889 NS</td>
</tr>
<tr>
<td>14</td>
<td>12.2</td>
<td>40.0</td>
<td>0.0075 **</td>
</tr>
<tr>
<td>15</td>
<td>7.3</td>
<td>25.7</td>
<td>0.0550 NS</td>
</tr>
<tr>
<td>16</td>
<td>4.9</td>
<td>31.4</td>
<td>0.0044 **</td>
</tr>
<tr>
<td>17</td>
<td>4.9</td>
<td>57.1</td>
<td>&lt;0.0001 ***</td>
</tr>
<tr>
<td>18</td>
<td>0.0</td>
<td>31.4</td>
<td>&lt;0.0001 ***</td>
</tr>
<tr>
<td>19</td>
<td>2.4</td>
<td>34.3</td>
<td>0.0003 ***</td>
</tr>
<tr>
<td>20</td>
<td>7.3</td>
<td>22.9</td>
<td>0.0991 NS</td>
</tr>
<tr>
<td>21</td>
<td>9.8</td>
<td>48.6</td>
<td>0.0002 ***</td>
</tr>
<tr>
<td>22</td>
<td>19.5</td>
<td>28.6</td>
<td>0.4224 NS</td>
</tr>
<tr>
<td>23</td>
<td>12.2</td>
<td>28.6</td>
<td>0.0892 NS</td>
</tr>
</tbody>
</table>

Data are the percentages of the control group (n = 41) and T1D group (n = 35) who responded (SI ≥ 3) to a particular ZnT8 peptide pool. Statistical comparisons used Fischer’s exact test.

* p < 0.05, ** p < 0.01, *** p < 0.001.

NS, not significant.
sociation at the 95% confidence level, with seven (pools 1, 5, 9, 17, 18, 19, and 21) also being significant at the 99.9% confidence interval (Table III). The same group of pools also showed the highest statistical significance when the data were stratified to include only those individuals who expressed at least one HLA-DRB1*03 or *04 allele (Supplemental Table III).

Pairwise analysis of the responses to individual peptide pools in PBMCs from subjects expressing HLA-DR4 but not HLA-DR3 revealed that 6 in 23 showed T1D association, with pools 1 and 17 having the greatest statistical significance (Supplemental Table III). Comparison with the results of the in vitro binding assay indicated that the positive responses to pools 1 and 17, which were each detectable in 9 of 19 (47%) subjects with T1D, might be explained by DR4-binding peptides (Fig. 3B). Consistent with the lower level of in vitro binding to HLA-DR3 (Fig. 3A), analysis of the responses to individual peptide pools in PBMCs from subjects expressing HLA-DR3 but not HLA-DR4 revealed only 4 of 23 that showed significant disease association (Supplemental Table III). The most significant of these (pool 21) gave positive responses in 4 of 6 (66.7%) subjects with T1D, but failed to elicit a positive response in all 12 control subjects (p = 0.0049). Consistent with the involvement of this molecule, pool 21 contains a peptide that showed modest in vitro binding to HLA-DR3 (Fig. 3A).

In addition to the disease association in individuals who express HLA-DR4 but not HLA-DR3, pool 17 also showed significant disease association in subjects expressing HLA-DR3 but not HLA-DR4 (Supplemental Table III). Overall this pool could detect 57.1% of T1D subjects with 95.1% specificity (Table III). However, the most effective assay was obtained when analysis was restricted to the six nonoverlapping pools that showed the most significant disease association (pools 1, 5, 9, 17, 19, and 21; Fig. 4). In the control group, the median number of positive pools was 0 (range, 0–2), compared with 2.0 (range, 0–6; p < 0.0001) in PBMCs from the patients. Using a randomly selected cut-off predefined as the mean + 3 SD of the control group (1.9 pools), the assay showed 74.3% sensitivity at 97.5% specificity (Fig. 4A). A similar result was obtained when only those individuals expressing one or more HLA-DQ2 or -DQ8 molecule were included (Fig. 4B), although in this case the calculated cut-off was marginally higher (2.03), giving an assay with 46.7% sensitivity at 100% specificity.

**Discussion**

The results of this initial study of cellular autoimmunity to ZnT8 clearly suggest that, like the other gold-standard humoral targets, ZnT8 is a significant target of autoimmune T cells in human T1D. Based on overall activity, >68% of patients but <8% of controls showed significantly expanded numbers of ZnT8-specific proinflammatory T cells in their peripheral blood. The presence of positive responses in some controls may appear surprising, but has often been reported in studies of human T cell responses to other diabetes autoantigens (reviewed in Ref. 1), and may be indicative of the inherent immunogenicity of the Ag. In addition, it should be noted that some of the control subjects are autoantibody negative, first-degree relatives of diabetic subjects, who might be more prone to islet autoimmunity than those with more protective genotypes. Using an established cut-off, all the newly diabetic subjects tested responded to at least one peptide from ZnT8, with the majority exhibiting elevated numbers of proinflammatory T cells specific to multiple noncontiguous regions of the protein, likely indicative of epitope-spreading. Moreover, the magnitude of the signal elicited by individual pools among responders was also considerably greater in the patients than controls. The time course of the ELISPOT assay is too short to allow significant maturaton of naive cells to occur (29). Consequently the responses we observed presumably reflect the presence of elevated pools of ZnT8-specific effector or memory T cells in the peripheral blood of the diabetic subjects, with the most likely explanation for this being that it is indicative of a role of ZnT8-specific T cells in diabetogenesis. However, as is also true for all other human T cell responses to diabetes autoantigens, the relationship between peripheral T cells and those in the target organ remains a subject of debate (30, 31). Nevertheless, consistent with an Ag-driven process, the peripheral frequency of ZnT8-specific, IFN-γ producing T cells after onset appeared greatest within 6 mo of clinical diagnosis, with individuals tested at later ages typically showing a much reduced response (data not shown). Given that cross-presentation of free 20mer peptides is typically inefficient (32), we assume that the responses we observed were predominantly, if not exclusively, derived from CD4+ T cells. However, because the library we used may contain some truncated peptides, we cannot entirely exclude the possibility that CD8+ T cells may also have contributed, although our recent studies using highly purified peptides suggest that this is unlikely to have significantly impacted the results we obtained.

When considered individually, 13 of the 23 dipeptide pools showed statistically significant disease association at the 95% confidence level. Although at first glance this may seem unexpectedly high, the human MHC is highly complex, with any individual expressing up to 12 different class II molecules, most if not all of which have the potential to bind multiple peptides from ZnT8. The existence of ZnT8-specific T cells restricted to many different class II molecules, both within an individual, and in the control and patient populations as a whole, likely explains both the breadth of the autoresponse we observed, and the fact that all of the pools gave positive responses in at least 11% of the patients tested. The degree of complexity of the response only increased when the components of the most reactive pools were tested individually in a separate cohort of patients (data not shown). To date we have only conducted direct binding studies with two of the

![FIGURE 4. T1D association of proinflammatory T cell reactivity to ZnT8. PBMCs from 35 subjects with a recent diagnosis of diabetes and 41 age-matched controls were analyzed by IFN-γ ELISPOT as described in Materials and Methods using 23 ZnT8 di-peptide pools, and stratified on the basis of the six nonredundant pools giving the most significant disease association (Table III). A. The number of selected peptide pools giving positive responses (SI > 3) in each subject is shown. B. The number of selected peptide pools giving positive responses in subjects who expressed at least 1 HLA-DR3/DQ2 or HLA-DR4/DQ8 haplotype is shown. The median and interquartile ranges of each group are indicated. The dotted line shows a cut-off for positivity based on the mean + 3 SD of the respective control group.](http://www.jimmunol.org/)

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HLA-DR molecules expressed by the subjects in our study. However, the level of T cell reactivity we observed appears consistent with in silico predictions that suggest that most of the HLA-DR molecules expressed by our subjects, including some of those deemed protective, have the capacity to bind peptides from multiple pools of the library. The bioinformatics-based predictions suggest that ZnT8 is inherently immunogenic, and the magnitude of the response we observe implies that its potential as a target of cellular immunity is realized in many patients, perhaps because ZnT8-specific T cells are subject to minimal negative selection. For tissue-specific Ags such as proinsulin, central tolerance is believed to depend on thymic and extra-thymic expression driven by transcriptional activators such as AIRE (33, 34) and Deaf1 (35). Interestingly, ZnT8 (slc30a8) was not identified among the genes selectively expressed by mouse thymic medullary epithelial cells (36), and we are unaware of any evidence of thymic expression in humans. Therefore, it is tempting to speculate that tolerance to ZnT8 is largely the result of immunologic ignorance, and once this is overcome a robust response may ensue.

It is possible that the actual diversity of the auto-response might be slightly exaggerated by our methodology, with the overlap of the constituent peptides in consecutive pools meaning that some of the disease-associated responses we observed might not be redundant. For example, part or all of the reactivity to pool 18 could be due to epitopes also in pools 17 and 19. Nonetheless, our data strongly suggest the presence of a minimum of nine disease-associated T cell epitopes in human ZnT8. Coincidentally, this value is similar to those obtained in studies using overlapping peptide libraries encompassing GAD65 (37) and the cytoplasmic domain of IA-2 (38). Because the HLA haplotypes exhibited by the two groups were not identical, we cannot discount the possibility that variations in detection limits within the two groups might have influenced the magnitude of the differences in overall reactivity that we observed. However, its contribution is clearly insufficient to account for the results we obtained, and a highly statistically significant disease association was also evident when our analysis was limited to nonoverlapping peptide pools (Figs. 1F, 4), when this concern no longer applies.

Our decision to use a library of overlapping peptides rather than focusing solely on those predicted to bind to high risk alleles was intended to ensure an unbiased analysis of the overall proinflammatory CD4+ response in patients and controls. However, the fine mapping of epitopes is highly problematic using this experimental approach, and currently we cannot be certain of the total number of immunodominant ZnT8 epitopes or of their restriction to any particular class II molecule. Nevertheless, our results clearly indicate that like ZnT8A, ZnT8-directed T cell autoreactivity is associated with, but not restricted to, both the DR4/DQ8 and DR3/DQ2 high-risk haplotypes. In particular the HLA-DR4 (0401) molecule, which was expressed by 19 in 35 (54.3%) of the diabetic group, appears to be a major element in ZnT8 autoimmunity, with several peptides that bind this molecule including ZnT8622 and ZnT815–29 (pool 1), ZnT8200–213 and ZnT8214–248 (pool 9), ZnT8260–274 (pool 17), ZnT8267–281 (pools 17 and 18), and ZnT8305–309 (pool 19) possibly containing disease-related epitopes. Similarly, ZnT815–169 (pool 10) and ZnT8323–337 (pool 21) may contain HLA-DR3 restricted epitopes.

In a previous study of autoreactivity to IA-2 and proinsulin, Arif et al. (14) reported a reciprocal polarization toward IL-10 or IFN-γ production for peripheral T cells from patients and controls specific for the same epitope. In the current study, we were unable to measure both IFN-γ and IL-10 responses to the entire peptide library in the majority of our study subjects because of the limited amount of blood available, although preliminary results from the subset tested for both cytokines revealed essentially equivalent ZnT8-specific IL-10 responses in both patients and controls (data not shown). Our identification of seven key disease-associated ZnT8 peptide pools will enable us to address this and other important questions relating to the role of ZnT8 in T1D pathogenesis, such as the temporal appearance and disappearance of ZnT8-specific T cells during disease progression, and whether ZnT8 is also a significant target of natural regulatory T cells. Our identification of seven key disease-associated ZnT8 peptide pools also provides the basis for the development of novel reagents that either alone or in combination could be used for Ag-specific therapeutic intervention to arrest the progression of the disease.

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Disclosures

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