

# Detection of GAD65-Specific T-Cells by Major Histocompatibility Complex Class II Tetramers in Type 1 Diabetic Patients and At-Risk Subjects

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**Soluble HLA-DR401 or -DR404 tetramers containing a peptide corresponding to an immunodominant epitope from human GAD65 were used to analyze peripheral blood T-cells of newly diagnosed type 1 diabetic patients and at-risk subjects. Peripheral blood mononuclear cells were expanded on antigen-presenting cells presenting GAD65 peptide and subsequently activated with specific plate-bound class II-peptide monomers. T-cell activation defined in flow cytometry by CD4<sup>high</sup> and/or CD25 markers were observed in all type 1 diabetic patients and some at-risk subjects, but not in normal control subjects. The activated T-cells stained positive with tetramers containing the GAD65 epitope 555-567. Tetramer-positive cells were CD4<sup>high</sup> T-cells with high avidity for an immunodominant GAD65 T-cell epitope. Phenotyping of T-cells utilizing HLA class II tetramers provides a new tool to characterize the autoimmune response in type 1 diabetes. *Diabetes* 51: 1375-1382, 2002**

**T**ype 1 diabetes is an autoimmune disease resulting from the destruction of insulin-producing  $\beta$ -cells of the pancreas. Both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells are involved in this process, which targets a number of proteins expressed in human islets. One of the best-characterized of these autoantigens is GAD65 (1,2). Most type 1 diabetic patients (70–80%) have autoantibodies against GAD65, which often appear years before the clinical onset of the disease, providing a useful predictive marker for the progression of autoimmune diabetes. The importance of GAD65 in the development of type 1 diabetes has also been demonstrated in diabetic NOD mice, a well-known animal model of diabetes (3–7). Studies on T-cell responses to GAD65 in immunized human major histocompatibility complex (MHC) class II transgenic mice and new-onset type 1 diabetic patients have identified several immunodominant regions from GAD65 (8), one of which has recently been shown to be

naturally processed, identified by using a combination of chromatography and mass spectrometry of peptides bound by HLA-DR401 molecules (9). Peptides corresponding to this epitope region (GAD 554-572) elicit a T-cell response in a majority of HLA-DR4 type 1 diabetic patients and in some at-risk subjects, indicating that this epitope represents one of the determinants recognized by CD4<sup>+</sup> T-cells during autoimmune events associated with diabetes. However, despite the identification of at least some of the immunodominant regions from GAD65, a detailed characterization of the development of the T-cell response during the progression of type 1 diabetes has been cumbersome because of limitations of the methods to detect and isolate a small number of antigen-specific T-cells in the peripheral blood.

New techniques, such as the use of multimeric peptide-MHC complexes and enzyme-linked immunospot (ELISpot) assay, have enabled the detection of autoreactive T-cells at a very low frequency. ELISpot techniques have been used in the identification of low-frequency autoreactive T-cells in multiple sclerosis (10,11). Although ELISpot analysis allows detection of individual T-cells directly from peripheral blood and determination of their antigen-specific cytokine release, the advantage of tetramer staining is that it provides the ability to isolate the autoreactive T-cells for further characterization. MHC class I and class II multimers (or tetramers) have been successfully used in enumeration of CD8<sup>+</sup> and CD4<sup>+</sup> T-cells, respectively, that are specific for viral antigens (12–17), but it has been more challenging to apply MHC class II tetramers in identifying CD4<sup>+</sup> T-cells in autoimmune diseases where the precursor frequency of antigen-specific T-cells is very low. In contrast to high-affinity T-cell responses to foreign antigens, circulating autoreactive T-cells may display low-to-moderate affinity for self antigens, which makes the staining of these T-cells by class II MHC tetramers cumbersome. We sought to circumvent this issue by taking advantage of the association between tetramer binding and T-cell activation. It has been shown in both humans and mice that upregulation of surface expression of CD4 identifies T-cells activated in an antigen-specific manner (18–20). Using this upregulation of the CD4 marker in newly diagnosed type 1 diabetic patients and at-risk subjects, we analyzed the T-cell activation profile resulting from the stimulation of peripheral blood mononuclear cells (PBMCs) by immobilized HLA-DR401 or HLA-DR404 monomer containing a specific GAD65 peptide corre-

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ELISpot, enzyme-linked immunospot; HSV, herpes simplex virus; IL-2, interleukin-2; MHC, major histocompatibility complex; OspA, outer surface protein A; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TcR, T-cell receptor.

sponding to the immunodominant epitope 555-567. All type 1 diabetic patients and some at-risk subjects displayed a highly activated CD25<sup>+</sup>/CD4<sup>high+</sup> subpopulation that contained 4–28% tetramer-positive cells, which were not found in normal control subjects. The presence of an activated tetramer-positive T-cell phenotype correlated with type 1 diabetes and possibly reflects the progression of the disease before clinical onset.

## RESEARCH DESIGN AND METHODS

Blood samples from patients with type 1 diabetes ( $n = 4$ , 14–25 years of age) were obtained 1–6 months postonset. At-risk subjects were positive for two or more autoantibodies (GAD65, the insulinoma-associated tyrosine phosphatase-like protein IA-2, and insulin autoantibody), except for two subjects who had a single autoantibody. All patients were being treated for diabetes at the Section of Endocrinology, Virginia Mason Medical Center, and the at-risk subjects (aged 8–22 years,  $n = 6$ ) were participants in the prediabetes screening program at the Virginia Mason Research Center. Healthy blood donors ( $n = 5$ ) were recruited from the hospital/research center staff. Only subjects positive for DR401 or DR404 were included in our study.

**Preparation of HLA-DR401 and DR404 monomers and tetramers.** The construction of the expression vectors for generation of the soluble DR\*0101/DRB1\*0401 has been described previously (16). Briefly, a site-specific biotinylation sequence was added to the 3' end of the DRB1\*0401 or DR404 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, purified, concentrated, and biotinylated. Specific peptide was loaded for 48–72 h, and tetramers were formed by incubating class II molecules with phycoerythrin (PE)-labeled streptavidin.

**Isolation and stimulation of PBMCs.** PBMCs were separated from 15–40 ml heparinized blood by gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway). Cells were resuspended in RPMI-1640 (Gibco/BRL, Rockville, MD) supplemented with 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 100 µg/ml penicillin/streptomycin, and 15% vol/vol pooled human serum at a density of  $5 \times 10^6$ /ml and cultured in the presence of a GAD65 555-567 (557I; NFIRMVISNPAAT) peptide at a concentration of 10 µg/ml. From some samples a control culture was set up with an myelin base protein (MBP) 84-102 (NPVVHFFKNIPTPTPPP) peptide that binds well to DR401 and -404. On days 6–10, the cells were transferred at a density of  $4 \times 10^6$ /ml onto a 48-well plate that had been absorbed with 10 µg/ml DR401 or -404 monomer in  $1 \times$  PBS for 2 h at 37°C. The class II monomer contained the same peptide used in the primary culture. Then, 1 µg/ml anti-CD28 antibody (BD/Pharmingen, San Jose, CA) was added into the culture, and a low dose of interleukin-2 (IL-2; 2.5 units/ml) was added 48 h later.

**Identification of T-cell activation and tetramer staining by flow cytometry.** On day 3 the stimulated cells were stained by 10 µg/ml of PE-labeled HLA-DR401 or HLA-DR404 tetramer for 3 h at 37°C and subsequently by fluorochrome-labeled anti-CD25 and anti-CD4 (BD/Pharmingen, San Jose, CA) for 30 min on ice. Cells were then washed with PBS containing 1% fetal bovine serum and analyzed using a Becton-Dickinson FACSCalibur flow cytometer. The calibration was performed by using cells stained by single-fluorochrome anti-CD4. Data analysis was performed by using WinMdi (Stanford University) and CellQuest (Becton Dickinson) software programs.

**Fluorescent single-cell sorting, T-cell cloning, and proliferation assay.** The top 1% of the CD4 staining intensity among the CD25<sup>+</sup>/CD4<sup>high</sup> cells from two at-risk subjects were single-cell sorted into 96-well plates using a FACS Vantage cell sorter (Becton Dickinson). Clones were expanded for 10 days by stimulation with irradiated unmatched PBMCs ( $1.5 \times 10^5$ /well), 5 µg/ml phytohemagglutinin, and 10 units/ml IL-2. Subsequently, the cells were stimulated by HLA-DR4<sup>+</sup> PBMCs pulsed with 10 µg/ml of GAD65 557I peptide and 10 units/ml IL-2, and on day 7 they were selected by growth for further expansion. The resting T-cells were tested for proliferation by stimulating  $2 \times 10^4$  T-cells with irradiated autologous PBMCs ( $5\text{--}10 \times 10^4$ /well) with and without a specific peptide. <sup>3</sup>H-thymidine incorporation was measured at 72 h.

## RESULTS

**T-cells from type 1 diabetic patients stimulated by GAD65 557I peptide show the CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype.** PBMCs from four new-onset type 1 diabetic patients were stimulated with GAD65 557I peptide for 6–10 days. This peptide, NFIRMVISNPAAT, has an F-to-I substitution at

position 557, which has been shown to enhance agonist activity for proliferation and cytokine release from DR4-restricted T-cells (20a). After the primary PBMC culture, the cells were stimulated with immobilized DR401 or -404 monomer containing GAD65 557I or irrelevant peptide, and soluble anti-CD28 antibody. On day 3, the cells were stained by fluorochrome-conjugated specific and control tetramers and anti-CD25 and anti-CD4 surface markers and analyzed by flow cytometry. All four patients displayed a highly activated T-cell subset expressing a CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype (Fig. 1A–D). This activation phenotype was not present in normal subjects (Fig. 1E–F).

**The T-cell activation profile is heterogeneous in at-risk subjects.** We investigated GAD65 epitope-specific T-cell activation in six at-risk subjects (Fig. 2) who were positive for either HLA-DRB1\*0401 (subject 5574, 7657, 7878, 6827, and 6899) or DRB1\*0404 (6212). Four subjects (6212, 5574, 7657, and 7878) were positive for two or more autoantibodies. One subject (6827) was positive for low levels of insulin autoantibodies and had impaired glucose tolerance, and one subject (6899) had only GAD65 autoantibodies. Three of the subjects were followed at two occasions (Fig. 2A–C). Subjects 7878, 6212, and 5574 (Fig. 2A–B and D) have a distinct subset of CD25<sup>+</sup>/CD4<sup>high+</sup> positive T-cells, as was observed in all our patients with type 1 diabetes. The sample from subject 7878 (Fig. 2A) shown on the left panel was drawn 5 months before the subsequent one shown on the right panel. The T-cell activation profile shows a dramatic difference between these two time points. The T-cells from the first sample show very little activation when stimulated with the GAD65 557I peptide, in contrast to the T-cells obtained a few months later, which express a distinct CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype. Whether this difference in the GAD65-specific T-cell response indicates progression to type 1 diabetes remains to be seen. The second at-risk subject, 6212 (Fig. 2B), displayed an activated CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype in the first sample (left panel), and no change was observed 7 months later (right panel). The third at-risk subject, 6827 (Fig. 2C), displays a decrease in the number of CD25<sup>+</sup> cells in the sampling 5 months later (right panel), but on both occasions the phenotype of these cells was CD4<sup>low</sup>. Also, subject 7657 (Fig. 2E) has CD25<sup>+</sup> T-cells, but they are predominantly the CD4<sup>low</sup> phenotype. Interestingly, the two subjects who display the least T-cell activation (Fig. 2C and F) have only single islet cell-specific autoantibody, suggesting a lower risk to develop type 1 diabetes.

**GAD65-specific cells in the CD25<sup>+</sup>/CD4<sup>high+</sup> population are identified by tetramer staining.** Figure 3 shows three examples of tetramer binding analysis, using HLA-DR401 GAD65 tetramers to stain cells gated on CD25<sup>+</sup>/CD4<sup>high+</sup> markers. Figure 3A shows tetramer staining of the sample from type 1 diabetic patient 7810 shown in Fig. 1A. Of the cells expressing CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype, 28.7% stained with the specific GAD65 tetramer. Binding to herpes simplex virus (HSV) p61-control tetramer was 0.8%. Figure 3B shows tetramer staining in at-risk subject 7878. The gating criteria were the same as in Fig. 3A. Of the CD25<sup>+</sup>/CD4<sup>high+</sup> cells, 5.2% bound the GAD65 tetramer. Figure 3C shows the lack of tetramer staining in at-risk subject 7657, who had an increased

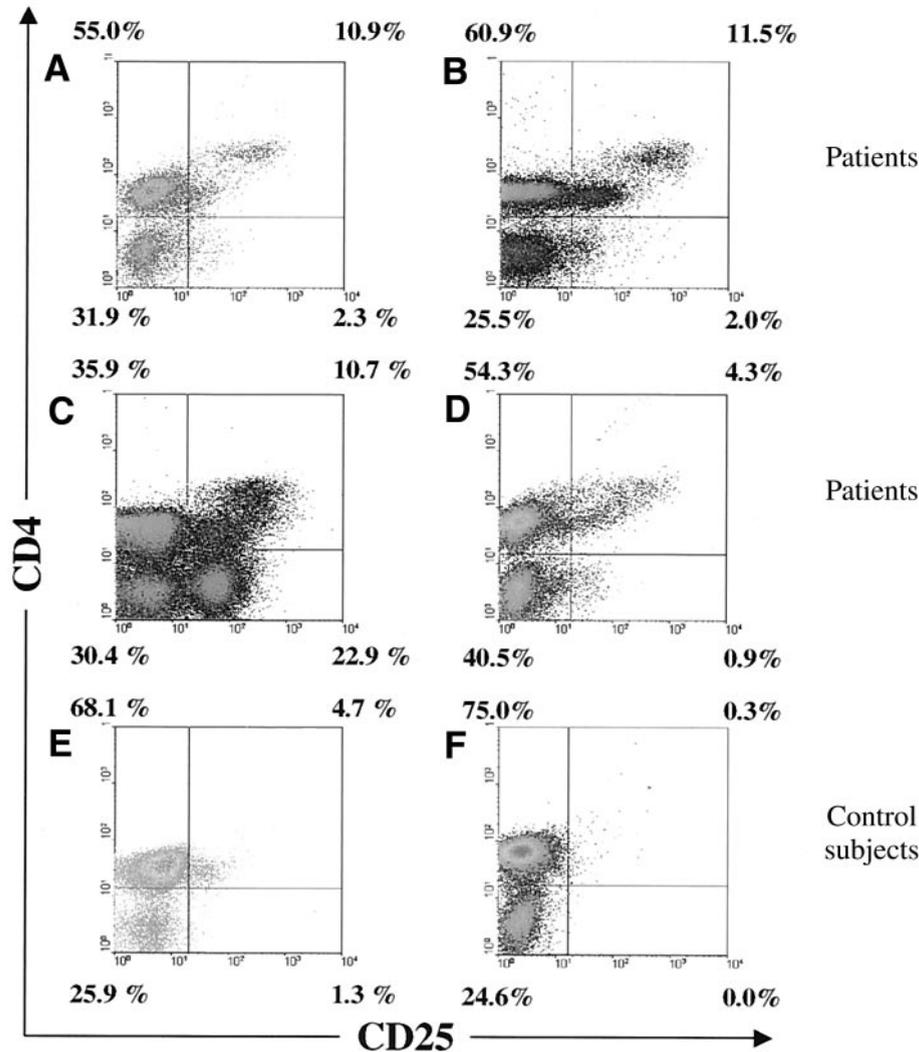


FIG. 1. GAD65-stimulated cells display CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype in type 1 diabetic patients. *A*, *C*, and *D*: Flow cytometry analysis of cells (obtained from newly diagnosed type 1 diabetic patients) that were restimulated with GAD65 5571 peptide on HLA-DR401 monomer. *A*: Patient 7810. *C*: Patient 7826 *D*: Patient 7858. *B*: Flow cytometry analysis of cells obtained from a newly diagnosed type 1 diabetic patient (7929) that were restimulated with GAD65 5571 peptide on DR404 monomer. In all panels, cells were gated on live lymphocyte cell populations in forward and side scatter. The cell number in the live population was variable because of the amount of PBMCs available for the analysis. The vertical axis shows CD4 fluorescence, and the horizontal axis shows the CD25 fluorescence. The percentage of CD25<sup>+</sup>/CD4<sup>+</sup> cells is shown on the upper right quadrant. HLA-DR401<sup>+</sup> normal subjects are shown in *E* (1010) and *F* (7842).

number of CD25<sup>+</sup>/CD4<sup>low</sup> cells (shown in Fig. 2*E*). Because this subject lacked the subset of CD4<sup>high+</sup> cells, the gating was set on the top 25% of the CD4 staining intensity among CD25<sup>+</sup>/CD4<sup>+</sup> cells, but no tetramer-positive cells were detected. Whether the activated CD25<sup>+</sup> cells are indeed antigen specific but have an affinity too low to stain by tetramer remains to be investigated. Overall, these findings indicate that CD4<sup>+</sup> T-cells that are able to bind GAD tetramers reside in the highly activated antigen-specific cell population characterized by the simultaneous high level of expression of CD25 and CD4.

**Correlation of CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype, tetramer staining, and type 1 diabetes.** Table 1 illustrates the relationship between tetramer staining and T-cell activation to GAD65 in our type 1 diabetic, at-risk, and normal subjects. All four new-onset diabetic patients had a highly activated CD25<sup>+</sup>/CD4<sup>high+</sup> cell population that was induced upon stimulation by GAD65 5571 peptide, and

4–28% of these cells also had the ability to bind the GAD65 tetramer. The same activation profile including positive tetramer staining was also observed in two at-risk subjects. In one of these subjects, the activated phenotype of CD4<sup>+</sup> T-cells was not present in an earlier sample, consistent with an emerging T-cell response toward this particular epitope. Heterogeneity in the GAD65-specific T-cell response among at-risk individuals, in terms of the expression of activation markers and tetramer staining that might be associated with the disease progression, is intriguing, but correlation with outcome requires long-term follow-up. Activation of T-cells to the GAD peptide, indicated by either CD25 or CD4<sup>high</sup> expression, was not detected in normal subjects. Also, when PBMCs from type 1 diabetic patients or at-risk subjects were stimulated by irrelevant peptide (MBP) in the primary culture and then exposed to control immobilized empty class II MHC, no activation or tetramer staining was observed (data not shown).

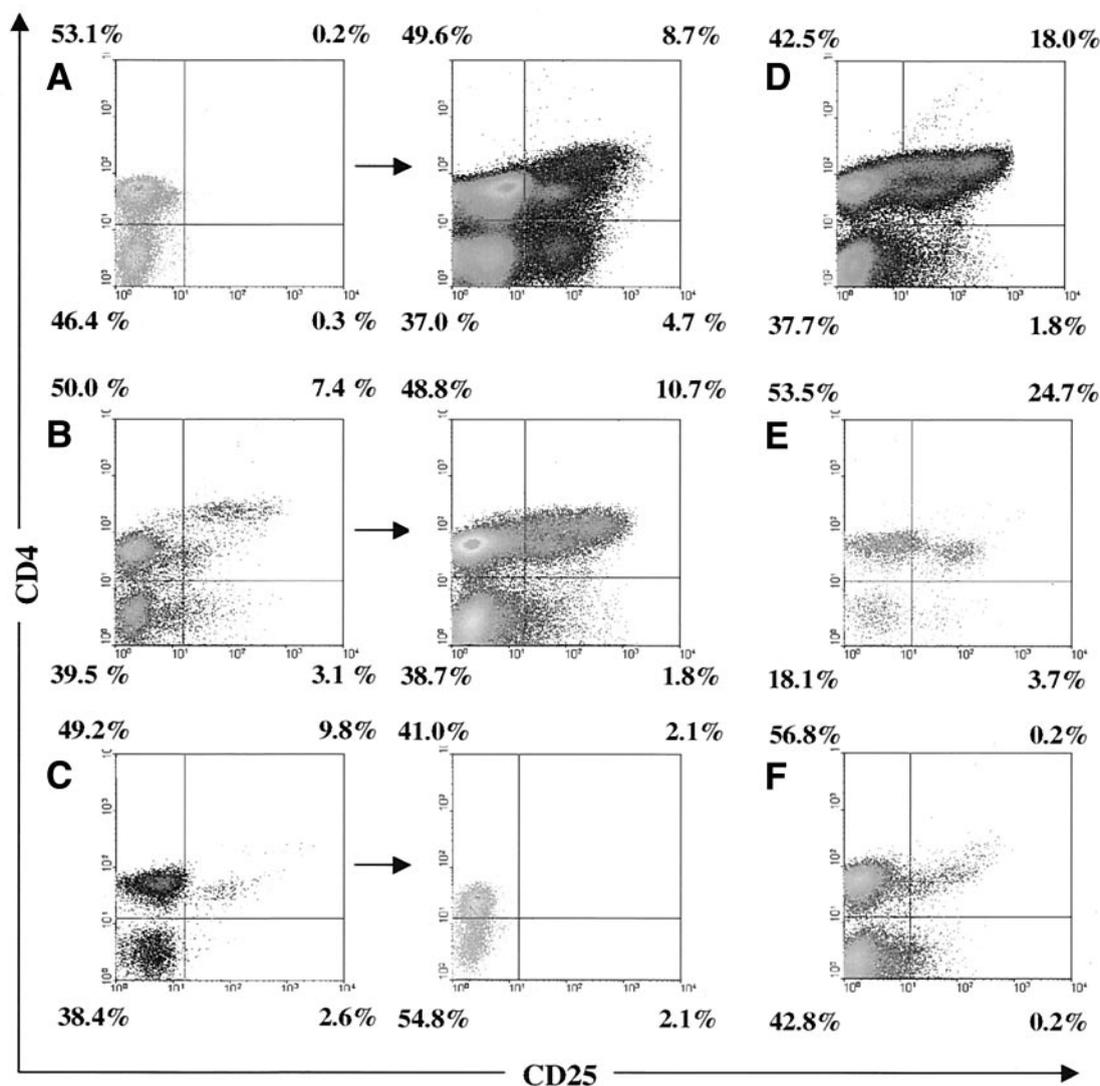


FIG. 2. GAD65-induced T-cell activation is heterogenous in at-risk subjects. Six at-risk subjects (A–F) were investigated for the presence of activated CD25<sup>+</sup>-CD4<sup>+</sup> T-cells on day 3 postrestimulation with immobilized HLA-DR401 or -DR404 monomer containing GAD65 557I peptide. The subjects were 7878 (A), 6212 (B), 6827 (C), 5574 (D), 7657 (E), and 6899 (F). PBMCs from at-risk subjects 7878, 6212, and 6827 were obtained at two different time points. Fresh samples from subjects 7878 and 6827 were analyzed at 5 months and those from subject 6212 at 7 months. In all panels cells were gated on a live lymphocyte cell population in forward and side scatter. The cell number in the live population was variable because of the amount of PBMCs available for the analysis. The vertical axis shows CD4 fluorescence, and the horizontal axis shows the CD25 fluorescence. The percentage of CD25<sup>+</sup>/CD4<sup>+</sup> cells is shown on the upper right quadrant.

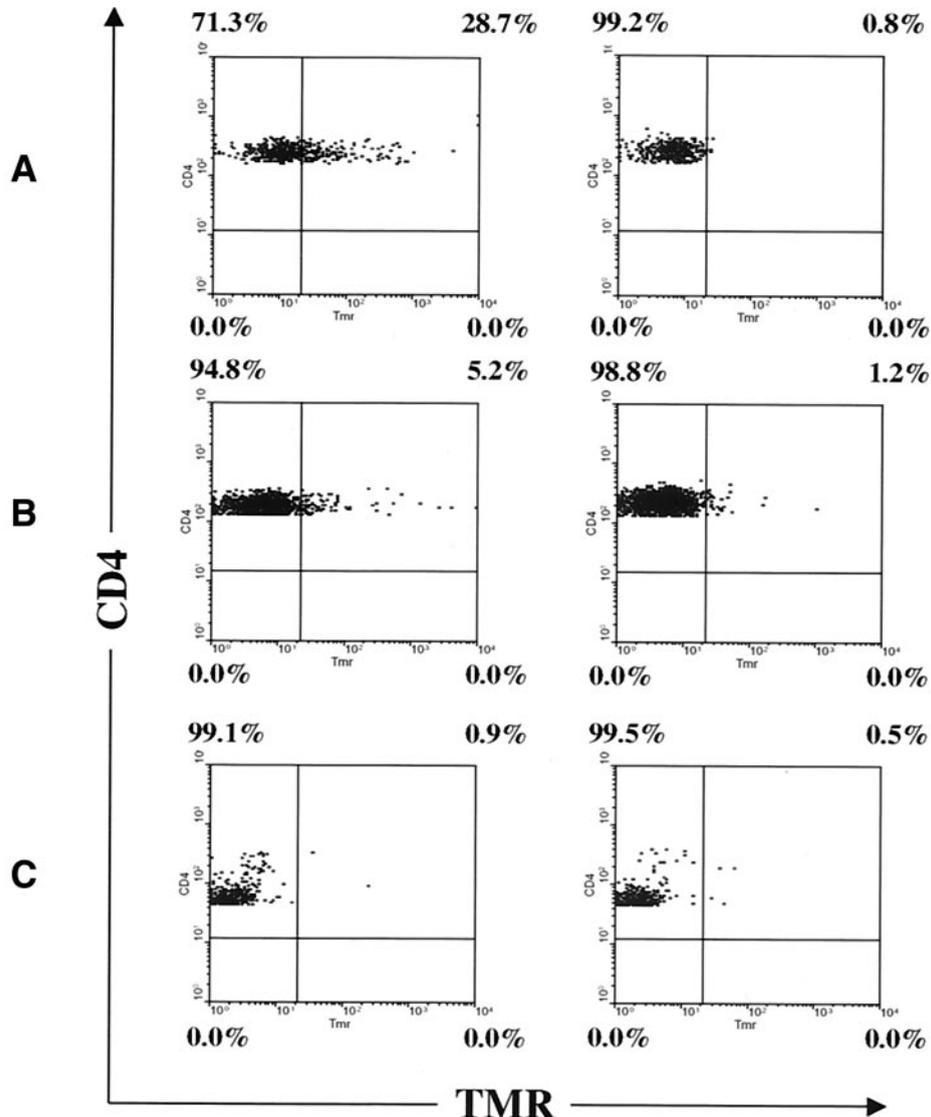
**CD25<sup>+</sup>/CD4<sup>high+</sup> sorted cells are GAD65 555-567-specific.** Specificity of the T-cell activation profile was examined in a more detailed fashion, using single-cell sorting of the CD25<sup>+</sup>/CD4<sup>high+</sup> cells from two at-risk subjects, 6212 and 5574, using flow cytometry to select cells from the top 1% of the CD4<sup>+</sup> staining intensity. Seven clones were obtained from both subjects, and these clones proliferated consistently in the presence of GAD65 557I peptide in replicate experiments. The T-cell proliferation in the presence of peptide was dose-dependent, and the clones responded to both the 557-I superagonist and the wild-type peptide GAD65 555-567 (Fig. 4). A clone isolated from subject 5574 stained strongly with GAD65 tetramer (Fig. 5).

#### DISCUSSION

The use of soluble class II MHC tetramers has enabled the identification of antigen-specific T-cells in type 1 diabetic patients and some at-risk subjects. Our approach takes

advantage of the appearance of highly activated T-cells expressing a CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype induced by immobilized class II MHC monomer containing the GAD65 peptide. This approach has enabled us to demonstrate in type 1 diabetic patients and some at-risk subjects a HLA-DR4 monomer-induced activation profile characterized by upregulation of CD4 on GAD65-specific T-cells. Almost all T-cells that stain with the specific tetramer reside in this population, and because this activation profile is not present in normal subjects, it could provide a useful tool for analysis of the T-cell response in autoimmune diabetes.

CD4<sup>+</sup> T-cell responses in recent-onset diabetic patients have been previously investigated in vitro using GAD peptides to induce proliferation of PBMCs. A major problem in detecting and quantitating autoreactive T-cells is their extremely low frequency in the peripheral blood of patients. Recent studies of patients with infections or autoimmunity, using class II tetramers, emphasize this point. In one study,



**FIG. 3.** Tetramer-binding GAD65-specific cells reside in the CD25<sup>+</sup>/CD4<sup>high+</sup> population. **A:** An example of the detection of tetramer-positive cells by flow cytometry in a CD25<sup>+</sup>/CD4<sup>high+</sup> cell population in type 1 diabetic patient 7810. **B:** Staining of cells from at-risk subject 7878, who displayed a distinct CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype upon stimulation by GAD65 peptide. **C:** Tetramer staining of the GAD65-stimulated cells from another at-risk subject, 7657, who displayed the CD25<sup>+</sup>/CD4<sup>low</sup> phenotype. **A–C:** The left panel represents the staining by a GAD65-557I tetramer and the right panel shows the staining by a control HSV-p61 tetramer. The cells were gated on a live lymphocytic cell population in forward and side scatter and CD25<sup>+</sup>/CD4<sup>high+</sup>. The cell number on both gates was variable because of the amount of PBMCs available for the analysis. **C:** The cells were gated on the top 25% of CD4 staining intensity among the CD25/CD4 double-positive cells because of the lack of a CD25<sup>+</sup>/CD4<sup>high+</sup> cell population in this subject. The vertical axis shows CD4 fluorescence and the horizontal axis shows the tetramer fluorescence. The percentage of tetramer<sup>+</sup>/CD4<sup>+</sup> cells is shown on the upper right quadrant.

the presence of collagen II- or glycoprotein 39-specific CD4<sup>+</sup> T-cells in synovial fluid of patients with rheumatoid arthritis was investigated (21). Although DR4 tetramers stained antigen-specific hybridomas, no tetramer-positive cells could be detected in synovial fluid of the patients. However, in a small number of DR401<sup>+</sup> patients, a low level of staining, slightly higher than background, was detected by glycoprotein 39 tetramer. It is possible that the number of T-cells recognizing these epitopes was too low for effective identification using class II tetramers. Also, the study was performed with patients who were 5 years postonset, and it is possible that the T-cell response to these particular epitopes from glycoprotein 39 and collagen II would have been stronger closer to disease onset. Another study demonstrated that class II tetramers can be used to enumerate

CD4<sup>+</sup> T-cells specific for *Borrelia* epitope outer surface protein A (OspA) from the synovial fluid and peripheral blood of HLA-DR401<sup>+</sup> patients with treatment-resistant Lyme arthritis (22). Although the frequency of tetramer-positive cells was very low, the authors could generate OspA-specific clones by sorting the tetramer-positive cells. In contrast, studies of CD4<sup>+</sup> T-cell responses to influenza or HSV 2 antigens have shown efficient detection with class II tetramers after in vitro expansion of responding cells (16,17).

The problems associated with the detection of rare autoreactive T-cells in the periphery by using tetramers has been addressed by Liu et al. (23) in a study on GAD-reactive CD4<sup>+</sup> T-cells in the NOD mouse model. T-cells from immunized mice were readily detectable with a I-A<sup>g7</sup> tetramer covalently linked to GAD peptides, but

TABLE 1  
CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype and detection of tetramer-positive cells in type 1 diabetic patients and at-risk and normal subject

Subject	CD4 <sup>high+</sup> / CD25 <sup>+</sup>	CD4 <sup>+</sup> / CD25 <sup>+</sup> *	Tetramer staining†	Type 1 diabetes
7810	Yes	10.9	28.7	Yes
7929	Yes	11.5	25.0	Yes
7826	Yes	10.7	4.1	Yes
7858	Yes	4.3	5.0	Yes
6212	Yes	7.4	8.6	At risk
7878	Yes	8.7	5.2	At risk
5574	Yes	18.0	5.4	At risk
7657	No	24.7	<1	At risk
6827	No	2.1	<1	At risk
6899	No	0.2	<1	At risk
7877	No	2.0	<1	No
7842	No	0.3	<1	No
1010	No	4.7	<1	No
7029	No	0.4	<1	No
3116	No	1.7	<1	No

Data are %. Number of CD25<sup>+</sup>/CD4<sup>+</sup> cells in total cell population gated on the live lymphocyte cell population in forward and side scatter; †number of tetramer-positive cells in total cell population gated on the live lymphocyte cell population in forward and side scatter and on CD25<sup>+</sup>/CD4<sup>high+</sup>.

those from islets or lymphoid organs of untreated NOD mice were not readily detectable, suggesting that immunization may expand antigen-specific T-cells or cells expressing relatively high-affinity T-cell receptors (TcRs), allowing better staining by tetramer. In our study the activation and in vitro amplification of GAD65-specific T-cells was crucial for detection of tetramer-positive cells. When tetramer staining was performed after a 10-day primary culture without secondary stimulation by GAD65-DR401 monomer, no tetramer-positive cells were detected (data not shown). We did not perform a direct comparison between antigen-presenting cells (APCs) and immobilized monomer stimulation because of the low number of cells available for analysis, but it is very likely that the DR401 monomer system provides a higher-density interaction between MHCs and TcRs. Problems associated with in vitro amplification include potential changes in the pheno-

type of the CD4<sup>+</sup> antigen-specific T-cells, preferential proliferation of high-affinity T-cells, and activation-induced cell death. In our T-cell culture system, a GAD65 557I peptide was used for better binding to DR401/404 and for the superagonist nature in the stimulation of T-cell clones specific for this minimal epitope. This high-binding peptide on a immobilized DR401/404 monomer may be one explanation for the efficient induction of the strongly activated CD25<sup>+</sup>/CD4<sup>high+</sup> cell population and expansion of these cells in the GAD65-responsive subjects. Notably, however, these CD25<sup>+</sup>/CD4<sup>high+</sup> T-cells, though expanded by GAD65 557I, also responded to the wild-type peptide GAD65 555-567, demonstrating true autoreactivity.

A correlation between T-cell activation, CD25<sup>+</sup>/CD4<sup>high+</sup> phenotype, and tetramer staining occurred in subjects who have type 1 diabetes or signs of diabetes-associated autoimmunity. Tetramer staining has been shown to be dependent on the activation state of CD4<sup>+</sup> T-cells in a study by Cameron et al. (24), in which they blocked staining by HA-DR1 oligomers by treatments that interfered with cytoskeletal rearrangements and endocytosis. Restimulation of antigen-specific T-cells with peptides in vitro can upregulate their CD4 expression. Liu et al. (23) demonstrated in their study that almost all the cells bound by I-A<sup>g7</sup>-GAD tetramer had upregulated their CD4 expression and become CD4<sup>high+</sup>, although a range in tetramer staining intensity was observed. This finding was extended to human systems in a study by Novak et al. (19). The physiological relevance of CD4 upregulation in TcR-MHC interaction is not fully understood, but it may contribute to signal amplification (25) or prolong the interaction between the TcR and MHC by affecting the dissociation of the complex (26).

One of our at-risk subjects had a high number of CD25<sup>+</sup>/CD4<sup>+</sup> cells with low levels of tetramer binding. It is possible that these cells are antigen-specific but possess low-affinity TcR. It has been demonstrated by Crawford et al. (27) that tetramer binding is directly proportional to TcR affinity and the level of TcR expression. Other alternative explanations are that the T-cells with high-affinity TcRs did not survive in the culture, or the CD25<sup>+</sup> cells represent regulatory T-cells prevalent in the prediabetic

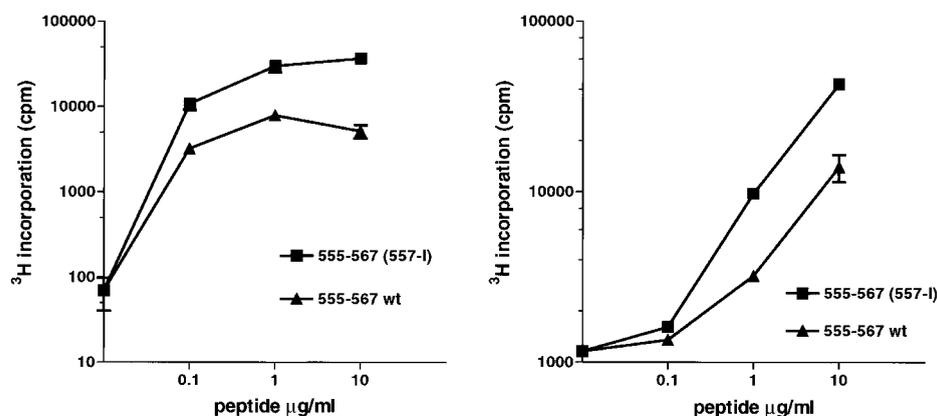


FIG. 4. Single-cell-sorted CD25<sup>+</sup>/CD4<sup>high+</sup> cells are GAD65 specific. Cells from an HLA-DR404-positive at-risk subject (6212) were stimulated with GAD65 as described above. Single-cell sorting was performed on the top 1% of the CD4 staining intensity in the cell population gated on forward and side scatter live lymphocyte cell populations and on CD25<sup>+</sup>/CD4<sup>high+</sup> cells. A representative T-cell proliferation of C15, one of seven T-cell clones isolated, is shown. Clone C15 was stimulated by autologous APCs (left chart) and BLS-DR404 B-LCL (right chart) pulsed with 0.1–10 µg/ml GAD65 557I or GAD65 555-567 wild-type (wt) peptide. Each dot represents the mean counts per minute of [<sup>3</sup>H]thymidine incorporation at 72 h in triplicate cultures. Error bars represent SE

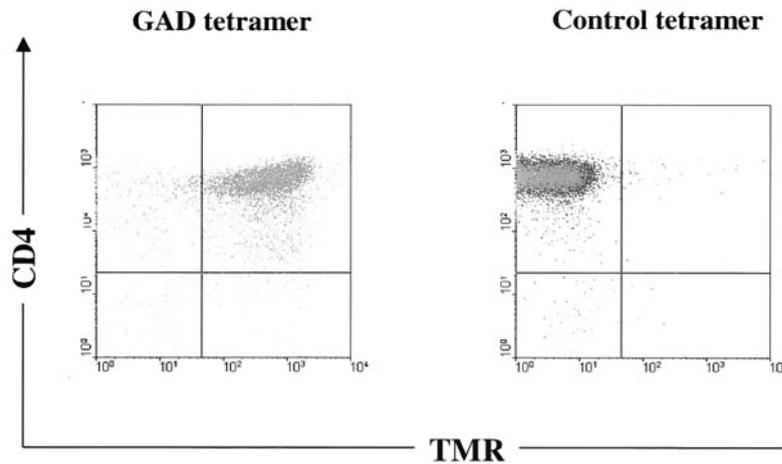


FIG. 5. Single-cell-sorted  $CD25^+/CD4^{\text{high}}$  cells are GAD65 tetramer-positive. Cells from an HLA-DR401-positive at-risk subject (5574) were stimulated with GAD65 as described above. Single-cell sorting was performed on the top 1% of CD4 staining intensity in the  $CD25^+$  live lymphocytic cell population. Clone 164 was stained by GAD65 557I tetramer (left panel) or HSV-p61 control tetramer (right panel). The vertical axis shows CD4 fluorescence, and the horizontal axis shows the tetramer fluorescence.

period and are possibly associated with a clinically non-progressive phenotype. Long-term prospective analysis of these at-risk subjects is needed to address this possibility.

The use of tetramers in the search of autoreactive T-cells is based on the presumption that the peptide on the tetramer corresponds to an immunodominant epitope of interest that is prevalent in the disease process. However, in type 1 diabetes, T-cell responses to several other epitopes have been reported, and it has been suggested that epitope spreading may occur during the progression of the disease (4,8,28,29). In addition, there are differences in the processing of epitopes between individuals and different APCs (30–32). All of these variables could create differences in the selection of autoreactive T-cells and could potentially limit the utility of the approach described here. Nevertheless, the use of tetramer techniques in the detection of autoreactive T-cells in type 1 diabetic patients and at-risk subjects is a powerful tool to gain insight into the mechanisms of molecular basis of autoimmunity. The phenotyping of T-cells should provide useful markers for progression of immune-mediated  $\beta$ -cell reactivity and could be used in clinical trials to evaluate the efficacy of the immunomodulatory therapies targeting intervention/prevention of type 1 diabetes.

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