A common non-synonymous single nucleotide polymorphism in the SLC30A8 gene determines ZnT8 autoantibody specificity in type 1 diabetes.

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**Objective:** Zinc transporter 8 (SLC30A8) is a major target of autoimmunity in human type 1A diabetes (T1D) and is implicated in type 2 diabetes (T2D) in genome-wide association studies. The T2D non-synonymous SNP affecting aa_325 lies within the region of highest ZnT8 autoantibody (ZnT8A) binding and prompted an investigation of its relationship to T1D.

**Research Design and Methods:** ZnT8A radioimmunoprecipitation assays were performed in 421 new onset T1D Caucasians using C-terminal constructs incorporating the known human aa_325 variants (Trp, Arg and Gln). Genotypes were determined by PCR-based SNP analysis.

**Results:** Sera from 224 subjects (53%) were reactive to Arg_325 probes, 185 (44%) to Trp_325, and 142 (34%) to Gln_325. 60 subjects reacted only with Arg_325 constructs, 31 with Trp_325 only and 1 with Gln_325 only. The restriction to either Arg_325 or Trp_325 corresponded to the inheritance of the respective C- or T alleles. A strong gene dosage effect was also evident as both Arg- and Trp restricted ZnT8A were less prevalent in heterozygotes than homozygous individuals. The SLC30A8 SNP allele frequency (75%C; 25%T) varied little with age of T1D onset or the presence of other autoantibodies.

**Conclusions:** The finding that diabetic autoimmunity can be defined by a single polymorphic residue has not been previously documented. It argues against ZnT8 autoimmunity arising from molecular mimicry and suggests a mechanistic link between the two major forms of diabetes. It has implications for antigen-based therapeutic interventions since the response to ZnT8 administration could be protective or immunogenic depending on an individual’s genotype.
Human type 1A diabetes (T1D) results from autoimmune destruction of pancreatic β-cells targeted at a restricted number of autoantigens, many of which show high β-cell specificity of expression (1). Susceptibility to the disease is associated with multiple genetic loci, most prominently HLA alleles encoding particular MHC class II glycoproteins (2).

ZnT8 is a newly-discovered target of T1D autoimmunity (3) localized to the insulin granule of pancreatic β-cell. It is encoded by SLC30A8, one of nine human genes for multispanning transmembrane proteins facilitating Zn\(^{2+}\) efflux from the cell and sequestration into intracellular compartments (4; 5). Recent genome-wide association studies (GWAS) demonstrate association of ZnT8 gene polymorphisms with human Type 2 diabetes (6-9) notably a nonsynonymous SNP encoding either Arg or Trp at aa325. The major, Arg 325-encoding C-allele confers a minor risk (odds ratio 1.07-1.18) of disease. In non-diabetic subjects with a family history of T2D the C-allele was associated with increased insulin sensitivity (10), increased circulating proinsulin/insulin ratio (11) and decreased insulin responses in IVGTT (12) indicating a dominant effect on insulin secretion and/or βcell mass.

We report here that the T1D autoimmune response to ZnT8 is focused on a few key epitopes, two of which are defined by the polymorphic aa325 residue. To our knowledge this is the first reported instance where a polymorphic variant determines the specificity of the autoimmune response. It indicates that the autoreactive B-lymphocyte repertoire is restricted to a few ZnT8 epitopes and is truly self-reactive as opposed to arising as a bystander response to a foreign antigen.

METHODS

Serum samples (n=421) were obtained within 2 weeks of T1D diagnosis from patients attending the Barbara Davis Center (median age 11.3 (0.6-58yr), 87% Caucasian, 6.3% Hispanic). The 150 controls (median age 13.1 (1-55yr), 72% Caucasian, 15.1% Hispanic) were parents and children in the DAISY general population cohort and parents of the sibling / offspring cohort (13). The male/female gender ratio in both groups was 0.8. Informed consent was obtained under approved Institutional Review Board oversight.

Genomic DNA was extracted from heparinized blood from 352 of the above T1D patients using standard procedures. Polymorphic variations in the SLC30A8 gene were determined by qPCR using Taqman probes and an ABI7000 (ABI, Waltham, MA) targeting the nonsynonymous SNP rs13266634, rs2466295 in the 3' untranslated region and rs6469675 in intron 2. Ascertainment rates were >99%.

ZnT8A radioimmunoprecipitation assays used \(^{35}\)S-Met-labeled in vitro transcribed and translated probes of hZnT8 C-terminal cytosolic segments (aa268-369) encoding the aa325 codon variants, CCG (Arg), TCG (Trp) and CAG (Gln) (Supplementary Fig 1). Assay procedures are detailed elsewhere (3; 14). ZnT8A assays data were normalized to a pan-reactive positive control sera (1:50) generated in rabbits to a glutathione-S-transferase / C-term Trp325 fusion protein and 16 human control sera in the same assay (3). Recombinant NUS-ZnT8 fusion proteins were generated in pET43.1 (EMD Biosciences, San Diego, CA), expressed in BL-21(DE3) E. coli and purified by Ni NTA agarose chromatography (Qiagen, Hilden, Germany). Synthetic 20-mer peptides were from Sigma Genosys, Woodlands, TX. For preabsorption, sera (5µl) were preincubated with 10µg protein or peptide in 40µl PBS at 20°C for 2h before addition of the radiolabeled antigen to initiate the assay. Results are expressed as mean±SD; statistical analyses were performed with the Prism 4 software package www.graphpad.com.

RESULTS

The present study was initiated to resolve a paradoxical finding that two constructs bearing the C-terminal antigenic region of ZnT8 with or without the N-terminus (Supplementary Fi g1) were recognized in a
differential fashion by subsets of T1D new onset sera (3). The constructs were derived from different cDNAs and subsequently shown to encode the Arg (C-probe) or Trp (NC-probe) variants of aa 325. To further explore this phenomenon, assays were performed on sera from newly-diagnosed T1D patients using C-terminal ZnT8 probes bearing Arg, Trp or Gln at aa325. 259 of 421 individuals (61.5%) reacted to at least one probe with the highest response recorded to the Arg variant (53.2%) followed by Trp (43.9%) and Gln (33.7%) (P<0.0001, Chi-square). Analysis of the overlap in responses (Fig 1A) show that some individuals react to the Arg or Trp probes alone and very rarely to Gln alone. 29.7% of individuals reacted to all 3 probes.

A comparison of the levels of autoantibody reactivity to the Arg and Gln probes (Fig 1B) showed that the majority of individuals either reacted equivalently to the probes (falling within the bounds of the diagonal of the XY plot ± 3SD) or responded to the Arg probe alone. Trp and Gln reactivities (Fig 1C) were similarly separated. Of the 34 patients who reacted equivalently to Arg and Trp probes (with the bounds of the diagonal ± 3SD of Fig 1D), 29 (85.3%) had an equivalent response as determined by the Gln probe. This indicated that for these individuals the aa at position 325 was not a determinant of autoantibody reactivity. A series of preabsorption experiments were therefore performed using peptides and recombinant proteins as competing ligands (Fig 2). Selected T1D sera that reacted with the Arg probe alone were blocked by recombinant NUS-C-term Arg325 protein but not by NUS-C-term Trp325 or NUS-C-term Gln325. Similarly, Trp-only responses were blocked by NUS-C-term Trp325 but not NUS-C-term Arg325 or NUS-C-term Gln325. Sera that reacted equivalently to Arg, Trp and Gln probes were blocked by any of the NUS-C-term ZnT8 recombinants. Overlapping 20-mer peptides spanning the ZnT8 C-terminal from 268-369 did not compete for reactivity suggesting that the epitopes were conformational rather than linear. Overall, these results suggested that ZnT8A reactivity could be accounted by 3 classes of conformational epitopes; one for which Arg325 was an essential determinant, a second Trp325-restricted and a third not affected by aa325.

The relationship between ZnT8 autoantibody reactivity and genetic variation at the SLC30A8 locus was examined using SNP (rs13266634) encoding the Arg/Trp325 variant and for two adjacent non-coding SNPs identified in the T2D GWAS study (6), rs2466295, located 259 bp distally in the 3’UTR and rs6469675, located 19635bp proximally in intron 2. The minor allele frequency (MAF) for rs13266634 in our T1D population of 0.266 (n=351) approximated the reference frequency of 0.256 (n=168) for Europeans in the NLM SNP database (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=13266634) and the distribution of genotypes (55.3% CC, 36.2% CT, 8.5% TT) was consistent with the Hardy Weinberg distribution (53.9, 39.0 7.1). Similar correspondences were observed of the MAF for rs6469675 (0.285 vs 0.220) and rs2466295 (0.361 vs 0.407).

The specificity of the ZnT8 autoantibody response reflected the rs13266634 genotype (Table 1) with little or no association being observed with the adjacent rs2466295 and rs6469675 SNPs. The ZnT8A response assessed by the Gln probe showed no significant variation with the rs13266634 genotype whereas responses to the Arg probe were highest in CC homozygotes, lowest in TT homozygotes and intermediate in the heterozygote group. Conversely, responses to the Trp probe were highest in TT homozygotes, lowest in CC and intermediate in heterozygotes. An even stronger relationship with genotype was seen in the groups having only Arg325- and Trp325-restricted responses. Arg325-only responses were observed only in individuals bearing the rs13266634 C-allele, with a 4.2 fold higher frequency in homozygotes than heterozygotes. With one exception, all Trp325-restricted responses were associated with the rs13266634 T-allele with a 10.2 fold higher frequency in homozygotes than heterozygotes. The single Gln325-restricted
ZnT8A patient (Fig 1A) was genotyped CC and confirmed by sequencing. The small number of sera that were positive for both Trp325− and Arg325−, but not Gln325 probes were associated with heterozygote genotypes (11 of 13 cases) as expected (data not shown). The prevalence of IAA, GADA or IA2A response did not change as a function of rs13266634 genotype.

The median age of onset of disease in the genotyped individuals was 11.2 yr (range 0.6-58) with more than half (57.3%) being diagnosed between 8 and 16, and 88.9% before age 18. A frequency distribution analysis based on binning at 4 year intervals showed no statistically significant difference in the representation of the CC, CT and TT genotypes at any age of onset, though a trend was observed for a higher CC and lower CT genotype frequency in the youngest onset group (0.6-4 yr) compared to that of a 5-15 year old reference group (Fisher Exact p=0.24, n=219) (Fig 3A).

The prevalence of ZnT8A measured with Gln325, Arg325 and Trp325 C-terminal probes increased with increasing age of onset, reached a plateau at 8-16 years, and then fell (Fig 3C and D). Arg325-restricted and Trp325-restricted responses were observed in all age groups but the low numbers of positive individuals did not permit ascertainment of changes in prevalence and levels of reactivity with age (Fig 3D). The autoantibody responses to insulin, GAD65 and IA-2 showed characteristic changes in prevalence relative to age of onset of disease, IAA prevalence being highest in younger onset patients, IA2A tending to be higher in adolescents than children and GADA showing little variation (Fig 3B).

DISCUSSION

The C-terminal domain of ZnT8 to which T1D autoantibodies bind (3) incorporates a conserved protein fold found in the large family of CDF efflux solute carriers and has orthologs in all cellular organisms (15). Autoantibodies to ZnT8 in human T1D patients however show little cross-reactivity to other human Zn transporters or even to mouse ZnT8, which is 82% identical in sequence. Even more remarkably we show here that the amino acid encoded by a common polymorphism in human ZnT8 at aa325 is a key determinant of two of the three major conformational epitopes in the protein. Given that antibody responses in any individual are polyclonal and the structural variation in the antibody repertoire that occurs between genetically identical individuals, such epitope restriction is remarkable and in light of this are termed iso-epitopes. Polymorphic variants of other diabetes-related autoantigens exist but to our knowledge none have been implicated as determinants of humoral autoreactivity, though clearly this bears further scrutiny.

The autoantibody responses to the ZnT8 Arg- and Trp-restricted isoepitopes segregated with the alleles encoding the respective variant amino acids, indicating that humoral T1D autoimmunity to ZnT8 is directed against self and not against non-self epitope determinants. This argues against the molecular mimicry hypothesis that suggests that autoimmunity is triggered by an initial immune response to a infectious agent that in turn triggers reactivity to self because of sequence homology between the pathological agent and a self protein (16-20). Our results favor the idea that ZnT8 autoreactivity arises because of a defect in induction of self tolerance since the mimicry model would more likely favor one isoepitope than another which in turn would be manifest as genetic dissociation of the encoding allele with the disease. The MAF of rs13266634 SNP in the T1D population under study was however similar to reference populations and no association of the SNP was seen with age of onset of diabetes or the prevalence of antibodies to ZnT8 or other diabetes autoantigens. We cannot however preclude a role for molecular mimicry in T cell recognition of antigenic peptides or in antigen presentation to CD4+ T-cells since antigen / antibody binding can directly influence the peptides presented from the antigen by virtue of altering intracellular proteolytic processing (21; 22).

While the rs13266634 genotype or ZnT8 isoepitope specificites may not
markedly affect T1D susceptibility or age of onset, their measurement will be important in a number of clinical settings. Since ZnT8 autoantibodies provide an independent marker of disease susceptibility in prediabetic individuals (3) measurements based on a single aa325 probe would underestimate ZnT8 autoimmunity by as much 20% given the differences in rs13266634 SNP allele frequency (12) and so affect inclusion in clinical trials. Given its high tissue specificity, ZnT8 is an attractive candidate as a component of a DNA or peptide based vaccine (23; 24) to prevent or retard the onset of T1D. In this context it is likely to be important to match the molecular form of the antigen to the recipient since mismatching the isoepitope might lead to immunization and acceleration of disease rather than induction of tolerance.

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### Table 1. Prevalence of autoantibody in relation to rs13266634 genotype.
Serum from each T1D subject was assayed with ZnT8 C-term probes incorporating Gln or Arg or Trp at aa325 or insulin, GAD65 or IA2. P values were calculated by a 3X2 Fisher exact test comparing the seropositivity (> index 0.02) to the number of subjects stratified by rs13266634 genotypes.

<table>
<thead>
<tr>
<th>Response</th>
<th>XX (351)</th>
<th>CC (194)</th>
<th>CT (127)</th>
<th>TT (30)</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
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<td>Any probe+</td>
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<td>128</td>
<td>66.0</td>
<td>70</td>
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<tr>
<td>All probes+</td>
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<td>63</td>
<td>32.5</td>
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<td>38.7</td>
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<tr>
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<td>71</td>
<td>36.6</td>
<td>61</td>
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<td>1</td>
<td>0.5</td>
<td>0</td>
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<td>Arg only</td>
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<td>45</td>
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<td>5.1</td>
<td>1</td>
<td>0.5</td>
<td>5</td>
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<td>89</td>
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<td>72.4</td>
<td>144</td>
<td>74.2</td>
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</table>
Figure 1. Relationship between autoantibody responses to Arg₃₂₅, Trp₃₂₅ and Gln₃₂₅ constructs.

A. Venn diagram illustrating the overlap of antibody detection with each of the polymorphic probes in the entire population (n=421) in the study. The prevalence in each sector is expressed as a percentage of the population total.

B. to D. Assays were performed on the same set of 117 new onset T1D individuals and stratified as indicated and the numbers in each sector shown in parentheses. The cutoffs for positive responses was set at 0.05 (vertical and horizontal lines). Responses judged to be equivalent are set by the boundaries indicated by the angled lines which correspond to a 3 SD excursion from the diagonal assuming an intrassay CV of 12.5% for each sample.

Data is expressed as the immunoprecipitation index (sample-control)/(positive sample (BUNE)-control).
Figure 2. Preabsorption of autoantibodies with recombinant proteins.

A. Single sera samples were selected from hCArg-restricted, hCTrp-restricted or samples that react equivalently with hCGln, hCArg and hCTrp probes. Samples were preincubated with 10µg of the indicated affinity purified NUS-C-term ZnT8 fusion protein for 2h at room temperature before addition of the designated labeled probe then processed by the usual procedure.

B. Panels of hCArg-restricted, hCTrp-restricted or aa325 unrestricted sera were individually preincubated with 10µg of the indicated affinity purified NUS-C-term ZnT8 fusion protein for 2h at room temperature before addition of the designated labeled probe then processed by the usual procedure.
**Figure 3.** Prevalence of genotypes, autoantibodies to ZnT8 and other antigens in relation to age of clinical diagnosis.

Data from 335 individuals were subdivided in groups 4 years apart and analyzed with respect to distribution of genotypes (A), presence of insulin, GAD and IA2 autoantibodies (B), ZnT8 autoreactivity as defined by Gln, Arg and Trp probes (C) and ZnT8 autoreactivity relative to isoepitope classification (D). Statistical significance relative to 5-15yr old reference group was determined by Fischer Exact test: * P<0.05; ** P<0.01.