



Testing for HLA/peptide tetramer-binding to the T cell receptor complex on human T lymphocytes

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ABSTRACT

HLA/peptide tetramers are frequently used for ex vivo monitoring of disease- or vaccine-induced T cell immune responses and for T cell epitope identification. However, when low-levels HLA/peptide tetramer-positive T cell populations are encountered, it is difficult to ascertain whether this represents a true T cell receptor (TCR)-mediated interaction or background signal. To address this issue, we have developed a method for both HLA class I and class II tetramer assays to confirm tetramer-binding to the TCR/CD3 complex. Preincubation of T cells with anti-CD3 mAb SPV-T3b and subsequent crosslinking interferes with the binding of HLA/peptide tetramers to the TCR/CD3 complex and thereby indicates to what extent HLA/peptide tetramer binds through interaction with TCR/CD3 complex. SPV-T3b pretreatment results in a 2- to 10-fold decrease in tetramer-binding intensity to antigen-specific CD8⁺ or CD4⁺ T cells, whereas background reactivity of HLA/peptide tetramers containing HIV-derived peptide in HIV-negative donors remained unchanged. SPV-T3b pretreatment forms a valuable tool to verify tetramer-based detection of antigen-specific T cells during the monitoring of immune responses in clinical studies.

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1. Introduction

HLA/peptide tetramers have become widely applied tools to detect antigen-specific human T cells. These tetramers detect antigen-specific T cells by binding to specific T cell receptors (TCR) expressed by the T cells and reagents can be generated to detect either antigen-specific CD4 or antigen-specific CD8 T cells. In immunomonitoring studies of patients with cancer or viral infections, HLA/peptide tetramers have been proven valuable to monitor the presence of specific immune responses against tumor antigens or pathogens [8,12,17,19,23,24,28,31]. Furthermore, high-throughout screening of cytotoxic T cell epitopes in pathogen genomes or other disease-associated genes by HLA/peptide tetramer staining has become a realistic option [2,29]. However, HLA/peptide tetramer analyses consistently detect higher numbers of antigen-specific T cells than other methods of detection, such as ELISPOT, cytokine flow cytometry and limiting dilution analysis, which are based on the activation and/or proliferation of T cells in response to stimulation with antigen [28,40]. Discrepancies in the levels of antigen-

specific T cells measured by HLA/peptide tetramers, as compared to the other methods might indicate that HLA/peptide tetramer analyses overestimate the size of the antigen-specific T cell population due to nonspecific reactivity of the HLA/peptide tetramer. On the other hand, the discrepancies may reveal an underestimation of the number of antigen-specific T cells detected by the other three methods due to unresponsiveness of T cells to stimulation, despite the expression of the specific TCR [16]. Therefore, validation of HLA/peptide tetramer analyses for reliable detection of antigen-specific T cells is essential for the applicability of HLA/peptide tetramer analyses in clinical studies [41,20].

We here describe a method to verify whether the detection of antigen-specific T cells by both HLA class I and class II tetramers, involves binding to the TCR/CD3 complex. We have investigated the ability of anti-CD3 and anti-TCR antibodies to interfere with the specific binding of HLA/peptide tetramers to T cells, and found that preincubation of T cells with monoclonal antibody (mAb) SPV-T3b and crosslinking with goat anti-mouse immunoglobulin (GAM-Ig) specifically decreases the specific binding of HLA/peptide tetramers to T cells by interaction with the TCR/CD3 complex from TCR-unrelated nonspecific binding of HLA/peptide tetramers. This method can be used to improve the accuracy of HLA/peptide tetramer-reactivity directly ex vivo, without in vitro expansion or activation.

Abbreviations: GAM-Ig, goat anti-mouse immunoglobulin; APC, allophycocyanin; PE, phycoerythrin; mAb, monoclonal antibody; MFI, mean fluorescence intensity

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2. Materials and methods

2.1. PBMC and HLA typing

Peripheral blood mononuclear cells (PBMC) were isolated from buffycoats from healthy donors of the Dutch Blood bank (Sanquin, Amsterdam, The Netherlands) by standard Ficoll density centrifugation. HLA-class I typing was performed by incubating PBMC with biotin-labeled antibodies (IgM) against HLA-A1, -A2 or -A3 (One Lambda, Canoga Park, CA) or control biotin-labeled IgM (BD Pharmingen, San Jose, CA) for 15 minutes on ice. Bound antibody was detected by incubation with Streptavidin-FITC (Immunotech, Beckman Coulter, Fullerton, CA) for 15 minutes, and flow cytometry analysis using a four-color FACS Calibur (Becton Dickinson, Pont de Claix, France). Dead cells were excluded by propidium iodide (PI) staining. HLA class II typing was performed by sequence based typing of HLA-DRA and HLA-DRB alleles (Sanquin, Amsterdam, The Netherlands).

2.2. Construction of HLA class I and class II/peptide tetramers

Synthetic peptides were produced at the Netherlands Cancer Institute by standard fluorenylmethoxycarbonyl chemistry and were >90% pure by analytical HPLC. Soluble allophycocyanin (APC)- or phycoerythrin (PE)-labeled HLA/peptide tetramers were generated as described [1]. HLA-A2 tetramers were produced containing the following HLA-A2-binding peptides: influenza A virus peptide (58–66) GILGFVFTL, modified MART-1 peptide (26–35, 27 A > L) ELAGIGILTV, gp100 peptide (280–288) YELPGPVTA, tyrosinase peptide (369–377) YMDGTMSQV, cytomegalovirus (CMV) peptide (495–503) NLVPMVATV. PE-labeled HLA-A2/HIV tetramer containing the p17 Gag derived SLYNTVATL peptide of the human immunodeficiency virus (HIV), kindly provided by dr. D. van Baarle (University Medical Center Utrecht, The Netherlands), was generated as described previously [34]. HLA-A1 tetramers were generated with the influenza A virus peptide (44–52) CTELKLSQY or tyrosinase peptide (243–251) KCDICTDEY. HLA-A3 tetramers were generated with influenza A virus NP peptide (265–273) ILRGSVAHK. HLA/peptide tetramers were tested for specific TCR binding using antigen-specific T cell clones and control T cell clones, as described [17]. HLA class II tetramers composed of HLA-DRA1*0101/DRB1*0401 molecules and influenza A virus hemagglutinin peptide (HA307–319, HLA-DR4/flu tetramer) were generated by the method described for murine MHC class II tetramers [25].

2.3. T cell culture

CD8⁺ cytotoxic T cell (CTL) clone INFA24 recognized the influenza A virus peptide (58–66) GILGFVFTL in HLA-A2 and was derived from the PBMC of an HLA-A2⁺ healthy donor by single cell sorting of T cells reactive with HLA-A2/flu tetramers, as described [36,42]. The CD8⁺ CTL clone AKR4D8 recognizes MART-1 (26–35) peptide EAA-GIGILTV in HLA-A2 and was derived from the PBMC of an HLA-A2⁺ melanoma patient by *in vitro* stimulation with the autologous melanoma cell line, as described previously [11,36]. The CD8⁺ CTL line ZWI29 recognizes the gp100 (280–288) peptide YLEPGPVTA in HLA-A2 and was isolated from the PBMC of an HLA-A2⁺ melanoma patient by sorting of polyclonal T cells that bound HLA-A2/gp100 tetramer by MoFlo High speed sorter (Cytomation, Fort Collins, CO). After expansion of this population, a second round of sorting of HLA-A2/gp100 tetramer-positive cells was performed at a density of 10 cells/well into a 96 well plate by MoFlo High speed sorter. Both CTL clones and CTL line ZWI29 were cultured by weekly stimulation with a feeder cell mixture consisting of 10⁶/ml irradiated (80 Gy) allogeneic PBMC and 10⁵/ml irradiated JY cells, supplemented with 100 ng/ml PHA (HA16, Burroughs Wellcome, Bechenham, UK) or antigenic peptide and 20 IU/ml recombinant human IL-2 (Chiron,

Amsterdam, The Netherlands) in Ysells medium as described [36,42]. CTL clone AKR4D8 and CTL line ZWI29 were immortalized by transduction with a retrovirus encoding the human telomerase reverse transcriptase (hTERT) gene, as described [11,18,36].

CD4⁺ T cells recognizing the influenza A virus hemagglutinin peptide (HA307–319) in HLA-DRA1*0101/DRB1*0401 molecules were cultured from the PBMC of an HLA-DRB1*0401⁺ healthy donor who had been immunized against influenza virus. PBMC were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) and cultured with the hemagglutinin peptide (HA307–319). CD4⁺ T cells recognizing the influenza A virus hemagglutinin peptide (HA307–319) in HLA-DRA1*0101/DRB1*0401 molecules were detected by binding the HLA-DR4/flu tetramer and decreased levels of CFSE labeling, as described [22]. Cloning of the tetramer-reactive CD4⁺ T cells was performed by single cell sorting of tetramer-reactive T cells and subsequent culture, as described for CD8⁺ T cell clones [36,42].

2.4. Antibodies and flow cytometry

Fluorochrome-labeled monoclonal antibodies (mAb) anti-CD3-FITC (Leu4, BD Pharmingen), anti-CD8 α -FITC (Leu2a, BD Pharmingen), anti-CD8 β -PE (Immunotech, Beckman Colter) and anti-CD4-APC (DAKO, Glostrup, Denmark) were used in flow cytometric analyses. Antibody incubations were performed PBS, 1% BSA, 0.05% sodium azide at 4 °C in 96 well round-bottom plates and cells were acquired in a four-color FACS Calibur. Viable lymphocytes were gated by forward and side scatter profile. Dead cells were excluded by propidium iodide (PI) staining. Data were analyzed with Cell Quest software (Immunocytometry systems, Becton Dickinson).

2.5. HLA/peptide tetramer-binding inhibition

HLA/peptide tetramer-binding inhibition assays were performed using purified anti-CD3 mAbs SPV-T3b [27] and OKT-3 (ATCC, Rockville, MD [21]) or anti-TCR mAbs WT31 [26] and T10B9 [37], or isotype control IgG (all obtained from BD Pharmingen). T cells or PBMC were preincubated with unlabeled SPV-T3b, OKT-3, WT31, T10B9 antibodies or with control IgG at concentrations ranging from 0.07–50 μ g/ml for 15 minutes at 4 °C in 96 well round-bottom plate in PBS, 1% BSA, 0.05% Sodium Azide (PBS/BSA) supplemented with 1% normal mouse serum (NMS). Cells were washed and incubated with Goat anti-mouse immunoglobulins IgA, IgG, IgM (GAM-Ig, dilution 1:60, ICN Biomedicals, Zoetermeer, The Netherlands) for 15 minutes 4 °C. After washing, the cells were incubated with 500 ng HLA/peptide tetramer per 10⁶ cells in PBS/BSA at 37 °C for 15 minutes. HLA/peptide tetramer-binding intensity was analyzed by flow cytometry. The levels of binding inhibition of tetramers by SPV-T3b pretreatment was analyzed by the decrease in mean fluorescence intensity (MFI) of the tetramer-reactive T cells. In analyses of PBMC, blocking of tetramer binding by SPV-T3b pretreatment resulted in a (partial) decrease in the percentage of tetramer-reactive cells in the tetramer-positive quadrant. To estimate the MFI of the total tetramer-positive population after SPV-T3b pretreatment (MFI_c), the MFI value of the tetramer-reactive T cells was corrected for the fluorescence intensity of the cells, in which tetramer binding was fully blocked (F_{neg}). This MFI_c was calculated by the formula: $MFI_c = (Tm_1 \times MFI_1 + (Tm_0 - Tm_1) \times F_{neg}) / Tm_0$, in which Tm₁ and MFI₁ are the percentage of tetramer-positive cells and the MFI value after SPV-T3b pretreatment, respectively, Tm₀ the percentage of tetramer-positive cells without SPV-T3b pretreatment or after control mIgG pretreatment and F_{neg} the fluorescence intensity (FI) of the quadrant setting discriminating tetramer-positive and negative cells. F_{neg} is taken as MFI of the tetramer-reactive T cells, in which tetramer-binding was fully blocked by SPV-T3b pretreatment.

3. Results

3.1. Identification of anti-CD3 or anti-TCR mAbs that compete with HLA/peptide tetramer-binding to human T cells

Bona fide identification of antigen-specific T cells using HLA/peptide tetramers requires the distinction between HLA/peptide tetramer binding to the TCR/CD3 complex and TCR-unrelated binding. To this end, we analyzed whether anti-TCR mAbs WT31 and T10B9, and anti-CD3 mAbs SPV-T3b and OKT3, which bind to the TCR/CD3 complex, interfered with HLA/peptide tetramer binding to antigen-specific T cells. All four antibodies bound to the HLA-A2/influenza-specific T cell clone INFA24 in a dose dependent fashion (Fig. 1A, left panel), which saturated at a concentration of 16 $\mu\text{g/ml}$, as measured by incubation of T cells with unlabeled antibody followed by goat anti-mouse (GAM) Ig-FITC antibody. When WT31, SPV-T3b or OKT3 mAbs were prebound to INFA24 T cells and cross-linked, subsequent HLA-A2/flu tetramer staining resulted in a decreased binding of HLA/peptide tetramer as compared to T cells without mAb preincubation (Fig. 1A, right panel). The extent of the tetramer-binding inhibition to the T-cells was dependent on the concentration of the TCR/CD3-reactive mAb during the preincubation. Although anti-CD3 mAb preincubation might induce T cell activation leading to activation-induced cell death, antibody preincubations did not affect the viability of the T cells, as measured by propidium iodide staining. mAb SPV-T3b was most effective in decreasing tetramer-binding to T cell clone INFA24, resulting in up to a four-fold decrease in mean fluorescence intensity (MFI). Preincubation with mAb T10B9 did not result in a decrease in tetramer binding, despite binding of this mAb to the T cells (Fig. 1A). These results show that binding of

anti-CD3 mAbs SPV-T3b and OKT3 interfered more with HLA/peptide tetramer binding than anti-TCR mAb WT31, while anti-TCR mAb T10B9 did not affect HLA/peptide tetramer binding at all. Based on the superior level of HLA/peptide tetramer-binding inhibition by mAb SPV-T3b preincubation, further analyses were performed with anti-CD3 mAb SPV-T3b.

Binding of HLA-A2/flu tetramers to the INFA24 T cells was not inhibited by preincubation with SPV-T3b alone but required subsequent cross-linking (Fig. 1B). Furthermore, HLA/peptide tetramer incubation prior to mAb incubation plus cross-linking did not significantly reduce tetramer binding intensity, which may be due to tetramer-induced internalization of the TCR/CD3 complex. SPV-T3b mAb pretreatment was most effective when SPV-T3b, GAM-Ig and HLA/peptide tetramer incubations were performed as three separate consecutive incubations. Since the incubations with anti-CD3 mAb and GAM-Ig were performed at 4°C and in the presence of sodium azide, anti-CD3 mAb-induced internalization of the TCR/CD3 complex is unlikely to occur. The reducing effect of SPV-T3b mAb pretreatment on HLA/peptide tetramer-binding may therefore result from steric hindrance or a conformational change in the CD3 complex by the immune complexes of anti-CD3 mAb and GAM-Ig antibodies that inhibit tetramer-binding to the TCR/CD3 complex.

Next, we analyzed the effect of SPV-T3b mAb pretreatment on the binding of specific and control tetramers using CTL clone INFA24, CTL clone AKR4D8 and CTL line ZWI29, specific for influenza peptide, MART-1 peptide (27–35) or gp100 peptide (280–288) in HLA-A2, respectively. SPV-T3b mAb pretreatment resulted in decreased binding of specific tetramers, measured as a ten-fold decrease in mean fluorescence intensity (MFI) by all three clones, while the background reactivity of control tetramers

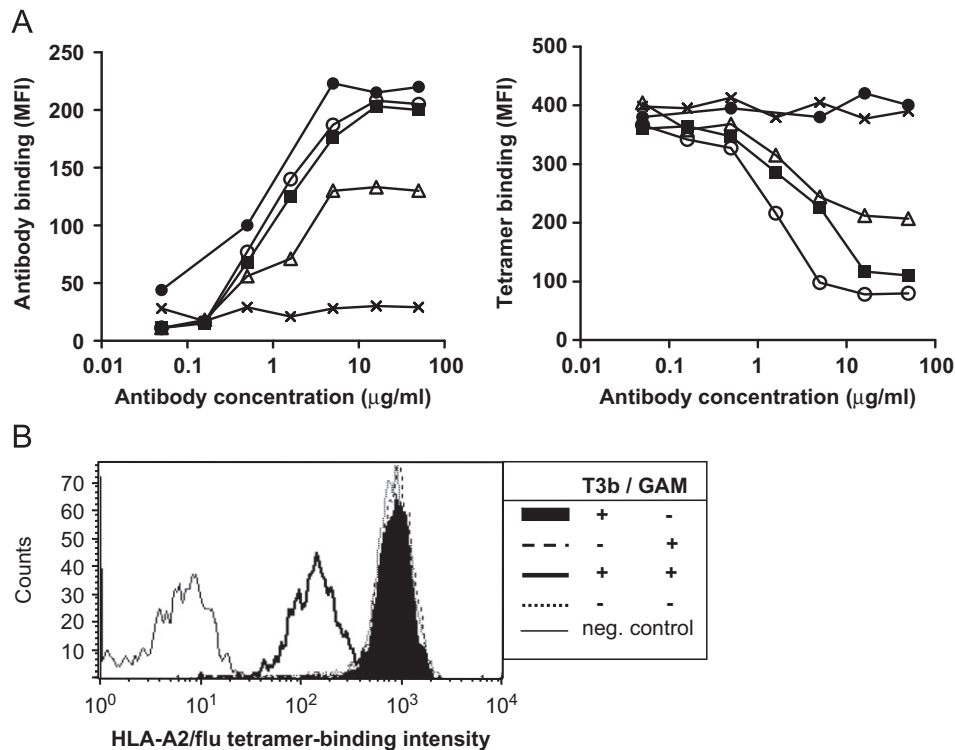


Fig. 1. Inhibition of tetramer-binding to T cells by preincubation with anti-CD3 and anti-TCR antibodies. **A.** CTL INFA24 was preincubated with 3-fold increasing concentrations ranging from 0.07–50 $\mu\text{g/ml}$ of anti-CD3 antibodies, SPV-T3b (○), OKT3 (■), and anti-TCR antibodies WT31 (△) and T10B9 (●) or control IgG (X), followed by incubations with GAM-Ig and HLA-A2/flu tetramer. *Left panel:* binding intensity (MFI) of anti-CD3 or anti-TCR antibodies or control IgG to CTLINFA24 T cells. *Right panel:* Binding intensity of HLA-A2/flu tetramer to CTLINFA24 following preincubation with anti-CD3 or anti-TCR antibodies or control IgG and GAM-Ig. Data are representative of two independent experiments. **B.** Crosslinking of bound mAb is required for inhibition of tetramer-binding. Graph shows the binding intensity of HLA-A2/flu tetramer to CTLINFA24 after preincubation with 5 $\mu\text{g/ml}$ SPV-T3b mAb with or without crosslinking by GAM-Ig, preincubation with GAM-Ig alone, or without antibody preincubation. Graph shows representative results from three experiments.

remained unchanged (Fig. 2). Pretreatment with anti-TCR mAb T10B9 and GAM-Ig did not decrease specific tetramer-binding, indicating that for all three TCR specificities tested mAb SPV-T3b, but not T10B9, specifically interfered with the tetramer-binding capacity of the TCR/CD3 complex.

3.2. SPV-T3b mAb pretreatment enables distinction between HLA/peptide tetramer-binding to T cells involving the TCR/CD3 complex and TCR-unrelated binding

We investigated the ability of SPV-T3b mAb pretreatment to discriminate among PBMC the antigen-specific T cells that bind HLA/peptide tetramer through the TCR/CD3 binding from those T cells that bind the HLA/peptide tetramer nonspecifically. PBMC of an HLA-A2+ donor (donor A) containing a clearly detectable influenza virus-reactive T cell population (0.64% of CD8+ T cells) was mixed

with PBMC of an HLA-A2+ donor without influenza-reactive T cells (donor B) at decreasing concentrations and the level of influenza-reactive T cells was tested by HLA-A2/flu tetramer analyses with SPV-T3b pretreatment or control IgG pretreatment (Table 1). As expected, the percentage of influenza-reactive T cells in donor A decreased with increasing dilution of the PBMC with donor B PBMC. SPV-T3b pretreatment of the samples prior to tetramer staining resulted in both a decrease in MFI and in the percentage of tetramer-positive cells in the upper right quadrant. The decrease in the percentage of tetramer-positive cells occurs when tetramer-binding was fully blocked and the MFI decreased to the level of tetramer-negative cells. To estimate the MFI of the total tetramer-positive population after SPV-T3b pretreatment (MFI_c), the MFI value of the tetramer-reactive T cells was corrected for the fluorescence intensity of the cells, in which tetramer binding was fully blocked (FI_{neg}), as described in the Methods section. As shown in Table 1, at the dilution of 12% donor A cells in the mixture, the tetramer staining

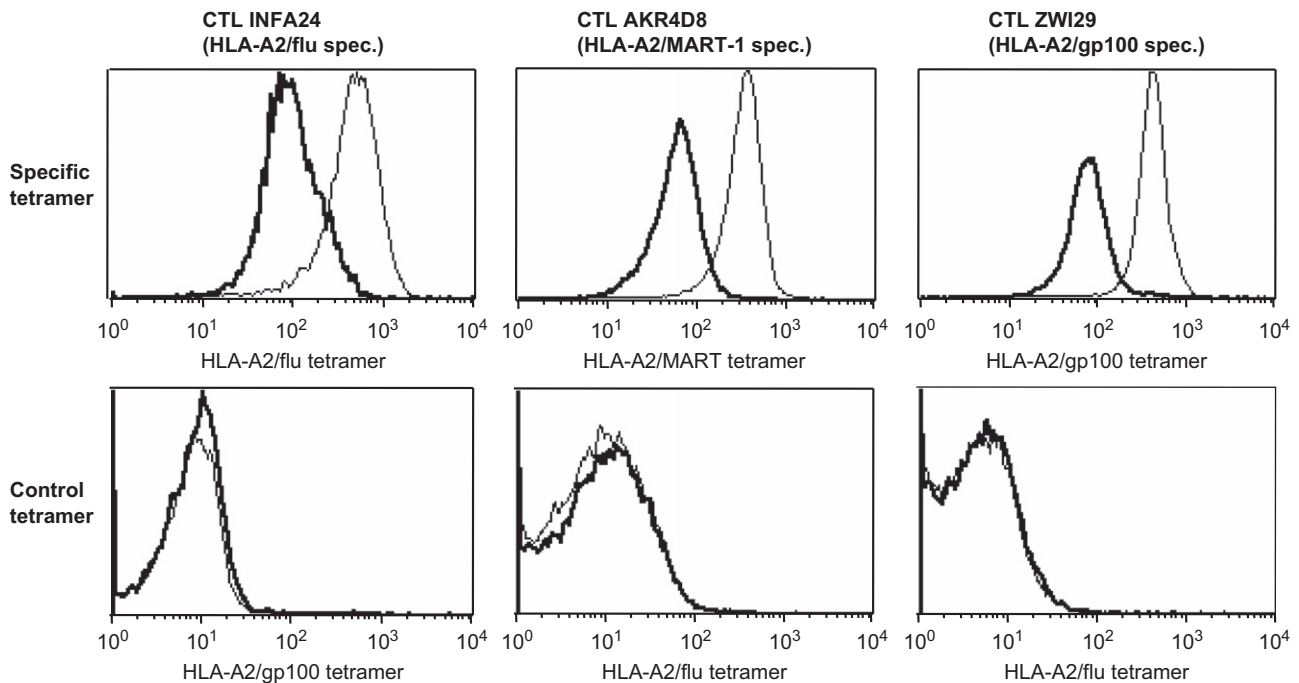


Fig. 2. SPV-T3b pretreatment exclusively decreases the binding of tetramers to antigen-specific T cells. CTLINFA24, CTLAKR4D8 and CTLZWI29 were incubated with the specific tetramers, HLA-A2/flu, HLA-A2/MART-1 and HLA-A2/gp100(280) respectively (upper panel, thin lines), and the effect of preincubation with 5 µg/ml SPV-T3b mAb and GAM-Ig was measured on the tetramer-binding intensity (upper panel, bold lines). Lower panel: SPV-T3b and GAM-Ig preincubation (bold lines) did not affect the reactivity of the T cells with control non-specific tetramers (thin lines). Data are representative of three independent experiments. Preincubation with the anti-TCR mAb T10B9 and GAM-Ig gave similar levels of tetramer-binding as the tetramer-binding without antibody preincubation.

Table 1
Sensitivity of detection of TCR/CD3-mediated tetramer binding in PBMC by SPV-T3b pretreatment.

Tetramer-reactivity in the CD8+ T cell population					SPV-T3b pretreatment				
No blocking									
% PBMC donor A	% PBMC donor B	Tetrameer	Tm ₀ ^a	MFI ₀ ^b	Tm ₁	MFI ₁	FI _q	MFI _{neg} ^c	Fold decrease
100	0	A2/flu	0.64	172	0.35	132	31	86	2.0
50	50	A2/flu	0.44	142	0.17	69	31	46	3.1
25	75	A2/flu	0.26	167	0.11	51	31	40	4.2
12	88	A2/flu	0.21	131	0.13	47	31	41	3.2
6	94	A2/flu	0.12	115	0.11	42	31	41	2.8
3	97	A2/flu	0.11	144	0.11	77	31	75	1.9
0	100	A2/flu	0.13	152	0.18	108	31	138	1.1

^a Tm₀ and Tm₁ indicate the percentages of tetramer-positive T cells of the CD8+ population without (Tm₀) or with SPV-T3b pretreatment (Tm₁), respectively.

^b MFI₀ and MFI₁ indicate the mean fluorescence intensity of the tetramer-positive T cells without (MFI₀) or with SPV-T3b pretreatment (MFI₁), respectively.

^c MFI_c indicates the mean fluorescence intensity of the tetramer-reactive T cell population after SPV-T3b pretreatment corrected for T cells with fully blocked tetramer-reactivity (FI_{neg}).

indicated a level of 0.21%, which was blocked 3-fold by SPV-T3b pretreatment, whereas the background reactivity in 100% donor B (0.13%), in which no specific T cells are present, was not affected by SPV-T3b pretreatment (1.1-fold). Table 1 shows that SPV-T3b pretreatment can be used to detect small populations of antigen-specific T cells in PBMC samples. SPV-T3b pretreatment decreases the MFI about 2-fold in samples with a known tetramer-reactive population, which justifies a 2-fold decrease in MFI as a threshold in checking the TCR/CD3-mediated tetramer-binding by SPV-T3b pretreatment.

We extended our SPV-T3b pretreatment analyses to check the detection of antigen-specific T cells in PBMC of 5 donors, using HLA-A1, HLA-A2 or HLA-A3 tetramers containing peptides derived from influenza A virus (flu), cytomegalovirus (CMV), MART-1, tyrosinase (Fig. 3 and Table 2). Fig. 3 shows a decrease in MFI of the tetramer-reactive cells in donor 1 for HLA-A2/CMV-, HLA-A2/MART-1- or HLA-A2/tyrosinase-reactive CD8+ T cells, whereas the reactivity with the HLA-A2/flu tetramer was not changed by the SPV-T3b pretreatment. These results are consistent with the antigen-specific proliferation upon stimulation with CMV-, MART-1 or tyrosinase peptide, but not influenza peptide, seen in this donor. This antigen-specific proliferation was apparent from an increase in tetramer-reactive T cells in the culture over time, whereas tetramer-reactivity remained unchanged in control PHA-stimulated cultures. In the panel of donors containing antigen-specific T cells, as verified by *in vitro* peptide stimulation, SPV-T3b pretreatment blocked tetramer-reactivities of the CD8+ T cell population. SPV-T3b pretreatment did not affect the background tetramer-reactivity of the CD8 negative population (Table 2). This background reactivity is unlikely to be mediated by the TCR/CD3 complex, as the CD8-negative population mostly comprised CD4+ T cells that do not recognize peptides presented in HLA-class I.

To further verify whether SPV-T3b pretreatment discriminates between TCR-mediated binding and unrelated binding in the CD8+ T cell population, we tested the reactivity of HLA-A2 tetramers containing the HIV-derived p17 Gag (SLYNTVATL) peptide on PBMC of three HLA-A2+ HIV-negative healthy donors, in whom no T cell reactivity to HIV antigens is expected to be present. Upon acquisition of large cell samples comprising more than 1×10^6 PBMC, a small but detectable level of HLA/peptide tetramer-reactivity was found in these donors. Importantly, this reactivity remained unaffected by SPV-T3b pretreatment (Fig. 4). In parallel analyses, the MFI of the population of HLA-A2/flu tetramer-reactive T cells in these donors decreased by SPV-T3b pretreatment, indicating that SPV-T3b pretreatment efficiently discriminates true TCR/CD3-mediated binding from background HLA/peptide tetramer-reactivity. Furthermore, these data show that the low-level reactivity of HLA/peptide tetramers with CD8+ T cell populations that lack the relevant antigen-specificity is not mediated through binding to the TCR/CD3 complex.

3.3. Testing of binding of HLA class II tetramers to the TCR/CD3 complex on T cells

Analyses of the immune response to vaccination are mostly performed based on the reactivity of the CD8+ T cells using HLA class I tetramers. However, successful activation of an effector CD8+ T cell response frequently involves the activation

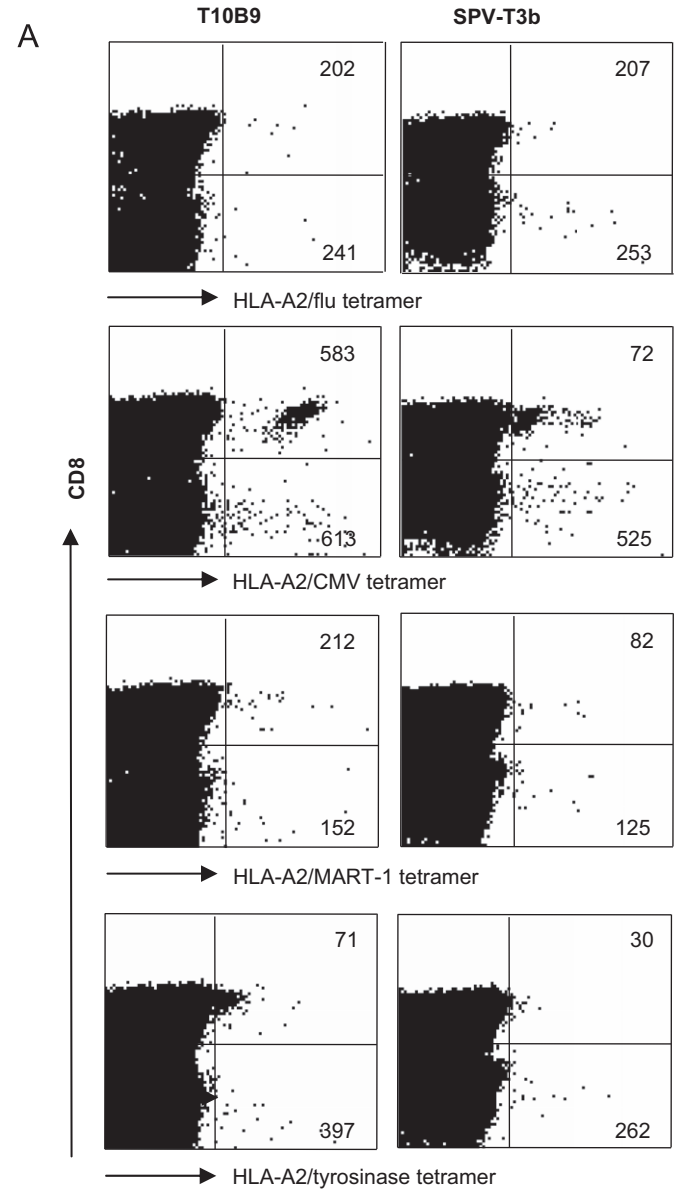


Fig. 3. SPV-T3b pretreatment enables to distinguish tetramer-binding to the TCR/CD3 complex from TCR unrelated binding among PBMC. A., PBMC from healthy HLA-A2+ donors were incubated with HLA-A2/flu, HLA-A2/CMV, HLA-A2/MART-1 or HLA-A2/tyrosinase tetramers (horizontal axis), after preincubation with anti-TCR mAb T10B9 (left panel) or anti-CD3 mAb SPV-T3b (right panel), followed by GAM-Ig. After tetramer incubation the cells were incubated with FITC-labeled anti-CD8 mAb, which is depicted on the vertical axis. Numbers in the quadrants indicate the MFI of tetramer-binding CD8+ T cells (upper right quadrants), or the MFI of tetramer-binding CD8- T cells (lower right quadrants), respectively. Graphs show that blocking of HLA-A2/CMV, MART-1 and tyrosinase tetramer-reactivity is exclusively found in the CD8+ population, but not in the CD8- population, indicating that the latter does not involve the TCR/CD3 complex. HLA-A2/flu tetramer reactivity was not blocked in the depicted donor. Data are representative of three experiments performed on PBMC of two different HLA-A2+ donors, and of one HLA-A2 negative donor. B., Graph shows an example of the eight-fold decrease in MFI of the HLA-A2/CMV tetramer following SPV-T3b pretreatment.

Table 2
Identification of HLA/peptide tetramer-binding T cells among donor PBMC by SPV-T3b pretreatment.

Tetramer-reactivity in the CD8+ T cell population										Tetramer-reactivity in the CD8-negative T cell population							
No blocking		SPV-T3b pretreatment								No blocking		SPV-T3b pretreatment					
Donor	HLA type	Tetramer	Tm ₀ ^a	MFI ₀ ^b	Tm ₁	MFI ₁	MFI _{neg}	MFI _c ^c	Fold decrease	Tm ₀	MFI ₀	Tm ₁	MFI ₁	MFI _{neg}	MFI _c	Fold decrease	
1	A2+	A2/CMV	1.425	583	1.500	72	43	72	8.1	0.147	613	0.129	591	43	525	1.2	
1	A2+	A2/ MART-1	0.077	212	0.020	189	43	82	2.6	0.019	152	0.009	214	43	125	1.2	
1	A2+	A2/tyr	0.553	71	0.022	66	29	30	2.4	0.024	397	0.023	262	29	262	1.5	
2	A1+ A2+	A1/flu	0.743	539	0.130	286	132	159	3.4	0.008	350	0.003	316	132	207	1.7	
2	A1+ A2+	A2/flu	0.340	400	0.151	83	40	59	6.8	0.175	113	0.292	199	40	199	0.6	
3	A1+	A1/flu	0.606	319	0.189	359	38	138	2.3	0.015	674	0.013	837	38	742	0.9	
3	A1+	A1/tyr243	0.925	242	0.237	105	38	55	4.4	0.189	119	0.119	315	38	212	0.6	
4	A3+	A3/flu	0.122	143	0.020	130	66	70	2.0	0.013	206	0.008	174	66	129	1.6	
5	A1+	A1/flu	0.158	151	0.070	90	59	73	2.1	0.072	138	0.044	100	59	84	1.6	
5	A1+	A1/tyr243	0.118	93	0.000	n.e.	45	45	2.1	0.077	113	0.058	124	45	104	1.1	
									Average decrease:						3.6	Average decrease:	1.2

^a Tm₀ and Tm₁ indicate the percentages of tetramer-positive T cells of the CD8+ population without (Tm₀) or with SPV-T3b pretreatment (Tm₁), respectively.
^b MFI₀ and MFI₁ indicate the mean fluorescence intensity of the tetramer-positive T cells without (MFI₀) or with SPV-T3b pretreatment (MFI₁), respectively.
^c MFI_c indicates the mean fluorescence intensity of the tetramer-reactive T cell population after SPV-T3b pretreatment corrected for T cells with fully blocked tetramer-reactivity (FI_{neg}).

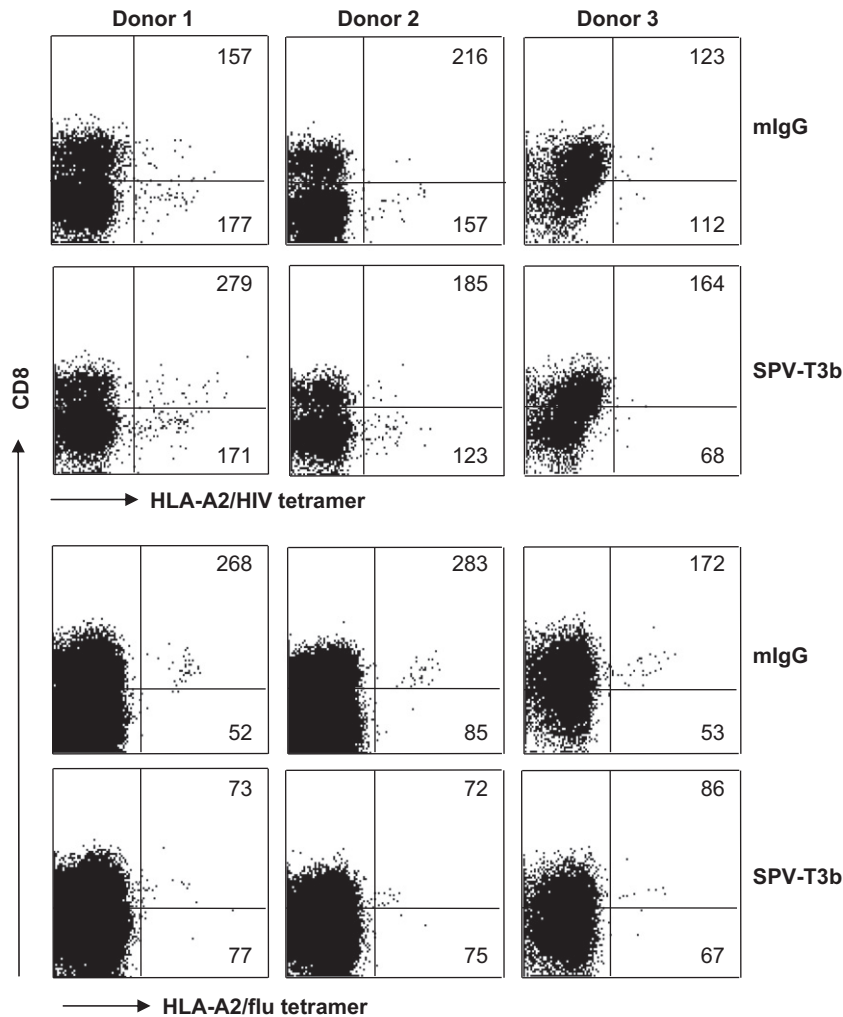


Fig. 4. Validation of SPV-T3b pretreatment on HIV tetramer-reactivity in HIV-negative donors. One million PBMC from three healthy HIV-negative HLA-A2+ donors were incubated with HLA-A2/HIV or HLA-A2/flu tetramers (horizontal axis), after preincubation with control murine IgG (upper panels of each tetramer analysis) or anti-CD3 mAb SPV-T3b (lower panels), followed by GAM-Ig. After tetramer incubation the cells were incubated with FITC-labeled anti-CD8 mAb, which is depicted on the vertical axis. Numbers in the quadrants indicate the MFI of tetramer-binding CD8+ T cells (upper right quadrants), or the MFI of tetramer-binding CD8- T cells (lower right quadrants), respectively. Graphs show that the observed HLA-A2/HIV tetramer-reactivity is not affected by SPV-T3b pretreatment, whereas HLA-A2/flu reactivity is decreased. Data are representative of three independent experiments.

of CD4⁺ T cells. An increasing number of epitopes recognized by CD4⁺ T cells has been identified over the last years, and for which class II tetramers may be generated [3,5,14,30,43,44]. As the background reactivity of HLA-class II tetramer may vary [13], extra testing for TCR/CD3-mediated binding is useful to improve reliable detection of antigen-specific CD4⁺ T cells.

We have tested our SPV-T3b pretreatment method on HLA class II tetramers, composed of the influenza virus hemagglutinin peptide (HA307-319) bound to HLA-DRA1*0101/DRB1*0401 molecules (HLA-DR4/flu tetramers). As a source of PBMC containing antigen-specific T cells, we used PBMC of a DRA1*0101/DRB1*0401-positive donor stimulated with the influenza virus hemagglutinin peptide (HA307-319), which resulted in an increase in HLA-DR4/flu tetramer-reactive T cells [22]. When the PBMC were labeled with CFSE prior to peptide stimulation, the decreased CFSE intensity of the HLA-DR4/flu tetramer-positive CD4⁺ T cells indicated that these cells had proliferated following peptide stimulation (not shown). SPV-T3b pretreatment decreased the MFI of HLA-DR4/flu tetramer-reactive cells, indicating that mAb

SPV-T3b also interfered with binding of HLA class II tetramers (Fig. 5A). As was observed for the HLA class I tetramers, pretreatment with mAb T10B9 did not inhibit HLA-class II tetramer-binding. To study the inhibition of tetramer-binding at the level of a single TCR specificity, we generated T cell clones from the HLA-DR4/flu tetramer-reactive T cell population. As shown in Fig. 5B, binding intensity of HLA-DR4/flu tetramers to T cell clones is almost ten-fold decreased by SPV-T3b pretreatment, indicating that HLA-DR4/flu tetramer bound to the T cells by interaction with the TCR/CD3 complex.

Taken together, the method of SPV-T3b mAb pretreatment described here allows one to determine whether HLA-class I and class II tetramer-binding to T cells are TCR-mediated, and our method is therefore a valuable tool for screening of polyclonal T cell populations in patient-derived samples.

4. Discussion

Monitoring of T cell responses in cancer patients following vaccination with tumor antigens frequently involves the analysis of small populations of tetramer-reactive T cells. In case of low frequency HLA/peptide tetramer-reactive T cells, the validity of the observed HLA/peptide tetramer staining is difficult to prove without *in vitro* stimulation and/or additional *in vitro* expansion [19]. We describe here a new method, SPV-T3b pretreatment, to test for binding of HLA class I/ or class II/peptide tetramers to the TCR/CD3 complex on T lymphocytes. This method enables to discriminate TCR/CD3-related binding of HLA/peptide tetramers from TCR-unrelated tetramer-binding to T cells, and thereby enhances reliable detection of antigen-specific T cells by HLA/peptide tetramer analyses. SPV-T3b pretreatment consists of preincubation of T cells with anti-CD3 mAb SPV-T3b followed by incubation with GAM-Ig prior to HLA/peptide tetramer incubations. Blocking of tetramer-binding by SPV-T3b pretreatment indicates the involvement of the TCR/CD3 complex in tetramer-binding.

HLA/peptide tetramers have mostly been validated by their specific binding to antigen-specific T cell clones or lines, and the absence of binding to HLA-matched control T cell clones [23,24,28]. However, analyses of polyclonal T cells, including PBMC, showed that HLA/peptide tetramer reactivity may also occur in a non-antigen specific fashion [15]. The antigen-specificity of HLA/peptide tetramer-binding T cells can be confirmed by *in vitro* peptide stimulation of the T cells, inducing proliferation of tetramer-reactive T cells, or by flow cytometric sorting of tetramer-binding T cells [16,33,32,12]. These validation methods depend on the *in vitro* growth capacity of the HLA/peptide tetramer-positive T cells, which may vary among antigen-specific T cells and introduce a bias in the T cell population analyzed. Alternatively, control samples of HLA mismatched donors have been used to estimate the nonspecific reactivity of HLA/peptide tetramers [23,28,35]. However, allospecificity of T cells in HLA-mismatched control samples may result in HLA/peptide tetramer-binding. Results of HLA mismatched control samples may therefore overestimate background levels of tetramer reactivity that is not fully relevant for analyses in HLA-matched patients, which do not have such alloreactivity. These observations underline the need for additional control measurements in HLA/peptide tetramer analyses to verify the antigen-specific binding of HLA/peptide tetramers. The new method of SPV-T3b pretreatment we describe here discriminates binding of HLA/peptide tetramers to the TCR/CD3 complex from TCR-unrelated binding, and thereby represents an internal control for HLA/peptide tetramer analyses.

The mechanism by which SPV-T3b pretreatment interferes with the binding of tetramers to T cells may include sterical

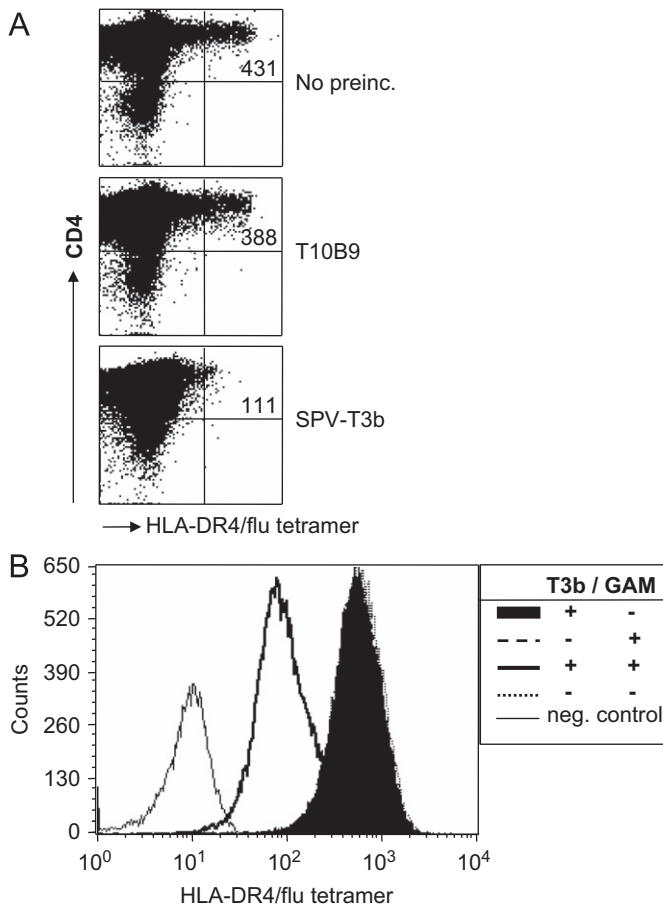


Fig. 5. SPV-T3b pretreatment decreases the binding intensity of HLA class II tetramers. **(A)** PBMC of an HLA-DRB1*0401+ healthy donor, which had been cultured with the Influenza virus hemagglutinin peptide (HA307-319), were incubated with the HLA-DR4/flu tetramer-reactive T cells (upper panel), or after preincubation with anti-TCR mAb T10B9 (middle panel), or anti-CD3 mAb SPV-T3b (lower panel) and GAM-Ig. After tetramer incubation the cells were incubated with APC-labeled anti-CD4 mAb, which is depicted on the vertical axis. Numbers in the upper right quadrants indicate the MFI of tetramer-binding CD4⁺ T cells. **(B)** Crosslinking of bound anti-CD3 mAb is required for inhibition of class II tetramer-binding. HLA-DR4/flu tetramer-reactive T cells from the PBMC in A. were cloned by single cell sorting and tested for specific HLA-DR4/flu tetramer-binding after preincubation with 5 μg/ml SPV-T3b mAb with or without crosslinking by GAM-Ig, preincubation with GAM-Ig alone, or without antibody preincubation. Graph shows typical results of two experiments of a representative CD4⁺ T cell clone.

hindrance of tetramers for binding the TCR/CD3 complex by bound anti-CD3 antibody immune complexes or the induction of a conformational change in the CD3 complex that reduces tetramer-binding. The observations that SPV-T3b pretreatment is effective at 4°C and in the presence of metabolic inhibitor sodium azide, and is not enhanced at 37°C indicate that blocking of internalization of TCR/CD3 complexes is unlikely to be responsible for the inhibitory effect of SPV-T3b pretreatment. Moreover, sterical hindrance or stabilization of tetramer-binding by bound antibodies has been shown for antibodies against the coreceptor CD8, which can either decrease or enhance CD8-dependent binding of certain MHC class I tetramers to human or murine T cells [4,6,7,10]. Surprisingly, antibodies T10B9 and WT31, which have been described to bind the TCR $\alpha\beta$ heterodimer and not to the CD3 complex [37,38], did not interfere with tetramer-binding upon crosslinking. For the anti-CD3-specific antibodies that were analyzed, interference with tetramer-binding depends on the mAb that is used. Unfortunately, as it is presently unknown which subunit of the CD3 complex forms the ligand of these anti-CD3 mAbs, it remains presently unknown which CD3 chain is the most effective target for tetramer-binding inhibition. Binding intensities of FITC-labeled anti-human CD3 mAbs HIT3a and UCHT1 were shown to be approximately 2-fold decreased when HLA/peptide tetramer were prebound to human antigen-specific T cells in one study [9]. However, only minor inhibition of HLA/peptide tetramer binding was observed after preincubation with the anti-CD3 mAbs, and the competing effect of mAb UCHT1 was not confirmed by others [7]. We have found that crosslinking of the anti-CD3 mAb is required for its effect on specific tetramer-binding performed at 37 °C, which was also reported to enhance the specificity of tetramer-binding, as compared to 4 °C [39].

In conclusion, our method of SPV-T3b pretreatment can contribute valuable data to studies of immunomonitoring of vaccinated patients, in which reactivity of T cells with HLA/peptide tetramers that can be blocked by SPV-T3b pretreatment represents the total antigen-specific T cell population, including unresponsive T cells.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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