

GAD65- and proinsulin-specific CD4⁺ T-cells detected by MHC class II tetramers in peripheral blood of type 1 diabetes patients and at-risk subjects

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Abstract

In type 1 diabetes the major loss of insulin producing beta-cells is caused by autoreactive T-cells specific for antigens expressed by the pancreatic islets. In this study we have analyzed the prevalence of glutamate decarboxylase 65 (GAD65)- and proinsulin-specific CD4⁺ T-cells in type 1 diabetes patients, at-risk subjects and in HLA-matched control children. Peripheral blood mononuclear cells were cultured in the presence of two different GAD65 peptides (555–567, 557I and 274–286) or with a proinsulin (B24–C36) peptide for 10–11 days. The autoreactive T-cells were detected using antigen specific-MHC class II tetramers by flow cytometry. Our results show that 11 of 18 (61%) type 1 diabetes patients and 7 of the 20 (35%) at-risk subjects were positive for one of the three GAD65 or proinsulin-containing tetramers, whereas only 2 of 21 (9.5%) controls had tetramer binding cells ($p = 0.0007$ type 1 diabetes vs. controls and $p = 0.0488$ at-risk subjects vs. controls, Chi-square test). Type 1 diabetes patients responded to all three peptides. At-risk subjects recognized also the GAD65 555–567 557I peptide, while none of the controls responded to it. In conclusion, type 1 diabetes patients and at-risk subjects have a significantly higher prevalence of GAD65- and proinsulin-specific CD4⁺ T-cells than the control subjects.

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1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by destruction of the beta-cells of the pancreas. It can take years until most of the beta-cells have been damaged and insulin secretion becomes insufficient to sustain normoglycemia. Autoantibodies specific for insulin, glutamate decarboxylase 65 (GAD65) and protein-tyrosine phosphatase 2 (IA-2) appear often during the preclinical phase of the disease and have

become a useful predictive marker for the progression of type 1 diabetes [1–3]. Less is known on the specificity of cellular responsiveness although the major loss of beta-cells is caused by autoreactive T-cells specific for antigens expressed in the pancreatic islets [4,5]. The development of T1D is also strongly associated with the major histocompatibility complex (MHC) class II genes. The genetic susceptibility in Caucasians correlates with the HLA-DR3-DQ201 and HLA-DR4-DQ302 haplotypes, where both HLA-DR and -DQ molecules affect the disease risk. Both these molecules may play an essential role in T1D through their presentation of islet cell specific-peptides to autoreactive CD4⁺ T-cells [6–8].

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The glutamic acid decarboxylase (GAD65) molecule is expressed in the beta-cells of the islets of Langerhans and in the central nervous system in humans [9]. Several T-cell epitopes of GAD65 have been described in type 1 diabetes [10]. A synthetic peptide consisting of amino acids 274–286 has been found to be recognized by DRB1*0401 transgenic mice [11] and a largely overlapping peptide 270–283 has been shown to stimulate T-cells isolated from a DR*0401 positive type 1 diabetes patient [12]. We have previously detected GAD65-specific CD4⁺ T-cells from peripheral blood of patients with type 1 diabetes that bind HLA-DR4 tetramers containing GAD65 555–567 peptide. This GAD65 555–567 peptide has been shown to be an efficiently processed immunogenic epitope in type 1 diabetes patients [13] and in DR*0401 transgenic mice [14].

Proinsulin is a precursor molecule that is processed into insulin and C-peptide in the beta-cells and it is the only known type 1 diabetes autoantigen expressed exclusively in the beta-cells. Autoantibodies to insulin (IAA) are an early marker of prediabetes which are frequently found especially in young children with T1D [15,16]. Multiple T-cell epitopes of proinsulin have been described and the region spanning the B–C junction has frequently been recognized in several studies. Proinsulin regions B14–C37 and C56–A72 have been shown to be recognized by diabetes patients [17] and a similar peptide (B24–C33) of mouse proinsulin has been described to stimulate T-cells from nonobese diabetic (NOD) mice [18]. Furthermore, peptide B24–C36 has been shown to stimulate T-cell responses in DR3 or DR4 positive individuals at risk for developing T1D [19]. Interestingly, this peptide has similarities with the GAD65 region 506–518, which might enhance the antigenic spreading of the T-cell reactivity as a result of cross-reactivity [19]. Martinez et al. have reported that the same proinsulin B24–C36 peptide can also simultaneously trigger CD4⁺ helper T-cell and CD8⁺ cytotoxic T-cell responses in NOD mice [20].

MHC class II tetramers are a useful tool for detection of autoreactive CD4⁺ T-cells in the peripheral blood. Tetramer MHC class II-peptide complexes bind antigen-specific CD4⁺ T-cells with a high degree of specificity and enable isolation of the T-cells for further characterization [13,21]. In the present study we have analyzed GAD65- and proinsulin-specific CD4⁺ T-cells in type 1 diabetes patients, at-risk subjects and HLA-genotype matched healthy controls using HLA-DR*0401 and -DR*0404 tetramers loaded with specific GAD65 peptides (epitope 555–567 557I or 274–286) and HLA-DR*0301 tetramers containing proinsulin peptide (epitope B24–C36). We also investigated whether GAD65- and proinsulin specific CD4⁺ T-cells in peripheral blood were detectable with MHC class II tetramers during the preclinical phase of the disease and whether these MHC class II tetramers could be a useful tool in the prediction of type 1 diabetes.

2. Materials and methods

Blood samples were obtained from type 1 diabetes patients ($n = 18$, median age 11.3 years); the date of diagnosis ranged

from 1 day to 8.5 years (median 4.5 years). At-risk subjects ($n = 20$, median age 6.3 years) were positive for islet cell autoantibodies (ICA) and one or more of biochemically defined autoantibodies (GAD65, IA-2, and insulin autoantibodies). The HLA-genotype matched healthy control subjects ($n = 21$, median age 5.5 years) were all autoantibody negative (Table 1). Only subjects positive for HLA-DRB1*0301, DR*0401 or DR*0404 were selected for this study. The at-risk and control subjects were participants in the Type 1 Diabetes Prediction and Prevention project (DIPP) in Turku, Finland. In the at-risk group 16 of the 20 subjects are participants in a placebo-controlled, double-blind intervention trial aimed at assessing whether it is possible to decrease the risk of progression to clinical T1D by daily nasal administration of human short-acting insulin [22]. Two at-risk subjects have developed diabetes since our study was completed (subject 23 and 34).

Typing for HLA-DR-DQ haplotypes was carried out using a lanthanide labeled oligonucleotide hybridization method as described earlier [23]. Control children participating in the DIPP study were originally selected based on the presence of HLA-DQB1*0302 and/or HLA-DQA1*05-DQB1*02 alleles/haplotypes and lack of protective alleles [22]. The typing was complemented with HLA-DR4 subtyping for those participating in this study.

2.1. Preparation of HLA-DR401, -DR404 and -DR301 monomers and tetramers

The construction of the expression vectors for the generation of the soluble DRB1*0401, DRB1*0404 and DRB1*0301 molecules have been described previously [24]. Briefly, a site-specific biotinylation sequence was added to the 3' end of the DRB1*0401, DRB1*0404 or DRB1*0301 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 were then purified, concentrated, and biotinylated. The desired peptide was loaded for 48–72 h, and tetramers were formed by incubating class II molecules with phycoerythrin (PE)-labeled streptavidin.

2.2. Peripheral blood mononuclear cell isolation and stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from 5–30 ml of heparinized blood by gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences, Uppsala, Sweden) and the cells were resuspended in RPMI-1640 (Invitrogen Corporation, Paisley, UK) supplemented with 3% glutamine (10 µl/ml) (Fluka Chemie, Stockholm, Sweden), 1 M sodium pyruvate (1 µl/ml) (Life Technologies, Paisley, UK), gentamycin sulfate 10 µg/ml (Biological Industries, Beit Haemek, Israel), 1 M HEPES-buffer (20 µl/ml) (Euroclone, West Yorks, UK) and 10% human serum (SPR, Helsinki, Finland) at a density of 5×10^6 /ml. The cells were cultured in the presence of GAD65 555–567 (557I; NFIRMVISNPAAT),

Table 1

Type 1 diabetes patients (T1D), at-risk subjects (AAB+) and HLA-genotype matched healthy controls in the study

Subject	T1D, AAB+, control	HLA-DQA1*	HLA-DQB1*	HLA-DRB1*	HLA-DR (deduced)	Age (years)	DM outbreak/prevention
1	T1D	NT	0301, 0302	0401, 0404	DR4, DR4	11	9 months
2	T1D	NT	0302, 0602	0401	DR4, DR15(2)	12	3 years, 9 months
3	T1D	NT	0302, 0501	0401	DR1, DR4	16	4 years, 7 months
4	T1D	03, 05	02, 0302	0401	DR3, DR4	13	2 years, 4 months
5	T1D	03, 05	02, 0302	0401	DR3, DR4	14	2 years, 2 months
6	T1D	03, 05	02, 0302	0401	DR3, DR4	12	8 years, 6 months
7	T1D	NT	0302, 0501	0401	DR1, DR4	3	10 months
8	T1D	NT	0302, 0501	0401	DR1, DR4	13	8 years, 5 months
9	T1D	NT	0302, 0604	0401	DR4, DR1302	11	7 years, 4 months
10	T1D	03, 05	02, 0302	0401	DR3, DR4	6	5 years
11	T1D	05	02, 0501		DR1, DR3	8	Less than a week
12	T1D	NT	0302, 0502	0401	DR4, DR16(2)	11	4 years, 7 months
13	T1D	NT	04, 0302	0401	DR4, DR8	11	4 years, 5 months
14	T1D	03, 05	02, 0303		DR3, DR3	11	5 years, 6 months
15	T1D	05	02, 02		DR3	11	5 years, 7 months
16	T1D	NT	0302, 0302	0404	DR4	16	6 years, 1 month
17	T1D	NT	02, 0302	0401	DR4	14	Less than a week
18	T1D	03, 05	02, 0302	0401	DR3, DR4	8	Less than a week
19	ICA+, GADA+	NT	0302	0401, 0404	DR4, DR4	14	Prevention
20	ICA+, GADA+	NT	02, 0302	0401	DR4	10	Prevention
21	ICA+, GADA+	NT	02, 0302	0401	DR4	7	
22A	ICA+, GADA+	03, 05	02, 0302	0404	DR3, DR4	6	Prevention
22B	ICA+, GADA+	03, 05	02, 0302	0404	DR3, DR4	7	Prevention
23*	ICA+, GADA+	NT	02, 0302	0401	DR4	5	Prevention
24	ICA+, GADA+, IA-2A+	NT	02, 0302	0401	DR4	9	Prevention
25	ICA+, GADA+, IA-2A+	03, 05	02, 0302	0404	DR3, DR4	6	Prevention
26A	ICA+, GADA+, IA-2A+	NT	0302	0401	DR4	6	
26B	ICA+, GADA+, IA-2A+	NT	0302	0401	DR4	6	
27	ICA+, IA-2A+	NT	02, 0302	0401	DR4	6	Prevention
28	ICA+, IA-2A+	NT	0302	0401	DR4	3	Prevention
29	ICA+, IA-2A+	NT	0302	0401	DR4	6	
30	ICA+, IAA+	NT	0302	0401	DR4	5	Prevention
31	ICA+, IAA+, GADA+	NT	02, 0302	0404	DR4	4	Prevention
32A	ICA+, IAA+, GADA+	NT	0302	0401	DR4	6	Prevention
32B	ICA+, IAA+, GADA+	NT	0302	0401	DR4	7	Prevention
33	ICA+, IAA+, GADA+, IA-2A+	NT	0302	0401	DR4	4	Prevention
34*	ICA+, IAA+, IA-2A+	03, 05	02, 0302	0401	DR3, DR4	3	Prevention
35	ICA+, GADA+, IA-2A+	05	02		DR3	5	Prevention
36	ICA+, IA-2A+	NT	0302, 0501	0401	DR1, DR4	10	Prevention
37	ICA+, IAA+, IA-2A+	NT	0302	0401	DR4	6	Prevention
38A	ICA+, IA-2A+	NT	02, 0302	0401	DR4	8	
38B	ICA+, IA-2A+	NT	02, 0302	0401	DR4	8	
39	Control	NT	0302	0404	DR4	8	
40	Control	NT	0302	0404	DR4	4	
41	Control	05	02		DR3	4	
42	Control	NT	0302	0401	DR4	4	
43	Control	NT	0302	0404	DR4	4	
44	Control	NT	0302	0404	DR4	6	
45	Control	03, 05	02, 0302	0401	DR3, DR4	6	
46	Control	05	02		DR3	6	
47	Control	NT	02, 0302	0404	DR4	5	
48	Control	03, 05	02, 0302	0401	DR3, DR4	9	
49	Control	NT	0302	0401	DR4	4	
50	Control	05	02		DR3	4	
51	Control	03, 05	02, 0302	0401	DR3, DR4	8	
52	Control	NT	02, 0302	0401	DR4	6	
53	Control	05	02, 0604		DR3, DR1302	5	
54	Control	NT	02, 0302	0404	DR4	8	
55	Control	NT	02, 0302	0404	DR4	10	
56	Control	NT	0302	0401	DR4	12	
57	Control	05	02		DR3	7	
58	Control	NT	0302	0401	DR4	5	
59	Control	NT	02, 0302	0404	DR4	7	

When two samples were subsequently tested from the same individual, the first sample is marked with an A and the follow up sample is marked with a B. At-risk subjects marked with an asterisk (*) have developed diabetes after this study was completed. ICA, islet-cell autoantibody; GADA, glutamate decarboxylase 65 autoantibody; IA-2A, protein-tyrosine phosphatase-2 autoantibody; IAA, insulin autoantibody; NT, not tested.

GAD65 274–286 (IAFTSEHSHFSLK) or proinsulin B24–C36 (FFYTPKTRREAED) peptides at a concentration of 10 µg/ml on a 24-well plate. After 10–11 days, the cells were transferred at a density of 1.5×10^6 /ml onto a 48-well plate that had been absorbed with 10 µg/ml DR*0401, DR*0404 or DR*0301 monomer in $1 \times$ PBS for 3 h at 37 °C. The monomers contained the same peptides used in the primary stimulation. The cells were cultured in the presence of 1 µg/ml of anti-CD28 antibody (BD Pharmingen, San Jose, CA, USA) during the secondary stimulation.

2.3. Tetramer binding and flow-cytometric analysis

On day 3 of the secondary stimulation one half of the cells were stained by 15 µg/ml of PE-labeled HLA-DR*0401/*0404 GAD65 555–567 557I, HLA-DR*0401 GAD65 274–286 or HLA-DR*0301 proinsulin B24–36 tetramers and the other half of the cells were stained by 15 µg/ml of PE-labeled control tetramers (HLA-DR*0401/*0404 herpes simplex virus (HSV) p61 465–484 (YGALDVDDFEFEQMFMTDAMG) or HLA-DR*0301 non-structural protein of influenza virus NSI p32–45 (FLDRLRRDQRSLRG) for 2.5 h at 37 °C followed by fluorochrome-labeled anti-CD4 and anti-CD25 (BD Pharmingen) for 20 min on ice. Cells were then washed in PBS containing 2% FCS and 0.1% NaN₃, and analyzed with a Becton Dickinson FACSCalibur flow cytometer. Data were analyzed with the CellQuest (Becton Dickinson) and WinMDI (Stanford University) software programs.

2.4. Statistical analysis

Distributions were tested with the Chi-square test, unless any expected value was less than five, when Fisher's exact test was used. The Mann–Whitney *U*-test was used to compare tetramer-binding levels between the groups.

3. Results

3.1. Detection of GAD65- and proinsulin-specific CD4⁺ T-cells in type 1 diabetes patients, at-risk and controls subjects with MHC class II tetramers

Peripheral blood mononuclear cells (PBMC) from 18 type 1 diabetes patients, 20 at-risk subjects (positive for two or more autoantibodies) and 21 HLA-matched healthy controls (autoantibody negative) were stimulated with GAD65 555–567 557I, GAD65 274–286 or proinsulin B24–C36 peptides based on their HLA-type for 10–11 days. The GAD65 557I peptide was used if the number of cells from a DR4⁺ subject was enough only for one culture. Additional stimulations with GAD65 274–286 peptide or proinsulin B24–C36 peptide were established from HLA-DR*0401 or -DR*0301 positive subjects, respectively, if the number of cells was sufficient.

The GAD65 555–567 557I peptide, NFIRMVISNPAAT, has a substitution at position 557 (F → I) at the P1 anchor residue that has been shown to efficiently stimulate proliferation and cytokine release from DR4-restricted T-cell clones [25].

GAD65 557I specific T-cell clones have also been shown to recognize both the wild type GAD65 555–567 peptide and the corresponding naturally processed epitope from GAD65 protein [13]. Because of the low frequencies of autoantigen epitope specific CD4⁺ T-cells in the peripheral blood, in vitro expansion with the peptides was necessary before the analysis by flow cytometry. After the primary stimulation the cells were stimulated with immobilized DR*0401, DR*0404 or DR*0301 monomers containing the same specific peptides as used in the primary stimulation and soluble anti-CD28 antibody. On day 3, the cells were stained by PE-labeled specific and control tetramers and anti-CD4 and anti-CD25 antibodies and analyzed by flow cytometry. Tetramer binding cells displayed a CD4^{high}/CD25⁺ profile and a representative staining is shown in Fig. 1. The top 20% of the CD4^{high}/CD25⁺ T-cells was gated and the frequency of tetramer binding cells was analyzed in this population of activated cells. As shown in Fig. 1A, 10% of the CD4^{high}/CD25⁺ cells from the type 1 diabetes patient 13 bind GAD65 557I tetramer. Staining with the control tetramer containing herpes simplex virus (HSV) p61 peptide was 0.9%. Fig. 1B shows tetramer staining of the cells from the at-risk subject 22A; 18.9% of the activated cells bound to GAD65 557I tetramer and 1.0% to the control tetramer. In Fig. 1C control subject 59 shows lack of tetramer binding cells.

3.2. Higher frequency of GAD65- and proinsulin-specific CD4^{high}/CD25⁺ T-cells in type 1 diabetes patients and in at-risk subjects than in control individuals

Fig. 2 shows the frequency of tetramer binding cells in type 1 diabetes patients (Fig. 2A), at-risk subjects (Fig. 2B) and in HLA-DR matched healthy controls (Fig. 2C). The positive tetramer staining value, $\geq 1.03\%$, was set on the 90th percentile for the tetramer staining in the control group. Our results show that 11 of 18 (61%) type 1 diabetes patients and 7 of the 20 (35%) at-risk subjects were positive for one of the GAD65 or proinsulin-containing tetramers, whereas only 2 of 21 (9.5%) controls had tetramer binding cells. The frequency of tetramer positive subjects was higher among type 1 diabetes patients and in at-risk subjects than in control subjects ($p = 0.0007$ and $p = 0.0488$, respectively) (Table 2A).

Also, the number of tetramer binding cells in the GAD65 or proinsulin activated CD4^{high}/CD25⁺ T-cells was higher in type 1 diabetes patients and in at-risk subjects than in control subjects. The tetramer staining in type 1 diabetes patients varied from 0.00 to 9.19% (median 0.60%). In the at-risk group the highest tetramer binding was as high as 53.60% (median 0.36%) and in the control group the tetramer positivity ranged from 0.00 to 2.84% (median 0.03%). The difference between type 1 diabetes patients and controls was statistically significant ($p = 0.02$).

In some occasions when sufficient numbers of PBMCs were available, the extra cells in the culture were analyzed at an additional time point: in this case the length of the culture was extended from standard 3 days to 6 days. Three type 1 diabetes patients became positive for GAD65 557I or GAD65 274–286 containing tetramers during this extended culture (data not

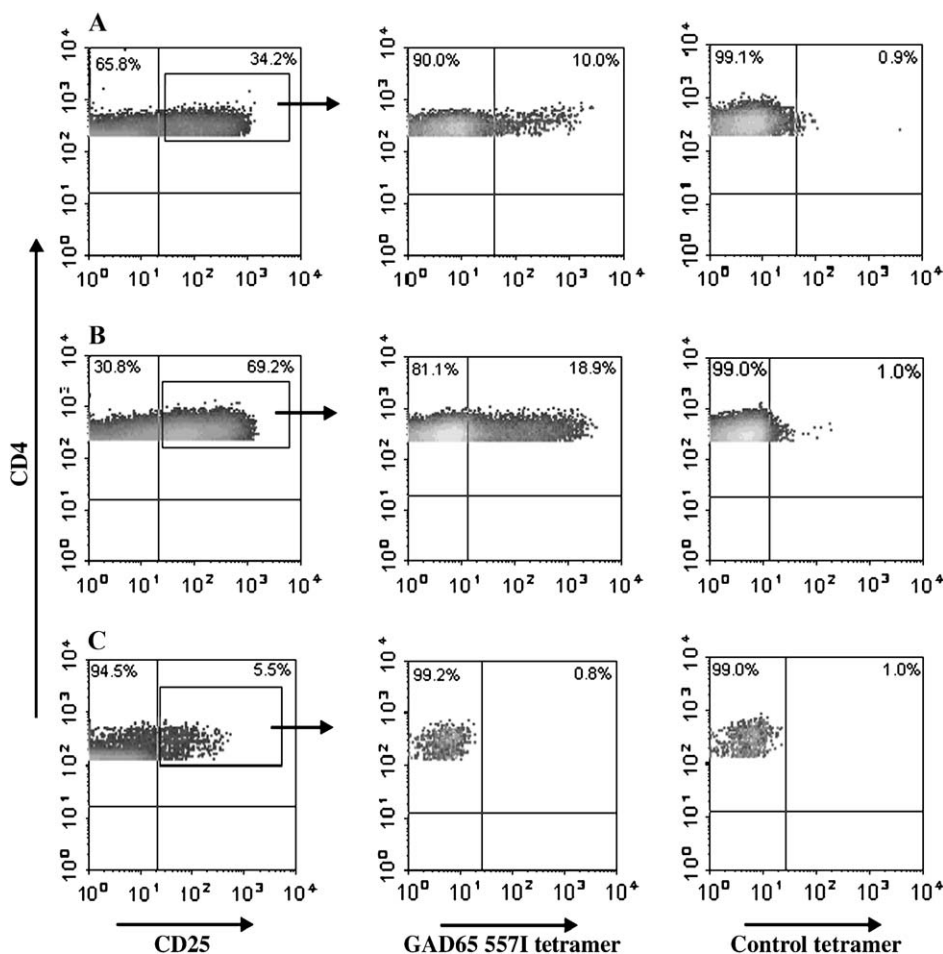


Fig. 1. GAD65 555–567 557I tetramer staining in T1D patient 13 (A), at-risk subject 22A (B) and control subject 59 (C). The cells were gated on live lymphocyte cell population in forward and side scatter. The top 20% of the $CD4^{\text{high}}/CD25^+$ T-cells (left) were gated, and the frequency of $CD4^{\text{high}}/CD25^+$ cells is shown on the upper right quadrant. The frequency of GAD65 555–567 557I (middle) and HSV p61 control (right) tetramer binding in the $CD4^{\text{high}}/CD25^+$ population is shown in the upper right quadrant. The cell number in the live populations was variable due to the amount of PBMCs available for the analysis.

shown). If we take into account tetramer positivity at any time point, day 3 and/or day 6, the total number of tetramer positive type 1 diabetes patients increases from 11 to 14 (78%).

3.3. GAD65 555–567 557I peptide activates $CD4^+$ T-cells in type 1 diabetes patients and in at-risk subjects but not in control individuals

Table 2B illustrates the distribution of GAD65 and proinsulin positive and negative tetramer binding in the three study groups. Four of the 18 tested T1D patients were positive for the GAD65 557I tetramer and five were positive for the proinsulin B24–C36 tetramer. Three T1D patients had GAD65 274–286 tetramer binding cells. Only one patient (subject 12) was positive for both GAD65 tetramers. Five out of seven type 1 diabetes patients who were diagnosed less than 2.5 years ago were positive either with proinsulin or GAD65 557I tetramers. Interestingly, the most frequent $CD4^+$ T-cell activator in the at-risk group was the GAD65 557I peptide (7 of 20 subjects), whereas none of the control subjects showed a positive tetramer staining after stimulation with this particular peptide. Four of these seven GAD65 557I

tetramer positive at-risk subjects (subjects 22, 25, 26 and 32) had also GAD65 autoantibodies, while three others, 28, 37 and 38, did not. Only one control subject responded to the GAD65 274–286 peptide and another showed a low positive staining with the proinsulin B24–C36 tetramer.

3.4. Follow-up analysis in the at-risk subjects

Twenty at-risk subjects were tested and six of them had tetramer binding $CD4^+$ T-cells in the first clinical sample. Three of these six have been analyzed at two occasions. Subject 22 displayed a high GAD65 557I positive tetramer binding profile at the first sampling, which was similar 10 months later (18%; 22A and 22B, respectively in Fig. 3A,B). Subject 26 showed a decrease of the GAD65 557I specific T-cells from 9.04% to 4.20% when analyzed 9 months later (26A, 26B in Fig. 3C,D). Both subjects were positive for islet-cell (ICA) and GAD65 autoantibodies at both time points and subject 26 had also IA-2 autoantibodies. No changes were seen in the autoantibody levels between the two time points. Another tetramer positive at-risk subject (subject 38) showed a very high GAD65 557I tetramer staining profile at the first

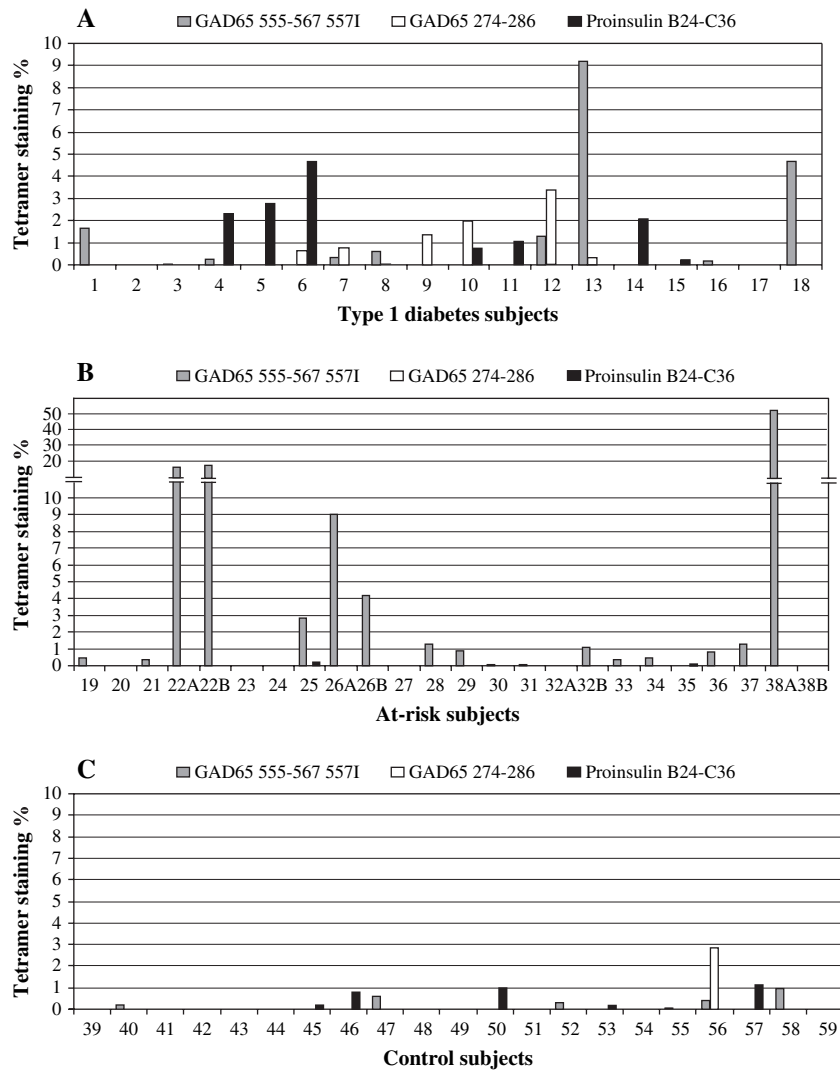


Fig. 2. Frequency of tetramer binding $CD4^{\text{high}}/CD25^+$ T-cells in type 1 diabetes patients (A), at-risk subjects (B) and HLA-genotype matched healthy controls (C). The bars show the percentage of tetramer staining cells for every subject, GAD65- or proinsulin tetramer staining minus control tetramer staining (background staining).

sampling (53.60%), but 3 months later no tetramer binding cells were detected (Fig. 3E,F). This subject had ICA and IA-2 autoantibodies, but no GAD65 autoantibodies. Once again no changes in the levels of autoantibodies between the two sampling dates were detected.

Only one originally negative subject was retested. In this case (subject 32) we saw a change from negative tetramer binding profile to a low positive one (1.06%) during a 12 month period (Fig. 3G,H). This particular subject expressed autoantibodies against ICA, GAD65 and insulin.

4. Discussion

Our study demonstrates that GAD65 and proinsulin tetramer binding $CD4^+$ T-cells are detectable more often in peripheral blood of type 1 diabetes patients and at-risk subjects than healthy individuals. HLA-DR4⁺ type 1 diabetes patients and at-risk subjects had T-cells specific for both GAD65 peptides tested (GAD65 555–567 557I and GAD65 274–286),

Table 2
Distribution of the prevalence of GAD65- and proinsulin tetramer binding in the three study groups

	Positive	Negative	%	<i>p</i> -value (chi-square)
A. Any tetramer				
T1D	11	7	61	0.0007
At-risk	7	13	35	0.0488
Controls	2	19	9.5	
B. Individual peptides				
	Positive	Positive	Positive	
T1D	4/15	3/8	5/8	
At-risk	7/19	0/1	0/2	
Controls	0/16	1/1	1/6	

The number of subjects with tetramer positive cells after stimulation with any peptide (A) and number of subjects with tetramer positive cells after stimulation with individual peptides (B). Definition of positive staining ($\geq 1.03\%$) was set on the 90th percentile of tetramer staining (with any tetramer) in the control group.

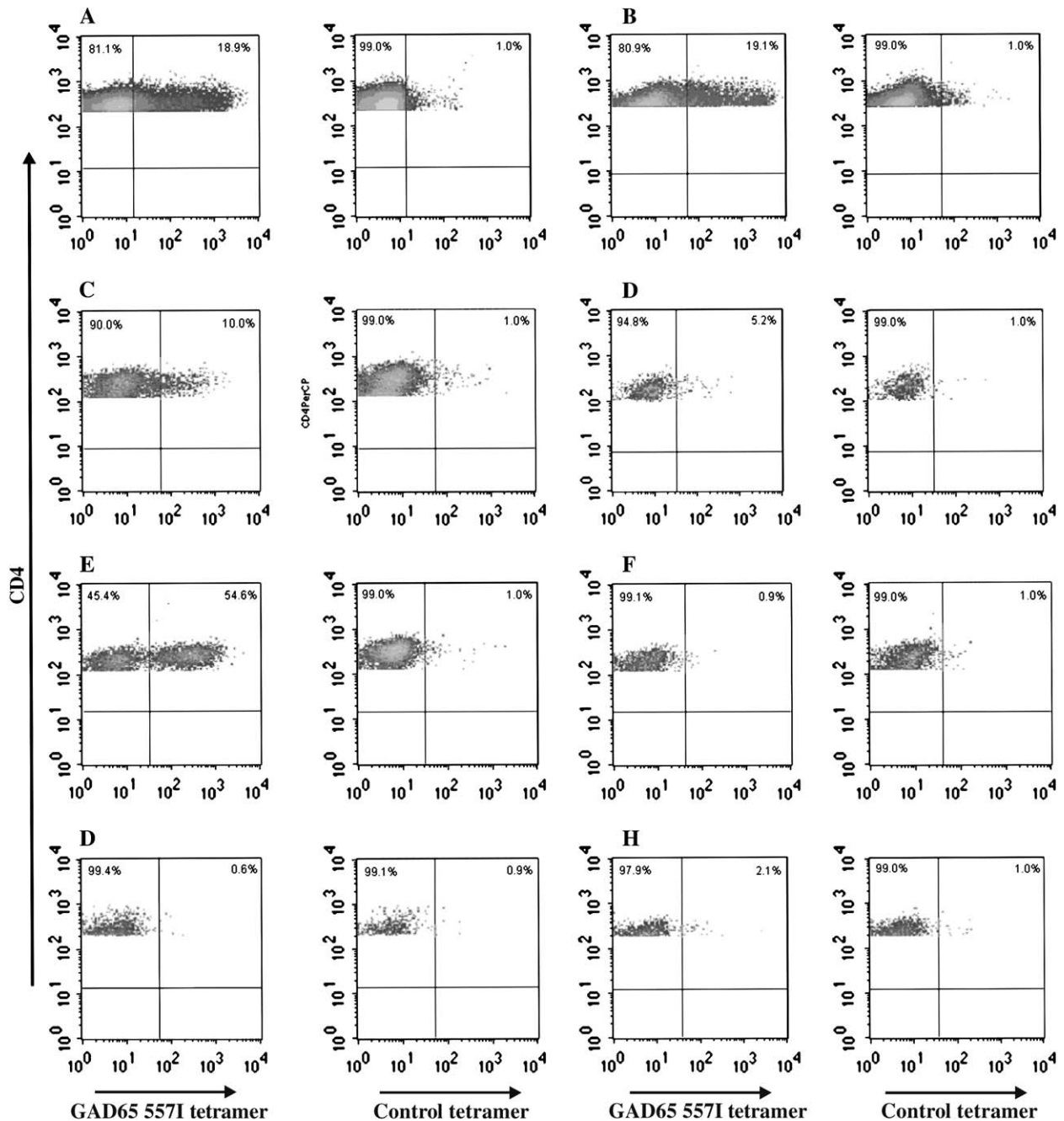


Fig. 3. Repeated tetramer analysis in at-risk subjects. Subject 22 at the first sampling (A), and 10 months later (B). Subject 26 at the first sampling (C), and 9 months later (D). Subject 38 at the first sampling (E), and 3 months later (F). Subject 32 at the first sampling (G), and 12 months later (H). The cells were gated on live lymphocyte cell population in forward and side scatter, and then on the top 20% of the CD4^{high}/CD25⁺ T-cells. The frequency of GAD65 555–567 557I (left) and HSV p61 control (right) tetramer binding in the CD4^{high}/CD25⁺ population is shown in the upper right quadrant. The cell number in the live populations was variable due to the amount of PBMCs available for the analysis.

while the majority of the DR3⁺ type 1 diabetes patients displayed response to a proinsulin peptide, B24–C36. Autoantibody positive children responded frequently to the GAD65 555–567 557I peptide, while none of the control subjects recognized it.

Autoantibodies specific for different islet cell proteins such as insulin, GAD65 and IA-2, are the most informative biological markers for the prediction of type 1 diabetes. In contrast, T-cell profiling in type 1 diabetes, and in human autoimmunity in general, is in its early stages. The development of new

techniques such as MHC class II tetramers now makes it possible to probe T-cell compartments for analysis of specificity for several autoantigens and epitopes. Precise definition of the MHC/peptide specificity and functional properties of CD4⁺ T-cell populations is required for mechanistic understanding of the disease process of type 1 diabetes. In order to design prevention therapies and for proper immunomonitoring we need to learn the specificity and phenotype of the T-cells that are prerequisite for the disease outcome and sufficient for the clinical disease.

In both human and NOD mice a small number of autoantigens including insulin (or proinsulin), GAD65 and IA-2, have been shown to be targets of the autoimmune attack [10,26]. Insulin is the only beta-cell specific autoantigen. In humans the strongest susceptibility conferred by a loci outside of MHC class II region is the insulin gene (Ins) variable number of tandem repeats (VNTR) regulatory region [27]. The disease associated polymorphism (class I repeats) correlates with reduced thymic expression of the Ins gene, which has been suggested to lead to defective negative selection and impaired induction of central tolerance to insulin and proinsulin [28,29]. In our study less than half of the type 1 diabetes patients were DR3⁺ but five out of eight of this group displayed T-cell response to the proinsulin epitope. Two very recent studies have highlighted the key role of proinsulin in NOD and human diabetes [30,31]. However, the role of GAD65 as a primary antigen cannot be excluded, at least in human type 1 diabetes. Even if insulin turns out to be a primary autoantigen involved in the early events of beta-cell destruction, GAD65 or another autoantigen could still play a crucial role in the amplification of the autoimmune process and the intermolecular epitope spreading. It is likely that several autoantigens and epitopes contribute to the disease process although the hierarchy of epitopes may vary along the progression of autoimmunity. The highest risk to progress to the clinical disease might correlate with the number of the T-cell epitopes recognized, which would be similar to the predictive value of having multiple autoantibodies. In this study type 1 diabetes patients displayed T-cell response to both GAD65 and proinsulin, but only one patient had reactivity to both autoantigens. However, only 11 patients were analyzed with multiple tetramers due to the limited sample size. T-cell response to the multiple autoantigens and potential epitope spreading during the preclinical phase of the disease process needs to be investigated in an extended study in a larger cohort of type 1 diabetes patients and at-risk subjects.

There are several technical constraints in the development of T-cell assays to identify autoreactive T-cells in the peripheral blood of type 1 diabetes patients. The difficulty to discriminate patients from normal subjects has been addressed in several individual studies and T-cell workshops [32,33]. Reliable detection of rare autoantigen specific T-cells in the peripheral blood has been challenging. We have estimated that only one in 30,000 or less CD4⁺ T-cells in peripheral blood are GAD65 reactive [24]. This low number of cells cannot be readily detected by flow cytometry and therefore our assay relies on in vitro expansion of antigen specific T-cells prior to the tetramer staining. Disadvantages of in vitro expansion include potential activation-induced apoptosis of the high avidity cells during the culture. The fact that T-cells with the highest avidity have been shown to be most readily detectable by tetramers suggests that the frequencies of T-cells determined in this study are conservative estimates [34,35]. It is also technically challenging to design the optimal culture conditions to facilitate the detection of antigen specific cells without confounding parameters such as unspecific by-stander proliferation and activation of regulatory cells.

In this study the presence of GAD65 specific T-cells were followed on two occasions in four at-risk subjects. Two subjects (subjects 22 and 26) displayed a consistent pattern of strong staining with a tetramer, and one individual (subject 32) became tetramer positive in the second sampling. However, in one at-risk child (subject 38) who had displayed the highest number of GAD65 557I tetramer binding cells in the study (53.60% of the CD4^{high}/CD25⁺ activated cells), disappearance of these GAD65 557I specific T-cells in the sample drawn 3 months later was observed. This kind of fluctuation in the level of circulating islet antigen specific T-cells is reminiscent of observations by Trudeau and colleagues [36] who demonstrated in NOD mice that murine CD8⁺ tetramer binding T-cells specific for the mimetic NRP epitope appear in the peripheral blood in cycles prior to the onset of hyperglycemia. Even mice that developed diabetes had undetectable levels of autoreactive T-cells in some blood samples. The authors suggest that these cycles possibly reflect clonal proliferation of antigen specific T-cells and target organ homing during the inflammatory process. This possibility is much more difficult to test in humans, but we can speculate that active beta-cell targeted immunity possibly leads to fluctuation and even periodic disappearance of circulating autoantigen specific T-cells in peripheral blood. Whether these kind of changes in the detectable levels of tetramer binding T-cells are a general phenomenon preceding the clinical onset of the disease remains to be investigated in a longitudinal study of a larger cohort of at-risk subjects with frequent sampling intervals.

Autoreactive T-cell responses in type 1 diabetes are very complex and much is still unclear about the target of the autoimmune attack. Epitope spreading may occur and there may be individual differences in the processing of these epitopes [37] that contribute to the progression of the type 1 diabetes. This study demonstrates that MHC class II tetramers are a promising tool for monitoring disease progression in the individuals at risk to develop type 1 diabetes and in the identification of autoreactive T-cells in type 1 diabetes patients.

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References

- [1] Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu DF, et al. Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. *Lancet* 1992;339:458–9.

- [2] Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, et al. Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science* 1983;222:1337–9.
- [3] Bingley PJ, Christie MR, Bonifacio E, Bonfanti R, Shattock M, Fonte MT, et al. Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. *Diabetes* 1994; 43:1304–10.
- [4] Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med* 1994;331:1428–36.
- [5] Panina-Bordignon P, Lang R, van Endert PM, Benazzi E, Felix AM, Pastore RM, et al. Cytotoxic T cells specific for glutamic acid decarboxylase in autoimmune diabetes. *J Exp Med* 1995;181:1923–7.
- [6] Ilonen J, Sjöroos M, Knip M, Veijola R, Simell O, Åkerblom HK, et al. Estimation of genetic risk for type 1 diabetes. *Am J Med Genet* 2002; 115:30–6.
- [7] Undlien DE, Friede T, Rammensee HG, Joner G, Dahl-Jorgensen K, Sovik O, et al. HLA-encoded genetic predisposition in IDDM: DR4 subtypes may be associated with different degrees of protection. *Diabetes* 1997;46:143–9.
- [8] Reijonen H, Nepom GT. Role of HLA susceptibility in predisposing to insulin-dependent diabetes mellitus. *Front Hormone Res* 1997;22: 46–67.
- [9] Karlens AE, Hagopian WA, Grubin CE, Dube S, Distchele CM, Adler DA, et al. Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. *Proc Natl Acad Sci U S A* 1991;88:8337–41.
- [10] Lieberman SM, DiLorenzo TP. A comprehensive guide to antibody and T-cell responses in type 1 diabetes. *Tissue Antigens* 2003;62:359–77.
- [11] Wicker LS, Chen SL, Nepom GT, Elliott JF, Freed DC, Bansal A, et al. Naturally processed T cell epitopes from human glutamic acid decarboxylase identified using mice transgenic for the type 1 diabetes-associated human MHC class II allele, DRB1*0401. *J Clin Invest* 1996;98: 2597–603.
- [12] Endl J, Otto H, Jung G, Dreisbusch B, Donie F, Stahl P, et al. Identification of naturally processed T cell epitopes from glutamic acid decarboxylase presented in the context of HLA-DR alleles by T lymphocytes of recent onset IDDM patients. *J Clin Invest* 1997;99:2405–15.
- [13] Reijonen H, Novak EJ, Kochik S, Heninger A, Liu AW, Kwok WW, et al. Detection of GAD65-specific T-cells by major histocompatibility complex class II tetramers in type 1 diabetic patients and at-risk subjects. *Diabetes* 2002;51:1375–82.
- [14] Patel SD, Cope AP, Congia M, Chen TT, Kim E, Fugger L, et al. Identification of immunodominant T cell epitopes of human glutamic acid decarboxylase 65 by using HLA-DR ($\alpha 1^*0101, \beta 1^*0401$) transgenic mice. *Proc Natl Acad Sci U S A* 1997;94:8082–7.
- [15] Ziegler AG, Hummel M, Schenker M, Bonifacio E. Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABY-DIAB Study. *Diabetes* 1999;48:460–8.
- [16] Kimpimäki T, Kupila A, Hämäläinen AM, Kukko M, Kulmala P, Savola K, et al. The first signs of beta-cell autoimmunity appear in infancy in genetically susceptible children from the general population: the Finnish Type 1 Diabetes Prediction and Prevention Study. *J Clin Endocrinol Metab* 2001;86:4782–8.
- [17] Narendran P, Williams AJ, Elsegood K, Leech NJ, Dayan CM. Humoral and cellular immune responses to proinsulin in adults with newly diagnosed type 1 diabetes. *Diabetes Metab Res Rev* 2003;19:52–9.
- [18] Chen W, Bergerot I, Elliott JF, Harrison LC, Abiru N, Eisenbarth GS, et al. Evidence that a peptide spanning the B-C junction of proinsulin is an early autoantigen epitope in the pathogenesis of type 1 diabetes. *J Immunol* 2001;167:4926–35.
- [19] Rudy G, Stone N, Harrison LC, Colman PG, McNair P, Brusica V, et al. Similar peptides from two beta cell autoantigens, proinsulin and glutamic acid decarboxylase, stimulate T cells of individuals at risk for insulin-dependent diabetes. *Mol Med* 1995;1:625–33.
- [20] Martinez NR, Augstein P, Moustakas AK, Papadopoulos GK, Gregori S, Adorini L, et al. Disabling an integral CTL epitope allows suppression of autoimmune diabetes by intranasal proinsulin peptide. *J Clin Invest* 2003; 111:1365–71.
- [21] Buckner JH, Holzer U, Novak EJ, Reijonen H, Kwok WW, Nepom GT. Defining antigen-specific responses with human MHC class II tetramers. *J Allergy Clin Immunol* 2002;110:199–208.
- [22] Kupila A, Muona P, Simell T, Arvilommi P, Savolainen H, Hämäläinen AM, et al. Feasibility of genetic and immunological prediction of type 1 diabetes in a population-based birth cohort. *Diabetologia* 2001;44:290–7.
- [23] Hermann R, Turpeinen H, Laine AP, Veijola R, Knip M, Simell O, et al. HLA DR-DQ-encoded genetic determinants of childhood-onset type 1 diabetes in Finland: an analysis of 622 nuclear families. *Tissue Antigens* 2003;62:162–9.
- [24] Novak EJ, Liu AW, Nepom GT, Kwok WW. MHC class II tetramers identify peptide-specific human CD4⁺ T cells proliferating in response to influenza A antigen. *J Clin Invest* 1999;104:R63–7.
- [25] Masewicz SA, Papadopoulos GK, Swanson E, Moriarity L, Moustakas AK, Nepom GT. Modulation of T cell response to hGAD65 peptide epitopes. *Tissue Antigens* 2002;59:101–12.
- [26] Wong FS, Janeway Jr CA. Insulin-dependent diabetes mellitus and its animal models. *Curr Opin Immunol* 1999;11:643–7.
- [27] Lucassen AM, Julier C, Beressi JP, Boitard C, Froguel P, Lathrop M, et al. Susceptibility to insulin dependent diabetes mellitus maps to a 4.1 kb segment of DNA spanning the insulin gene and associated VNTR. *Nat Genet* 1993;4:305–10.
- [28] Pugliese A, Zeller M, Fernandez Jr A, Zalcberg LJ, Bartlett RJ, Ricordi C, et al. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. *Nat Genet* 1997; 15:293–7.
- [29] Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, et al. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. *Nat Genet* 1997;15:289–92.
- [30] Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, et al. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature* 2005;435:220–3.
- [31] Kent SC, Chen Y, Bregoli L, Clemmings SM, Kenyon NS, Ricordi C, et al. Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope. *Nature* 2005;435:224–8.
- [32] Roep BO, Atkinson MA, van Endert PM, Gottlieb PA, Wilson SB, Sachs JA. Autoreactive T cell responses in insulin-dependent (Type 1) diabetes mellitus. Report of the first international workshop for standardization of T cell assays. *J Autoimmun* 1999;13:267–82.
- [33] Peakman M, Tree TI, Endl J, van Endert P, Atkinson MA, Roep BO. Characterization of preparations of GAD65, proinsulin, and the islet tyrosine phosphatase IA-2 for use in detection of autoreactive T-cells in type 1 diabetes: report of phase II of the Second International Immunology of Diabetes Society Workshop for Standardization of T-cell assays in type 1 diabetes. *Diabetes* 2001;50:1749–54.
- [34] Savage PA, Boniface JJ, Davis MM. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity* 1999;10: 485–92.
- [35] Reichstetter S, Ettinger RA, Liu AW, Gebe JA, Nepom GT, Kwok WW. Distinct T cell interactions with HLA class II tetramers characterize a spectrum of TCR affinities in the human antigen-specific T cell response. *J Immunol* 2000;165:6994–8.
- [36] Trudeau JD, Kelly-Smith C, Verchere CB, Elliott JF, Dutz JP, Finegood DT, et al. Prediction of spontaneous autoimmune diabetes in NOD mice by quantification of autoreactive T cells in peripheral blood. *J Clin Invest* 2003;111:217–23.
- [37] Reijonen H, Elliott JF, van Endert P, Nepom G. Differential presentation of glutamic acid decarboxylase 65 (GAD65) T cell epitopes among HLA-DRB1*0401-positive individuals. *J Immunol* 1999;163:1674–81.