T1DGC AUTOANTIBODY WORKSHOP

Changes in Zinc Transporter 8 Autoantibodies Following Type 1 Diabetes Onset: The Type 1 Diabetes Genetics Consortium Autoantibody Workshop

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Zinc transporter 8 autoantibodies (ZnT8A) were analyzed in sera from 1,504 subjects as part of the Type 1 Diabetes Genetics Consortium (T1DGC) Autoantibody Workshop. For these participants with type 1 diabetes (T1D), samples were collected within 3 years of T1D diagnosis. ZnT8A were detected in 862 subjects (57.3%), with the highest frequencies and median titers being associated with the shortest duration of disease. ZnT8A were present at similar frequencies in non-Hispanic whites, non-Hispanic blacks, and Hispanics, but significantly less prevalent in those of Asian ancestry. Sera containing ZnT8A selectively recognizing at least one of the *SLC30A8* single nucleotide polymorphisms (encoding ZnT8A) were detected in all populations; however, Trp-specific sera were much less frequent in non-Hispanic blacks, consistent with the anticipated lower frequency of the *SLC30A8* rs13266634 T allele in African American populations. ZnT8A positivity was associated with HLA-DQ8, but this was primarily due to the *DRB1*0404*-DQ8 haplotype. This was in contrast to autoantibodies to IA-2 that were strongly associated with DRB1*0401-DQ8. These effects appeared essentially independent of racial or ethnic background. The *DRB1*0401*-DQ8 and *DRB1*0404*-DQ8 haplotypes were associated with T1D subjects positive for GAD65, IA-2, and ZnT8A. In contrast to *DRB1*0401*-DQ8, there was no significant association of *DRB1*0404*-DQ8 with single or dual autoantibody positivity. The *DRB1*0404*-DQ8 haplotype was also associated with T1D subjects whose sera recognized both polymorphic variants of zinc transporter 8, an effect not seen for *DRB1*0401*-DQ8.

The insulin secretory granule zinc transporter SLC30A8 (ZnT8) is a major target of cell-mediated (1–3) and humoral (4–8) autoimmunity in human type 1 diabetes (T1D). ZnT8 autoantibodies (ZnT8A) are present in 60–80% of Caucasian patients with T1D at onset (5,8) and, together with autoantibodies to insulin (IAA), GAD65 (GADA), and IA-2 (IA-2A), can confirm diagnosis of autoimmune diabetes in over 94% of subjects. In genetically at-risk first-degree relatives of T1D cases followed prospectively, ZnT8A appear at a median age of 3–4 years (4,8) and generally persist until onset of clinical disease. In young individuals, ZnT8A typically develop later than IAA and GADA and, together with IA-2A and ZnT8A positivity, can identify subjects with prediabetes at greatest risk of rapid progression to clinical T1D (6,7). Postonset ZnT8A titers decline rapidly, likely reflecting the continuing loss of β-cell mass (9–11).

Epitope mapping experiments have revealed the presence of at least one autoantibody binding site in the ZnT8 N-terminal intracellular domain that precedes the

six transmembrane–spanning regions and at least two distinct sites in the 95aa C-terminal cytosolic domain comprising residues 275-369 (8,12–14). *SLC30A8* exon 13, which encodes amino acid residues 318-369, is the site of single nucleotide polymorphism (SNP) rs13266634 (C/T), a nonsynonymous substitution in the first base of the codon encoding residue 325 that results in either an Arg or Trp at this position. This residue determines the binding specificity of a subset of ZnT8A (14). Genome-wide association studies (GWAS) have shown that the major allele (encoding Arg) is associated with increased susceptibility to type 2 diabetes (15–18). No direct association between this SNP and T1D risk has been detected (19). Presence of the rs13266634 major allele may confer an increased susceptibility of developing autoimmune diabetes at a younger age (20) and homozygosity at this locus can stratify risk in ZnT8A^{+}, but not ZnT8A^{-}, individuals (4). The second base of the codon encoding residue 325 is the site of rs16889462 (G/A), a second nonsynonymous SNP that encodes a Gln at this position. This allele is virtually absent in individuals of European descent but is detectable at a low minor allele frequency in sub-Saharan African, African American, and Asian populations (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=16889462).

A key concern of current human T1D research is to develop improved metrics that can stratify risk in subjects with prediabetes, identify those individuals most likely to benefit from particular therapeutic interventions, or act as secondary biomarkers of therapeutic efficacy. Given that autoimmune diabetes is believed to result from an inappropriate response to an environmental trigger in the context of genetic susceptibility that leads, initially, to immune dysregulation and, ultimately, to insulin insufficiency (21–23), it is of paramount importance to fully understand the extent to which genetic variation influences the behavior of the most commonly used biochemical markers of disease.

The Type 1 Diabetes Genetics Consortium (T1DGC) is an international study whose primary aims are to discover genes that modify diabetes risk and to provide an expanded genetic and sample resource for research (24). Within the T1DGC (https://www.niddkrepository.org/studies/t1dgc/), there are sera from more than 1,500 T1D-affected individuals that were collected within 3 years of clinical onset—a time period over which ZnT8A typically persist (10,11). The significant sample size and genetically diverse population represented by the T1DGC can both provide increased statistical power over our previous studies and permit identification of regional environmental influences that might otherwise confound the results obtained.

We now report a detailed study of genetic associations between positivity for ZnT8A and other biochemical markers of islet autoimmunity close to clinical onset. Moreover, given the common association of T1D with other autoimmune endocrinopathies (25), we investigated associations between ZnT8A and markers of Addison disease (steroid 21-hydroxylase autoantibodies [21-OHA]), autoimmune thyroiditis (thyroid peroxidase autoantibodies [TPOA]), autoimmune gastritis (autoantibodies to the α-subunit of ATPase 4 [ATP4A-A]), and celiac disease (transglutaminase autoantibodies [TGA]).

RESEARCH DESIGN AND METHODS

Subjects

The T1DGC sample and data repository is derived from affected sibling pairs, trio families, cases and control subjects from four regional networks (Asia-Pacific, European, North American, and U.K.) (24). Previous studies have shown that ZnT8A prevalence and their titers fall rapidly after T1D onset, with an average half-life approximating 90 days (9–11). Consequently, an inclusion criterion for our study was that the serum sample had been drawn within 3 years of clinical diagnosis of T1D. At the time that this study commenced the repository contained 6,749 sera from subjects with T1D, of which 1,504 met our entry criteria (420 year 0; 596 year 1; 488 year 2). Demographics of the included subjects are shown in Supplementary Table 1. For the purpose of racial/ethnic comparisons, non-Hispanic whites (NHWs) are defined as whites of European descent who failed to self-identify as Hispanic and include both subjects who specifically self-identified as non-Hispanic and those who did not specifically express an ethnicity. Similarly, non-Hispanic blacks (NHBs) are defined as blacks who failed to self-identify as Hispanic.

Genotyping

Eight HLA loci (*HLA-A, -B, -C, -DRB1, -DQA1, -DQB1, -DPA1,* and *-DPB1*) were genotyped at four-digit resolution in all T1DGC affected sibling pair and trio families and cases and control subjects, as previously described (26). SNP markers in the *INS* (−23HphI, rs689) and *CTLA4* (T17A, rs231775) genes were also genotyped for all of the 1,504 subjects in our substudy.

Autoantibody Assays

T1DGC laboratories performed assays for GADA, IA-2A, 21-OHA, TGA, and TPOA using established assays (27). ATP4A-A and ZnT8A were assayed using in-house radioimmunoprecipitation assays, as previously described (8–11,14,28). For ZnT8, three ^{35}S-radiolabeled probes were generated, each containing two copies of the C-terminal domain (amino acids 275-369) joined by a flexible linker (Pro-Ser-Thr-Pro-Pro-Gly-Ser-Gly-Gly-Gly-Ala-Ser). Probes varied solely with respect to the amino acids at position 325 in each monomer. Thus, probe CR/CW is a heterodimer of ZnT8 C-terminal domains containing sequentially Arg$_{325}$ and Trp$_{325}$, while CR/CR and CW/CW are homodimers containing two copies of Arg$_{325}$ or Trp$_{325}$, respectively. Aliquots of each sera (2.5 μL) were incubated overnight at 4°C with labeled probe (nominally 20,000 cpm) and immune complexes were captured with protein A Sepharose (GE Healthcare, Piscataway, NJ), extensively washed, and bound radioactivity quantified by liquid scintillation counting. To allow for standardization, each assay plate also contained eight human negative control samples and two positive controls, comprising a rabbit polyclonal anti-ZnT8 and a human reference serum that was pan-reactive for the three ZnT8 probes. Each sample was assayed in duplicate and results are expressed as immunoprecipitation indices of the means (sample − negative control)/(rabbit standard − negative control). The threshold for positivity for each assay was determined from the 99th percentile of a series of >200 sera from subjects without T1D matched for age, sex, and race, run alongside >100 "new-onset" sera from T1D patients attending the Barbara Davis Center for Diabetes (Aurora, CO). The derived indices were 0.0129 (CR/CW), 0.0132 (CR/CR), and 0.0110 (CW/CW). Discordant replicates that spanned the cutoff value were routinely repeated. Unless otherwise specified, ZnT8A refers

to results obtained with the CR/CW probe.

Classification of "unrestricted," "Arg-only," "Trp-only," or "Hetero-only" sera was based on the concordance in signals between the CR/CW, CR/CR, and CW/CW probes. Thus, unrestricted sera are positive for all probes, Arg-only immunoreactivity is defined as CR/CR >0.0132 and CR/CW >0.0129 but CW/CW ≤0.0110, and Trp-only sera as CR/CR ≤0.0132 but CW/CW >0.0110 and CR/CW >0.0129. ZnT8 is naturally dimeric (29), raising the possibility that in rs13266634 heterozygous individuals epitopes that selectively recognize ZnT8 heterodimers may exist. Such Hetero-only sera are defined as CR/CW >0.0129 but CW/CW ≤0.0110 and CR/CR ≤0.0132.

Statistical Analysis

The relationship of individual *SLC30A8* (ZnT8A) variants to age, age of diabetes onset, duration of diabetes, sex, and classical HLA alleles, *INS* and *CTLA4* SNPs, was examined using logistic regression with robust variance estimators. Duration and ethnicity were included as covariates and familial correlations were included to accommodate the pedigree structure. A multinomial regression model was used in association analyses of the number of islet autoantibodies. Screening for genetic associations was based upon a single SNP in each of *INS* and *CTLA4* and 38 common (minor allele frequency >5%) HLA alleles. HLA class II haplotypes were reconstructed using MENDEL (version 12.0) (30,31) to assign the parental origin of HLA-*DRB1*, -*DQA1*, and -*DQB1* alleles. In most cases, parental origin of the alleles at all three loci was assigned unambiguously from family data including both parents and unaffected siblings. HLA haplotypes with unphased alleles were inferred based upon known linkage disequilibrium (LD) within populations.

Stepwise logistic regression was used to distinguish HLA allele associations that are likely secondary to LD with other HLA alleles. Regression coefficients, odds ratios (ORs), standard errors, significance levels (Wald test), and prevalence estimates were calculated using STATA (version 11.2; StataCorp LP; College Station, TX). A stringent criterion for statistical significance of genetic association tests was taken as $P < 0.00125$ (0.05/40), using a Bonferroni correction that assumes all of the 38 common HLA alleles are independent. However, given the strong LD of all eight HLA loci, a significance level of 0.005 (0.05/10) was considered suggestive evidence of association.

RESULTS

ZnT8A were detected in 862 (57.3%) of the 1,504 T1DGC subjects who had sera drawn within 3 years of clinical T1D onset. Prevalence of ZnT8A decreased from 64.5% in subjects with 0–1 year's duration of diabetes to 51.0% in subjects with 2–3 years' duration. This decrease in prevalence was mirrored by the duration-dependent decline in the median indices of ZnT8A+ subjects from 0.23 (range 0.013–0.919) in the "year 0" group to 0.09 (range 0.013–0.882) in the "year 2" group ($P = 0.0001$). ZnT8A showed an expected age-dependent bimodal distribution, being less prevalent in individuals aged 0–5 years or over 20 years of age at blood draw, as compared with those aged 6–20 years (8,32,33). Prevalence of ZnT8A was similar in males (57.4%) and females (57.3%, $P = 1.00$). ZnT8A were less common among T1D cases of Asian ancestry (26.0%, $P < 1.0 \times 10^{-8}$), with similar frequencies observed among NHW (59.7%), Hispanic (60.5%), and NHB (57.9%) groups (Table 1). Of the 862 ZnT8A+ subjects, 66.9% were unrestricted,

21.8% were Arg-only, 9.3% were Trp-only, and 2.0% were Hetero-only. There were no significant racial/ethnic differences in the pattern of autoreactivity among ZnT8A+ subjects ($P = 0.29$) (Table 2). The relative frequency of unrestricted autoreactivity decreased from 68.6% in subjects with less than 1 year's duration of T1D to 59.4% with 2–3 years' duration ($P = 0.001$). There were corresponding increases in the relative frequencies of Arg-only (from 23.3% to 24.9%), Trp-only (from 7.8% to 10.4%), and Hetero-only (from 0.4% to 5.2%) subjects (Supplementary Table 2).

Among the 1,504 T1DGC subjects, 61.5% were positive for GADA and 66.8% positive for IA-2A. The prevalence of GADA, IA-2A, and ZnT8A varied with race/ethnicity, although there were marked differences in the patterns of variation. GADA were more widespread among NHBs (70.0%, $P = 3.9 \times 10^{-4}$) and IA-2A were more frequent among Hispanics (83.2%, $P = 2.6 \times 10^{-4}$) and less common among Asians (34.0%, $P = 1.4 \times 10^{-9}$) (Fig. 1). Like ZnT8A, the frequency of IA-2A was similar in males and females; however, GADA showed a female bias (67.9% vs. 55.7%, $P = 0.003$). In contrast to the distinct decline in ZnT8A prevalence with duration of disease (Table 1), the prevalence of IA-2A was relatively stable over the time range studied, decreasing from 67.6% in subjects with 0–1 year's duration to 65.8% in subjects with 2–3 years' duration ($P = 0.83$) (Supplementary Table 3). A decline in the prevalence of GADA, from 67.6% in subjects with 0–1 year's duration to 56.6% in subjects with 2–3 years' duration, was also observed ($P = 0.003$) (Supplementary Table 4).

Despite these differences, the vast majority (94.5%) of the 862 subjects who were ZnT8A+ were also positive for either GADA or IA-2A, with over half (58.3%) positive for both specificities.

Table 1—Frequency of ZnT8A positivity in T1DGC subjects recruited within 3 years of clinical diagnosis

Group	n	Prevalence	Male	Female	P*	0–1 year	1–2 years	2–3 years	P†
		Characteristics				*Diabetes duration*			
Total	1,504	57.31	57.36	57.26	1.000	64.52 (0.23)	57.38 (0.145)	51.02 (0.09)	0.0002 / 0.0001
NHW	991	59.74	59.78	59.69	1.000	67.16 (0.275)	59.85 (0.14)	53.42 (0.09)	0.003
Hispanic	167	60.48	57.69	62.92	0.528	68.52 (0.25)	58.57 (0.18)	53.49 (0.17)	0.281
NHB	240	57.92	57.26	58.54	0.896	62.16 (0.14)	56.47 (0.12)	55.56 (0.09)	0.666
Asian	100	26.00‡	32.69	18.75	0.170	27.27 (0.035)	31.58 (0.05)	20.00 (0.095)	0.515
Other	6	66.67	50.00	100	0.467	100 nd	50 nd	50 nd	nd

Data are *n*, %, or % (median ZnT8A+ index). nd, not determined. *, sex association; †, association with disease duration; ‡, $P = 7.47 \times 10^{-9}$ relative to other racial/ethnic subgroups.

Table 2—Relative frequency of ZnT8A stratified by homodimer reactivity

Group	n	Unrestricted	Arg-only	Trp-only	Hetero-only
Total	862	66.94 (583)	21.81 (184)	9.28 (78)	1.97 (17)
NHW	592	65.54 (388)	21.62 (128)	10.98 (65)	1.86 (11)
Hispanic	101	72.28 (73)	20.79 (21)	5.94 (6)	0.99 (1)
NHB	139	73.38 (102)	20.14 (28)	2.88 (4)	3.60 (5)
Asian	26	69.23 (18)	19.23 (5)	11.54 (3)	0
Other	4	50.00 (2)	50.00 (2)	0	0

Data are n or % (number of positive subjects). Results are expressed as the percentage of ZnT8A+ subjects in each group.

This concordance appeared independent of the pattern of immunoreactivity to the different ZnT8 polymorphic variants (87.5% among Trp-only subjects; 92.6% among Arg-only subjects). The association between ZnT8A and IA-2A (detected in 83.2% of ZnT8A$^+$ subjects vs. 44.7% ZnT8A$^-$ subjects [$P = 1.2 \times 10^{-8}$]) appeared stronger than that for GADA (detected in 69.7% of ZnT8A$^+$ subjects vs. 50.5% of ZnT8A$^-$ subjects [$P = 1.3 \times 10^{-8}$]). Concordance rates for islet antibody positivity among 171 sibling pairs from 167 families were 69.6% for ZnT8A, 70.8% for GADA, and 77.2% for IA-2A, which is significantly higher than expected for random pairing of the 342 subjects ($P = 2.2 \times 10^{-6}$, $P = 2.3 \times 10^{-7}$,

$P = 1.3 \times 10^{-9}$, respectively). However, there was no evidence for familial clustering of immunoreactivity to specific *SLC30A8* SNPs, with sibling concordance of 56.0% among 75 ZnT8A$^+$ sibling pairs—a value very similar to the expected concordance for random pairs of 150 subjects (53.8%).

Although T1D is often associated with other organ-specific autoimmune endocrinopathies, there was minimal evidence of association of either ZnT8A or IA-2A with markers of Addison disease (21-OHA), autoimmune thyroiditis (TPOA), pernicious anemia (ATP4A-A), or celiac disease (TGA), notwithstanding modest increases in TGA frequencies from 4.7% to 7.3% between subjects with and

without ZnT8A ($P = 0.04$) and from 4.4% to 7.1% between subjects with and without IA-2A (data not shown). In contrast, GADA were strongly associated with a subset of other organ-specific autoantibodies including TPOA (12.9% among GADA$^-$ subjects vs. 22.6% in GADA$^+$ subjects, $P = 2.7 \times 10^{-6}$) and ATP4A-A (10.8% among GADA$^-$ subjects vs. 16.9% in GADA$^+$ subjects, $P = 0.002$), despite the near-equal frequencies of TGA between subjects with or without GADA (Supplementary Table 5).

Association analyses were performed to assess the effects of HLA alleles and haplotypes on the presence of islet autoantibodies. Common alleles and haplotypes of *HLA-A, -B, -Cw, -DRB1, -DQA1, -DQB1, -DPA1*, and *-DPB1* were screened for association with each of ZnT8A, GADA, and IA-2A. All three markers were strongly associated with DR and DQ alleles known to confer the highest risk for T1D; however, the pattern of association differed markedly for the different autoantibodies (Fig. 2). Whereas GADA showed a strong positive association with *DRB1*0301*-DQ2 (OR 1.46, $P = 1.2 \times 10^{-4}$), IA-2A showed an equally strong negative association with this haplotype (OR 0.70, $P = 3.9 \times 10^{-4}$). Similarly, the DQ8 association with ZnT8A was with *DRB1*0404*-DQ8 (OR 1.97, $P = 0.001$), while the DQ8 association with IA-2A was with *DRB1*0401*-DQ8 (OR 2.31, $P = 9.1 \times 10^{-12}$). In both cases, the DR4 alleles significantly increased the risk for ZnT8A beyond that conferred by DQ8. There was no evidence of association between DQ8 and GADA, although this specificity was strongly associated with *DPA1*0103* (OR 1.42, $P = 1.1 \times 10^{-4}$) and *DPB1*0301* (OR 1.55, $P = 0.001$). These associations were not seen for either ZnT8A or IA-2A. There was also no evidence for association of the highest T1D risk DQ2/DQ8 genotype with either ZnT8A (OR 1.10, $P = 0.459$), GAD65A (OR 1.21, $P = 0.116$), or IA-2A (OR 1.25, $P = 0.103$). Similarly, there was no evidence of association with SNPs in the T1D-associated candidate genes *INS* (ZnT8: OR 0.88, $P = 0.188$; GAD65A: OR 0.90, $P = 0.300$; IA-2A: OR 1.14, $P = 0.211$) or *CTLA4* (ZnT8: OR 1.09, $P = 0.282$; GAD65A: OR 1.01, $P = 0.927$; IA-2A: OR 1.16, $P = 0.083$).

In addition to the strong positive (risk) associations with HLA class II haplotypes, suggestive negative (protective) associations were observed for ZnT8A with *DQB1*0301* (OR 0.63, $P = 0.004$), for
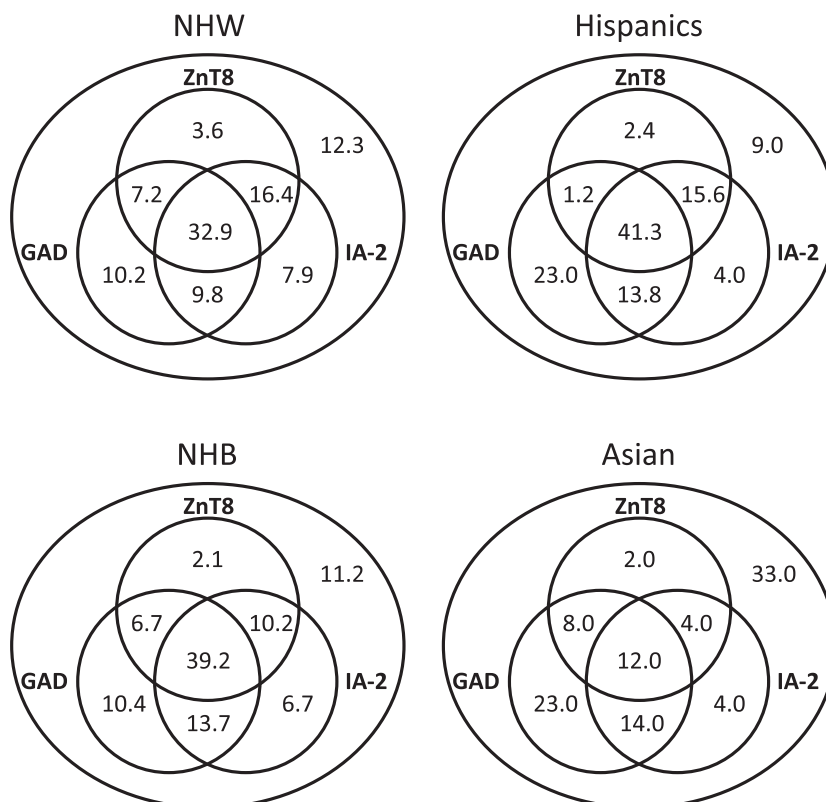

Figure 1—Venn diagrams showing the frequency of GADA, IA-2A, and ZnT8A among T1DGC subjects stratified by racial/ethnic group.
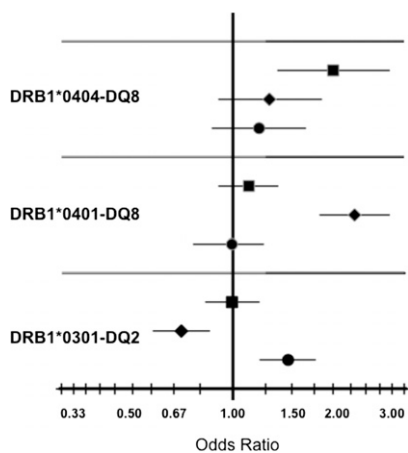
Figure 2—OR plots illustrating positive associations between specific HLA-DR-DQ haplotypes and individual T1D autoantibodies. ORs and confidence limits for the 1,504 included subjects are shown. GAD65A (circles), IA-2A (diamonds), and ZnT8A (squares).

Figure 3—OR plots illustrating negative associations between specific HLA-DR-DQ haplotypes and individual T1D autoantibodies. ORs and confidence limits for the 1,504 included subjects are shown. GAD65A (circles), IA-2A (diamonds), and ZnT8A (squares).

GAD65A with DQ5 (*DQA1*0101-DQB1*0501*: OR 0.60, P = 0.002), and for IA-2A with A*2402 (OR = 0.66, P = 0.002), *DQA1*0102* (OR 0.61, P = 0.001), DQ5 (OR 0.64, P = 0.005), and *DRB1*0101* (OR 0.70, P = 0.001). The *DRB1*0101* association is based upon LD with DQ5 (P = 0.117 when added to a model that included DQ5) (Fig. 3). The ZnT8A association with *DQB1*0301* was driven by the DQ7.5 haplotype (*DQA1*0501-DQB1*0301*: OR 0.59, P = 0.027), with no evidence for association of ZnT8A with DQ7.3 (*DQA1*0301-DQB1*0301*: OR 0.88, P = 0.615).

Of the three major haplotypes containing *DQA1*0102* (*DQA1*0102-DQB1*0602*, *DQA1*0102-DQB1*0502*, and *DQA1*0102-DQB1*0604*), the *DQA1*0102-DQB1*0602* haplotype had the strongest association with IA-2A (OR 0.37, P = 0.014), although all three haplotypes were relatively uncommon in this data set (MAF <3%). Although they did not reach significance, there were also trends toward negative associations of ZnT8A and GADA with *A*2402*, of ZnT8A with HLA-DQ5, and of IA-2A with HLA-DQ7.5, consistent with a recent report (34).

Association analyses were also conducted based on the number of islet autoantibodies present, a metric known to predict T1D risk more accurately than the presence of any single autoantibody (35). The groups defined by the number of autoantibodies for which they were seropositive showed consist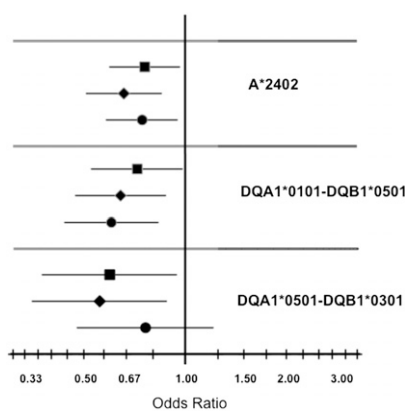ent trends for age of onset and duration, modest differences by sex, and racial/ethnic differences limited to a lower frequency of antibody positivity among Asian ancestry (maximum P = 0.012 across all categories of antibody positivity) (Table 3). Stratifying by age, single autoantibody positive subjects formed the youngest group with the earliest onset of T1D (P < 0.01 compared with GADA⁻, IA-2A⁻, and ZnT8A⁻ subjects), followed by those with two autoantibodies (P < 0.01), those who were GADA⁺, IA-2A⁺, and ZnT8A⁺, and finally the oldest individuals in the triple-autoantibody positive group (also with the most recent onset of diabetes) (P = 0.006 comparing duration for three vs. no autoantibodies).

The frequency of DQ2/DQ8 was significantly increased among subjects with one or more of GADA, IA-2A, or ZnT8A when compared with GADA⁻, IA-2A⁻, and ZnT8A⁻ subjects (OR 1.84 to OR 2.05, 0.002 ≤ P ≤ 0.006). This feature of DQ2/DQ8 was the same as for the *DRB1*0401*-DQ8 haplotype (OR 1.73 to OR 2.09, $8.2 \times 10^{-5} \leq P \leq 0.005$). The frequency of *DQA1*0102-DQB1*0602* was significantly decreased among subjects with one or more islet autoantibodies (OR 0.14 to OR 0.50, $1.6 \times 10^{-7} < P < 0.012$). In contrast, the effect of the *DRB1*0301*-DQ2 haplotype was restricted to the group with only one autoantibody, while the effect of the *DRB1*0404*-DQ8 haplotype was restricted to those with three islet autoantibodies (OR 1.88, P = 0.026, with no autoantibody as baseline; OR 1.82,

P = 0.007, with one autoantibody as baseline) (Table 3). A negative association was observed between the number of antibody specificities and *HLA-A*2402* (OR 0.98, P = 0.917 for one antibody; OR 0.63, P = 0.015 for two antibodies; OR 0.57, P = 0.003 for three antibodies). The association between *DRB1*0404*-DQ8 and the persistent expression of multiple islet autoantibodies was reflected in the increased frequency of this haplotype in ZnT8A⁺ subjects reacting to both the CR/CR and CW/CW probes as compared with Arg-only or Trp-only subjects (exact test of independence P = 0.019), an effect not seen for the *DRB1*0401*-DQ8 or *DRB1*0301*-DQ2 haplotypes (or DQ2/DQ8 genotype) (Supplementary Table 6).

CONCLUSIONS

Autoantibodies to islet antigens are currently the most reliable biomarkers of T1D (36) and, as such, are generally central to defining entry criteria for clinical trials designed to prevent or delay onset of overt disease (37). However, it is abundantly clear that there are significant differences between individuals in their humoral autoresponses to specific autoantigens. Such differences may be the result of dissimilar temporal or environmental factors but may also reflect genetic variation. If the contribution of genetics could be better understood, the utility of these key biomarkers could be improved. The goal of the current study was to identify genetic markers associated with the generation of ZnT8A in the subjects enrolled by the T1DGC as part of the T1DGC Autoantibody Workshop and compare them to markers linked to GADA and IA-2A.

Previous studies have demonstrated that ZnT8A titers decline significantly after onset of T1D, often in parallel with the loss of C-peptide (9–11). This decline suggests that ZnT8A might be a surrogate indicator of residual β-cell mass, yet it also limits its utility as a biomarker in individuals with long-standing disease. Accordingly, we restricted our analyses to subjects with disease duration of less than 3 years at blood draw. Consistent with expectations, both the overall frequency and median titer of ZnT8A in positive subjects were significantly reduced in the samples provided 2–3 years after onset relative to those taken during the first year of T1D. The decline was most

Table 3—Association analyses between HLA and number of islet autoantibodies (GADA, IA-2A, and ZnT8A)

	Number of islet autoantibodies								
	1			2			3		
	OR	CI	P	OR	CI	P	OR	CI	P
DR4(01)-DQ8	1.73	1.18–2.55	0.005	1.99	1.38–2.87	0.005	2.09	1.45–3.00	8.5×10^{-5}
DR4(04)-DQ8	1.06	0.56–1.99	0.857	1.25	0.70–2.23	0.444	1.88	1.08–3.20	0.26
DR3(01)-DQ2	1.60	1.17–2.18	0.003	1.40	1.04–1.90	0.028	1.23	0.90–1.68	0.194
DQ2/DQ8	2.05	1.31–3.20	0.002	1.92	1.24–2.96	0.004	1.84	1.19–2.86	0.006
A*2402	0.98	1.68–1.41	0.917	0.63	0.44–0.92	0.015	0.57	0.39–0.83	0.003

Significance levels are calculated from multinomial regression using 0 autoantibodies as baseline, and including age or age at onset (but not both), duration, and race/ethnicity as covariates.

evident in the NHW and Hispanic groups, who also had the highest median titers in year 0. The significantly lower prevalence of ZnT8A among T1D participants of Asian ancestry (26.0%) in the T1DGC, a subgroup derived predominantly from the southern Asian peninsula and Indian subcontinent, is remarkably consistent with previous studies reporting ZnT8A positivity in 27.8% of Japanese (38) and in 24.1% of Chinese patients with T1D (39). However, a more recent study in a second Japanese cohort reported marked variability across clinical subtypes of T1D, ranging from 0% among patients with fulminant T1D to 77% among young-aged (<10 years) patients with acute onset T1D (33).

The C-terminal domain of SLC30A8 (ZnT8) contains multiple autoantibody epitopes (13,14) that can broadly be separated into two classes depending on the significance of residue 325. Class "A" epitopes have an absolute dependence on residue 325 and are directly related to the subject's rs13266634 genotype and antibodies that recognize them can bind one of the homodimeric probes, but not both. Sera defined as Arg-only or Trp-only exclusively contain antibodies to class A epitopes. In contrast, class "B" epitopes are independent of residue 325 and likely involve ZnT8 surfaces that do not include this residue (13) and antibodies that recognize them can bind both types of homodimers as well as the heterodimer. Any one ZnT8A^{+} sera may contain antibodies recognizing either only class A, only class B, or a mixture of both. Thus, unrestricted sera may contain antibodies exclusively to class B epitopes, a mixture of class A and class B, or, in the case of individuals heterozygous at rs13266634, a mixture of class A epitopes of each variant. The latter individuals may additionally contain Hetero-only antibodies. Within an individual subject, each antibody specificity will behave

in a related but independent manner and after T1D onset may decline at distinct rates. Evidence to support such independence was obtained in a Swedish study that examined ZnT8A decline after onset (11), and two lines of evidence from the current study give additional support to this conclusion. First, there was no evidence for familial clustering of immunoreactivity to specific SLC30A8 variants beyond chance, despite the shared environmental exposures and increased genetic sharing from affected sibling pairs. Second, together with the decline in titer, there was a distinct disease duration-related decrease in the proportion of unrestricted sera in the ZnT8A^{+} subjects. As our previous postonset study gave no evidence for the acquisition of new specificities through epitope spreading during this phase of the disease (10), the most likely explanation is that the postonset decline in ZnT8A is associated with a simplification of the epitope specificities that remain detectable. There was, however, an unexpectedly large duration-dependent increase in the frequency of Hetero-only sera, suggesting that antibodies to these epitopes are preferentially preserved.

A key feature of this study was the inclusion of subjects drawn from multiple locations and ethnic backgrounds. Although a strength, this heterogeneity could be considered a weakness, as unidentified ancestral subgroups can increase the risk of detecting spurious associations. The T1DGC Autoantibody Workshop resource contrasts with the less diverse local populations investigated in the majority of previous studies of ZnT8A (4,8,9,11,38,39,40,41,42); in particular, we were able to document for the first time the behavior of ZnT8A in an African ancestry population (232/240 NHB subjects attended North American clinics). Overall, there was a similar

prevalence to the NHW subjects, although the median titer of ZnT8A in positive subjects was lower and there appeared to be a slower rate of decline. Consistent with the much lower frequency of rs13266634 T allele in African American populations, the frequency of Trp-only sera was reduced relative to other populations. The relative low number of autoantibody-positive NHB subjects precluded a definitive analysis of HLA associations; however, the overall trends in these subgroups were similar to those in the entire cohort, with positive associations of ZnT8A with the DRB1*0404-DQ8 haplotype, of IA-2A with the DRB1*0401-DQ8 haplotype, and of GADA with the DRB1*0301-DQ2 haplotype. Overall, the consistency of our results with previously reported HLA associations in IA-2A, GAD65A, and ZnT8A, together with similar prevalence estimates in ZnT8A across subgroups (excepting Asian ancestry), adds strength to our novel findings showing differential effects of DR4-DQ8 haplotypes and possible negative associations with DQ5, DQ7.5, and DQ6.2.

diagnostic use of ZnT8A. No other potential conflicts of interest relevant to this article were reported.

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