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Zinc Transporter 8 Autoantibodies and Their Association With *SLC30A8* and *HLA-DQ* Genes Differ Between Immigrant and Swedish Patients With Newly Diagnosed Type 1 Diabetes in the Better Diabetes Diagnosis Study

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We examined whether zinc transporter 8 autoantibodies (ZnT8A; arginine ZnT8-RA, tryptophan ZnT8-WA, and glutamine ZnT8-QA variants) differed between immigrant and Swedish patients due to different polymorphisms of *SLC30A8*, *HLA-DQ*, or both. Newly diagnosed autoimmune (≥1 islet autoantibody) type 1 diabetic patients (n = 2,964, <18 years, 55% male) were ascertained in the Better Diabetes Diagnosis study. Two subgroups were identified: Swedes (n = 2,160, 73%) and immigrants (non-Swedes; n = 212, 7%). Non-Swedes had less frequent ZnT8-WA (38%) than Swedes (50%), consistent with a lower frequency in the non-Swedes (37%) of *SLC30A8* CT+TT (RW+WW) genotypes than in the Swedes (54%). ZnT8-RA (57 and 58%, respectively) did not differ despite a higher frequency of CC (RR) genotypes in non-Swedes (63%) than Swedes (46%). We tested whether this inconsistency was due to HLA-DQ as 2/X (2/2; 2/y; y is anything but 2 or 8), which was a major genotype in non-Swedes (40%) compared with Swedes (14%). In the non-Swedes only, 2/X (2/2; 2/y) was negatively associated with ZnT8-WA and ZnT8-QA but not ZnT8-RA. Molecular simulation showed nonbinding of the relevant ZnT8-R peptide to DQ2, explaining in part a possible lack of tolerance to ZnT8-R. At diagnosis in non-Swedes, the presence of ZnT8-RA rather than ZnT8-WA was likely due to effects of *HLA-DQ2* and the *SLC30A8* CC (RR) genotypes. ***Diabetes*** **61:2556–2564, 2012**

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More than 90% of childhood type 1 diabetes (T1D) in Caucasian populations are classified as autoimmune diabetes in association with HLA class II genes (1). The autoimmune response involves production of islet autoantibodies, and their types and number assist in prediction (2,3) and classification (4) of T1D.

Recently, the zinc transporter 8 (ZnT8) was described as a target of autoimmunity in childhood T1D (5,6) and adult-onset autoimmune diabetes (7). The ZnT8 is a 41-kDa membrane protein of β-cell secretory granules and a member of the zinc transporter (ZNT)/SLC30 subfamily of the cation diffusion facilitator family (8). ZnT8 is thought to play an essential role in insulin crystallization and secretion through permitting cellular efflux of zinc (8,9). Two single nucleotide polymorphisms (SNPs) of the ZnT8 gene *SLC30A8* determine single amino acid (aa) variation at position 325 of the cytosolic segments of ZnT8: *1*) rs13266634, codes for either arginine (CGG) or tryptophan (TGG), and *2*) rs16889462, codes for glutamine (CAG) (5,10). The single aa polymorphism at position 325 of ZnT8 identifies three antigenic variants: arginine (ZnT8-R), tryptophan (ZnT8-W), or glutamine (ZnT8-Q).

Autoantibodies against the ZnT8 (ZnT8A) were found in 63% of T1D patients, 2% of healthy controls, and 3% of type 2 diabetic (T2D) patients (6). ZnT8A were also detected among 26% of T1D patients who were negative for other islet autoantibodies (6). Therefore, it has been suggested that adding ZnT8A would detect >95% of T1D patients (11). Furthermore, ZnT8A were detected in 81% of children who progressed to T1D in the BABYDIAB study (12), indicating their importance in prediction of autoimmune childhood diabetes. This progression to diabetes was found to be associated with the CC genotype of *SLC30A8*, which was also associated with younger (<5 years), newly diagnosed T1D patients (13). However, genomewide association studies did not yet confirm the association between *SLC30A8* and T1D (14). Nevertheless, the *SLC30A8* is associated with T2D (15) because the C allele was found to confer 14 and 16% increased risk for T2D in Europeans and Asians, respectively (16). Furthermore, 46% of European nondiabetic offspring of T2D patients was found homozygous for CC genotype of the *SLC30A8* and prone to diabetes (17). In T1D patients, the C allele (R) of *SLC30A8* genotype was associated with higher stimulated C-peptide

levels (18) during the first year following diagnosis. This suggests that the two variants (RR and WW) of ZnT8A may reflect different clinical outcomes.

Recent data suggest that DQ molecules can modulate autoimmune response through differential bindings to islet autoantigen fragments (19). These data showed that the binding of the insulin antigenic peptide to HLA-DQ molecules may induce regulatory or proinflammatory responses of T cells depending on which DQ molecule is involved in this binding. In vitro studies showed that the peptide pools containing the whole 369-aa ZnT8 sequence are targeted by autoreactive T cells, especially in DR3-DQ2 and DR4-DQ8 carriers (20), indicating their importance in ZnT8 presentation. However, it is still unclear whether T-cell epitope differs from the B-cell epitope defined by the autoantibody-binding site. Glutamic acid decarboxylase (GAD65)-specific B cells and the autoantibodies they secrete were reported to affect the autoimmune T-cell repertoire by downregulating T-cell epitopes in an immunodominant area while boosting epitopes in another part of the autoantigen (21).

In Sweden, 12–15% of children <18 years have non-Swedish backgrounds. Immigrant T1D patients in Sweden present different HLA-DQ and islet autoantibody associations from native Swedish patients (22). Therefore, we studied the three ZnT8A variants (ZnT8-RA, ZnT8-WA, and ZnT8-QA) and their *SLC30A8* and HLA-DQ associations among immigrant (non-Swedish) and Swedish patients with newly diagnosed autoimmune (≥1 autoantibody) T1D. We also investigated the T1D-susceptible HLA-DQ peptide-binding motifs within the 369-aa ZnT8, focusing on the region around aa polymorphism at position 325.

RESEARCH DESIGN AND METHODS

Study design. Participants were recruited from the national Better Diabetes Diagnosis (BDD) study in Sweden. The BDD study design was previously described (22). The World Health Organization criteria for diagnosis and classification of T1D were used to determine clinical diagnosis (23,24); however, we included only patients with autoimmune T1D who were positive for at least one islet autoantibodies. Dried blood spots (DBS) for HLA-DQ typing, blood for SLC30A8 genotyping, and serum samples for islet autoantibodies (GAD65A, islet antigen-2 autoantibodies [IA-2A], insulin autoantibodies [IAA], ZnT8-RA, ZnT8-WA, and ZnT8-QA) were used.

The ethnic origin was defined by country of birth of parents and grandparents and was obtained from a questionnaire.

The Karolinska Institute Ethics Board approved the BDD study (2004/1:9).

Study population. A total of 3,686 newly diagnosed patients (<18 years) with childhood diabetes in the BDD study during the period May 2005 to January 2011 were recruited. We identified a total of 2,964 patients with autoimmune T1D for whom autoimmune status and country of birth were known. Three subgroups were identified based on the origin of all parents and grandparents: Swedes (2,160; 73%), non-Swedes (212; 7%), and Swedish-mixed origins (592; 20%) (Fig. 1). Non-Swedes were defined as patients whose parents and grandparents were all born outside Sweden, and Swedes were patients whose parents and grandparents were all born in Sweden. The classification of non-Swedish origin relied on the definition used by the Swedish Central Bureau of Statistics of foreign background: "any person born outside Sweden or born in Sweden to parents who in turn both were born outside Sweden." Two main geographical aggregates were observed within the non-Swedish subgroup, the Middle East and North Africa (including Somalia: 58%) and South-East Europe (mainly former Yugoslavia: 24%). Only 9% of the non-Swedes could be considered as immigrants from European countries with relatively high T1D risk (e.g., U.K. and Denmark); however, some of these patients had mixed European/non-European origins. In a previous analysis (22), we reported that the mixed group did not show significant differences in the frequencies and associations of immunogenetic markers from the Swedish patients, and therefore, the mixed group was excluded in the current analysis.

The majority (86%) of autoimmune T1D patients were diagnosed before 15 years of age and almost 18% diagnosed before the age of 5 years. Males

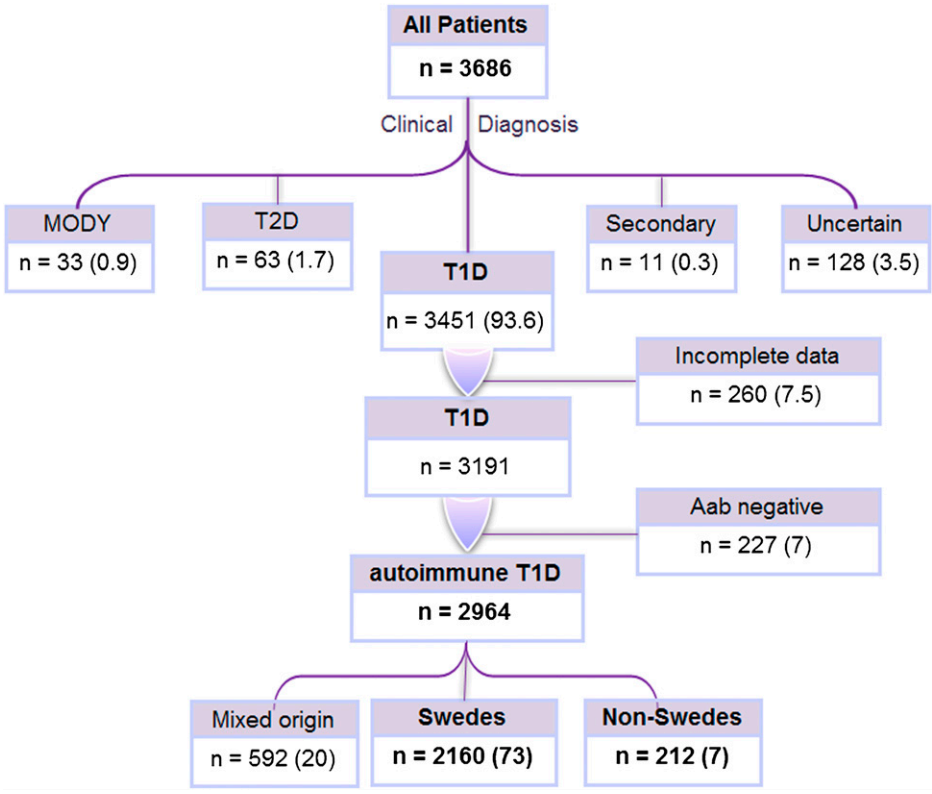

FIG. 1. Classification of patients according to country of birth of parents and grandparents. A total of 2,964 patients with autoimmune (≥1 autoantibody positive) T1D were included in this analysis. Non-Swedes (immigrants) accounted for 7% of patients, Swedes accounted for 73%, whereas 20% had mixed origins. Aab, autoantibody. (A high-quality color representation of this figure is available in the online issue.)

TABLE 1
Frequencies of islet autoantibodies and HLA-DQ genotypes in non-Swedes and Swedes

	Origin (country of birth)	
	Non-Swedes	Swedes
n (%)	212 (7)	2,160 (73)
Mean age (SD) (years)	9.4 (4.1)	9.8 (4.4)
Males [*n* (%)]	105 (50)	1,187 (55)
Autoimmunity [*n* (%)]		
IA-2A	139 (66)	1,728 (80)*
GAD65A	150 (71)	1,306 (61)*
IAA	64 (30)	749 (35)
ZnT8A (≥2/3)	131 (62)	1,499 (69)
ZnT8-RA	120 (57)	1,244 (58)
ZnT8-WA	81 (38)	1,083 (50)*
ZnT8-QA	64 (30)	729 (34)
Multiple Aab (≥2/6)	165 (78)	1,814 (84)
HLA-DQ [*n* (%)]		
2/8	37 (18)	660 (31)†
8/X‡		
8/8	13 (6)	258 (12)
8/y	32 (15)	639 (30)
2/X§		
2/2	33 (16)	73 (3)
2/y	50 (24)	236 (11)
X/X	44 (21)	269 (13)†

*ZnT8-WA and IA-2A were less frequent in non-Swedes than Swedes ($P = 0.001$, $P < 0.0005$), respectively, whereas GAD65A were more frequent in non-Swedes than Swedes ($P = 0.003$). †Non-Swedes predominately had DQ2 ($P < 0.0005$) compared with Swedes who had DQ8 ($P < 0.0005$) and DQ2/8 ($P < 0.0005$). ‡DQ8/X (03:02/X) includes DQ8/8 and DQ8/y; y any other haplotype except DQ2. §DQ2/X (05:02/X) includes DQ2/2 and DQ2/y; y any other haplotype except DQ8. Aab, autoantibody.

(1,879; 55%) had slightly higher (10.1; SD = 4.45) mean age of diagnosis than females (9.4; SD = 4.24) (Table 1).

Islet autoantibody analysis

ZnT8 autoantibodies. Serum samples were analyzed for ZnT8 autoantibodies (ZnT8A)—arginine (ZnT8-RA), tryptophan (ZnT8-WA), and glutamine (ZnT8-QA)—using the radioligand binding assay as previously described (25). Briefly, the COOH-terminal constructs of ZnT8 were prepared using a Phusion site-directed mutagenesis kit (Finnzymes Oy, Espoo, Finland). The [^{35}S]methionine-labeled antigens were incubated overnight at 4°C with duplicate serum samples followed by precipitation of immune complexes with protein A-Sepharose (PAS; Amersham Biosciences, Uppsala, Sweden). The antibody-bound radioactivity was counted in a β-counter (1450 MicroBeta TriLux Microplate Scintillation-Luminescence Counter; PerkinElmer, Boston, MA), and concentrations of antibodies were estimated from a known standard curve and analyzed in GraphPad Prism 4.0 software (GraphPad). Our assays showed comparable precision (intra-assay coefficient of variation [CV] was 5.5% for ZnT8-RA, 5.3% for ZnT8-WA, and 4.9% for ZnT8-QA) and reproducibility (interassay CV was 13.8% for ZnT8-RA, 6.7% for ZnT8-WA, and 11.0% for ZnT8-QA). In the Diabetes Autoantibody Standardization Program (DASP) 2010 workshop (26), our laboratory was among the top-ranking laboratories in assays performance with workshop sensitivity of 52% and specificity of 100% for ZnT8-RA, 50 and 100% for ZnT8-WA, and 38 and 100% for ZnT8-QA, respectively.

GAD65A and IA-2A. Recombinant GAD65 and IA-2 were labeled with [^{35}S]methionine (GE Healthcare Life Sciences, Amersham, U.K.) by in vitro-coupled transcription and translation in the TNT SP6 coupled reticulocyte lysate system (Promega, Southampton, U.K.) as described (27). Full-length cDNA coding for human GAD65 in the pTNT vector (Promega) (pThGAD65) or the intracellular domain (aa 603–980) of IA-2 in the pSP64 Poly(A) vector (Promega) (IA-2ic) was used (28). GAD65A and IA-2A were analyzed in a radioligand binding assay (27) in samples eluted from DBS. The DBS discs were incubated overnight at 4°C in 80 μL Tris-buffered saline with Tween 20 with shaking to elute antibodies. In the autoantibody assays, 30 μL DBS eluate was incubated with 24,000 counts per minute (cpm) of ^{35}S-labeled GAD65 or IA-2 in Tris-buffered saline with Tween 20 in a final reaction volume of 60 μL. The samples were transferred to filtration plates (Millipore, Solna, Sweden) and free ^{35}S-labeled GAD65 or IA-2 separated from antibody bound with PAS (Zymed Laboratories, San Francisco, CA). Supermix scintillation cocktail (PerkinElmer) was added and the radioactivity of antibody bound ^{35}S-labeled GAD65 or IA-2 counted in a Wallac Microbeta Trilux (PerkinElmer) β counter. GAD65A and IA-2A levels were expressed as units per milliliter derived from the World Health Organization standard 97/550 (29). Samples were considered positive if GAD65A levels were >50 U/mL and IA-2A levels >10 U/mL. The intra-assay CV for duplicates in the GAD65A assay was 7% and in the IA-2A 11%. In the DASP 2010 workshop, our laboratory was among the top-ranking laboratories for GAD65A in workshop sensitivity (80%) and specificity (99%) and the top-ranking laboratory for IA-2A in workshop sensitivity (60%) and specificity (99%).

IAA. Noncompetitive method: serum samples (7 μL) were added to duplicate wells of a 96-well microplate, and 36 μL of [^{125}I]insulin (30) with an activity of 60,000 cpm/well was added, then incubated on a shaker at 4°C for 48 h. PAS in a 40% slurry (50 μL) was added to a filter plate and washed three times with 200 μL Tris buffer using a Micro-Plate Strip Washer (BioTek ELx50; BioTek Instruments, Bedfordshire, U.K.). Supermix scintillation solution (50 μL) was added to the wells after the plate had dried for 15 min. The radioactivity was measured in a β counter (Wallac Micro Beta Trilux; PerkinElmer).

Competitive method: Positive samples for IAA were further analyzed using a competitive method. Serum samples (7 μL) were added to four wells on a 96-well plate. To these wells, 36 μL of ^{125}I-insulin with an activity of 60,000 cpm/well was added, with 0.072 IU (or 2 IU/mL) unlabeled insulin (Actrapid; Novo Nordisk) added in the last two wells. The plates were incubated and examined under similar conditions as in the noncompetitive method. IAA levels were calculated as relative units and were related to positive controls. Positivity for IAA was set to 1.0 relative units. The competitive method was used to verify false-positive binding in the noncompetitive assay. However, in subsequent analysis, the competitive assay was used.

Our assays showed comparable precision (intra-assay CV was 6.0% for IAA) and reproducibility (interassay CV was 13.2%). Our laboratory has since its inception participated in the DASP (31) and classified among the top-ranking laboratories in performance for IAA in workshop sensitivity (26%) and specificity (100%).

***SLC30A8* genotyping.** The plasmid Max isolation kit (Qiagen) was used to isolate DNA from whole blood of the newly diagnosed diabetes patients according to the manufacturer's instructions. The *SLC30A8* genotyping (SNP rs13266634) was performed with the use of an allele-specific assay (KASPar; KBio-science; http://www.kbioscience.co.uk/) as previously described (32). The *SLC30A8* genotypes were grouped into CC and CT+TT genotypes.

HLA typing. The HLA-DQA1 and B1 were typed using sequence-specific oligonucleotide probes on DBS used directly for PCR amplification of DQA1 and DQB1 alleles as described (33) using a DELFIA Hybridization assay (PerkinElmer). The first set of probes defines the presence of HLA-DQB1*02, 0302, 0301, 0602, 0603, and 0604. The second set of probes defines the presence of additional DQB1 alleles. HLA-DQA1 probes defines the DQA1*0201, 03, and 05 alleles. The HLA-DQ genotypes were grouped based on the presence of DQ8 (A1*03:01-B1*03:02) and DQ2 (A1*05:01-B1*02:01) haplotypes into four groups: *1*) DQ2/8 (patients carry DQ2 and DQ8), *2*) DQ8/X (either homozygous DQ8/8 or heterozygous DQ8/y, where y is any haplotype except DQ8 or DQ2), *3*) DQ2/X (either homozygous DQ2/2 or heterozygous DQ2/y, where y is any haplotype except DQ2 or DQ8), and *4*) DQX/X (neither DQ8 nor DQ2). DQ6.4 was recognized as a risk allele within nine high-risk genotypes in the BDD cohort and therefore was further analyzed as DQ6.4 allele regardless of previous grouping where it could be grouped under DQ2/X, 8/X, or X/X.

Epitope scanning and molecular simulation of HLA-DQ–peptide epitope complexes. The ZnT8 molecule (all three variants) was scanned for epitopes to HLA-DQ2, HLA-DQ8, HLA-DQ2trans, and HLA-DQ8trans using the established motifs of these alleles (19,34–38). It was shown that there were two sets of epitopes fulfilling the motifs for alleles HLA-DQ2 and -DQ8 in the polymorphic region around residue 325 [i.e., peptide 319–327 **V**A**T**A**AS**(***RWQ***)D**S** and peptide 321–329 **T**A**AS**(*RWQ*)**DSQV** (core nonamers, polymorphic residues in italics in parentheses, anchors in boldface)]. In the first epitope, the p7R variant is a nonbinder to HLA-DQ2. In silico molecular simulation of the complexes of HLA-DQ alleles and peptides were performed as previously described (19,38). Briefly, the complexes of HLA-DQ2–gliadin peptide (1s9v.pdb), and HLA-DQ8–insulin B11–23 peptide (1jk8.pdb) were used as base molecules for the respective HLA-DQ2 and -DQ8 complexes; in the case of complexes with the DQ2trans and DQ8trans alleles, models of the trans alleles were built by superposition of the two DQcis structures and selection of two combinations. In all cases, the peptide coordinates used were those of insulin B11–23, as the gliadin peptide contained four prolines within the core nonamer sequence, an unlikely circumstance for T1D autoantigens. The most suitable rotamers were chosen in the case of the antigenic peptide residues, and the energy minimization process consisted of 1,000 steps of steepest gradient and another 1,000 steps of the conjugate gradient using the program Discover of Accelrys (San Diego, CA) on an Octane or a Fuel instrument of Silicon

Graphics (Fremont, CA). Figures of modeled structures were made using the DSViewer Pro and Discover of Accelrys.

Statistical analysis. SPSS 18 statistical package (SPSS, Chicago, IL) was used for statistical analysis. Pearson χ^2 test of independence (and Yates' correction for continuity value when applied) was used to assess relationships among ZnT8A, *SLC30A8*, and HLA. Logistic regression models were used to assess whether any of the *SLC30A8* and HLA-DQ genotypes were independently associated with ZnT8A and country of birth.

RESULTS

ZnT8A. Among the autoantibody-positive T1D patients (n = 2,964), a total of 2,021 (68%) were positive for at least one ZnT8A variant, and 880 (30%) were positive for all three variants. ZnT8A were least common <5 years and the highest frequency observed between 10 and 15 years in both non-Swedes and Swedes. In general, Swedish patients had frequently two or more ZnT8A (69%) than non-Swedes (62%; P = 0.02) (Table 1). This difference was due to a higher frequency of ZnT8-WA in Swedes (50%) than non-Swedes (38%; P = 0.001) (Fig. 2).

***SLC30A8* genotypes and ZnT8A.** The distribution of *SLC30A8* genotypes differed between non-Swedes and Swedes. The T2D-associated CC genotype was more common among non-Swedes (63%) than Swedes (46%; $P < 0.0005$) (Table 2). Compared with CT+TT, the CC genotype was more frequent in non-Swedes at younger age of diagnosis (<5 years; odds ratio [OR] = 2.5, 95% CI = 1.1–5.6; P = 0.02; 5–10 years; 2.87, 1.6–5.1; P = 0.0002) but there were no age differences in the Swedes.

The CC genotype was associated with ZnT8-RA in both non-Swedes (67%; P = 0.001) and Swedes (72%; $P < 0.0005$). Similarly, CT+TT genotype was associated with ZnT8-WA in non-Swedes (54%; P = 0.003) and Swedes (66%; $P < 0.0005$).

ZnT8A and HLA-DQ genotypes. In non-Swedes, HLA-DQ 2/X was both the main genotype (40%) and more common than in Swedes (14%; P = 0.0005). In non-Swedes, DQ2/X was negatively associated with multiple (≥2/3) ZnT8A (P = 0.02), ZnT8-WA (P = 0.008), and ZnT8-QA (P = 0.03) but not ZnT8-RA (P = 0.26). DQ2/8, which was more frequent in Swedes (37%) than in non-Swedes (18%; P = 0.005), could not explain the low frequency of ZnT8-WA in the non-Swedes (P = 0.38).

DQ8/X, however, was associated with multiple ZnT8A (P = 0.016) as well as with all three variants, ZnT8-RA (P = 0.04), ZnT8-WA (P = 0.03), and ZnT8-QA (P = 0.01).

In Swedes, all three ZnT8A variants were associated with DQ2/8 ($P < 0.0005$) and DQ8/X ($P < 0.0005$). However, DQ2/X did not show any association. DQ6.4 (59% of all DQ6.4 in the Swedes were DQ8/6.4) showed positive association with all three ZnT8A variants ($P < 0.0005$).

***SLC30A8* and HLA-DQ genotypes.** As expected from the autoantibody results, non-Swedes who carry DQ2/X

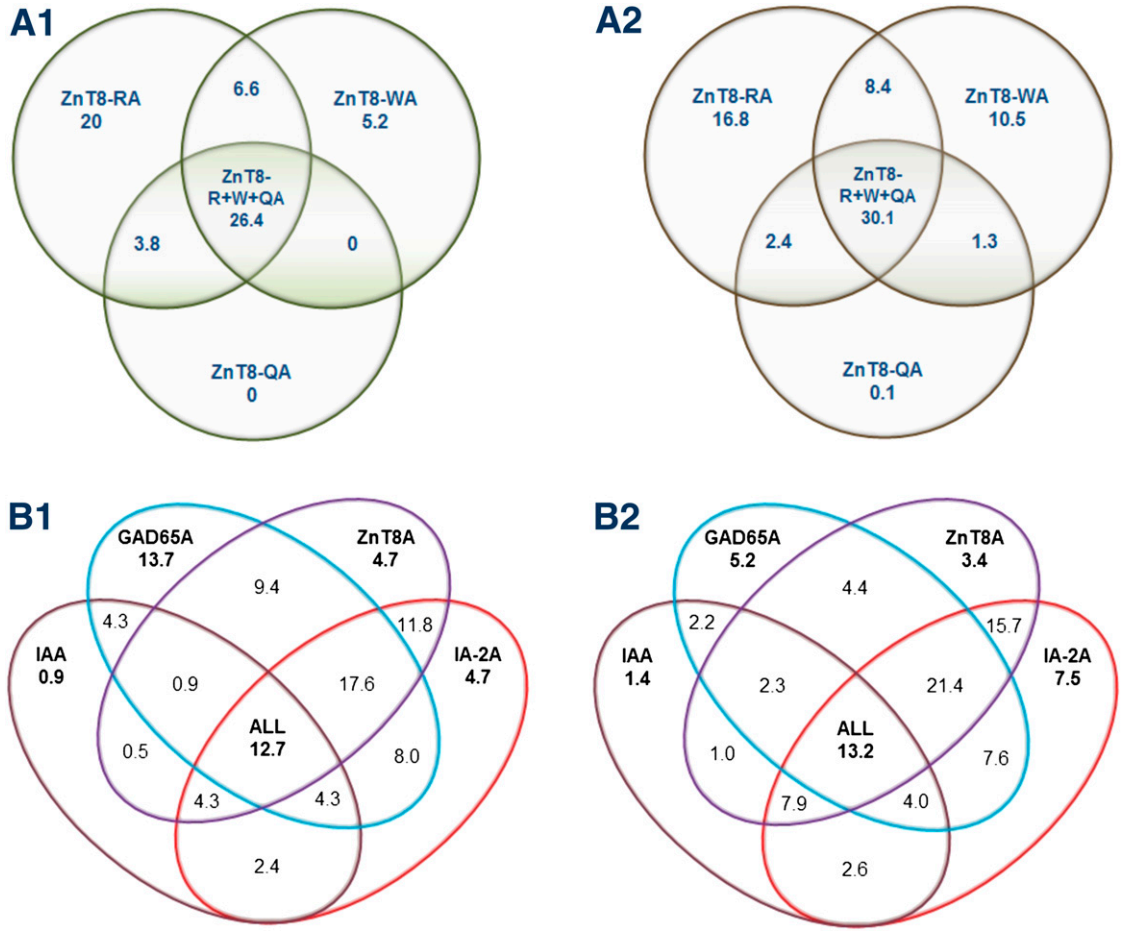

FIG. 2. Venn diagrams of islet autoantibodies. *A*: Frequencies and codetection (percent positive) of ZnT8A in A1: non-Swedes (n = 212) and A2: Swedes (n = 2,160). *B*: ZnT8A (≥1 ZnT8 autoantibodies) were detected in 4.7% of non-Swedes (B1) and 3.4% of Swedes (B2) who were negative for conventional autoantibodies. Unlike Swedes, non-Swedes develop ZnT8A more frequently with GAD65A rather than IA-2A. (A high-quality color representation of this figure is available in the online issue.)

TABLE 2
The *SLC30A8* (SNP rs13266634) genotypes in non-Swedes and Swedes

	Non-Swedes [*n* (%)]			Swedes [*n* (%)]		
Age-group (years)	CC	CT+TT	Total	CC	CT+TT	Total
<5	20 (67)	10 (33)	30 (100)	123 (44)	154 (56)	277 (100)
5 to <10	46 (72)	18 (28)	64 (100)	250 (47)	281 (53)	531 (100)
10 to <15	32 (53)	28 (47)	60 (100)	324 (47)	360 (53)	684 (100)
15–18	7 (58)	5 (42)	12 (100)	93 (42)	130 (58)	223 (100)
Total	105	61	166	790	925	1715

The CC genotype predominated the non-Swedes ($P < 0.0005$), whereas the CT+TT genotypes predominated the Swedes ($P < 0.0005$). Unlike Swedes, the CC compared with CT+TT in non-Swedes was more frequent in younger patients (<5; OR 2.5, 95% CI 1.1–5.6; $P = 0.02$; and 5–10; 2.87, 1.6–5.1; $P = 0.0002$).

compared with all other DQ genotypes had a higher frequency of CC (75%) than CT+TT (25%; $P = 0.009$) genotypes In Swedes, however, 57% of the DQ8/X carriers compared with all other DQ genotypes had the CT+TT genotype ($P = 0.02$) (Fig. 3). These findings were explained by the heterozygous genotype DQ2/y (y is any haplotype but 2 or 8) in non-Swedes ($P = 0.002$) and DQ8/y (y is any haplotype but 2 or 8) in Swedes ($P = 0.03$) but not by the homozygous DQ2/2 and DQ8/8 carriers, respectively (Table 3). The above findings remain statistically significant only in the non-Swedes after correcting the *P* values for multiple comparisons. To test for possible interactions, the RR in non-Swedes and Swedes were compared using an online calculator (http://hutchon.net/compareRR.htm). The ratio of relative risk for codetection of HLA-DQ2 and CC genotype in non-Swedes and Swedes was 1.35 (95% CI 1.06–1.71; Z-score = 2.467; $P = 0.0136$). However, the ratio of relative risk for codetection of HLA-DQ8 and CT+TT was 0.7 (0.33–1.52, Z-score = −0.894; $P = 0.37$). Logistic regression models showed that having a CC genotype of the *SLC30A8* (1.95, 1.4–2.7; $P = 0.0005$) and DQ2/X (1.6, 1.0–2.5; $P = 0.04$) was associated with non-Swedish origins.

HLA-DQ–ZnT8 peptide bindings. The analysis for ZnT8 motifs for DQ8, DQ2, and DQ6.4 showed that bindings of DQ2 epitopes throughout the whole ZnT8 369-aa peptide (including 325R/W/Q) were more abundant than DQ8 and DQ6 epitopes (Table 4). There were seven very good and many other intermediate epitopes for DQ2 compared with two strong and several intermediate epitopes for each of DQ8 and DQ6.4. However, none of the strong epitopes involved the polymorphic 325RWQ position within 319–329R/W/Q, but there were six intermediate binders for DQ8, three

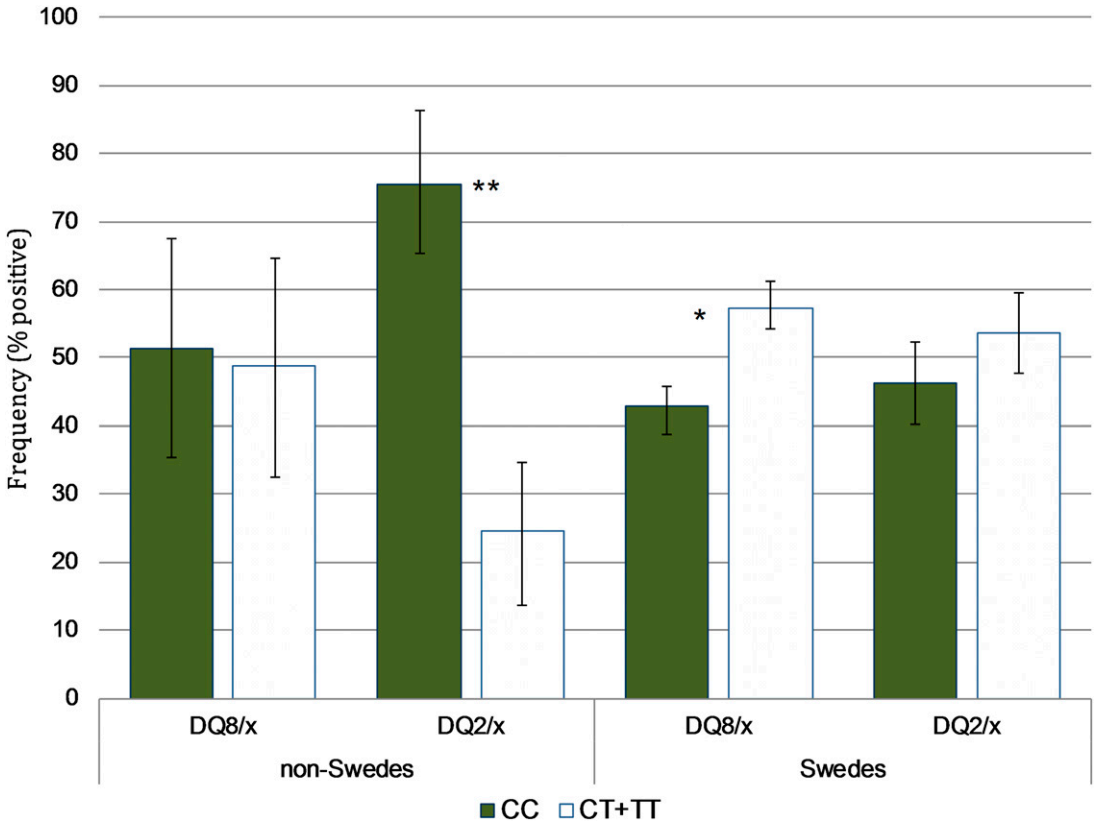

FIG. 3. Codetection of *SLC30A8* with HLA-DQ genotypes. In non-Swedes, DQ2/X was detected more frequently with CC ($P = 0.009$), and in Swedes, DQ8/X was detected more frequently with CT+TT (*$P = 0.02$) when compared with all other DQ genotypes. (A high-quality color representation of this figure is available in the online issue.)**

TABLE 3
The *SLC30A8* genotypes in relation to HLA-DQ genotypes

	SLC30A8 genotypes* [*n* (%)]					
	Non-Swedes			Swedes		
HLA-DQ genotypes	CC	CT+TT	*P* value	CC	CT+TT	*P* value
All	104 (63)	61(37)	0.002	787 (46)	926 (54)	0.001
2/8	18 (60)	12 (40)	0.68	262 (50)	261 (50)	0.026†
2/2	15 (60)	10 (40)	0.89	27 (46)	31 (54)	0.94
2/y‡	34 (85)	6 (15)	0.002	93 (46)	108 (54)	0.95
8/8	4 (40)	5 (60)	—	94 (44)	120 (56)	0.51
8/y‡	14 (56)	13 (44)	0.18	208 (42)	289 (58)	0.03
X/X	19 (56)	15 (44)	0.32	103 (47)	117 (53)	0.81

The predominate CC genotype among non-Swedes was detected in association with HLA-DQ2/y ($P = 0.002$) but not DQ2/2, whereas the CT+TT genotypes were more frequent in Swedes with HLA-DQ8/y ($P = 0.03$) but not DQ8/8. In this analysis, the DQ2/y in non-Swedes and DQ8/y in Swedes were compared with all other DQ genotypes in the respective group. *Results for *SLC30A8* were not available for all cases. †This *P* value reflects differences in non-DQ2/8 carriers. ‡y in DQ2/y is any haplotype other than DQ8 and in DQ8/y is any haplotype other than DQ2.

for DQ6.4, and only two combinations for DQ2 (Table 4). The epitopes 319–327, including tryptophan (W) at position 7 (VATAAS***W***DS), and 319–327, including glutamine (Q) at position 7 (VATAAS***Q***DS), but not arginine (R), may also bind weakly to DQ2, indicating that it selectively binds to epitopes of W and Q but not the R variant (Fig. 4). Of the strong binding epitopes, the nearest to the 325R/W/Q position were 344–351: MHSLTIQM for DQ8 and 352–360: ESPVDQDPD for DQ2. The in silico molecular simulation photo of HLA-DQ2 in complex with ZnT8 peptides 319–327p7Q/W are shown in Fig. 4*B* and *C*. Of the five different anchors (p1, p4, p6, p7, and p9) and the one shelf (p3), we note that p4A is a weak anchor, whereas all others are good to very good anchors.

DISCUSSION

In this study, we demonstrated that offspring of immigrant (non-Swedes) parents and grandparents in Sweden develop ZnT8A with genetic associations different from those

TABLE 4
ZnT8 epitopes restricted to T1D susceptible HLA-DQ haplotypes

Haplotype	Sequence*	Residue numbers	Comments on binding to HLA-DQ heterodimers*
HLA-DQ2	**YA*F*TLES**V**E**	18–26	Excellent
	EE*L*ESGGM**Y**	43–51	GG may be a problem making the peptide too flexible
	EK*G*ANE**Y**A**Y**	61–69	p4A weak, other anchors very good
	ER*L*LYP**D**Y**Q**	164–172	Good
	FV*H*A**L**G**D**L**F**	218–226	p4A weak, other anchors very good
	ES*P*V**D**Q**D**P**D**	352–360	p6Q weak, other anchors very good
	VD***Q***D**P**D**C**L**F**	355–363	Very good
	VA*T*AAS*W*DS	319–327W	p4A weak, other anchors good
	VA*T*AAS*Q*DS	319–327Q	p4A weak, other anchors good
	VA*T*AAS*R*DS	319–327R	Nonbinder
	TA*A*S*R*D**S**Q**V**	321–329R	p1**T** medium strength
	TA*A*S*W*D**S**Q**V**	321–329W	p1**T** medium strength
	TA*A*S*Q*D**S**Q**V**	321–329Q	p1**T** medium strength
HLA-DQ8	**I**CF**I**F**M**I**A**E**	80–88	Very good
	MHS**L**T**I**Q**M**E**	344–352	Very good
	VATAAS*R*D**S**	319–327R	p7R acceptable, p9S weak
	VATAAS*W*D**S**	319–327W	p7W acceptable, p9S weak
	VATAAS*Q*D**S**	319–327Q	p7Q acceptable, p9S weak
	TAAS*R*D**S**Q**V**	321–329R	p1**T** medium strength, p9V weak
	TAAS*W*D**S**Q**V**	321–329W	p1**T** medium strength, p9V weak
	TAAS*Q*D**S**Q**V**	321–329Q	p1**T** medium strength, p9V weak
HLA-DQ6.4	**F**I*F***S**I**L**V**L**A**	253–261	Very good
	IL*S*AH**V**A**T**A**	314–322	Very good
	VA*T*AAS*R*DS**	319–327R	Nonbinder
	VA*T*AAS*W*DS**	319–327W	Nonbinder
	VA*T*AAS*Q*DS**	319–327Q	Nonbinder
	TA*A*S*R*D**S**Q**V**	321–329R	p1**T** weak, other anchors good to very good
	TA*A*S*W*D**S**Q**V**	321–329W	p1**T** weak, other anchors good to very good
	TA*A*S*Q*D**S**Q**V**	321–329Q	p1**T** weak, other anchors good to very good

HLA-DQ2 and -DQ6.4 have a p3 shelf, indicated by italicized residue. *Only the well-binding epitopes are listed for the entire molecule. There is, however, a complete analysis of all possible epitopes around the 325W/Q/R polymorphic residue, shown in italics wherever appropriate. Only core nonamers of epitopes are shown with anchors p1, p4, p6, p7, and p9 in boldface. Total numbers of the nonshown weak and intermediate epitopes are as follows: 43 for HLA-DQ2, 14 for HLA-DQ8, and 15 for HLA-DQ6.4.

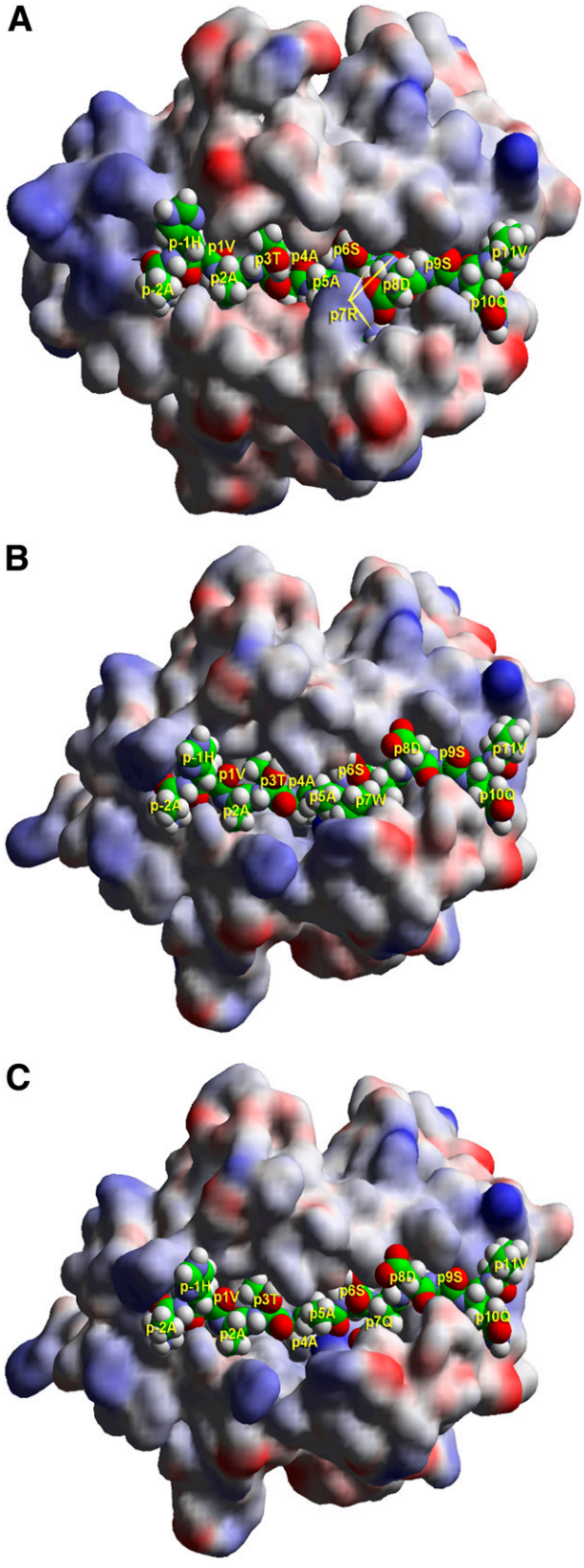

FIG. 4. ZnT8 epitopes in complex with HLA-DQ alleles. *A*: T-cell receptor view of the modeled structure of the T1D-susceptible HLA-DQ8 allele (A1*03:01-B1*03:02), in complex with the ZnT8 peptide 317–329, AHVATAAS*R*DSQV (anchors underlined, polymorphic residue in italics). The ZnT8 peptide is shown in Van der Waals solid surface form (atom color code: carbon, green; oxygen, red; nitrogen, blue; hydrogen, white; sulfur, yellow), whereas the α1β1 domain of the HLA-DQ molecule is shown in Van der Waals surface form with atom charges (positive, blue; negative, red; neutral, gray, and with appropriate scales of gray for situations in between). The polymorphic residue 325Arg occupies pocket 7, for which it is eminently suited in this allele. *B*: T-cell receptor view of the modeled structure of the T1D-susceptible HLA-DQ2 allele (A1*05:01-B1*02:01), in complex with the ZnT8 peptide 317–329, AHVATAAS*W*DSQV (anchors underlined, polymorphic residue in italics). Color conventions as in *A*. The polymorphic residue 325Trp occupies pocket 7, for which it is suited in this allele; HLA-DQ2 cannot tolerate arginine in any of its pockets. *C*: T-cell receptor view of the modeled structure of the T1D-susceptible HLA-DQ2 allele (A1*05:01-B1*02:01), in complex with the ZnT8 peptide 317–329, AHVATAAS*Q*DSQV (anchors underlined, polymorphic residue in italics). Color conventions as in *A*. The polymorphic residue 325Gln occupies pocket 7, for which it is well-suited in this allele. Note that there are slight rearrangements of both peptide residues and HLA-DQ residues because of the p7Trp→Gln substitution around the site of the substitution.

in Swedish patients. Non-Swedes tended to have a higher frequency of the T2D-associated CC genotype of the *SLC30A8* than Swedes (P = 0.0005) and, as expected, ZnT8-RA rather than the ZnT8-WA. Our data also showed that the DQ2 haplotype had more epitope binding sites to ZnT8 than the DQ8 haplotype. The ZnT8-W and -Q but not *R*-containing peptides could bind weakly to the A1*05:B1*02-containing DQ heterodimer, which may explain the differences in ZnT8A frequencies between non-Swedes and Swedes. Finally, among the non-Swedes but not the Swedes, the CC genotype was associated with younger age of diagnosis.

The importance of ZnT8/*SLC30A8* in diabetes is increasing because they appear to have dual roles: ZnT8 is an autoantigen in T1D (39), and the C allele of *SLC30A8* is associated with T2D but not T1D (14,40). The high frequency (>63%) of ZnT8A in T1D compared with <3% of T2D patients (5) highlights the importance of these autoantibodies as disease markers in T1D, although their exact role in pathogenesis is yet to be fully understood. It is an enigma why the two *SLC30A8* SNPs are associated with T2D but not T1D despite the fact that the SNPs are associated with the risk of developing T1D with ZnT8A.

The observed differences in the ZnT8A between non-Swedes and Swedes were related to differences in their genetic heritage. This difference may not be solely explained by the predominance of the *SLC30A8* CC genotype in non-Swedes. Data from the international HapMap project (41) showed that the CC genotype is more frequent in non-Caucasian African populations than Europeans and Asians. In our cohort, ~60% of non-Swedes originate from Middle Eastern and African countries, which may explain, in part, why the CC genotype is more prevalent in these patients. The C allele was previously found to be associated with younger age of onset of T1D patients (13). Interestingly enough, we detected this age variation only among non-Swedes but not among the Swedes. More interestingly, the DQ2/X (2/2 or 2/y), which was the main HLA-DQ genotype in non-Swedes, showed negative association with ZnT8-WA (P = 0.008) but not with ZnT8-RA and was abundant in the CC ($P < 0.009$) carriers only. Of interest, this finding was evident only with the carriers of the heterozygous DQ2/y ($P < 0.002$) but not the homozygous DQ2/2 individuals and was not influenced by the DQ2/8 genotype. Therefore, we proposed that the lower frequency of ZnT8-WA in non-Swedes may be explained both by the low frequency of the CT+TT (RW+WW) and the high frequency of DQ2/X. This phenomenon was not observed in the Swedes. Previous studies showed that DQ8 was associated mainly with ZnT8-RA (42), and DQ6.4 was associated with ZnT8-RA and ZnT8-WA

(43). Therefore, the DQ6.4 association with ZnT8A, especially ZnT8-WA, may be independent from DQ2 as seen in non-Swedes but potentiated by DQ8 as seen in Swedes, although the DQ6.4 associations in the non-Swedes were limited by the number of patients. The ethnic variation in the associations of HLA-DQ genotypes and autoantibodies may also reflect similar associations in healthy subjects from the general populations (44). However, studying these associations will require mass screening of healthy individuals.

The stronger DQ2 associations in non-Swedes may reflect different affinities of DQ2 for the respective antigenic peptides around the polymorphic 325RWQ position. Our ZnT8 epitope binding data showed that the overall number of epitopes binding to DQ2 exceeds the number of epitopes binding to DQ8 and also to DQ6.4, although DQ8 had more intermediate bindings around the polymorphic 325RWQ position. The higher binding affinity of DQ2 may in fact be related to its highly versatile pockets (19,34,35) that can bind several aliphatic, aromatic, and acidic-polar residues (abundant in ZnT8). These properties are different from DQ8, which has a narrower preference spectrum of residues for its pockets. The epitope binding analysis close to the 325RWQ position, showed that DQ2 had weak binding affinity toward the tryptophan- (W) and glutamine- (Q) but no binding to the arginine (R)-containing epitope (nonapeptide 319–327): VATAAS*W*DS, VATAAS*Q*DS, and VATAAS*R*DS (Fig. 4). These data may suggest that DQ2 preferably binds, albeit weakly, to W- and Q-containing peptides involving the polymorphic site aa 325, thereby reducing the chance of an immune response against these variants through central tolerance. By contrast, DQ8 and 6.4 carriers are less likely to promote tolerance mechanisms against ZnT8 variants. Taken together, the lower frequency ZnT8-WA and ZnT8-QA may be due to the moderating effects of DQ2 through tolerance. It should be stressed that these findings are based on in silico simulation experiments that do not allow distinction between autoantibody epitopes and T-cell epitopes in these ZnT8 variants. Therefore, further studies are needed to explore in detail whether T-cell epitopes in the trimolecular complex also represent autoantibody epitopes, especially in light of studies suggesting that T-cell epitopes may be modulated by autoantibodies (21).

The C allele of *SLC30A8* (rs13266634) was found to be associated with β-cell dysfunction but not insulin resistance (45). In contrast, the zinc-transporting system in β-cells, in particular ZnT8, was shown to be sensitive to cytokine (interleukin-1β)-induced apoptosis (46). Furthermore, recent findings suggest that patients with recent onset T1D (<6 months) expressed significantly higher autoreactive T cells against ZnT8 compared with controls (20). Upon processing of proinsulin to insulin, zinc in the β-cell granules may associate with insulin (47). The above studies suggest that ZnT8/*SLC30A8* may predispose to T1D through two mechanisms: *1*) autoimmune and inflammatory mechanisms involved in islet autoimmunity, and *2*) compromising β-cell function. Therefore, our findings may suggest that the role of ZnT8 in T1D, especially in patients of non-European descent, is determined largely by HLA-DQ alleles but also by *SLC30A8* genotypes. The antigen presentation of DQ–ZnT8 complexes may induce tolerance or promote autoimmune response against the ZnT8-containing β-cells depending on a variety of factors, such as strength of peptide binding and cognate T-cell receptor binding, as well as respective level of expression of HLA-DQ and autoantigen in central antigen-presenting cells (APC), peripheral APC, or both. There has been no reported expression of ZnT8 in the thymus or peripheral APC, as has been the case for the acetylcholine receptor protein (autoantigen for myasthenia gravis), another integral membrane protein, albeit at the cell surface and not in a secretion granule such as ZnT8 (48,49). Further studies on larger and more homogenous groups of T1D patients from non-European Caucasian populations may provide insights on the contribution of *SLC30A8* and other T2D genes in the risk for T1D. Studies addressing the recognition of HLA-DQ–restricted and ZnT8-specific T cells in patients with T1D should allow further understanding of autoimmune responses against the aa 325 polymorphic site of ZnT8. A major limitation in our analysis is the nonhomogeneity of the non-Swedes. However, we were able to recognize aggregates of immigrants sharing geographic, genetic, and cultural characteristics. Despite this limitation, the differences in immunogenetic factors under study remained statistically significant.

Non-Swedish patients develop T1D predominately with ZnT8-RA rather than ZnT8-WA likely due to immune tolerance to the (W)-containing peptide brought about by HLA-DQ2 rather than DQ8. In these patients, the CC genotype of *SLC30A8* may further contribute to the genetic predisposition to T1D. Based on these findings, we speculate that HLA-DQ molecules may modulate autoimmune response in T1D depending on their peptide-binding affinities.

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A.J.D. researched the data and wrote the manuscript. F.V.-S., H.E.-L., and S.A.I. researched data. A.C., G.F., and J.L. researched data, contributed to discussion, and reviewed the manuscript. B.L., U.S., I.K., and E.Ö. reviewed and edited the manuscript. C.M. and G.P.B. researched data. G.K.P. researched data and contributed to discussion. L.G. and Å.L. contributed to discussion and reviewed and edited the manuscript. A.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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