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# Increased in vivo frequency of IA-2 peptide-reactive IFNγ$^+$/IL-4$^-$ T cells in type 1 diabetic subjects

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**Abstract**

Active T cell recognition of islet antigens has been postulated as the pathogenic mechanism in human type 1 diabetes, but evidence is scarce. If T cells are engaged, they are expected to display increased clonal size and exhibit a T helper (Th)1/Th2 differentiation state. We used a peptide library that covers tyrosine phosphatase IA-2, a target antigen expressed in pancreatic beta cells, to probe 8 diabetic patients and 5 HLA-matched controls. When tested in a high resolution IFNγ/IL-4 double color ELISPOT assay directly ex vivo, the number of IA-2-reactive IFNγ producing cells was 17-fold higher in patients than in controls and IL-4 producing cells were not present. An average of 9 peptides was recognized in the patients vs. one in the controls. Determinant recognition primarily involved CD4$^+$ cells and showed high variability among the patients. Furthermore, anti-CD28 antibody signal enhances quantitative assessment of effector T cells in T1D patients. In vitro expansion with peptides and IL-2 results in detection of responding cells in the controls and loss of disease specificity of the T cell response. Together these data provide strong evidence for the active targeting of IA-2 by Th1 memory effector cells in human type 1 diabetes.


*Keywords:* Cytokines; IA-2/peptides/epitopes; T-lymphocytes; Type 1 diabetes

## 1. Introduction

Type 1 diabetes (T1D) is thought to result from the autoimmune destruction of the insulin-producing islet cells of the pancreas [1,2]. The autoimmune hypothesis is based on the fact that patients develop autoantibodies and T cells against islet cell antigens years before the actual manifestation of the disease [3–6]. In animal models of T1D, T cells have been shown to mediate the disease [7–9]. Although islet cell-specific T cells have been the prime focus of efforts to monitor the autoimmune process in human T1D, it is challenging to gain insights into their function in vivo, for several reasons. First, antigen-specific T cells are thought to occur in low frequency in the peripheral blood [10]. Second, the detection of T cells in functional assays requires that the antigen be known. In human T1D, however, the target antigen is not yet known, and there are several equally attractive candidates for this role [5,11–13]. Third, when T cells are tested in functional assays, the antigen presenting cells (APC) present in the blood will dictate the rules of antigen presentation. The APC in blood, resting B cells and macrophages are likely to generate different antigenic determinants after processing of the protein antigen than the APC in the inflamed target organ which belong to different cell lineages and in

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addition, are pre-activated [14]. Fourth, T cells belong to different cytokine effector classes and recently evidence emerged in murine models that in addition to T helper (Th)1 cells, Th2 cells are candidates for mediating islet cell destruction [15]. Finally, based on rodent models of T1D, in addition to the CD4 cell dependent disease development, contributions of CD8 cells are also essential [7,16].

For this study, we selected a tyrosine phosphatase (IA-2) expressed in islet cells and in the brain, which was initially identified by screening an islet cell cDNA library with sera from T1D patients [17]. Antibodies against IA-2 react against the cytoplasmatic portion of the protein and are preferentially found in T1D patients with HLA-DR4 phenotype [18–20]. Their presence appears to be associated with a more rapid progression to diabetes in high-risk subjects [21]. Proliferation assays with peripheral blood mononuclear cells (PBMC) demonstrated reactivity against IA-2 after 5 days' in vitro stimulation [5,22,23], and in T cell lines in T1D patients [24]. During the in vitro culture, proliferation and differentiation of select cell populations might occur, which alters the frequencies and Th1/Th2 differentiation states. Ideally, testing should, therefore, be done directly ex vivo within a time period too short to permit proliferation or differentiation of the antigen-activated T cells. Short term cytokine recall assays (24–48 h in duration) may be used for this purpose, because naïve T cells do not produce type 1 or type 2 cytokines such as IFNγ or IL-4 [25]. Several days are required for proliferation and differentiation before naïve T cells gain the ability to express these cytokines [26]. However, high resolution analysis of clonal size and of the in vivo Th1/Th2 differentiation states of the IA-2-reactive T cells has not been performed.

Here we studied the autoimmune T cell response to IA-2 in PBMC using high resolution cytokine ELISPOT assays [27]. After the addition of antigen, the specific T cells become activated and (if they have been pre-primed to the Th1/Th2 memory state in vivo) they start secreting IFNγ/IL-4 with maximal production within 24/48 h [28]. In such ex vivo ELISPOT assays, the cytokine is captured around the secreting cell before proliferation occurs and before the cytokine is diluted in the supernatant or bound by receptors which renders the assay unique sensitivity. Leaving a spot-shaped fingerprint for each secreting cell, the numbers of antigen-induced spots, therefore, reflect the frequencies of the antigen-specific Th1/Th2 cells in vivo.

Our data provide clear evidence for a wide array of IA-2 peptides actively targeted in vivo by Th1 polarized $CD4^+$ T cells in T1D.

## 2. Materials and methods

### 2.1. Patients

A total of 13 HLA-DRB1*04, DQB1*0302 positive individuals were analyzed (Table 1): 8 patients with T1D (median age 25 years, median duration of insulin treatment 11.5 months) and 5 healthy control subjects without family history of T1D (median age 30 years). Informed consent was obtained from all individuals after the nature and possible consequences of the studies were fully explained. Studies were performed in accordance with the Declaration of Helsinki.

### 2.2. HLA-typing and determination of autoantibodies

HLA typing of DRB1* and DQB1* alleles was performed by C. Loeliger in University Hospital Eppendorf (Hamburg, Germany) by the use of a locus-specific PCR amplification procedure as described elsewhere [29].

Table 1
Characteristics of study subjects

| | ID | Sex | Age (yr) | T1D (months) | HLA- | | Antibodies | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | DRB1* | DQB1* | GADA (KU/l) | IA-2A (KU/l) | IA (μU/l) | ICA (JDFU) |
| T1D | #1 | M | 21 | 3 | 0401, – | 0302, – | **7** | **182** | **1147** | 0 |
| | #2 | M | 34 | 32 | 0401, 0701 | 0302, 02 | **4** | 0 | **207** | 0 |
| | #3 | F | 34 | 240 | 0401, 0401 | 0302, 0302 | 1 | 1 | 41 | 0 |
| | #4 | F | 24 | 46 | 0401, 1201 | 0302, 0301 | **10** | **4** | **198** | 0 |
| | #5 | F | 19 | 7 | 0401, 1701 | 0302, 02 | **53** | ND | ND | **60** |
| | #6 | M | 24 | 12 | 0401, 1601 | 0302, 0502 | **7** | **6** | **8920** | 0 |
| | #7 | M | 23 | 1 | 0401, 0301 | 0302, 02 | ND | ND | ND | ND |
| | #8 | F | 25 | 11 | 0401, 0701 | 0302, 02 | **34** | 1 | **13,717** | 0 |
| Controls | #1 | F | 32 | | 0401, 0407 | 0302, 0301 | 0 | 1 | 0 | 0 |
| | #2 | F | 25 | | 0401, 1501 | 0302, 0602 | 0 | 0 | 0 | 0 |
| | #3 | F | 24 | | 0401, 0401 | 0302, 0302 | 0 | 0 | 69 | 0 |
| | #4 | M | 30 | | 0402, 0405 | 0302, – | 0 | 1 | 0 | 0 |
| | #5 | F | 40 | | 0401, 0301 | 0302, 02 | 0 | 0 | 0 | 0 |

The numbers in bold represent positive antibody titers. GADA, glutamic acid decarboxylase autoantibodies; IA-2A, tyrosine phosphatase autoantibodies; IA, insulin antibodies; ICA, islet cell autoantibodies.

The antibody assays have been performed by M. Schlosser in Institute of Pathophysiology Karlsburg (University of Greifswald, Germany) as previously described in detail [30,31]. All subjects were analyzed for the presence of insulin antibodies (IA), antibodies against glutamic acid decarboxylase (GADA), islet tyrosine phosphatase (IA-2A) and cytoplasmic islet cell antibodies (ICA). Antibody assays have been evaluated in the 1st Diabetes Antibody Standardization Program (DASP), proficiency evaluation 2001 of the Immunology of Diabetes Society (IDS) and the Centers for Disease Control and Prevention (CDC) achieving high sensitivity and specificity. GADA and IA-2A levels were expressed as arbitrary Karlsburg units per liter (KU/l, cut off 3.9 KU/l and 1.85 KU/l, respectively), IA is given in μU/l (cut off 195.9 μU/l) and ICA is expressed in JDF units (cut off 20 JDFU).

### 2.3. Peptides and antigens

Sixty-two overlapping IA-2 peptides, spanning the intracellular part of the molecule from position 601 to 979 according to the primary IA-2 structure [17], were synthesized on a Syro multiple peptide synthesizer (MultiSynTech, Witten, Germany) using Fmoc/*t*-butyloxycarbonylchemistry. They were 18 amino acids long and overlapped by 12 amino acids. All peptides were purified by preparative HPLC and the purity of the peptides was in the range of 90–95%. The integrity of the final products was confirmed by electrospray mass spectrometry.

In each assay, control antigen tetanus toxoid (TT; Connaugh Laboratories, Toronto, Canada) and phytohemagglutinin M (PHA; Difco, Michigan, USA) were included. All antigens and peptides did not contain significant levels of endotoxin as determined by the Limulus lysate assay (<0.06 EU/ml at 10 μg peptide/ml).

### 2.4. 1° ELISPOT assays

Two color assays were performed as described previously [32]. Briefly, ImmunoSpot M50 plates (Cellular Technology Limited, Cleveland, OH) were first blocked with bovine serum albumin (10 g/l in PBS) for 1 h and then coated overnight at 4 °C with the IFNγ- and the IL-4-specific capture antibodies (Endogen, Woburn, MA at 2 μg/ml, and 8D4-8, BD Pharmingen at 4 μg/ml). PBMC were plated (150,000 per well) in duplicates in complete RPMI medium. Peptides were added at 14 μM final concentration. All cultures contained anti-CD28 antibody at 1 μg/ml final concentration (CD28.2, BD Pharmingen). In select experiments, OKT-4 or OKT-8 monoclonal antibodies (both from ATCC, and grown from hybridoma, at 20 μg/ml final concentration) were added for blocking CD4 or CD8 T cell responses (Fig. 2b). After 48 h, the cells were discarded (or transferred for secondary ELISPOT assays, see below), the ELISPOT plates were washed, and the biotinylated anti-IFNγ-detection antibody (M701, Endogen, biotinylated in our laboratory) was added at 0.75 μg/ml PBS/BSA/Tween (10 g/l BSA with 0.5% Tween) along with anti-IL-4 detection antibody MP4-25D2. After an overnight incubation at 4 °C, and 3 washes with PBS/Tween, streptavidine–horseradish peroxidase (Dako, Denmark, 1:2000 dilution) and in PBS/BSA/Tween were added for 2 h at room temperature. After washing, the spots were developed using an AEC solution (Pierce Pharmaceuticals, Rockford, IL). The AEC stock solution was prepared by dissolving 10 mg AEC in 1 ml *N*,*N*-dimethyl formamide (Fischer Scientific, Fair Lawn, NJ). For the actual development, 1 ml of this AEC stock solution was freshly diluted in 30 ml of 0.1 M sodium-acetate buffer (pH 5.0), filtered (0.45 μm), and mixed with 15 μl $H_2O_2$. One hundred microliters of this solution was plated per well. The "red spots" were developed for 13 min. Then the plates were flicked and the BCIP/NBT Phosphatase Substrate System (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added at 100 μl of the stock per well, at room temperature to develop the "blue" IL-4 spots. After 23 min, the reaction was stopped by rinsing with tap water. This enzymatic reaction yielded 1–3-fold inter-assay variations in generated spot size. Therefore, spot sizes were internally controlled for each experiment, and data points to be compared were generated in the same experiment. The plates were air-dried overnight before subjecting them to image analysis by using a Series 1 ImmunoSpot Image Analyser (Cellular Technology). Results are expressed as spot forming cells per 150,000 PBMC (mean of two adjacent peptide/antigen stimulated wells) after subtracting the mean of unstimulated wells (medium background). The average medium background response did not exceed 10 spots. In each assay, PHA and control antigen TT were included revealing for PHA on average >150 spots and for TT 82 ±56 spots.

### 2.5. 2° ELISPOT assays

PBMC were plated as in primary ELISPOT assays described above. After the first 48 h in vitro activation-culture with peptide, 50 μl of complete medium was added to each well, the cells were resuspended by gentle pipetting and transferred well by well into regular Costar 96 well flat bottom plates (Corning Inc., Corning, NY). To recover the remaining cells from the ImmunoSpot plates, 100 μl medium was added to each well, again followed by gentle resuspension, and well by well transfer to the Costar plate. Cells were washed 2 times with complete medium by spinning the Costar plate at 600 rpm (60 g) for 5 min. Then the cells were resuspended in complete medium containing IL-2 (2 U/ml). The cells were cultured in an incubator for 5 days. On the next day (day 8 of in vitro culture), cells were washed and

resuspended in IL-2 medium. Four days later (day 12), cells were washed 3 times and resuspended in 100 μl complete medium. These cells were transferred well by well for secondary testing into a pre-coated ELISPOT plate. Freshly thawed autologous PBMC were added as APC (100,000 cells per well) along with the respective peptide used for the primary stimulation of each split culture. The subsequent two color ELISPOT assay was performed as described above for the primary testing.

## 3. Results

### 3.1. Increased ex vivo IA-2 peptide reactivity in T1D patients

We synthesized a panel of 62 peptides (18 amino acids long) that covered the IA-2 sequence in steps of 6 amino acids. PBMC were isolated from 8 T1D patients and from 5 HLA-DRB1*04/DQB1*0302-matched healthy donors (Table 1). The PBMC were cryopreserved within 4 h of isolation and samples of patients and controls were thawed and assayed in parallel. To be able to test an extended panel of peptides on each sample, two adjacent peptides were pooled for stimulation of the PBMC. During a 48 h ex vivo peptide challenge, the peptide-induced production of IFNγ was measured by ELISPOT assays showing scattered responses to a few peptides in patients and in controls (Table 2). In the presence of the specific signal enhancing anti-CD28 antibody, IA-2 peptides induced IFNγ producing cells in the PBMC of 6 of the 8 patients tested (Table 3). Pooled peptides 9/10 induced IFNγ producing cells ($>10$ per 150,000 PBMC plated per well) in 4 of the 8 patients but in none of the HLA-matched healthy controls. Also peptides 11/12, 27/28, 51/52, 55/56, and 61/62 were recognized in 4 of the 8 patients, with variations in individual patients. Of these peptides only one, 61/62, was recognized in one of the 5 controls. Peptide pool 39/40 was targeted in 5 of the patients, but in none of the controls. In controls, scattered recognition of few IA-2 peptides was seen. While the patients responded to an average of 9 peptides, only one IA-2 peptide on average was recognized in the controls (Table 3 and Fig. 1a).

Positive peptides in the patients induced on average $>62$ spots within the 150,000 PBMC tested, compared to 24 spots for the average positive peptide in the controls (Table 3 and Fig. 1b). Therefore, the number of cells recognizing IA-2 peptides was 2.6-fold higher for patients. The combined recognition of 9 times as many peptides with a 2.6-fold higher cell number (corresponding to the sum of all peptide-induced spots) yielded an average of 561 IA-2 peptide-reactive cells per patient vs. an average of 33 IA-2 peptide-reactive cells in the controls (Table 3 and Fig. 1c). This is a 17-fold difference.

The reactivity to control antigen tetanus toxoid and to PHA did not differ between patients and controls (data not shown).

### 3.2. The IA-2 peptide-reactive cells are type 1-polarized CD4 cells

From patient #1, we obtained sufficient frozen PBMC for repeated testing including the assessment of the CD4/CD8 lineage of the IA-2 peptide-reactive cells. The results of the first experiment (SIs, calculated by dividing the mean number of spots per peptide by the mean number of spots in the background control) are compared with the results of a second experiment retesting another aliquot of this patient's frozen PBMC while systematically covering the second half of the complete peptide library (Fig. 2a). Peptides 43/44, 49/50, and 57/58 that tested negative in the first assay, also did not trigger IFNγ producing cells in the second experiment. The 11 other peptide pools retested, all of which scored positive in the first experiment, also induced IFNγ production with similar stimulation indices (SI) in the repeat experiment. For a third testing with the remaining frozen cells, we randomly selected some of the peptides that scored positively in the first 2 experiments (47/48, 59/60 and 61/62), along with two that scored negatively (43/44 and 49/50). For all 3 experiments, comparable SI were obtained for these peptides (the medium background was lower in this third repeat experiment). In this third repeat experiment, we included wells with anti-CD4 and anti-CD8 antibodies at concentrations that we previously established as ideal for blocking the activation of the respective T cell lineage. The response to all 3 positive peptides was completely blocked by anti-CD4, but not by anti-CD8 antibody (Fig. 2b).

Except for samples from patients 5 and 6, that were subjected to single-color IFNγ ELISPOT testing, all other patients and controls were tested in a two color IFNγ, IL-4 assay. While IL-4 secreting cells in the 10 per 150,000 frequency range were detected in PBMC of healthy donors after challenge with recall antigens (showing that the IL-4 assay detects this cytokine when produced), no IL-4 spots over background ($SI < 2$ with background $<3/150{,}000$) were induced by the IA-2 peptides in the patients or the controls. Therefore, if IL-4 producing IA-2-specific cells are present in the diabetes patients, they are far outnumbered by the IFNγ producing cells (Table 3). The IA-2-specific T cell repertoire in the patients is therefore highly type 1 polarized.

### 3.3. Biased detection of peptide-reactive cells after in vitro expansion

As stated above, before we introduced anti-CD28 antibodies into the test system, we could not consistently detect IA-2 peptide-induced cytokine (IFNγ or IL-4)

Table 2
Direct ex vivo measurements of IA-2 peptide-induced IFNγ producing cells in PBMC

| IA-2 peptides | | Patients | | | | | | | | Controls | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | position | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 |
| 01+02 | 601 - 624 | -- | -- | -- | -- | -- | -- | 14 | -- | -- | -- | -- | -- | 22 |
| 03+04 | 613 - 639 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 35 | 40 |
| 05+06 | 625 - 648 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 72 | -- | 49 |
| 07+08 | 637 - 660 | -- | -- | -- | -- | -- | -- | 26 | -- | -- | -- | -- | -- | -- |
| 09+10 | 649 - 672 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 11+12 | 661 - 684 | 13 | -- | -- | -- | -- | -- | -- | -- | 26 | -- | -- | 15 | -- |
| 13+14 | 673 - 696 | -- | -- | -- | -- | -- | -- | 39 | -- | -- | -- | -- | -- | 29 |
| 15+16 | 685 - 708 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 29 | -- |
| 18+19 | 703 - 726 | -- | -- | -- | -- | -- | -- | 18 | -- | -- | 12 | -- | -- | -- |
| 20+21 | 715 - 738 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 13 | -- | -- |
| 22+24 | 726 - 756 | -- | -- | -- | -- | -- | -- | | -- | -- | -- | -- | -- | -- |
| 25+26 | 745 - 768 | -- | -- | -- | -- | -- | -- | 29 | -- | -- | -- | -- | -- | -- |
| 27+28 | 757 - 780 | -- | -- | -- | -- | -- | -- | -- | -- | 15 | -- | -- | -- | -- |
| 29+30 | 769 - 792 | -- | -- | -- | -- | -- | -- | 17 | -- | 37 | 26 | -- | -- | -- |
| 31+32 | 781 - 804 | -- | -- | -- | -- | -- | -- | | -- | -- | -- | -- | -- | -- |
| 35+36 | 805 - 828 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 37+38 | 817 - 840 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 39+40 | 829 - 852 | -- | -- | -- | -- | -- | -- | -- | -- | -- | 25 | -- | -- | -- |
| 41+42 | 841 - 864 | -- | -- | -- | -- | -- | -- | 19 | -- | -- | -- | 14 | -- | -- |
| 43+44 | 853 - 876 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 45+46 | 865 - 888 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 47+48 | 877 - 900 | -- | -- | -- | -- | -- | -- | | -- | -- | -- | -- | -- | -- |
| 49+50 | 889 - 912 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 51+52 | 901 - 926 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 53+54 | 913 - 936 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 55+56 | 925 - 948 | -- | -- | -- | -- | -- | -- | -- | -- | 24 | -- | -- | -- | -- |
| 57+58 | 937 - 960 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 59+60 | 949 - 972 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 42 | -- |
| 61+62 | 961 - 979 | -- | -- | -- | -- | -- | -- | 13 | -- | 26 | 65 | -- | -- | -- |
| Nr. of pos. Peptides | | 1 | 0 | 0 | 0 | 0 | 0 | 8 | 0 | 5 | 4 | 3 | 4 | 4 |
| | | | | | $\bar{x}$= 1 | | | | | | | $\bar{x}$= 4 | | |
| Mean Nr. of spots/pos. peptide | | | | | $\bar{x}$= 21 | | | | | | | $\bar{x}$= 31 | | |
| Σ of peptide induced IFN-γ spots | | 13 | 0 | 0 | 0 | 0 | 0 | 175 | 0 | 128 | 128 | 99 | 121 | 140 |
| | | | | | $\bar{x}$= 188 | | | | | | | $\bar{x}$= 616 | | |

Peptides #17, 23, 33 and 34 were not tested. Results are expressed as spot forming cells per 150,000 PBMC after subtracting the medium background. To increase the visual transparency of data, numbers <10 are not specified (--) and those exceeding 10 are highlighted by a black box. The raw data shown for the individual peptides are summarized for every subject. The mean value for the groups is also indicated.

Table 3
Direct ex vivo measurements of IA-2 peptide-induced IFNγ producing cells in PBMC after addition of anti-CD28

| IA-2 peptides | | Patients | | | | | | | | Controls | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | position | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 |
| 01+02 | 601 - 624 | 131 | -- | -- | -- | 11 | -- | -- | -- | -- | -- | -- | -- | -- |
| 03+04 | 613 - 639 | 27 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 28 | -- |
| 05+06 | 625 - 648 | 147 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 07+08 | 637 - 660 | 15 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 09+10 | 649 - 672 | 43 | -- | -- | -- | 23 | 30 | 11 | -- | -- | -- | -- | -- | -- |
| 11+12 | 661 - 684 | 33 | -- | >150 | -- | -- | 35 | 12 | -- | -- | -- | -- | -- | -- |
| 13+14 | 673 - 696 | 100 | -- | >150 | -- | -- | -- | 15 | -- | -- | 28 | -- | -- | -- |
| 15+16 | 685 - 708 | 37 | -- | >150 | -- | 25 | -- | -- | -- | -- | -- | -- | 13 | -- |
| 18+19 | 703 - 726 | 112 | -- | -- | -- | 51 | 30 | -- | -- | -- | -- | -- | -- | -- |
| 20+21 | 715 - 738 | 13 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 22+24 | 726 - 756 | 73 | -- | -- | -- | -- | 24 | 15 | -- | -- | -- | -- | -- | -- |
| 25+26 | 745 - 768 | 47 | -- | >150 | -- | 21 | -- | -- | -- | -- | -- | -- | -- | 39 |
| 27+28 | 757 - 780 | 104 | -- | >150 | -- | 13 | -- | 13 | -- | -- | -- | -- | -- | -- |
| 29+30 | 769 - 792 | 28 | -- | -- | -- | -- | -- | 13 | 11 | -- | 22 | -- | -- | -- |
| 31+32 | 781 - 804 | 95 | -- | -- | -- | 21 | -- | 11 | -- | -- | -- | -- | -- | -- |
| 35+36 | 805 - 828 | 88 | -- | >150 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 37+38 | 817 - 840 | 156 | -- | 37 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 39+40 | 829 - 852 | 138 | -- | >150 | -- | 15 | 14 | 15 | -- | -- | -- | -- | -- | -- |
| 41+42 | 841 - 864 | 35 | -- | >150 | -- | -- | -- | 15 | -- | -- | -- | -- | -- | -- |
| 43+44 | 853 - 876 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 45+46 | 865 - 888 | 17 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 47+48 | 877 - 900 | 74 | -- | 17 | -- | -- | -- | 16 | -- | -- | -- | -- | -- | -- |
| 49+50 | 889 - 912 | -- | -- | >150 | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| 51+52 | 901 - 926 | 151 | -- | >150 | -- | 11 | -- | 14 | -- | -- | -- | -- | -- | -- |
| 53+54 | 913 - 936 | -- | -- | | -- | -- | -- | | -- | -- | -- | -- | -- | -- |
| 55+56 | 925 - 948 | 114 | -- | >150 | -- | 20 | -- | 16 | -- | -- | -- | -- | -- | -- |
| 57+58 | 937 - 960 | -- | -- | -- | -- | -- | 14 | -- | -- | -- | -- | -- | -- | -- |
| 59+60 | 949 - 972 | 62 | -- | >150 | -- | -- | -- | -- | -- | -- | 18 | -- | -- | -- |
| 61+62 | 961 - 979 | 55 | -- | >150 | -- | 32 | -- | 25 | -- | -- | 17 | -- | -- | -- |
| Nr. of pos. | | 25 | 0 | 15 | 0 | 11 | 6 | 13 | 1 | 0 | 4 | 0 | 2 | 1 |
| Peptides | | $\bar{x}$= 9 | | | | | | | | $\bar{x}$= 1 | | | | |
| Mean Nr. of spots/pos. peptide | | $\bar{x}$= 62 | | | | | | | | $\bar{x}$= 24 | | | | |
| Σ of peptide induced IFN-γ spots | | 1895 | 0 | >2004 | 0 | 242 | 147 | 191 | 11 | 0 | 85 | 0 | 41 | 39 |
| | | $\bar{x}$= 561 | | | | | | | | $\bar{x}$= 33 | | | | |

Peptides #17, 23, 33 and 34 were not tested. Results are expressed as spot forming cells per 150,000 PBMC after subtracting the medium background (same as in Table 2). For the small volume plates used, more than 150 IFNγ spots per well were confluent and no longer clearly countable. These results are indicated as >150. When calculating average peptide-induced spot numbers, such results were set to equal 150, representing an underestimation of the actual numbers. Peptides that induced >10 IFNγ producing cells in ≥4 of the 8 patients are marked in gray.

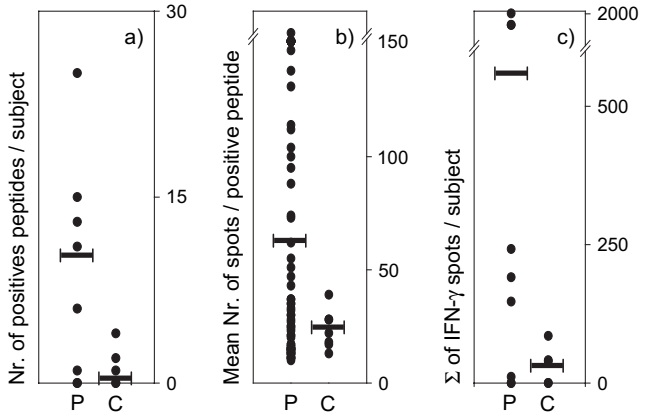


Fig. 1. Cumulative ex vivo IFNγ ELISPOT results of IA-2 peptide reactivity. a) The number of peptides that induced >10 spots per 150,000 PBMC is shown for each subject with the mean indicated for patients (P) and controls (C). These data summarize the diversity of determinant recognition. b) The cumulative number of IFNγ spots induced by the different positive peptides in an individual is shown, along with the mean of the groups. These data summarize the cumulative clonal mass of peptide-reactive cells. c) The mean number of spots per positive peptide is shown for each individual with the mean of the groups indicated. These data reveal the clonal size with which the individual peptides are recognized. Table 2 shows the derivation of the numbers in a–c.

producing cells in PBMC. Therefore, we applied in vitro amplification prior to testing in a secondary ELISPOT assay. We first activated the PBMC with the IA-2 peptides in a primary ELISPOT assay (analogous to the experiment shown in Table 3), but after 2 days, instead of discarding the cells for development of the ELISPOT plates, we transferred the peptide stimulated cells well by well to regular 96 well plates and cultured them with IL-2 for an additional 10 days. After this 12 day culture, the cells were retested for reactivity to the inducing peptides in a secondary ELISPOT assay using autologous APC. All 5 controls and patients #1, 2, and 4 were tested in this fashion. The results obtained with anti-CD28 antibody in the primary ex vivo assay (Table 3) were compared to the ones obtained in the secondary assay. Fig. 3 summarizes the results for those peptides that induced IFNγ producing cells in ≥4 of the 8 patients, but in none of the 5 controls with the exception of a weak response to one peptide in one control subject (peptides highlighted in gray in Table 3). As shown for the controls in the upper panels, IFNγ secreting cells were detected in the secondary assay in all of the 5 controls. Each individual developed responses to 1–7 of the peptides. Therefore, the tested peptides are potential antigenic determinants in the controls as well (likely utilizing the shared class II molecules as restriction elements). However, in the controls these peptides are recognized only after in vitro culture. After the 12 day culture, IFNγ producing cells emerged in response to 4 of the 7 peptide pools in patient #4, who did not respond directly ex vivo (lower panels). In the cases where peptides induced IFNγ producing cells ex vivo, the frequencies were increased for 2/6 peptide pools, but decreased in 10 cases for 7 peptide pools after the 12 day culture. Moreover, the striking differences that were seen directly ex vivo between patients and controls (Fig. 1) were largely lost after the culture (data not shown).

## 4. Discussion

In the present study we were looking for evidence of the T cell hypothesis, according to which IA-2-specific cytokine producing cells of patients with T1D occur in higher frequencies and show a different state of Th1/Th2 activation than the cells of healthy HLA-matched controls, because they were primed in vivo during the spontaneous autoimmune response. In order to detect the entire in vivo primed IA-2-specific repertoire, we used peptide antigens that can bind directly to MHC molecules and be presented without the need of further processing by blood borne APCs. The peptide length chosen, 18-mers, which seems to occur naturally after processing of IA-2 [33], is too long for direct binding to MHC class I molecules. However, it is ideal for class II binding, and hence favors the detection of CD4 cells. To further enhance antigen presentation by the resting APC in the test system, which mimics APC function in the inflamed target organ, we included anti-CD28 antibodies in the culture, which specifically enhances costimulation [34]. To account for Th1 and Th2 cells, we measured IFNγ and IL-4 production simultaneously in two color ELISPOT assays [32]. Direct ex vivo measurements were compared with results obtained after short term in vitro expansion.

We show that the number of IA-2-reactive IFNγ producing cells was 17-fold higher in patients than in controls, whereas IL-4 secretion was not detectable. An average of 9 peptides was recognized in the patients vs. only one in the controls implying active targeting of IA-2 by Th1 cells in human T1D. Recognition of several of the peptides was shared among the patients, consistent with their shared HLA haplotype. However, none of these determinants was immune dominant. The data reproduce and extend findings reported by Peakman et al. who defined naturally processed epitopes of IA-2 eluted from HLA-DRB1*0401 molecules of IA-2-pulsed B cells [33]. These peptides have been shown to be 12–24 amino acids long and to be recognized in DR4 positive T1D patients, but not in DR4 negative patients or healthy controls. Notably, the patients responded with increased proliferation to the 5 eluted peptides with considerable heterogeneity. In face of this apparent heterogeneity of IA-2 peptide recognition suggested by this study, and to cover a wide spectrum of determinants, we opted for a comprehensive peptide scanning strategy. In our study

a)

| Peptide | Experiment 1 | 2 | 3 |
|---|---|---|---|
| 31+32 | 10.6 | 4.7 | nd |
| 35+36 | 9.8 | 2.2 | nd |
| 37+38 | 17.3 | 4.1 | nd |
| 39+40 | 15.3 | 6.2 | nd |
| 41+42 | 3.9 | 7.9 | nd |
| 43+44 | 1.0 | 1.0 | 1.0 |
| 45+46 | 1.9 | 2.4 | nd |
| 47+48 | 8.2 | 8.5 | 8.0 |
| 49+50 | 1.0 | 1.0 | 1.0 |
| 51+52 | 16.8 | 1.9 | nd |
| 53+54 | 1.0 | 1.0 | nd |
| 55+56 | 12.7 | 5.1 | nd |
| 57+58 | 1.0 | 1.0 | nd |
| 59+60 | 6.9 | 7.5 | 9.0 |
| 61+62 | 6.1 | 7.8 | 18.0 |
| (Medium) | (9) | (10) | (1) |

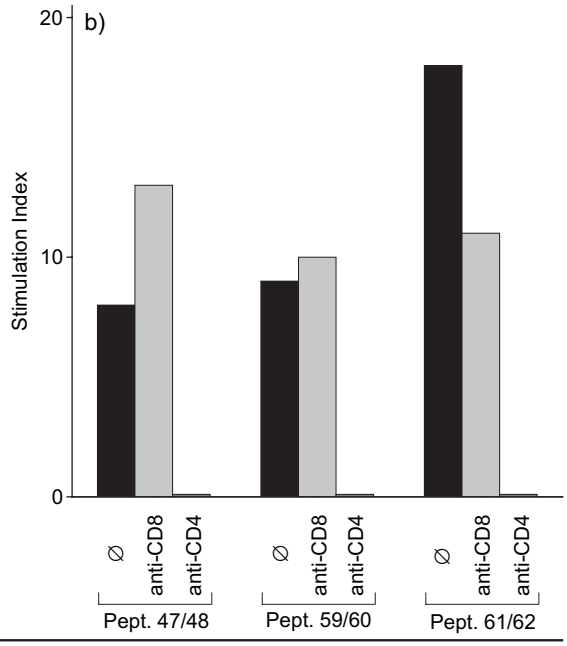


Fig. 2. Reproducibility of IA-2 peptide-induced IFNγ ELISPOT measurement on PBMC and their definition as CD4 cell derived. a) The SI for experiment 1 was calculated for T1D patient #1 from the raw data shown in Table 2. Results of retesting a comprehensive panel of peptides, or of select peptides are shown for experiments 2 and 3, respectively. The number of background spots in the medium control is specified for each experiment in parentheses. SI > 3 were considered as positive, SI ≤ 3 as negative. Data that were reproduced as positive or negative are highlighted in gray. b) In experiment 3, anti-CD4 or anti-CD8 antibodies were included as specified to test for the inhibitability of the peptide pool 47/48-, 59/60-, and 61/62-induced IFNγ spot formation.

the number of IFNγ secreting cells was increased in 7 peptide pools; 4 of these 7 (9/10, 11/12, 27/28, 61/62) included the naturally processed determinants that this group has identified by proliferation. The region of IA-2 covered by peptides 27/28 has also been recognized by T cell lines derived from DRB1*0401 positive T1D patients [24]. In addition to these 4 peptides, however, 23 more peptide pools were recognized, albeit in a lower proportion of the patients. Overall, peptide recognition followed random patterns reminiscent of codominant determinant recognition. This diversity of peptides recognized in the patients is not surprising considering the 379 amino acid long IA-2 segment studied (residues 601–979) and the fact that in addition to the accounted DRB1*04 molecule, all the other DR, DQ, and DP molecules expressed in the patients are attractive candidates for restricting IA-2-specific responses as shown in well defined murine models of experimental allergic encephalomyelitis [35] and in patients with multiple sclerosis [36]. Our data, therefore, do not permit the distinction whether the IA-2-reactive T cells are primary effector cells or second wave cells engaged in the organ-specific amplificatory spreading reaction. The fact that these islet antigen-derived peptides were not targeted in the HLA-matched healthy controls, however, suggests a disease related priming.

To assess the CD4/CD8 lineage of the IA-2 peptide-reactive cells, we blocked the activation of the respective T cell lineage with anti-CD4 and anti-CD8 antibodies. While these data point towards prevalent CD4 cell activity in T1D, it should be noted that the peptide length chosen (18-mers) favors MHC class II binding and, hence, CD4 cell detection. This blockade was CD4 lineage specific, because the same concentration of the anti-CD4 antibody did not inhibit the activation of CD8 cells that recognized HLA-A2 restricted EBV and CMV peptides. The anti-CD8 antibody selectively inhibited the latter response (data not shown).

The growth characteristics of the peptide-reactive T cells i.e. their Th1/Th2 activation state appeared to be heterogeneous. While T cells in the controls seemed to readily expand, the in vivo primed T cell clones of the patients might have undergone extensive proliferation in vivo reaching replicative senescence. Such aged cells are known to maintain their ability to produce cytokine (and hence to function as effector cells), but to lose their proliferative potential [37]. The drop in frequencies of peptide-reactive T cells after in vitro peptide stimulation, therefore, might provide additional evidence that these cells have been actively engaged in the autoimmune responses in vivo. Overall, the fine specificity of the repertoires detected after the 12 day culture differed fundamentally from the ex vivo results.

On the whole, the elevated frequency of IA-2 peptide-reactive CD4 cells in PBMC in our study, and their direct ex vivo production of IFNγ provide clear

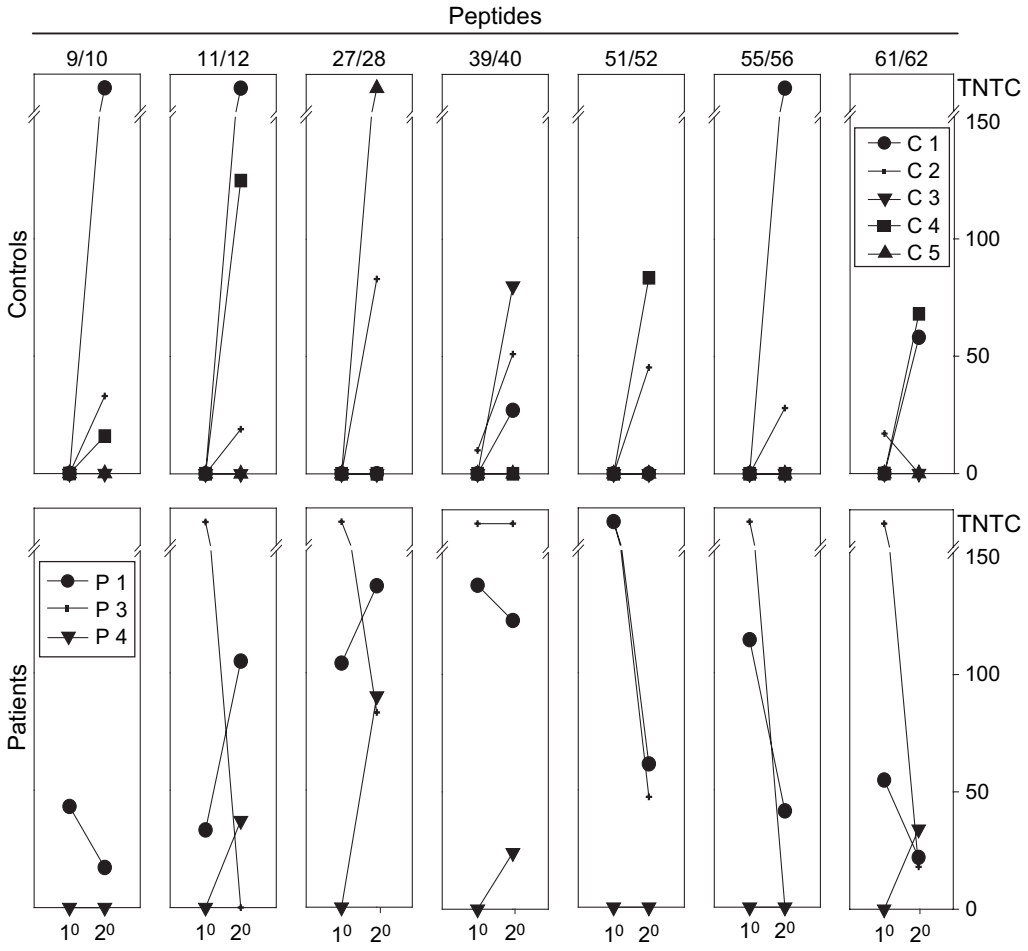


Fig. 3. Comparison of frequencies of IA-2-peptide-induced IFNγ producing cells directly ex vivo and after 12 days in culture in vitro. Results for the peptides that scored positive in ≥4 of the 8 patients (highlighted in gray in Table 3) are shown. The panels on the top show results for the 5 controls, panels at the bottom show the results for the 3 patients that were also tested after the 12 days' in vitro culture. Different symbols are used to distinguish the subjects as specified for controls by the insert in the outer right panel and for patients in the outer left panel. The frequencies of cytokine producing cells ex vivo, in the presence of anti-CD28 antibody are shown for each peptide and subject on the left side of each panel (1° ELISPOT testing). This value is linked to the number of spots counted after 12 days of in vitro cell culture (right side of each panel; 2° ELISPOT testing). Not exactly countable results are indicated as TNTC (>150 spots and too numerous to count).

evidence that these cells had been primed in vivo. The diverse fine specificity of the IA-2-specific cells argues against them having been engaged in a cross-reactive infection, in which case a limited repertoire would be seen that would be confined to a single or few determinants shared between the infectious agent and the autoantigen. The diverse repertoire is consistent, however, with endogenous priming by determinant spreading. These high resolution measurements provide evidence for an active autoimmune T cell process in humans with T1D.

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