

# GAD65-Specific CD4<sup>+</sup> T-Cells with High Antigen Avidity Are Prevalent in Peripheral Blood of Patients With Type 1 Diabetes

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**Negative selection of self-reactive T-cells during thymic development, along with activation-induced cell death in peripheral lymphocytes, is designed to limit the expansion and persistence of autoreactive T-cells. Autoreactive T-cells are nevertheless present, both in patients with type 1 diabetes and in at-risk subjects. By using MHC class II tetramers to probe the T-cell receptor (TcR) specificity and avidity of GAD65 reactive T-cell clones isolated from patients with type 1 diabetes, we identified high-avidity CD4<sup>+</sup> T-cells in peripheral blood, coexisting with low-avidity cells directed to the same GAD65 epitope specificity. A variety of cytokine patterns was observed, even among T-cells with high MHC-peptide avidity, and the clones utilize a biased set of TcR genes that favor two combinations, V $\alpha$ 12- $\beta$ 5.1 and V $\alpha$ 17-V $\beta$ 4. Presence of these high-avidity TcRs indicates a failure to delete autoreactive T-cells that likely arise from oligoclonal expansion in response to autoantigen exposure during the progression of type 1 diabetes. *Diabetes* 53:1987–1994, 2004**

Central tolerance mechanisms known as negative selection are designed to preferentially delete autoreactive T-cells during maturation of the immune system. Nevertheless, in human autoimmune diseases, peripheral T-cells with autoantigen specificity demonstrate that such selection is imperfect. In type 1 diabetes, autoreactive CD4<sup>+</sup> T-cells specific for several islet proteins can be expanded from peripheral blood, including cells that recognize epitopes from hGAD65 in the context of the diabetes-associated HLA class II molecules DR0401 and DR0404 (1–6). Previous studies of animal models of autoimmunity have suggested several potential mechanisms to account for this type of escape from T-cell tolerance. First, there is an avidity threshold for negative selection, by which low-avidity cells escape deletion and can populate the peripheral lymphoid compartment; a prediction of this mechanistic pathway is that peripheral autoreactive cells should be of generally

low avidity with respect to self-antigens. Second, antigenic epitopes derived during antigen presentation can be recognized as immunodominant or as cryptic, depending on whether they are processed efficiently or not, respectively. In this context, central tolerance to cryptic epitopes may be lacking, and a prediction of this pathway is that autoreactive cells should preferentially recognize cryptic epitopes that become presented in the periphery. A third mechanism is suggested by recent studies of an NOD mouse model of CD8<sup>+</sup> T-cell-mediated islet immunity, in which enrichment of a highly autoreactive CD8<sup>+</sup> T-cell population in the islets has been described, likely resulting from avidity maturation of the immune response (7). This model is consistent with a progressive focusing of the T-cell repertoire over time during autoimmunity, in which high-avidity responses are amplified from low-frequency precursors as a result of oligoclonal expansions in response to repeated autoantigen exposure.

Central selection mechanisms in human type 1 diabetes must be inferred from analysis of the peripheral lymphocyte pool, an approach made possible by the use of soluble class II peptide tetramers, which bind the antigen-specific T-cell receptor (TcR) on CD4<sup>+</sup> T-cells. We have previously identified hGAD65-specific CD4<sup>+</sup> T-cells expanded from peripheral blood of patients with type 1 diabetes that bind specific GAD-HLA-DR4 tetramers that contain GAD555-567 peptide, which has been shown to be an efficiently processed immunodominant epitope in patients with type 1 diabetes (8) and DR401 transgenic mice (9). This epitope was presented efficiently on the surface of GAD65-transfected DR401+ cells as demonstrated by elution and mass spectrometry of peptides bound to DR401 molecule (10). We now describe the characterization of several GAD65 555-567-specific T-cells, which likely represent escape from negative selection and may be important to the pathogenesis of autoimmunity.

MHC class II tetramers are useful probes of CD4<sup>+</sup> TcR specificity and avidity (11–13). When used at nonsaturating concentrations, tetramer binding is a straightforward measure of structural avidity, and binding strength correlates with the dissociation rate of particular TcR–MHC peptide complex formation on the T-cell surface (14,15). The tetramer-positive CD4<sup>+</sup> T-cells analyzed in this study express a diverse range of antigen interactions, with high-avidity cells coexisting with low- or moderate-avidity cells directed to the same MHC peptide specificity, even in a single subject. Within a group of high-avidity CD4<sup>+</sup>

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APC, antigen presenting cell; PBMC, peripheral blood mononuclear cell; PMHC, peptide-MHC class II complex; TcR, T-cell receptor.

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T-cells, a variety of cytokines are expressed, although there seems to be biased TcR utilization, most likely resulting from preferential oligoclonal expansion.

## RESEARCH DESIGN AND METHODS

DR401-positive patients with type 1 diabetes (<2 years from diagnosis;  $n = 5$ ) were recruited at the Diabetes Clinical Research Unit at Benaroya Research Institute. Healthy HLA-matched blood donors ( $n = 5$ ) were recruited from normal volunteers. T-cell cloning was performed from subject 0102-239 (DRB1\*0401, DQB1\*0302/DRB1\*0101, DQB1\*0501), whose type 1 diabetes was diagnosed at the age of 15, and subject 0702-592 (DRB1\*0404, DQB1\*0302/DRB1\*0301, DQB1\*0201), whose diabetes was diagnosed at the age of 17. Peripheral blood mononuclear cells (PBMCs) were purified from peripheral blood samples that were obtained under informed consent 2 and 4 years, respectively, after diagnosis. Subject 0102-239 was positive and subject 0702-592 was negative for GAD65 autoantibodies at the time the blood sample was drawn.

**Preparation of HLA-DR0401 and DR0404 monomers and tetramers.** The construction of the expression vectors for generation of soluble DR0401 (DRA\*0101/DRB1\*0401) and DR0404 (DRA\*0101/DRB1\*0404) molecules has been described previously (16). Briefly, a site-specific biotinylation sequence was added to the 3' end of the DRB1\*0401 or DRB1\*0404 leucine zipper cassette, and the chimeric cDNA was subcloned into a Cu-inducible *Drosophila* expression vector. DR-A and DR-B expression vectors were cotransfected into Schneider S-2 cells; the class II monomers then were purified, concentrated, and biotinylated. The desired peptide was loaded for 48–72 h, and tetramers were formed by incubating class II molecules with phycoerythrin-labeled streptavidin.

**Isolation and stimulation of PBMCs.** PBMCs were separated from 15 to 40 ml of heparinized blood by gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway) and expanded in vitro in the presence of specific antigen, as previously described (8). Briefly, cells were cultured with RPMI 1640 containing 10% vol/vol pooled human serum at the density of  $5 \times 10^6$ /ml, in the presence of a GAD65 555-567 (557I) (NFIRMVISNPAAT) peptide at a concentration of 10  $\mu$ g/ml. On day 10, the cells were transferred at a density of  $4 \times 10^6$ /ml onto a 48-well plate that had been adsorbed with 8  $\mu$ g/ml DR0401 or DR0404 monomer that contained GAD65 557I peptide (pMHC) in  $1 \times$  PBS for 3 h at 37°C. A total of 1  $\mu$ g/ml anti-CD28 antibody (BD/Pharmingen, San Jose, CA) was added to the media, and the cells were incubated for an additional 3–6 days. Some PBMCs were cultured in the presence of GAD65 555-567 (wild-type) peptide for 12–14 days without subsequent stimulation with DR4-GAD65 monomer.

**Tetramer binding, proliferation, and cytokine secretion assays of stimulated PBMCs and T-cell clones.** The stimulated cells from the PBMC culture were stained using 10  $\mu$ g/ml phycoerythrin-labeled HLA-DR0401 or 0404 tetramer for 3 h at 37°C and subsequently with fluorochrome-labeled anti-CD25 and anti-CD4 (BD/Pharmingen) for 30 min on ice. Cells were then washed with PBS that contained 1% FBS and analyzed using a Becton Dickinson FACSCalibur flow cytometer. Data analysis was performed using WinMdi (Stanford University) and CellQuest (Becton Dickinson) software. The GAD65 557I peptide-stimulated cells were single-cell sorted into 96-well plates using a FACSvantage cell sorter (Becton Dickinson). Sorted clones were expanded for 10 days by stimulation with irradiated unmatched PBMCs ( $1.5 \times 10^5$ /well), 5  $\mu$ g/ml phytohemagglutinin, and 10 units/ml IL-2 for two cycles, followed by stimulation with HLA-DR4-matched PBMCs pulsed with 10  $\mu$ g/ml GAD65 557I peptide and 10 units/ml IL-2. On days 10–12, clones were selected on the basis of growth for further expansion. Resting T-cells ( $5 \times 10^4$ ) were tested for specificity by stimulation with irradiated HLA-DR4-matched PBMCs ( $1 \times 10^5$ /well) with and without a specific peptide in the culture. The peptides used in the assay were GAD65 555-567 (557I) (NFIRMVISNPAAT) and/or GAD65 (555-567 NFFRMVISNPAAT). Recombinant GAD65, which was used in some experiments, was a gift from Dr. Peter van Endert (Hôpital Necker, Paris, France) (2). Proliferation as measured by [<sup>3</sup>H]thymidine incorporation was tested after 72 h in culture. The restriction elements of the T-cell clones were confirmed by testing proliferation induced by DR0401 or 0404 transfected type 1 bare-lymphocyte-syndrome cell lines (BLS-1) pulsed with GAD65 peptide. Cytokine secretion by the cells was measured at 48 h after stimulation by Cytometric Beads Array assay (BD Biosciences Pharmingen) according to the manufacturer's instructions. T-cell clones were tested for tetramer binding by staining with 10  $\mu$ g/ml GAD65 or control tetramer for 1 h at 37°C followed by fluorochrome-conjugated antibody on ice for 30 min. Clones BRI-5.325 and BRI-5.307 were incubated with soluble tetramers (5  $\mu$ g/ml) for 3 h at 37°C and subsequently stained with antigen presenting cell (APC)-labeled anti-CD69 monoclonal antibodies (Pharmingen). After washing with FACS buffer, samples were acquired by flow

cytometry and the percentage increase in CD69<sup>+</sup> cells was calculated as follows:

$$\frac{\% \text{ CD69}^+ \text{ cells} - \text{basal } \% \text{ CD69}^+ \text{ cells}}{100 - \text{basal } \% \text{ CD69}^+ \text{ cells}}$$

where the percentage of CD69<sup>+</sup> cells in basal conditions was 53–58% for both clones.

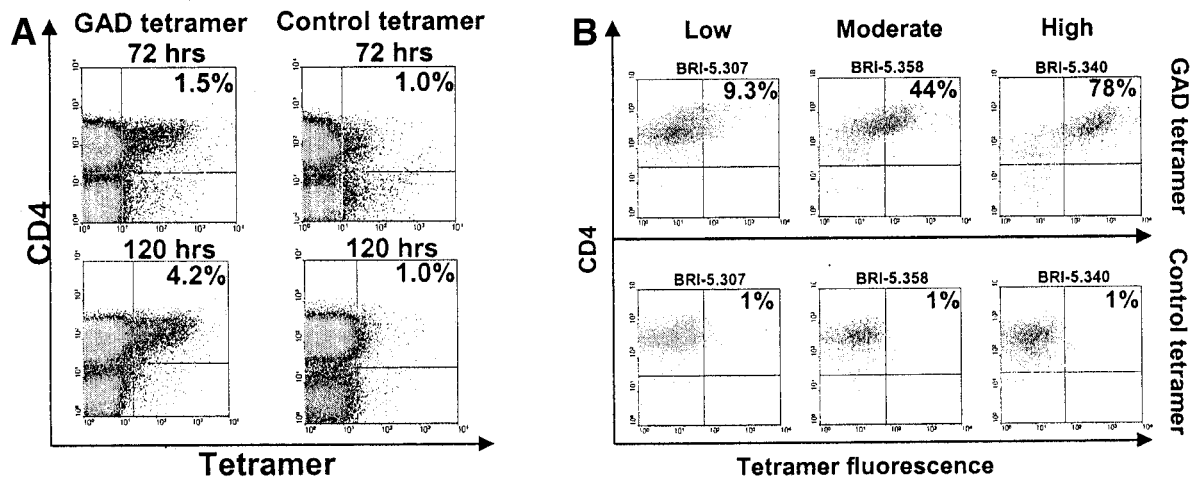
**TcR typing.** TcR V $\beta$  chains were determined by using Beckman-Coulter IO Test BetaMark TCR  $\beta$  Repertoire Kit, which contains 24 V $\beta$ -specific fluorescent-labeled antibodies and anti-human TcR monoclonal V $\beta$  6.7 FITC-labeled antibody (Endogen). The stained cells were analyzed by flow cytometry. TcR V $\alpha$  chains and those V $\beta$  chains that did not stain with any TcR V $\beta$ -specific monoclonal antibody in the flow cytometry analysis were evaluated by PCR (17). Briefly, total RNA was isolated from  $1 \times 10^6$  T-cells using Qiagen Rneasy Mini kit followed by first-strand cDNA synthesis (Applied Biosystems GeneAmp RNA PCR kit). A particular TcR variable gene family was amplified from cDNA using a specific V-region oligonucleotide as a 5' primer and a common C-region oligonucleotide as a 3' primer. The PCR products were run in a 1.6% agarose  $1 \times$  TBE gel, and the specificity was evaluated. Amplified cDNA transcripts were sequenced using Applied Biosystems BigDye version 1.1.

## RESULTS

**High- and low-avidity CD4<sup>+</sup> T-cells to GAD65 coexist in peripheral blood.** Fluorescent HLA-DR0404 tetramers that contained the GAD65 557I peptide were used to analyze PBMCs from subject 0702-592. Because of the low precursor frequency of these T-cells in the peripheral blood, in vitro expansion with the same GAD65 peptide was necessary before the analysis by flow cytometry. In Fig. 1A, profiles of peripheral blood cells analyzed by GAD65 tetramer staining are shown, 72 h or 120 h after incubation of the PBMCs with recombinant plate-bound pMHC complexes. Several HLA-DR4 haplotypes (e.g., containing DRB1\*0401, \*0404, or \*0405) are associated with type 1 diabetes, and the introduction of a substitution at position 557 (F $\rightarrow$ I) at the P1 anchor residue generates an agonist peptide that binds efficiently to all of these DR4 subtypes (data not shown) and stimulates proliferation and cytokine release from DR4-restricted T-cell clones (4).

A small but distinct subpopulation of CD4<sup>+</sup> T-cells that bound the DR0404 tetramer that contained GAD65 557I but not a similar DR0404 tetramer that contained a control peptide was observed. The majority of the tetramer-positive cells expressed high levels of CD4, consistent with recent activation by the plate-bound pMHC complexes. These CD4-high cells also stained with the CD25 activation marker, as previously described (8,18). Cell sorting by flow cytometry was performed, selecting the CD4-high/CD25<sup>+</sup> population that was also tetramer-positive. Of 68 clones generated by cell sorting and tested for antigen-specific proliferation, 20 CD4<sup>+</sup> T-cell clones were expanded, and all responded specifically to both specific antigen stimulation and tetramer staining. These T-cell clones displayed a range of tetramer-binding profiles, consistent with the heterogeneous tetramer-binding pattern of the polyclonal T-cells (Fig. 1B). Individual T-cell tetramer binding strength (low, moderate, or high) was a stable phenotype for each clone as determined by multiple tetramer staining experiments over 6–12 months.

CD4<sup>+</sup> T-cell clones that were isolated with tetramers that contained the GAD557I peptide showed antigen-specific proliferation with GAD65 555-567 wild-type peptide (Fig. 2A) and also recognized a naturally processed epitope from recombinant GAD65 protein in a dose-dependent manner (Fig. 2B). Tetramer-induced activation,



**FIG. 1.** T-cells display a range of tetramer binding. **A:** DR0404-GAD65 and DR0404-HSV control tetramers were used to stain *in vitro*-amplified PBMCs from subject 0702-592 after restimulation with DR0404-GAD65 monomer. In all cases, tetramer staining was evaluated on the viable lymphocytic population as gated based on the forward/side scatter profile. The frequency of CD4/tetramer-positive cells is noted in the upper-right quadrant. **B:** Single-cell sorting of T-cells was performed at 72 h after restimulation with DR0404-GAD65 monomer. A range of staining intensities was detected as well as representative T-cell clones displaying low (5–30%; 6 of 20), moderate (30–60%; 5 of 20), and high (60–100%; 9 of 20) binding to GAD65 tetramer.

as measured by CD69 upregulation, validated the utility of tetramer staining intensity as a measure of TcR avidity (Fig. 2C): tetramer high clones displayed higher sensitivity for tetramer stimulation as compared with tetramer low ones.

Proliferation profiles for a representative set of the clones are shown in Fig. 3. There was a partial correlation between functional sensitivity and tetramer binding. This correlation was less evident at the highest antigen concentration, likely as a result of the excess of the peptide. At suboptimal concentration of the peptide, the clones with high tetramer binding showed the strongest antigen responses. Cytokine profiles were heterogeneous (data not shown). In particular, there was no correlation between TH1/TH2-type cytokine bias and the strength of tetramer binding. Similar responses were seen for all clones in the presence of GAD65 555-567 (557F, wild-type) peptide, consistent with the interpretation that this P1 residue substitution is a site for MHC but not TcR interaction. Ten unique TcR sequences were found among the clones (Table 1). It is interesting that TcR V $\alpha$  and V $\beta$  chain usage was nonrandom, with two combinations (V $\alpha$ 12-V $\beta$ 5.1 and V $\alpha$ 17-V $\beta$ 4) represented more than once, with unique CDR3 regions.

**Oligoclonal T-cell expansion from primary PBMCs.** The GAD65 peptide 555-567 is presented in the context of multiple class II molecules and has been shown to stimulate T-cells in the context of both DRB1\*0404- and HLA-DRB1\*0401-positive APCs (8). When PBMCs from patient 0102-239 (HLA-DRB1\*0401) were analyzed for tetramer binding after *in vitro* amplification with antigen, a discrete cohort of strongly DR0401 tetramer-positive CD4<sup>+</sup> cells were seen, which were not present in the T-cell sample lacking antigen stimulation or in the activated population stained with a control DR0401 tetramer. Compared with the analysis shown in Fig. 1, this patient's tetramer binding assay showed a much more homogeneous high-avidity profile. Cell sorting was per-

formed, gating on CD4-high/CD25<sup>+</sup> and on tetramer-positive cells. A total of 117 T-cell clones were analyzed, and all were tetramer positive (Fig. 4B).

Twenty clones were selected for further analysis of functional and structural properties. All responded to specific antigen with high sensitivity (Fig. 5A), and all utilized the identical TcR, containing both V $\alpha$ 12 and V $\beta$ 5.1. It is interesting that this particular TcR chain combination was also detected in two GAD65-specific T-cell clones isolated from patient 0702-592 with type 1 diabetes, as described above (Table 1), and also, on a different occasion, from one multiple autoantibody-positive at-risk subject (data not shown). All of these T-cell clones displayed high antigen sensitivity and bound GAD65 tetramer. Despite the oligoclonal response in patient 0102-239 with type 1 diabetes, the functional phenotype of these T-cell clones was not uniform. As shown in Fig. 5B, two patterns were present: some of the clones displayed a characteristic Th1 profile, and others displayed a Th0 profile. These profiles seemed to be stable properties of particular clones, as they did not change over time and were identical for either the native peptide sequence or the modified sequence with an optimized P1 anchor residue 557I.

**TcR-V $\beta$  5.1 is prevalent among the T-cells that bound to GAD65 tetramer.** We analyzed whether a biased usage of TcR-V $\beta$  can be identified in PBMCs of five patients with newly diagnosed type 1 diabetes. The CD4<sup>+</sup> cells were stimulated with GAD65 555-567 (wild-type) peptide and autologous antigen-presenting cells for 12–14 days and analyzed for GAD65 tetramer and TcR-V $\beta$  staining by flow cytometry. A representative example of the staining is shown in Fig. 6. GAD65 tetramer-positive T-cells were observed in all patients with type 1 diabetes but not in normal subjects (Fig. 6A). A total of 50–98% of the GAD65 tetramer-binding T-cells were positive for V $\beta$ 5.1 chain, suggesting that this particular V $\beta$  chain is frequently used among T-cells that are specific for GAD65 555-567 epitope (Fig. 6B).



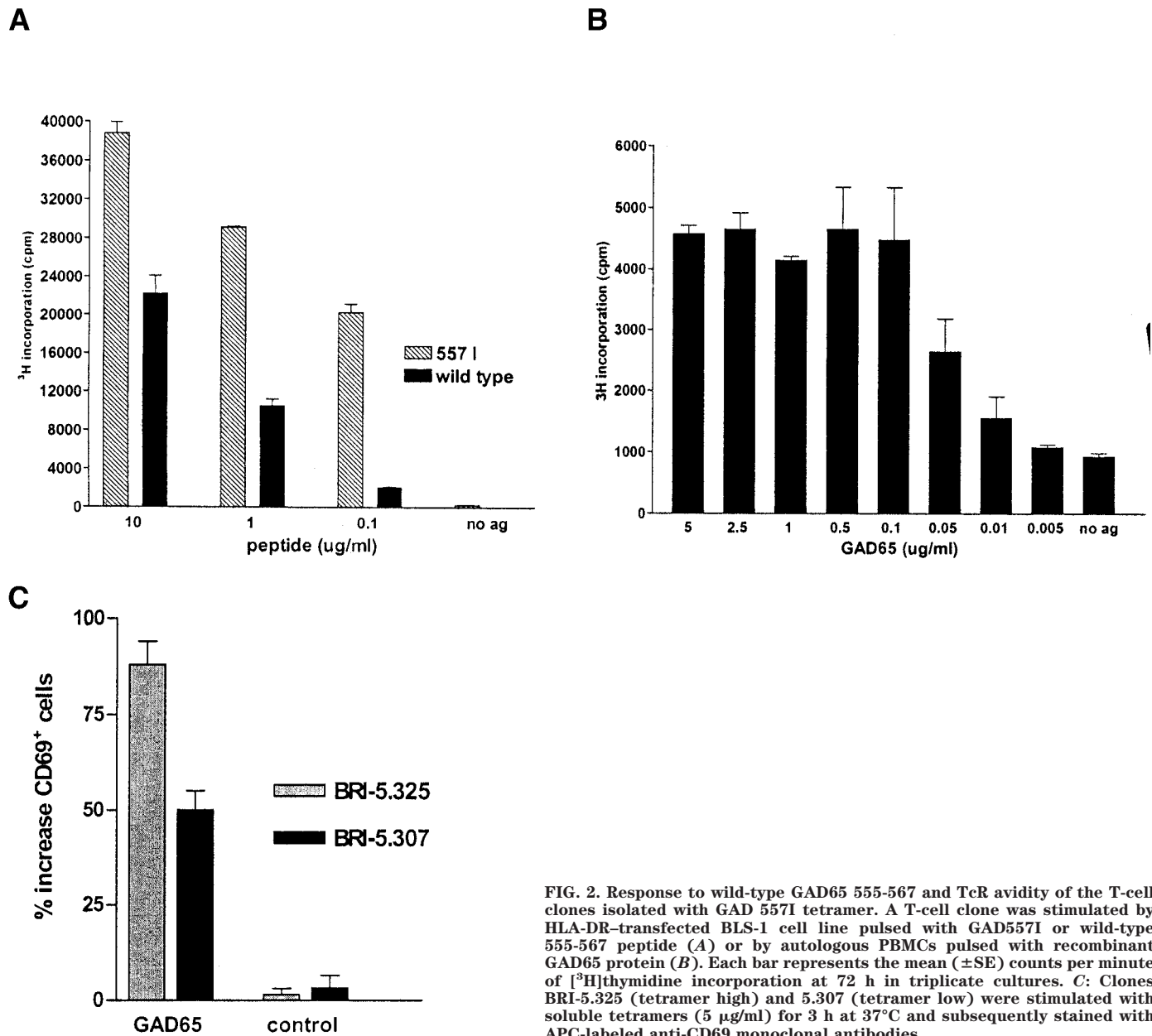


FIG. 2. Response to wild-type GAD65 555-567 and TcR avidity of the T-cell clones isolated with GAD 557I tetramer. A T-cell clone was stimulated by HLA-DR-transfected BLS-1 cell line pulsed with GAD557I or wild-type 555-567 peptide (A) or by autologous PBMCs pulsed with recombinant GAD65 protein (B). Each bar represents the mean ( $\pm$ SE) counts per minute of [ $^3$ H]thymidine incorporation at 72 h in triplicate cultures. C: Clones BRI-5.325 (tetramer high) and 5.307 (tetramer low) were stimulated with soluble tetramers (5  $\mu$ g/ml) for 3 h at 37°C and subsequently stained with APC-labeled anti-CD69 monoclonal antibodies.

## DISCUSSION

We used GAD65-induced T-cell activation and tetramer staining to analyze autoantigen-specific T-cells from peripheral blood of patients with type 1 diabetes and identified high-avidity profiles associated with particular TcR usage patterns. The same TcR V $\alpha$  $\beta$  composition displaying high structural and functional avidity for GAD65 was identified in T-cell clones from both patients with type 1 diabetes, and it was a dominant clonotype of high-avidity T-cells with restricted TcR repertoire in subject 0102-239.

Amplification of specific TcR upon activation with a diabetogenic mimotope peptide in mice has been described (19). In humans with disease-associated HLA molecules, a spectrum of potentially autoreactive T-cells with a range of avidities exist, but selective amplification of high-avidity cells may lead to skewing of this spectrum during the progression of disease, so high-avidity cells become more predominant. Type 1 diabetes of patients in

our study was diagnosed 2–4 years before the isolation of T-cell clones from their peripheral blood, demonstrating persistence of such high-avidity T-cells in the circulation. The persistence of these T-cells years after the onset of the disease, when the dose of stimulating islet antigen is most likely minimal, illustrates the potential longevity of the autoreactive memory response. It is also possible that limited regeneration of the  $\beta$ -cells or presence of residual  $\beta$ -cell mass could provide a source of antigen that could maintain a population of high-avidity T-cells that are capable of being reactivated at low doses of antigen. Subject 0702-592 was negative for GAD65 autoantibody at the time of the blood draw 4 years after diagnosis, suggesting that GAD65-specific T-cell response can be more persistent than positivity for autoantibodies, at least in some individuals. When we analyzed the tetramer-positive cells in peripheral blood of subject 0702-592 drawn 6 months earlier, a similar tetramer staining profile was

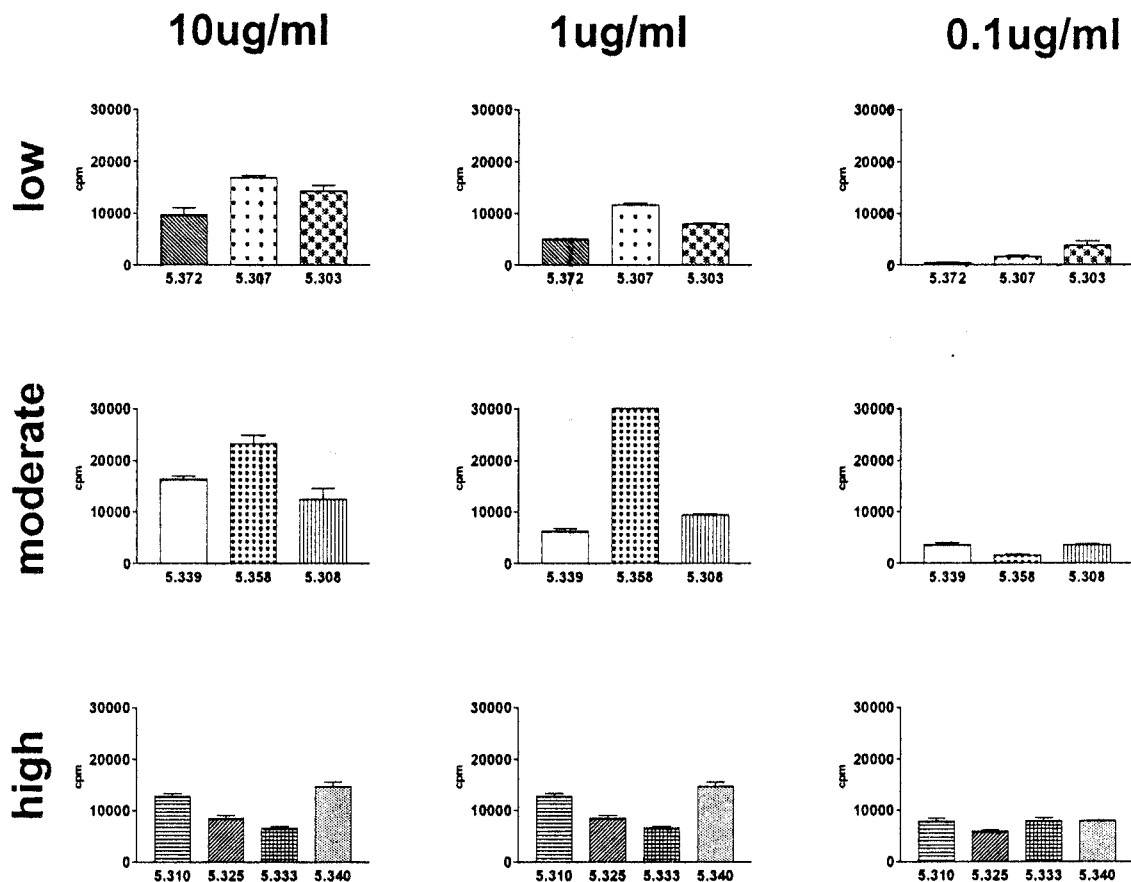


FIG. 3. Proliferation of T-cells with distinct tetramer binding. Proliferative response of T-cell clones with low, moderate, and high tetramer binding are shown. Each clone (BRI-5.303 to 5.372) was stimulated by GAD65 557I peptide in the presence of irradiated HLA-DR0404-matched PBMCs. Each bar represents the mean ( $\pm$ SE) counts per minute of [ $^3$ H]thymidine incorporation at 72 h in triplicate cultures.

found. However, we cannot exclude possible fluctuations of autoreactive T-cell populations in peripheral blood. A recent study by Trudeau et al. (20) on detection of class I tetramer-positive synthetic peptide mimotope-reactive T-cells in the NOD mouse suggested distinct cycles in the appearance of autoreactive cells in the peripheral blood. In human diseases other than diabetes, presence of dominant clonotypes in the peripheral blood of patients with rheumatoid arthritis (21–24) and melanoma (25–28) has been documented.

Several studies have demonstrated a correlation be-

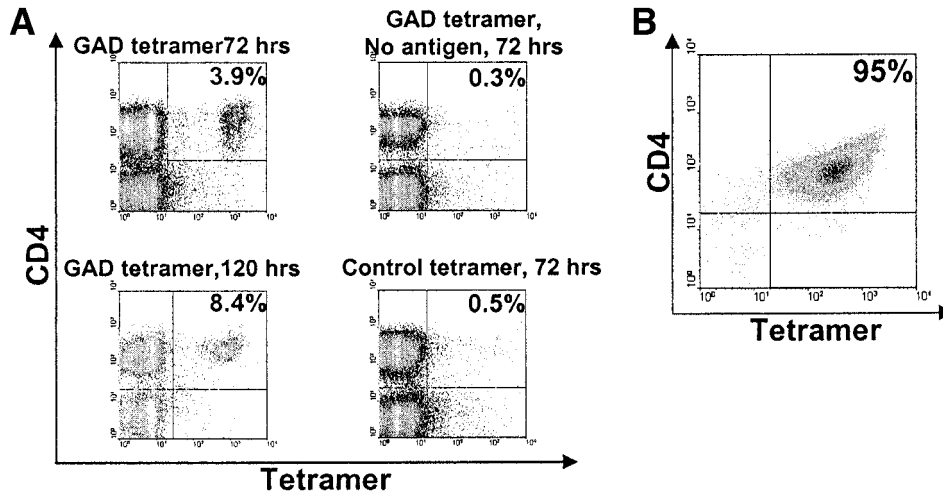
TABLE 1  
TcR usage, GAD tetramer staining, and cytokine profiles of the T-cell clones isolated from a patient with type 1 diabetes

Clone	TcR	Tetramer	Cytokines
339	V $\alpha$ 12 $\beta$ 5.1	++	Th0
358	V $\alpha$ 12 $\beta$ 5.1	++	Th0
307	V $\alpha$ 13 $\beta$ 8	+	Th1
372	V $\alpha$ 1 $\beta$ 8	+	Th1
303	V $\alpha$ 18 $\beta$ 6.7	++	Th0
340	V $\alpha$ 17 $\beta$ 4	+++	Th0
308	V $\alpha$ 17 $\beta$ 4	+++	Th2/0
310	V $\alpha$ 17 $\beta$ 4	+++	Th0
325	V $\alpha$ 17 $\beta$ 4	+++	Th0
333	V $\alpha$ 17 $\beta$ 4	+++	Th1

+, 5–20%; ++, 20–60%; +++, 60–100%.

tween tetramer staining and antigen sensitivity, the measures of structural and functional avidity used in this study (29–33). In an earlier study of the functional sensitivity and tetramer-binding characteristics, two HSV-specific HLA-DQ0602-restricted clones showed a direct correlation between the two properties, likely attributed to the kinetics of dissociation of the TcR-pMHC complex (15). In our current work, we observed a correlation between the tetramer-binding intensity and functional avidity in a set of clones that displayed differential tetramer binding. Moreover, the intensity of tetramer binding paralleled the magnitude of tetramer signaling, as assessed by CD69 upregulation.

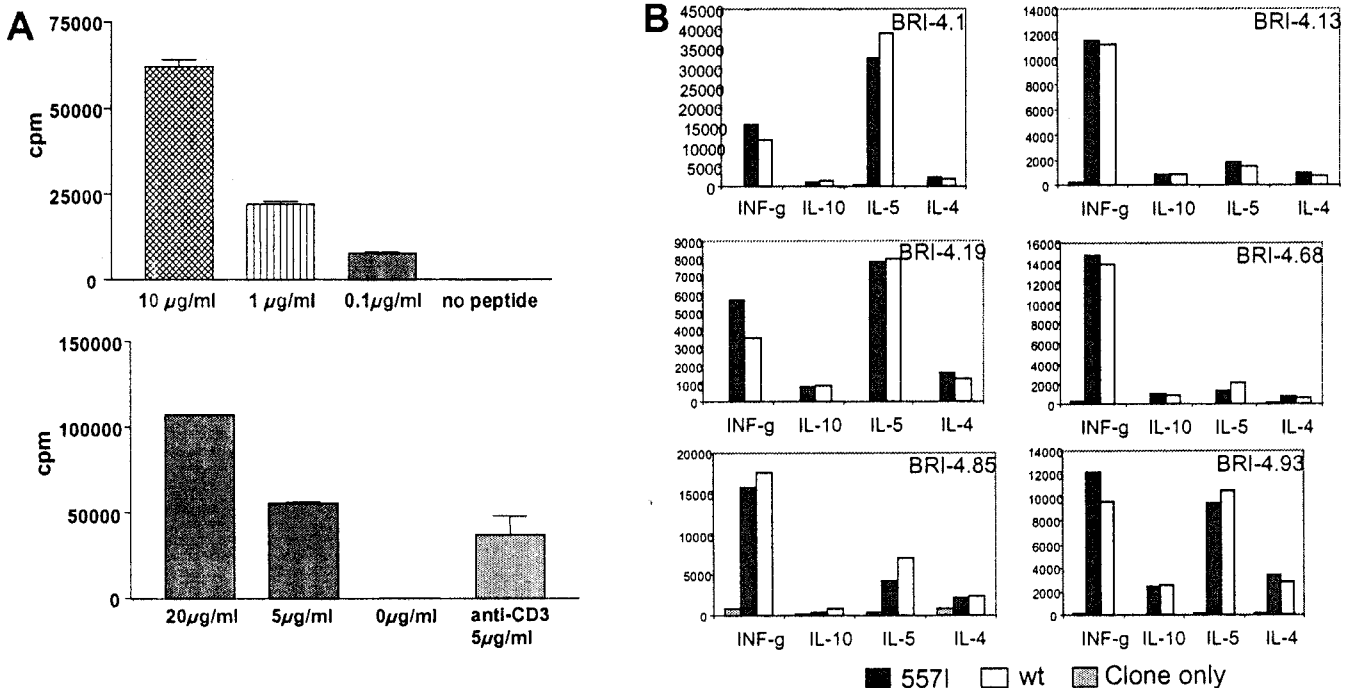
It is interesting that high-avidity T-cells using TcR V $\alpha$ 12-V $\beta$ 5.1 were observed in both patients with type 1 diabetes, and in one of them, this T-cell phenotype was dominant. Oligoclonal responses have been demonstrated in NOD mouse (34,35) but not in human diabetes. In the NOD mouse as well, a population of autoreactive pathogenic T-cells is represented by clonotypes that share similar TcR-V $\alpha$  chains (7). Dominant TcR V $\beta$  requirements for self-antigen recognition by CD4 $^+$  T-cells have been reported in multiple sclerosis (36). There have been several studies on the preferential V $\beta$ -chain usage by peripheral or islet-infiltrated T-cells in type 1 diabetes, but they have not provided evidence for dominance or antigen specificity of TcR V $\beta$  (37–39). The usage of TcR V $\beta$ 5.1 by several of our



**FIG. 4.** Oligoclonal high-avidity T-cells in PBMCs. *A:* DR0401-GAD65 and DR0401-HSV tetramers were used to stain *in vitro*-amplified PBMCs from subject 0102-239 after restimulation with DR0401-GAD65 monomer. Staining of unstimulated PBMCs with DR0401-GAD65 tetramer and control HSV tetramer to GAD65-stimulated cells is also shown. *B:* The single-cell sorting of the T-cells from patient 0102-239 with type 1 diabetes was performed as described above. A representative example (clone BRI-4.1) of binding to GAD65 tetramer is shown.

T-cell clones and peripheral GAD65-reactive CD4<sup>+</sup> T-cells of patients with type 1 diabetes is interesting, because an increase in the expression of this TcR V $\beta$  chain has been demonstrated in islet-infiltrating cells isolated from a postmortem pancreas of a patient with newly diagnosed type 1 diabetes, although antigen specificity of the dominant T-cells remained unresolved (40). It is possible that the preferential recognition of the peptide bound to multimeric soluble MHC class II by the V $\alpha$ 12, V $\beta$ 5.1 receptor may correlate with T-cells that display a high affinity to soluble tetramers and are less dependent on costimulation

(41). This is in line with findings of Viglietta et al. (42), who demonstrated that GAD65-reactive T-cells from patients with type 1 diabetes but not from normal subjects are independent of costimulation through the CD28/B7 pathway. Compared with naïve T-cells, memory T-cells are less dependent on costimulation (43), and it is possible that high-affinity cells that need less antigen and costimulation are activated earlier and have faster expansion kinetics (44). In the NOD mouse model, Tian et al. (45) showed that autoimmunity was first detected to V $\beta$ -cell determinants that had the highest precursor frequency and highest



**FIG. 5.** Proliferation and cytokine release of high-avidity oligoclonal T-cells. *A:* Proliferation of a representative T-cell clone from patient 0102-239 with type 1 diabetes was induced by GAD65 5571 peptide with irradiated HLA-DR0401-matched PBMCs (*top*) or with plate-bound HLA-DR0401 monomer at 5 and 20  $\mu$ g/ml (*bottom*). Each bar represents the mean counts per minute  $\pm$ SE of [<sup>3</sup>H]thymidine incorporation at 72 h in triplicate cultures. *B:* Cytokine production of six T-cell clones stimulated in the presence of GAD65 5571 and wild-type (wt) GAD65 555-567 peptides (10  $\mu$ g/ml) was evaluated from culture supernatants collected at 48 h from triplicate wells and then pooled.

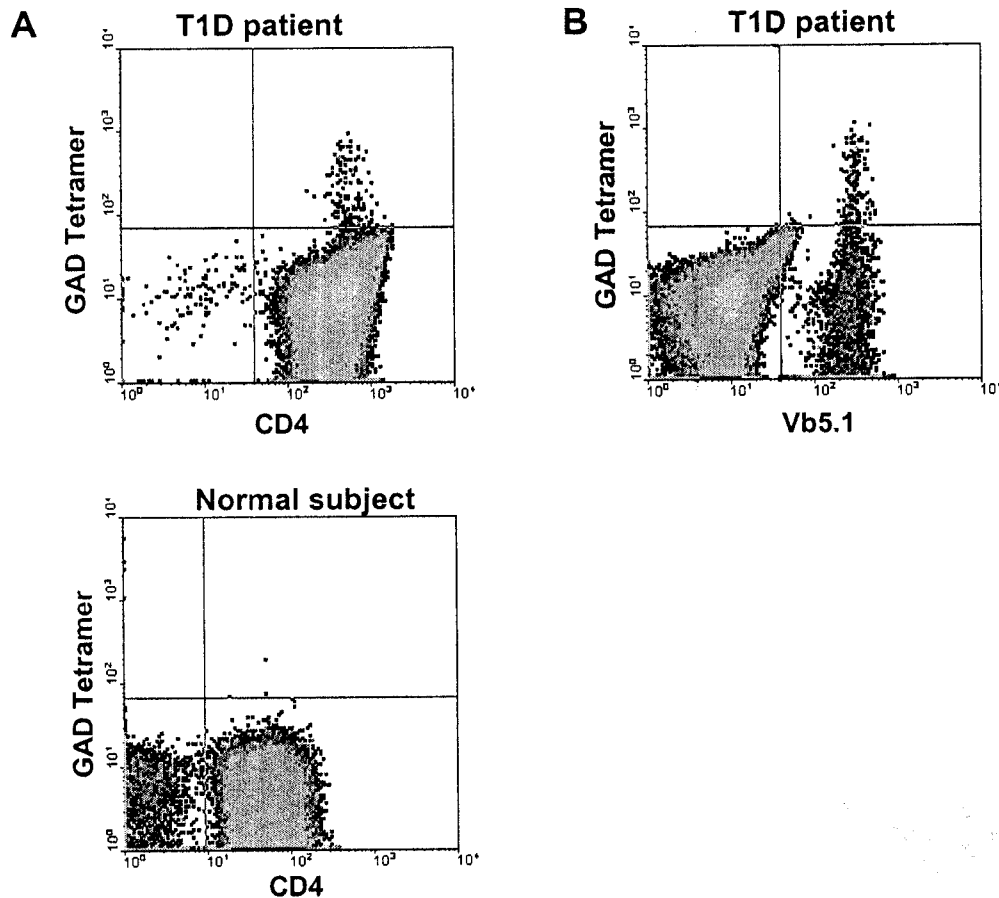


FIG. 6. TcR-V $\beta$  5.1 usage of GAD tetramer binding T-cells. An example of DR0401-GAD65 tetramer binding (A) and TcR-V $\beta$ 5.1 use (B) by antigen-stimulated PBMCs. Tetramer was used to stain PBMCs cultured in vitro with the GAD65 555-567 (wild-type) peptide for 12–14 days. Flow cytometry of the viable lymphocytic population as gated based on the forward/side scatter profile is shown.

proportion of high-avidity T-cells. Expansion of these high-avidity T-cells may create a persistent pool of autoreactive T-cells that can be subsequently detected from peripheral blood by specific tetramers. A retrospective study on the prevalence of these high-avidity T-cells and their role in the progression of type 1 diabetes in the subjects who are at risk to develop diabetes is under way. The presence of autoreactive T-cells with potential preferential usage of TcR to this important diabetes autoantigen may serve as both a potential marker for disease progression and a target for immune manipulation in autoimmune diabetes.

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