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*J Immunol* 2006; 176:2781-2789; doi: 10.4049/jimmunol.176.5.2781

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Islet-Specific Glucose-6-Phosphatase Catalytic Subunit-Related Protein-Reactive CD4\(^+\) T Cells in Human Subjects

Junbao Yang,* Nancy A. Danke,* DeAnna Berger,* Sandra Reichstetter,* Helena Reijonen,* Carla Greenbaum,* Catherine Pihoker,† Eddie A. James,* and William W. Kwok\(^2*\)‡

Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) is recognized as a major autoantigen for autoimmune type 1 diabetes (T1D) in the NOD mouse model. This study was undertaken to examine CD4\(^+\) T cell responses toward IGRP in human subjects. The tetramer-guided epitope mapping approach was used to identify IGRP-specific CD4\(^+\) T cell epitopes. IGRP\(_{247-259}\) were identified as DRA1*0101/DRB1*0401-restricted epitopes. IGRP\(_{13-25}\) and IGRP\(_{226-238}\) were identified as DRA1*0101/DRB1*0301-restricted epitopes. IGRP-specific tetramers were used to evaluate the prevalence of IGRP-reactive T cells in healthy and T1D subjects. More than 80% of subjects with either DRB1*0401 or DRB1*0301 haplotype have IGRP-specific CD4\(^+\) T cell responses for at least one IGRP epitope. IGRP-specific T cells from both healthy and T1D groups produce both γIFN and IL-10. DRA1*0101/DRB1*0401 IGRP\(_{247-259}\)-Restricted T cells also show cross-reactivity to an epitope derived from liver/kidney glucose-6-phosphatase. The detection of IGRP-reactive T cells in both type 1 diabetic subjects and healthy subjects and recent reports of other autoreactive T cells detected in healthy subjects underscore the prevalence of potentially autoreactive T cells in the peripheral immune system of the general population. The Journal of Immunology, 2006, 176: 2781–2789.

Type 1 diabetes (TID)\(^3\) is an autoimmune disease in which T cell-mediated autoimmune responses eventually lead to the destruction of insulin-producing β cells in the pancreatic islets (1). Susceptibility to TID in humans is strongly associated with the HLA class II loci. The DRB1*0401 (DR0401)-DQB1*0302 and DRB1*0301 (DR0301)-DQB1*0201 haplotypes are disease susceptible, whereas the DRB1*1501-DQB1*0602 haplotype offers disease protection (2). The NOD mouse (NOD) develops autoimmune diabetes spontaneously, and has been well established as a model for studying diabetes (3). Studies in NOD mice have suggested that both CD4\(^+\) and CD8\(^+\) T cells are involved in the pathogenesis of autoimmune diabetes (4). A high percentage of islet-infiltrating CD8\(^+\) T cells in NOD mice are specific for a peptide mimotope NRP (5). In subsequent reports, the NRP mimotope was revealed to be an epitope derived from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (6). Progression of autoimmune diabetes in NOD mice is also correlated with an increase in the avidity of NRP/IGRP-reactive T cells, and administration of NRP/IGRP peptides has an antidiabetogenic effect (7, 8). These results suggest that IGRP is a major autoantigen and plays a role in islet destruction in the NOD model. More recently, CD4\(^+\) T cells specific for IGRP were also identified in NOD mice (9). In adoptive T cell transfer experiments, IGRP-specific CD4\(^+\) T cells delayed the onset of diabetes, implying that IGRP-specific CD4\(^+\) T cells acted as regulatory T cells (Treg).

IGRP is a member of the glucose-6-phosphatase (G6Pase) family of proteins. Other members of this family include G6Pase-α and ubiquitously expressed G6Pase catalytic subunit-related protein (UGRP or G6Pase-β). Expression of IGRP is restricted to the islet. G6Pase-α is found in all gluconeogenic tissues, including the liver, kidney, and intestine, whereas UGRP is widely expressed (10, 11). IGRP shows 50% sequence homology to G6Pase-α and 35% sequence homology to UGRP. The G6Pase catalytic subunit couples with glucose-6-phosphate transporter to form a G6Pase complex that hydrolyzes glucose-6-phosphate to glucose. This complex is involved in both the glycogenolysis and gluconeogenesis pathways and plays a major role in the control of blood glucose homeostasis (12). Recently, the enzymatic function of the IGRP has also been demonstrated. Membrane fractions from cells that overexpressed IGRP converted glucose-6-phosphate to glucose (13). It is speculated that IGRP is the major enzyme that is responsible for G6Pase activity in the islet. Like other G6Pases, IGRP is a glycoprotein anchored in the endoplasmic reticulum by multiple transmembrane helices. The major portion of the protein is embedded in the membrane, with short lumens and cytoplasmic loops (14).

The precise role of IGRP in human TID is unclear. The TID susceptibility locus IDDM7 was mapped within the IGRP locus (11, 15), suggesting the possibility that IGRP is a disease susceptibility gene. Studies in human subjects have identified GAD65, insulin, and IA-2 as the major TID autoantigens, and CD4\(^+\) T cells that are directed against each of these proteins have been described (16–21). However, the prevalence of IGRP-specific CD4\(^+\) T or CD8\(^+\) T cells in human subjects has not yet been reported. In this study, we examined CD4\(^+\) T cell responses to IGRP in both healthy and TID subjects using MHC class II tetramers. The tetramer-guided epitope mapping (TGM) approach (22) was used to identify human IGRP epitopes. The prevalence of these IGRP epitopes in healthy and TID subjects was also examined.

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Received for publication September 15, 2005. Accepted for publication December 30, 2005.

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\(^*\)This work was supported by Juvenile Diabetes Foundation International and the Immune Tolerance Network.

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\(^\ddagger\)Abbreviations used in this paper: TID, type 1 diabetes; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; Treg, regulatory T cells; G6Pase, glucose-6-phosphatase; UGRP, ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein; TGM, tetramer-guided epitope mapping.
Materials and Methods

Healthy and TID subjects

DR0301 and/or DR0401 TID subjects <3.5 years after diagnosis were recruited at the Diabetes Clinical Research Unit at Benaroya Research Institute (Seattle, WA). Healthy DR0301 and/or DR0401 donors were recruited from normal volunteers with consent. A total of 10 TID subjects and 10 healthy subjects were recruited for this study. The HLA status of these groups is shown in Table I. The mean age of the patient group was 22 ± 9 years, whereas the mean age of the healthy group was 30 ± 10 years.

Peptides and tetramers

A panel of 43 overlapping IGRP peptides (p1 to p43) were synthesized on polyethylene pins with 9-fluorenylmethylcarbonyl chemistry by MIMOTOPES. These peptides, each 20-aa in length with a 12-aa overlap between adjacent peptides covered the entire IGRP protein. Peptides were dissolved in DMSO, and peptide pools were generated by mixing five consecutive peptides. There were a total of nine IGRP peptide pools. The individual peptides—IGRP13–25 QHLQKDYRAYYTF, IGRP23–35 YTFLNFMSNVGDP, IGRP206–220 RVLNIDLWLSWPVL, IGRP228–240 KGLGVDLLWTLEK, G6Pase-α28–29 KGLGVDLLVTEK, G6Pase-α30–30 KGLGVDLLVTEK with a corresponding set of individual peptide tetramers. Only preparations that give acceptable staining of the reference clone and low background staining when were loaded with 0.2 mg/ml of either pooled IGRP peptides or individual peptides. Loadings were conducted at 37°C for 72 h in the presence of 10 μg/ml tetramers in staining. The sequence of Flu MP169–181 is PLIRHEN-IGRP peptides. Loadings were conducted at 37°C for 72 h in the presence of 10 μg/ml tetramers in staining. The sequence of Flu MP169–181 is PLIRHEN-IGRP226–238 RVLNIDLLWSVPI, IGRP247–259 DWIHIDTTPFAGL, G6Pase-α28–29 KGLGVDLLVTEK, G6Pase-α30–30 KGLGVDLLVTEK—

Validation of tetramer reagents

Soluble DR0401 molecules were produced from Schneider cells using a similar approach. Empty biotinylated HLA-DR0401 or DR0301 monomer was loaded with 0.2 mg/ml of either pooled IGRP peptides or individual IGRP peptides. Loadings were conducted at 37°C for 72 h in the presence of 2.5 mg/ml (0.25%) n-octyl-β-D-glucopyranoside and 1 mM Pefabloc SC (Sigma-Aldrich). Peptide-loaded HLA-DR monomers were tetramerized with PE- or allophycocyanin-conjugated streptavidin (BioSource International) at a molar ratio of 8:1, respectively.

DR0301/Flu MP169–181 and DR0401/Flu HA306–318 were used as control tetramers in staining. The sequence of Flu MP169–181 is PLIRHEN-RMVLAS and Flu HA306–318 is PRYVKQNTLKLAT.

TID and tetramer staining

The TGEM approach for epitope identification has been described in detail previously (22). Briefly, CD4+ T cells were isolated from donor PBMCs by no-touch CD4+ isolation kit (Miltenyi Biotec). To augment T cell responses, CD4+CD25+ T cells were removed from total CD4+ T cells by FACs sorting (24). Two million purified CD4+CD25+ T cells were seeded into wells of a 48-well plate, which had been coated with adherent cells (to be used as APCs) from the same donor. Cells were stimulated with 10 μg/ml of pooled peptides in RPMI 1640 supplemented with 10% pooled PBMC supernatant every 3 days with fresh medium and IL-2. On day 14, the cultured cells were stained with pooled tetramers. Cells that gave positive staining with pooled tetramers were identified and subsequently stained with a corresponding set of individual peptide tetramers. For other experiments in detecting IGRP-reactive T cells, 2 × 106 unfraccionated CD4+ T cells or 3 × 105 PBMC were plated out per well onto a 48-well plate. Cells were stimulated with either one or two IGRP peptides per well (10 μg/ml per peptide). Cells were cultured as described above.

Tetramer staining was conducted with 2 × 105 cells and 10 μg/ml tetramers in 100 μl of T cell medium at 37°C for 2 h followed by anti-CD4 staining at 4°C for 15 min. Cells were analyzed on a FACSCalibur (BD Biosciences). In most experiments, the background tetramer staining was <0.2%, and a staining of 0.4% or higher was considered to be positive. In some experiments, cells that were positive for a particular tetramer were single-cell sorted by using a BD Biosciences FACSVantage. Sorted cells were expanded with 1.5 × 105 unmatched, irradiated PBMC per well as feeders with 2.5 μg/ml PHA and IL-2.

Cytokine assay

CD4+ T cells were stimulated with IGRP peptides as described above and assayed on day 14. For the cytokine assay, 1 × 106 T cells were incubated with 10 μg/ml tetramers, 10 U/ml IL-2, and 10 μg/ml anti-CD28. After 3 h of incubation, IFN-γ and IL-10 secretions were determined by use of a cytokine secretion capture assay (Miltenyi Biotec). Briefly, cells were washed twice in PBS and incubated in 100 μl of medium on ice for 5 min with an Ab-Ab conjugate directed against both CD45 and the cytokine (i.e., IFN-γ or IL-10). Prewarmed medium was added to a final volume of 2 ml, and cells were incubated at 37°C for 45 min under gentle rotation to allow cell surface capture of secreted cytokines. Cells were washed once in PBS and then stained for 15 min on ice with allop hydroxycoamin-conjugated Ab directed against the cytokine of interest and PerCP-conjugated anti-CD4 Ab.

T cell proliferation assay

T cells were plated in 96-well plates at a concentration of 103/well. Irradiated DR0301 or DR0401-expressing BLS-1 lines were used as APCs and plated at 2.5 × 104/well (25). Peptides were added at various concentrations. Assays were performed in triplicate wells in 150 μl of T cell medium. After a 3-day incubation at 37°C, 1 μCi [3H]thymidine/well was added. Cells were harvested, and tritium uptake was measured after 15 h of further incubation.

Results

Detection of IGRP-specific CD4 T cells and identification of DR0301- and DR0401-restricted IGRP T cell epitopes

The TGEM approach was used to detect IGRP-specific T cells for the identification of DR0301 and DR0401-restricted IGRP T cell epitopes. A set of 43 overlapping peptides spanning the entire IGRP protein, each 20 residues in length with an offset of 8 residues, was synthesized. The peptides were divided up into 9 pools.

Table I. DR0301 and DR0401 donors

<table>
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<tr>
<th>Subject no.</th>
<th>HLA-DR genotype</th>
<th>Duration of disease in months</th>
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<tbody>
<tr>
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<tr>
<td>2</td>
<td>0301/0401</td>
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<table>
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<th>Subject no.</th>
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<tr>
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Pools 1–8 consisted of 5 peptides each, whereas pool 9 consisted of 3 peptides. These pooled peptides were used to stimulate CD4⁺CD25⁻ T cells from a DR0301 healthy subject and a DR0401 healthy subject. As described in Materials and Methods, CD4⁺CD25⁻ T cells were depleted because CD4⁺ T cells are more responsive to antigenic challenge when these regulatory cells are absent (24). Fourteen to 16 days after the peptide stimulation, cells were examined by staining with DR0301 or DR0401-pooled IGRP peptide tetramers. Results of tetramer staining of cells from the DR0301 subject are shown in Fig. 1A, and results of staining cells from the DR0401 subject are shown in Fig. 1B. For the DR0301 subject, positive tetramer staining was observed with DR0301 pool 1 and pool 6 tetramers (Fig. 1A). For the DR0401 subject, positive staining was observed with DR0401 pool 1 and pool 7 tetramers (Fig. 1B). Single peptide tetramers were generated with individual peptides from the positive pools, and the staining was repeated. For the DR0301 subject, tetramers with peptide p2 from pool 1 and peptide p29 from pool 6 gave positive staining (Fig. 2A) (peptide p30 gave a staining above background; however, the result was irreproducible), and for the DR0401 subject, tetramers with peptide p3 from pool 1 and peptide p31 from pool 7 gave positive staining (Fig. 2B). These experiments were repeated with two additional DR0301 T1D subjects (samples were obtained within 16 mo after disease onset) and one DR0401 subject.
The results from five T1D and five healthy DR0301 subjects are tide-specific DR0301 tetramers 14 days after in vitro stimulation and DR0401/IGRP247–259 tetramers were also assembled. IGRP-(IGRP225–244) have a DR0301 binding motif (26) as underlined, projects with IGRP13–25 and IGRP226–238 peptides. The presence of tetramers, confirming that peptide IGRP23–35 encompassed the p3 epitopes, respectively, and peptide IGRP247–259 encompassed the DR0301-restricted p2 and p29, respectively, were single-cell cloned by sorting for tetramer-positive T cells. Similarly, DR0401-restricted T cells that were specific for p31 were also cloned. Attempts to clone DR0401/p3-specific T cells failed.

The IGRP residues corresponding to p2, p29, p3, and p31 peptides are shown in Table II. Peptides p2 (IGRP8–27) and p29 (IGRP225–244) have a DR0301 binding motif (26) as underlined, and peptides p3 (IGRP17–36) and p31 (IGRP241–260) have a DR0401 binding motif (27) as underlined. Peptides that were 13 aas in length containing the putative binding motifs from p2, p29, p3, and p31 were then synthesized. These peptides were IGRP13–25, IGRP226–238, IGRP23–35, and IGRP247–259, respectively (Table II). DR0301/IGRP13–25, DR0301/IGRP226–238, DR0401/IGRP23–35, and DR0401/IGRP247–259 tetramers were also assembled. IGRP-specific T cell clones isolated as indicated above were stained with the respective tetramers generated with the long and short peptides (Fig. 3). Staining of T cell clones with tetramers generated from the shorter peptides confirmed that peptides IGRP13–25 and IGRP226–238 encompassed the DR0301-restricted p2 and p29 epitopes, respectively, and peptide IGRP247–259 encompassed the DR0401-restricted p31 epitopes. Proliferation assays also confirmed the specificities of these T cell clones (data not shown). T cells that were specific for p3 were not isolated, but a p3-stimulated T cell line could be easily stained with IGRP23–35-loaded tetramers, confirming that peptide IGRP23–35 encompassed the p3 epitopes (Fig. 3D).

**Prevalence of IGRP CD4+ T cells in healthy and TID subjects**

The prevalence of IGRP13–25 and IGRP226–238-specific T cells in DR0301 healthy and T1D subjects was examined by stimulating PBMC or total CD4+ T cells from DR0301 healthy and T1D subjects with IGRP13–25 and IGRP226–238 peptides. The presence of IGRP13–25 and IGRP226–238 T cells were evaluated by IGRP peptide-specific DR0301 tetramers 14 days after in vitro stimulation. The results from five T1D and five healthy DR0301 subjects are shown in Fig. 4, A and B, respectively. All five DR0301 T1D subjects have significant T cell responses toward both the IGRP13–25 and IGRP226–238 epitopes (Fig. 4A). For the DR0301 healthy subjects, robust IGRP-reactive T cells toward both epitopes were also detected in some subjects. There was also one subject in which the responses toward both epitopes were minimal (subject number 15). Similarly, the prevalence of IGRP247–259 and IGRP23–35-specific T cells in DR0401 subjects was evaluated with IGRP peptide-specific DR0401 tetramers. The results from five T1D and five healthy DR0401 subjects are shown in Fig. 5. The majority of DR0401 T1D subjects have T cell responses toward at least one IGRP epitope. For the healthy group, most of the T responses are directed toward the IGRP247–259 epitope. One subject in the T1D group and one subject in the healthy group did not respond to either epitope. Although the sample set was too small to evaluate whether there might be a significant difference in the magnitude of responses and the extent of intramolecular determinant spreading between the healthy and T1D groups, the data demonstrated convincingly that IGRP-specific T cells were present in both healthy and T1D subject subjects. An unexpected finding of these experiments was that IGRP T cell responses could be detected in the majority of the healthy subjects, even without the depletion of CD4+CD25+ Treg.

**FIGURE 3.** Fine mapping of DR0301 and DR0401-restricted IGRP epitopes. A. Staining of DR0301-restricted IGRP p2 clone with DR0301/p2 and DR0301/IGRP226–238 tetramers. B. Staining of DR0301-restricted IGRP p29 clone with DR0301/p29 and DR0301/IGRP226–238 tetramers. C. Staining of DR0401-restricted IGRP p31 clone with DR0401/p31 and DR0401/IGRP247–259 tetramers. D. CD4+ T cells from a DR0401 subject stimulated with peptide p3, the cells were then stained with DR0401/p3 and DR0401/IGRP23–35 tetramers. A DR0301/Flu MP169–181 tetramer was used as an irrelevant control tetramer in A and B. A DR0401/Flu HA306–318 tetramer was used as the negative control in C and D.

<table>
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<tr>
<td>p2</td>
<td>IGRP8–27</td>
<td>GVLIIQHOLDQKDYRAYTYFLN</td>
<td>IGRP13–25 QHLDQKDYRAYTYF</td>
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<tr>
<td>p29</td>
<td>IGRP225–244</td>
<td>LRVLNIIDLLMSPIAKRWCA</td>
<td>IGRP226–238 RVLNIIDLLMSPI</td>
</tr>
<tr>
<td>p3</td>
<td>IGRP17–36</td>
<td>KDIQRAYTVFLNMSNVDPFR</td>
<td>IGRP23–35 YTLPFLNMSNVDP</td>
</tr>
<tr>
<td>p31</td>
<td>IGRP241–260</td>
<td>KWCANPDNHIDTPFGAVL</td>
<td>IGRP247–259 DWHDIPFGAVL</td>
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</table>

* Peptides p2 (IGRP8–27) and p29 (IGRP225–244) have a DR0301 binding motif, and peptides p3 (IGRP17–36) and p31 (IGRP241–260) have a DR0401 binding motif.
* The proposed p1, p4, and p9 anchor residues are underlined.
* Truncated peptides that were 13 aa in length containing the respective binding motifs from p2, p29, p3, and p31 were shown. These peptides were IGRP13–25, IGRP226–238, IGRP23–35, and IGRP247–259, respectively.
Cytokine profiles of IGRP-reactive T cells in healthy and T1D subjects

Because frequencies of autoreactive T cells are usually very low, we have not attempted to assay for the cytokine profiles of CD4⁺ IGRP-reactive T cells directly ex vivo. We did compare the cytokine profiles of DR0401/IGRP247–259-reactive T cells after in vitro expansion in both healthy and T1D subjects. Examples of these assays are shown in Fig. 6. For T1D subjects, the percentage of γ-IFN-positive cells within the tetramer-positive population

FIGURE 4. Detection of IGRP autoreactive T cells in DR0301 T1D and healthy subjects. A, PBMC or CD4⁺ T cells from DR0301 T1D subjects were stimulated with peptides IGRP13–25 and IGRP226–238. Cells were analyzed by flow cytometry 14 days poststimulation with anti-CD3 and anti-CD4 mAbs, DR0301/IGRP13–25, and DR0301/IGRP226–238 tetramers. Results from five T1D subjects (numbers 1–5) are shown. B, PBMC or CD4⁺ T cells from DR0301 healthy subjects were stimulated with peptides IGRP13–25 and IGRP226–238. Cells were analyzed by flow cytometry 14 days poststimulation with anti-CD3 and anti-CD4 mAbs, DR0301/IGRP13–25, and DR0301/IGRP226–238 tetramers. Results from five healthy subjects (numbers 11–15) are shown. DR0301/Flu MP169–181 tetramers were used as irrelevant control tetramers in both A and B.

FIGURE 5. Detection of IGRP autoreactive T cells in DR0401 T1D and healthy subjects. A, PBMC or CD4⁺ T cells from DR0401 T1D subjects were stimulated with peptides IGRP247–259 and IGRP23–35. Cells were analyzed by flow cytometry 14 days poststimulation with anti-CD3 and anti-CD4 mAbs, DR0401/IGRP247–259, and DR0401/IGRP23–35 tetramers. Results from five T1D subjects (numbers 6–10) are shown. B, PBMC or CD4⁺ T cells from DR0401 healthy subjects were stimulated with peptides IGRP247–259 and IGRP23–35. Cells were analyzed by flow cytometry 14 days poststimulation with anti-CD3 and anti-CD4 mAbs, DR0401/IGRP247–259, and DR0401/IGRP23–35 tetramers. Results from five healthy subjects (numbers 16–20) are shown. DR0401/Flu HA306–318 was used as irrelevant control tetramers in both A and B.
ranged from 33 to 40%, with an average of 36 ± 4%, and the percentage of IL-10-positive cells within the tetramer-positive population ranged from 14 to 33%, with an average of 26 ± 8% for four subjects. For healthy subjects, the percentage of γ-IFN-positive cells ranged from 11 to 41%, with an average of 25 ± 14%, and the percentage of IL-10-positive cells ranged from 5 to 16%, with an average of 11 ± 5% for three subjects. Although the sample size was too small to conclude any significant difference in cytokine profiles between the two groups, it is notable that the tetramer-positive T cells from healthy subjects and T1D produce both γ-IFN (suggesting an effector phenotype) and IL-10 (suggesting a regulatory phenotype).

Cross-reactivity between IGRP epitopes and G6Pase epitopes

IGRP is one member of the G6Pase family. The other members of this family of proteins are G6Pase-α and UGRP. IGRP protein shows roughly 50% aa sequence homology to other G6Pases. Amino acid alignment indicated that the homology between IGRP23–35 and the corresponding region in G6Pase-α and UGRP is minimal. In contrast, amino acid alignment indicated IGRP247–259 sequence has extensive homology to the corresponding sequences with the other G6Pases. The IGRP247–259 sequence is DWIHIDTPFAGL, whereas the corresponding sequence for G6Pase-α249–261 and UGRP239–251 are EWVHDTPFASL and EWHVDSRPFASL, respectively (Table III). IGRP247–259 and G6Pase-α249–261 bound to DR0401 with an IC50 of 0.1 μM and 0.3 μM, respectively, in an in vitro peptide binding assay (data not shown). Because UGRP239–251 did not bind to DR0401 in the same assay, this peptide was not investigated further. To examine whether T cells that responded to IGRP247–259 could cross-react with G6Pase-α249–261, IGRP247–259 clones were stimulated with either IGRP247–259 or G6Pase-α249–261 peptide using BLS-DR0401 transfectants as APC. Strong proliferation was observed in all of the 10 clones examined. Representative results from three different clones are shown in Fig. 7A. In addition, DR0401-restricted CD4+ T cells stimulated with IGRP247–259 could be stained with both DR0401/IGRP247–259 and DR0401/G6Pase-α249–261 tetramers (Fig. 7B).

Similarly, we examined whether DR0301-restricted T cells that responded to IGRP epitopes could cross-react with homologous G6Pase epitopes. No homology occurs between IGRP13–25 and the corresponding regions of both G6Pase-α and UGRP, but limited homology was observed between IGRP226–238 and the corresponding regions in G6Pase-α and UGRP. The peptide sequence for IGRP226–238 is RYLNIDLLWSVPL. The corresponding sequences in G6Pase-α228–240 and UGRP218–230 are KGGLVDLLWTLEK and FTLGLDSLWSIL, respectively (see Table III). Five different DR0301-restricted IGRP226–238-specific T cells were tested for their reactivity toward IGRP226–238, G6Pase-α228–240, and UGRP218–230. Peptides in proliferation assays using BLS-DR0301 as APC. Although all clones proliferated upon stimulation with the cognate IGRP peptide, no cross-reactivity was observed with the other two peptides. Representative results from three of these clones are shown in Fig. 7C. In another series of experiments, CD4+ T cells from a DR0301 subject were stimulated with IGRP226–238. Fourteen days poststimulation, these cells were stained with DR0301/IGRP226–238, DR0301/G6Pase-α228–240, and DR0301/UGRP218–230 tetramers. A strong staining signal was observed with the DR0301/IGRP226–238 tetramers; however, no staining signal was observed with DR0301/G6Pase-α228–240 tetramers and DR0301/UGRP218–230 tetramers (Fig. 7D).

In summary, cross-reactivity was observed between IGRP247–259 and G6Pase-α249–261 for T cells from DR0401 subjects, but no homology or cross-reactivity was observed between other corresponding epitopes within IGRP and UGRP. Limited homology was observed between IGRP226–238, G6Pase-α228–240, and UGRP218–230. However, cross-reactivity was not observed with either of these homologous epitopes for T cells from DR0301 subjects.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>IGRP247–259</td>
<td>DWIHIDTPFAGL</td>
</tr>
<tr>
<td>G6Pase-α249–261</td>
<td>EWVHDTPFASL</td>
</tr>
<tr>
<td>UGRP239–251</td>
<td>EWVHDSPFASL</td>
</tr>
<tr>
<td>IGRP226–238</td>
<td>RYLNIDLLWSVPL</td>
</tr>
<tr>
<td>G6Pase-α228–240</td>
<td>KGGLVDLLWTLEK</td>
</tr>
<tr>
<td>UGRP218–230</td>
<td>FTLGLDSLWSIL</td>
</tr>
</tbody>
</table>

*The proposed p1, p4, and p9 anchor residues for IGRP are underlined.
*Residues of G6Pase-α and UGRP sequences that are identical to the corresponding sequence in IGRP are in bold italics.
FIGURE 7. Cross-reactivity of IGRP-reactive T cells. A, Proliferation assays of DR0401-restricted IGRP clones with either IGRP<sub>247–259</sub> peptide or G6Pase-<sub>α239–250</sub> peptide, with BLS-DR0401 as APC. Assays for three different clones are shown. B, CD4<sup>+</sup> T cells from a DR0401 T1D subject were stimulated with 10 μg/ml IGRP<sub>247–259</sub> peptide. Fourteen days poststimulation, cells were stained with DR0401/IGRP<sub>247–259</sub> APC and DR0401/G6Pase-<sub>α239–250</sub> PE. C, Proliferation assays of DR0301-restricted IGRP clones with either IGRP<sub>226–238</sub> peptide, G6Pase-<sub>α228–240</sub> peptide, or UGPR<sub>218–230</sub> peptide, using BLS-DR0301 as APC. Assays for three different clones are shown. D, CD4<sup>+</sup> T cells from a DR0301 T1D subject were stimulated with 10 μg/ml IGRP<sub>226–238</sub> peptide. Fourteen days poststimulation, cells were stained with DR0301/IGRP<sub>226–238</sub> DR0301/G6Pase-<sub>α228–240</sub> and DR0301/UGRP<sub>218–230</sub> tetramers.

Discussion

In previous studies, TGEM was used successfully to identify CD4<sup>+</sup> T cell epitopes within viral Ags (22). In this context, TGEM provided a rapid and consistent approach for MHC-specific epitope identification and the isolation of MHC-specific T cell clones. However, epitope mapping using tetramers relies on the binding of tetramers to high-avidity T cells. It has been previously demonstrated that many high-avidity self-reactive T cells are deleted within the thymus, and that self-reactive T cells that are found in the periphery tend to bind weakly to tetramers (28, 29). Therefore, it was unclear whether the TGEM approach could be used to identify T cell epitopes for autoantigens. In the current study, the TGEM approach was successfully used to identify a set of CD4<sup>+</sup> T cell epitopes for an islet-specific autoantigen IGRP for both DR0301 and DR0401 individuals. IGRP<sub>13–25</sub> and IGRP<sub>226–238</sub> were identified as DR0301-restricted epitopes, whereas IGRP<sub>35–51</sub> and IGRP<sub>247–259</sub> were identified as DR0401-restricted epitopes. Because tetramer-based assays rely on high-avidity interactions, it is possible that additional low-avidity epitopes also exist. There is also the possibility that some of the epitopes identified are not naturally processed epitopes. Nevertheless, these results indicate that the TGEM approach is valid for the discovery of autoimmune epitopes.

This study demonstrated the prevalence of CD4<sup>+</sup> IGRP-specific T cells not only in T1D subjects, but also in healthy individuals that carry the DR0301 or DR0401 haplotypes. DR0301-restricted IGRP-specific T cells were detected in 100% of the healthy subjects and T1D subjects that were examined. DR0401-restricted IGRP-specific T cells were detected in 80% of the subjects that were examined. In contrast to our earlier report on GAD65-reactive T cells, which demonstrated that the depletion of CD4<sup>+</sup>CD25<sup>+</sup> Treg significantly enhanced responses to a GAD65 peptide in healthy subjects (24), responses to IGRP peptides could be detected in most healthy and T1D subjects even without CD4<sup>+</sup>CD25<sup>+</sup> Treg depletion. Based on these results, the specific role of IGRP-responsive CD4<sup>+</sup> T cells in autoimmunity is unclear. Differences between patients and healthy subjects may be subtle, or it is possible that certain aspects of the tetramer assay mask these differences. In any case, the detection of IGRP-restricted T cells within DR0301 and DR0401 patients and normal volunteers confirm that high-avidity cells with autoimmune potential exist and can escape into the periphery. Therefore, the presence of high-avidity autoreactive T cells alone must be insufficient to induce autoimmunity.

The current study also investigated the cytokine profile of IGRP-restricted T cells isolated from T1D subjects and healthy controls. Secretion of γ-IFN and IL-10 by IGRP-stimulated CD4 cells was measured using a Miltenyi capture assay. Tetramer-positive T cells isolated from patients and controls after in vitro expansion secreted both γ-IFN and IL-10. Whether IGRP-reactive T cells produce both cytokines in vivo has not been determined. The detection of γ-IFN (an inflammatory cytokine) and IL-10 (a suppressive cytokine) secreting cells as observed in these experiments suggests that IGRP-specific CD4 cells can play multiple roles. We speculate that naïve IGRP-specific T cells in the peripheral immune system have the plasticity to become either proinflammatory pathogenic T cells or Treg. In a proinflammatory environment, naïve IGRP CD4<sup>+</sup> T cells differentiate toward a Th1 pathway and perpetuate the inflammatory condition. Because CD4<sup>+</sup> T cells are required for both the priming and maintenance of CD8<sup>+</sup> T cells, IGRP-specific CD4<sup>+</sup> T cells could then provide help for CD8<sup>+</sup> IGRP-specific T cells that have cytotoxic function. Although a definitive report of the presence of IGRP-specific CD8<sup>+</sup> T cells in human subjects has not yet been published, it is likely that these T
cells will be identified in the near future. In a noninflammatory state, CD4+ IGRP T cells have a more regulatory function. Interactions between immature DC and IGRP-specific T cells could cause the IGRP-reactive T cells to exhibit suppressive function, which would inhibit the activity of effector T cells.

IGRP is one member of a family of homologous G6Pase proteins. Extensive homology between IGRP and other family members of the G6Pase proteins led us to investigate T cell cross-reactivity between homologous regions of the G6Pase protein family members. The homologous epitopes for G6Pase-α and UGRP are summarized in the Results. For the DR0401-restricted T cells, cross-reactivity between the IGRP epitope and G6Pase-α epitope was observed. However, no cross-reactivity was observed for UGRP. Cross-reactivity for the DR0301-reactive T cells was not detected for either protein. The cross-reactivity of IGRP and G6Pase-α-specific T cells in DR0401 subjects raises the possibility that G6Pase-α-expressing tissues could become the target of IGRP-specific (and, by cross-reaction, G6Pase-α-specific) T cells.

The identities of the primary autoantigens in T1D pathogenesis are still being defined. In mouse models, the most extensively studied Ags include insulin and GAD65. For example, it has been shown in NOD mice that the presence of a specific insulin epitope is essential for the development of disease (30). Recent experiments in NOD mice indicated that CD8+ T cells from the islet infiltrates of NOD mice can recognize IGRP, and that an increase in the avidity of these T cells is associated with progression of disease (5–7). Others described the presence of CD4+ IGRP-specific T cells in mice (9). Thus, IGRP could be a major target for islet cell destruction in the NOD model. T cells from the peripheral blood of human T1D subjects have also been studied extensively, implicating GAD65, IA-2, phogrin, insulin, and islet amyloid polypeptide as autoantigens (16–20, 31–33). A recent report also described the detection of insulin-specific T cells in pancreatic lymph nodes of T1D subjects (34). The detection of CD4+ IGRP-specific T cells in peripheral blood of human subjects in this study adds IGRP to the list of autoantigens implicated in T1D.

The role of IGRP-specific T cells in T1D is an active area of research. Recent experiments in mice demonstrated that IGRP-specific CD4+ T cells in NOD mice delay the onset of autoimmune diabetes in an adoptive T cell transfer animal model (9). The detection of IGRP-specific T cells in both healthy and T1D subjects and the secretion of IL-10 by these cells in this study suggest that IGRP-specific T cells could have a similar regulatory role in humans. However, it should be noted that CD4+ GAD65-reactive T cells have been demonstrated to have both pathogenic and regulatory roles in T cell transfer experiments in murine models (35–37). Our group and others (24, 38) have detected GAD65-reactive T cells in both healthy and T1D subjects. The presence of autoimmune T cells toward both insulin and IA-2 in both healthy and T1D subjects have also been reported (21). Thus, it is likely that antigen-specific effector and Treg are present in the periphery for a variety of autoantigens, and that it is the interplay of these two populations that will determine whether clinical autoimmunity or tolerance is the outcome. It should also be noted that the observation of autoantigen-specific T cells in healthy individuals is not confined to T1D. For example, T cells specific for desmoglein-3 have been detected in both healthy subjects and patients with pemphigus vulgaris (39). Similarly, T cells specific for myelin basic protein and myelin oligodendrocyte glycoprotein can be detected in healthy subjects and patients with multiple sclerosis (40–42). All of these observations attest to the prevalence of autoreactive T cells both in healthy subjects and in patients with a variety of different autoimmune diseases. Hauben et al. (43) have suggested that although a prolonged autoimmune response is pathogenic, a transient autoimmune response could actually be beneficial to the host. They also suggested that such a “benign” autoimmune response may be an essential step for the induction of adaptive Treg to control autoimmunity. The current findings demonstrating the presence of IGRP-autoreactive T cells in healthy subjects appear to support this benign autoimmunity hypothesis. Clearly, more investigation is needed to determine whether IGRP-specific CD4+ T cells are primarily effector cells that accelerate or trigger disease, or regulatory cells that down-regulate the inflammatory process associated with disease progression.

In summary, we reported in this study the identification and isolation of tetramer-positive IGRP-specific CD4+ T cells from human subjects for the first time. IGRP epitopes could be readily mapped using the TGEM approach, even without the removal of CD25+ Tregs. Cross-reactivity was observed between IGRP and G6Pase-α epitopes for DR0401-restricted T cells. The equal prevalence of autoreactive T cells in healthy subjects and T1D subjects and their production of both IL-10 and -IFN support the hypothesis that autoreactive T cells with different functional roles coexist. Additional studies aimed at understanding the significance of Ag-specific effector and Treg and the differences between transient benign autoimmunity and prolonged autoimmunity will be crucial.

Acknowledgments
We thank Aru Arumuganathan and Kelly Geubtner for technical assistance and Matt Warren for secretarial assistance.

Disclosures
The authors have no financial conflict of interest.

References


