Clinical Cancer Research

High-throughput Screening of Human Tumor Antigen–specific CD4 T Cells, Including Neoantigen-reactive T Cells

Carla Costa-Nunes[1], Amélie Cachot[2], Sara Bobisse[2], Marion Arnaud[2], Raphael Genolet[2], Petra Baumgaertner[1], Daniel E. Speiser[1], Pedro M. Sousa Alves[3], Federico Sandoval[3], Olivier Adotévi[4], Walter Reith[5], Maria Pia Protti[6], George Coukos[2], Alexandre Harari[2], Pedro Romero[1], and Camilla Jandus[2]

Abstract

Purpose: Characterization of tumor antigen–specific CD4 T-cell responses in healthy donors and malignant melanoma patients using an *in vitro* amplified T-cell library screening procedure.

Patients and Methods: A high-throughput, human leukocyte antigen (HLA)-independent approach was used to estimate at unprecedented high sensitivity level precursor frequencies of tumor antigen- and neoantigen-specific CD4 T cells in healthy donors and patients with cancer. Frequency estimation was combined with isolation and functional characterization of identified tumor-reactive CD4 T-cell clones.

Results: In healthy donors, we report frequencies of naïve tumor-associated antigen (TAA)-specific CD4 T cells comparable with those of CD4 T cells specific for infectious agents (*Tetanus toxoid*). Interestingly, we also identified low, but consistent numbers of memory CD4 T cells specific for several TAAs. In patients with melanoma, low frequencies of circulating TAA-specific CD4 T cells were detected that increased after peptide-based immunotherapy. Such antitumor TAA-specific CD4 T-cell responses were also detectable within the tumor-infiltrated tissues. TAA-specific CD4 T cells in patients displayed a highly polyfunctional state, with partial skewing to Type-2 polarization. Finally, we report the applicability of this approach to the detection and amplification of neoantigen-specific CD4 T cells.

Conclusions: This simple, noninvasive, high-throughput screening of tumor- and neoantigen-specific CD4 T cells requires little biologic material, is HLA class II independent and allows the concomitant screening for a large number of tumor antigens of interest, including neoantigens. This approach will facilitate the immunomonitoring of preexisting and therapy-induced CD4 T-cell responses, and accelerate the development of CD4 T-cell–based therapies.

Introduction

The realization that human tumors express T-cell–defined tumor associated antigens (TAA) has been the cornerstone to develop T-cell–based cancer immunotherapies. In this context, strategies comprising cancer vaccines, adoptive T-cell transfer therapies, and mAbs have been tested in the clinical setting aiming to improve antitumor T-cell responses (1). Some of these approaches have recently led to impressive clinical responses, resulting in a rapidly increasing number of immunotherapy agents approved by the FDA and European Medicines Agency for the treatment of different types of solid tumors and hematologic malignancies (2–4).

In this scenario, increasing evidence highlights the crucial roles played by CD4 T cells in antitumor immunity (5). Tumor infiltration by Th1-polarized CD4 T cells has been associated with better survival in patients with different types of tumors, while the presence of cells with Th2 and Treg phenotypes most often correlates with poor prognosis (6). Hence, the heterogeneity and plasticity within CD4 T-cell subsets provide highly interesting new therapeutic perspectives. For instance, infusion of autologous tumor-specific Th1 CD4 T cells in a patient with melanoma resulted in a durable clinical response (7). Moreover, recent observations show that CD4 T cells are exquisitely sensitive to sensing and recognition of "neoepitopes," a category of antigens that hold great promise in cancer immunotherapy (8–11).

[1]Department of Oncology UNIL CHUV, University of Lausanne, Lausanne, Switzerland. [2]Ludwig Institute for Cancer Research and Department of Oncology, University of Lausanne, Lausanne, Switzerland. [3]GSK, Wavre, Belgium. [4]University Bourgogne Franche-Comté, INSERM, EFS BFC, UMR1098, Besançon, France. [5]Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, Geneva, Switzerland. [6]Tumor Immunology Unit, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy.

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C. Costa-Nunes and A. Cachot are the co-first authors of this article.

P. Romero and C. Jandus are the co-last authors of this article.

Current address for P.M. Sousa Alves: PDC*line Pharma, Liege, Belgium; and current address for F. Sandoval, Product Development – Oncology (PDO) F. Hoffmann-La Roche Ltd, Basel, Switzerland.

Corresponding Authors: Camilla Jandus, Ludwig Institute for Cancer Research and Department of Oncology, University of Lausanne, Lausanne, Chemin des Boveresses 155, Epalinges 1066, Switzerland. Phone: 412-1652-5993; Fax: 412-1692-5995; E-mail: Camilla.jandus@chuv.ch; and Pedro Romero, Department of Oncology UNIL CHUV, Chemin des Boveresses 155, Epalinges CH-1066, Switzerland. E-mail: pedro.romero@hospvd.ch

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Translational Relevance

Major challenges in the monitoring of tumor-specific CD4 T cells in humans are due to their very low frequency and the absence of sensitive tools for their detection. Here, we used a high-throughput, HLA type-independent approach to estimate at unprecedented high sensitivity level precursor frequencies of tumor-specific CD4 T cells, combined with their isolation and functional characterization. In healthy donors, we report frequencies of naïve tumor-specific CD4 T cells comparable with those of CD4 T cells specific for infectious agents and low, but consistent numbers of memory tumor antigen–specific CD4 T cells. In patients with cancer, low frequencies of circulating tumor antigen–specific CD4 T cells were detected, with a trend for increased levels after peptide-based immunotherapy. Using this simple, noninvasive strategy requiring little biologic material, valuable information can be gained on preexisting and therapy-induced T-cell responses for a large number of tumor-associated antigens, including neoantigens, for rapid clinical translation.

To target tumor-specific CD4 T cells in cancer immunotherapy, knowledge on their natural abundance is required. Yet, several obstacles have hindered the measurement of the frequency of circulating antigen-specific CD4 T cells in humans. Those include extensive HLA class II polymorphism, low frequencies of antigen-specific CD4 T cells and technical difficulties in generating tools for antigen-specific CD4 T-cell identification (12). Attempts have been made using various approaches which include peptide-MHC class II tetramer-based enrichment protocols (13, 14), multicytokine quantification upon antigen stimulation (15, 16) or T-cell library microculture procedures (17, 18). Overall, in humans, it has been speculated that the frequency of most individual antigen-specific naïve CD4 T-cell populations might approximately be around 2 cells per million CD4 T cells, with the upper limit being of 10–20 cells (12). In contrast, memory populations specific for infectious agents are expected to be of several hundred cells per million in peripheral blood of healthy adults (17, 19, 20). Using standard methods, not surprisingly, tumor-specific cells are mainly undetectable in treatment-naïve patients and in healthy donors (21–25). We and others reported on increased frequencies of tumor-specific CD4 T cells in patients with melanoma following immunotherapy (16, 21, 22, 26, 27). Nevertheless, consistent high-throughput and sensitive identification of tumor-reactive CD4 T cells remains challenging.

Here, we used a high-throughput, HLA-independent approach to estimate at unprecedented high sensitivity level precursor frequencies of tumor antigen–specific CD4 T cells, combined with their functional characterization. In healthy donors, we report frequencies of naïve tumor antigen–specific CD4 T cells comparable with those of CD4 T cells specific for infectious agents and low, but consistent numbers of memory tumor antigen–specific CD4 T cells. In patients with cancer, low frequencies of polyfunctional and polyclonal circulating tumor-specific CD4 T cells were detected, with a trend for increased levels after peptide-based immunotherapy. Overall, this approach greatly facilitates the immunomonitoring of preexisting and therapy-induced CD4 T-cell responses and will accelerate the development of CD4 T-cell–based therapies for rapid clinical translation, including TCR gene transfer and screening for neoantigen-specific T cells.

Materials and Methods

PBMC isolation

Peripheral blood from healthy donors was collected at the Blood Transfusion Center, Lausanne, Switzerland. Collected peripheral blood from patients with stage III/IV melanoma was obtained from the Department of Oncology, University Hospital (CHUV, Lausanne, Switzerland; NCT00112242), under the approval of the Lausanne University Hospital's Institute Review Board. Written informed consent was obtained from all healthy subjects and patients, in accordance with the declaration of Helsinki. Blood was diluted with PBS or RPMI1640 (Gibco), mononuclear cells were purified by centrifugation over Ficoll-Plaque Plus (Amersham Biosciences) and washed three times with RPMI1640. Lymphocytes were directly used or cryopreserved in RPMI1640 containing 40% FCS and 10% DMSO. Vials containing 5×10^7 lymphocytes from healthy volunteers, 1×10^7 from patients with melanoma (both PBCMs and pre-immunization tumor-infiltrated lymph nodes, TILN) and patients with ovarian cancer (PBMC) were stored in liquid nitrogen.

Isolation of CD4 T cells and CD14 monocytes for antigen screening of T-cell libraries

CD14 and CD4 cells were collected from purified mononuclear cells derived from whole blood of healthy volunteers or patients with melanoma after positive selection with antibody-coated magnetic microbeads (Miltenyi Biotec). Subsequently, CD4 T-cell subsets were sorted to 98% purity on a FACSAria (BD Biosciences) with anti-CD3 APC-A750 (UCHT1, Beckman Coulter), anti-CD4 PE-Cy7 (SFCI12T4D11, Beckman Coulter), anti-CCR7 BV421 (G043H7, BioLegend), anti-CD45RA ECD (2H4LDH11LDB9, Beckman Coulter), and dead cells were excluded by DAPI (Molecular Probes) staining. CD14 cells were immediately frozen for subsequent use for the screening of the polyclonally expanded CD4 T-cell libraries at day 14.

Amplified T-cell libraries

Sorted CD4 T cells were cultured in medium RPMI1640 (Gibco) supplemented with 2 mmol/L glutamine, 1% (vol/vol) nonessential amino acids, 50 μmol/L 2β-mercaepthanol, penicillin (50 U/mL) and streptomycin (50 μg/mL), and 8% human serum (Blood transfusion center, Bern, Switzerland; complete medium). T cells (2,000 naïve and 1,000 memory cells/well) were stimulated with 1 μg/mL phytohemagglutinin (PHA; Remel) in the presence of irradiated (30 Gy) allogeneic feeder cells (2.5×10^4 per well) and human recombinant hrIL2 (500 IU/mL) in a 96-well plate, as reported previously (17). Multiple individual microcultures (up to 192) were seeded in 96-well plates. At day 7 postculture, each individual well was transferred into a 48-well format plate, and at day 10 each individual well is further transferred into 24-well plates. Antigenic screening of each individual polyclonally expanded CD4 T-cell library was assayed at day 14 of culture by stimulating 2.5×10^5 T cells/well with previously isolated autologous $CD14^+$ monocytes (2.5×10^4), which were pulsed for 6 hours with Tetanus Toxoid (Novartis), Keyhole Lympet Hemocyanin (Biosearch Technologies), NY-ESO-1 (GSK, Belgium), Melan-A (kindly provided by Ludwig Institute for Cancer Research, New York), Melanoma Associated

Antigen (MAGE)-A3 (GSK), PRAME (GSK), Carcinoembryonic antigen (Abcam), Prostatic Acid Phosphatase (Abcam), hTERT (kindly provided by Prof. O. Adotévi, INSERM), and MBP (kindly provided by Prof. O. Adotévi, INSERM, Paris, France) recombinant proteins at 5 μg/mL, or left unpulsed, and washed before the coculture with T cells. CD4 T cells were washed 4 times with PBS and rested at 37°C for at least 4 hours. Read-out assessed as proliferation by Thymidine (Hartmann Analytic) incorporation was measured on day 4 following an overnight incubation with 0.5 μCi/w ^{3}H. In this read-out setting, where each individual well is screened against several purified proteins, each protein is in itself the negative control of the others. Responding cultures were defined on the basis of a threshold of Stimulation Index (SI)>5 and were isolated for secondary screening. On the basis of the number of nonresponding wells over the total number of initially seeded wells, precursor frequencies were calculated following a Poisson distribution (15, 17).

Generation of specific CD4 T-cell lines from expanded T-cell libraries

Antigen-specific CD4 T lymphocytes were isolated by activation-guided cell sorting using FACSAria II or FACSAria III. Primary library cultures were stimulated during 3 days in the presence of 5 μg/mL of protein and autologous monocytes (10 T cells:1 CD14 cell) at 37°C. Cell surface detection of activation markers was performed by extracellular staining for anti-CD3 APC (UCHT1, Beckman Coulter), anti-CD4 PE-Cy7 (SFCI12T4D11, Beckman Coulter), anti-CD25 PE (4E3, Miltenyi Biotec), anti-CD134 PE (ACT35, BioLegend), and anti-CD278 A488 (C398.4A, BioLegend). Dead cells were excluded by DAPI (Molecular Probes) staining added prior to sorting. Collected sorted cells were expanded to generate cell lines from up to 10,000 sorted cells that were stimulated with 1×10^5 irradiated allogeneic PBMCs and 1 μg/mL PHA in T-cell culture medium containing 100 U/mL rhIL2. Every 18–20 days, cells were restimulated with allogeneic irradiated PBMCs, 1 μg/mL PHA (Remel), and 100 U/mL hrIL2. After expansion, primary cultures were stimulated for secondary screening and fine-antigenic specificity assessment.

Synthetic peptides

Peptides were synthesized by Dr. C. Servis (Protein and Peptide Chemistry Facility, UNIL, Epalinges, Switzerland), by standard solid-phase chemistry on a multiple peptide synthesizer (Applied Biosystems) using F-moc for transient NH_2-terminal protection and were analyzed by mass spectrometry. All peptides were >90% pure as indicated by analytic HPLC. Lyophilized peptides were diluted in pure DMSO at 10 mg/mL or aliquots of 1 mg/mL in 10% DMSO were prepared and stored at −80°C.

Peptide stimulation of CD4 T-cell lines

Expanded CD4 T-cell lines were stimulated between days 10 to 15 of culture with overlapping peptides spanning the responding protein initially screened. Pools of peptides were prepared in complete medium to stimulate 1×10^5 cells per condition with 1 μg/mL or 10 μg/mL of each peptide in a final volume of 100 μL for 24 hours at 37°C. For every line tested, an unstimulated and a PHA-stimulated conditions were included with corresponding %DMSO. After stimulation, supernatants were collected and stored at −80°C for further analysis.

TCR characterization of isolated reactive CD4 T-cell lines

Expanded CD4 T-cell lines were stained for TCR Vβ identification as follows: anti-CD3 PerCp-Cy5.5 (UCHT1, BioLegend), anti-CD4 A700 (RPA-T4, BioLegend), anti-TCR Vβ1 PE-Vio770 (REA662, Miltenyi Biotec), anti-TCR Vβ2 APC-Vio770 (REA654, Miltenyi Biotec), anti-TCR Vβ3 FITC (REA646, Miltenyi Biotec), anti-TCR Vβ4 PE (Immunotech), anti-TCR Vβ5.1 PE (IMMU157, Beckman Coulter), anti-TCR Vβ5.3 APC (REA670, Miltenyi Biotec), anti-TCR Vβ6.7 FITC (Immunotech), anti-TCR Vβ7.1 FITC (Immunotech), anti-TCR Vβ7.2 APC (3C10, BioLegend), anti-TCR Vβ8 PE (Immunotech), anti-TCR Vβ9 PE (MKB1, BioLegend), anti-TCR Vβ11 APC (REA559, Miltenyi Biotec), anti-TCR Vβ12 FITC (VER2.32.1, Beckman Coulter), anti-TCR Vβ13.1 PE (Immunotech), anti-TCR Vβ13.2 PE (H132, BioLegend), anti-TCR Vβ13.6 APC (REA554, Miltenyi Biotec), anti-TCR Vβ14 PE-Vio770 (REA557, Miltenyi Biotec), anti-TCR Vβ16 APC-Vio770 (REA556, Miltenyi Biotec), anti-TCR Vβ17 PE (E17.5F3.15.13, Beckman Coulter), anti-TCR Vβ21.3 FITC (Immunotech), anti-TCR Vβ22 PE (IMMU546, Beckman Coulter), anti-TCR Vβ23 APC (REA497, Miltenyi Biotec), and anti-TCR Vβ24 FITC (Immunotech). Cells were labeled with dead cell marker DAPI (Molecular Probes) before acquisition.

TCR sequencing

mRNA was isolated using the Dynabeads mRNA DIRECT Purification Kit (Thermo Fisher/Ambion) and then amplified using the MessageAmp II aRNA Amplification Kit (Thermo Fisher/Ambion) with the following modifications: 500 ng of total RNA was used as starting material. The *in vitro* transcription was performed at 37°C for 16 hours. First-strand cDNA was synthesized using the Superscript III (Thermo Fisher) and a collection of TRBV-specific primers. TCRs were then amplified by PCR (20 cycles with the Phusion from NEB) with a single primer pair binding to the constant region and the adapter linked to the TRAV/TRBV primers added during the reverse transcription. A second round of PCR (25 cycles with the Phusion from NEB) was performed to add the Illumina adapters containing the different indexes. The TCR products were purified with AMPure XP beads (Beckman Coulter), quantified, and loaded on the MiniSeq instrument (Illumina) for deep sequencing of the TCRβ chain. The TCR sequences were further processed using *ad hoc* Perl scripts to: (i) pool all TCR sequences coding for the same protein sequence; (ii) filter out all out-of-frame sequences, and (iii) determine the relative abundance of each distinct TCR sequence. TCRs with only 1 read were not considered for the analysis.

Cytokine quantification

Cytokine quantification in cell-free supernatants was performed by an IFNγ ELISA (huIFNγ Cytoset kit, Biosource Europe SA) or using a Mesoscale Discovery (MSD) multiplexed assay for 10 cytokine panels (IFNγ, IL10, IL12, IL13, IL1β, IL2, IL4, IL6, IL8, and TNFα) or (Eotaxin-3, GM-CSF, IFNγ, IL4, IL5, IL10, IL13, IL17A, IL22, and TNFα) following manufacturer's instructions.

Tumor cell recognition

NY-ESO-$1_{87\text{-}99}$–specific CD4 T cells (restricted by HLA-DR7) or Melan-$A_{25\text{-}36}$–specific CD4 T cells (restricted by HLA-DQ6) were cocultured at 1:1 ratio with HLA-DR7$^+$ (GEFI, GEF II, kindly provided by Prof Maria Pia Protti, Milan, Italy) or HLA-DQ6$^+$ (Me 275, generated in house from primary patients' material) melanoma cell lines. All cell lines were periodically tested for

Mycoplasma contamination and confirmed negative by PCR with *Mycoplasma*-specific primers (5′-ACTCCTACGGGAGGCAG-CAGTA-3′ and 5′-TGCACCATCTGTCACTCTGTTAACCTC-3′). To enforce constitutive MHC class II expression, GEFI and Me 275 tumor cell lines were transduced with a promoter modified retroviral vector pQCXIP (Clontech Corp) containing the human isoform 3 of the CIITA gene under the control of a hPGK promoter and a puromycin-resistant gene. Virus preparation was performed according to the standard protocols. After selection, cell surface MHC class II expression was assessed by flow cytometry using an anti-HLA-DR antibody (L243, BioLegend).

To induce the processing and presentation of the diverse antigens of interest, the GEFI and Me 275 cell lines were transfected with mRNA encoding minigenes containing the NY-ESO-$1_{87\text{-}99}$ and Melan-$A_{25\text{-}36}$ epitopes. Each minigene contained an antigen flanked by 9 a.a. of the natural sequence. A standard DNA vector including a T7 promoter, the minigenes linked by a glycine-serine–rich segment and sequences for correct HLA class II presentation was codon optimized and provided by GeneArt. After plasmid linearization, *in vitro* mRNA transcription was performed using the mMachine T7Ultra Transcription kit (Invitrogen). The synthetic mRNA was then electroporated into GEFI and Me 275 cell lines using the Neon transfection System (Invitrogen, Life Technologies), with the following parameters: 1,170 V, 30 ms, and 2 pulses. The GEFII tumor cell line is endogenously expressing NY-ESO-1 and MHC class II. Transfected and untransfected cells were then plated into a 96-well plate and cultured in absence or presence of the tumor-specific CD4 T clones isolated from the libraries. Supernatants from all conditions were harvested after 24 hours and IFNγ-specific ELISA was performed using the Human BD OptEIA ELISA set (BD Biosciences).

Statistical analysis

Precursor frequencies were calculated on the basis of the number of negative wells according to the Poisson distribution and expressed per million cells. Comparisons of responsiveness to different antigens were conducted by two-way ANOVA, as appropriate, and two-group comparisons (i.e., healthy-donors vs. patients) were conducted by Student *t* test. Prevaccine to post-vaccine comparisons were conducted by paired *t* test.

Statistical analyses were done using GraphPad Prism (version 7). For all analyses, a $P < 0.05$ was statistically significant and labeled by *, <0.01 by **, <0.001 by ***, and <0.0001 by ****. No significant differences were labeled with ns.

Results

Identification of tumor antigen–specific CD4 T cells in healthy individuals using amplified T-cell libraries

The CD4 T-cell library method (17) was adopted with the aim of estimating precursor frequencies of rare circulating human tumor-specific CD4 T cells (Fig. 1A). After two weeks of polyclonal expansion of individual microcultures, every single amplified CD4 T-cell culture was screened for multiple specificities, as previously reported for infectious agents (17). Maintenance of the initial T-cell repertoire during the polyclonal expansion period was monitored by assessing Vβ usage by flow cytometry at different time points and by measuring TCRα and β clonotype diversity *ex vivo* and over the culture period (Supplementary Fig. S1).

First, aliquots of naïve (defined as $CD45RA^+$ and $CCR7^+$ cells in the CD4 T cell gate) and memory (defined as $CD45RA^-$ $CCR7^{+/-}$) CD4 T cells were sorted from peripheral blood of healthy donors and libraries were generated from up to 19 individuals. Responsiveness to TAAs including Melan-A/MART-1 (melanoma antigen recognized by T cells 1), CEA (carcinoembryonic antigen), PAP (prostatic acid phosphatase), NY-ESO-1 (New York esophageal squamous cell carcinoma1), MAGE-A3 (melanoma-associated antigen 3), PRAME (preferentially expressed antigen in melanoma), and hTERT (human telomerase reverse transcriptase) was assessed. The bacterial antigen Tetanus toxoid (TT) and the marine Keyhole limpet hemocyanin (KLH) protein were used as positive and negative controls for memory T-cell antigens, respectively (Fig. 1B).

In the naïve compartment, estimated frequencies of TAA-specific CD4 T cells ranged from 0 to 97 specific cells per million, being similar to those of naïve T cells responsive to TT (Fig. 1B and C; Supplementary Table SI). In contrast, in the memory compartment, low but consistent TAA-specific cells were detected at 0 to 76 specific cells per million, while CD4 T cells specific for the recall antigen TT were up to nearly 3,000, in line with previous reports using the same method (refs. 17, 28; Fig. 1C; Supplementary Table SI). In contrast to TT-specific CD4 T cells, no significant difference was observed by comparing the magnitudes of naïve and memory CD4 T-cell frequencies for any given TAA tested (Supplementary Fig. S2). Unexpectedly higher frequencies of hTERT-specific CD4 T cells were detected in both naïve and memory CD4 T-cell libraries (0 to 347 per million within the naïve and 0 to 539 per million within the memory; Fig. 1D; Supplementary Table SI). Interestingly, for some donors, hTERT-specific responses were at similar frequencies as the ones observed for the other TAA, while in some others they significantly exceeded them (Fig. 1D). To further substantiate the specificity of these cells, hTERT-reactive CD4 T-cell lines were generated from the positive microcultures and quantification of IFNγ secretion upon specific stimulation confirmed the reactivity to the hTERT protein, for both naïve- and memory-derived T-cell lines (Supplementary Fig. S3A). Next, recognition of previously identified telomerase-derived CD4 T-cell epitopes, referred as universal cancer peptides (UCP), was tested for the generated hTERT-specific lines (Supplementary Table SII). UCP were previously shown to bind to the most common HLA class II present in the Caucasian population, and UCP-specific CD4 T cells were identified in patients with metastatic cancer (29). None of the tested hTERT-specific lines responded to any of the four tested UCP epitopes, suggesting that the expanded hTERT-specific cell lines are reactive against other regions of the 1,132 amino acid long hTERT protein (Supplementary Fig. S3B). Additional analyses using HLA-matched target cells, endogenously expressing telomerase would be needed to definitely demonstrate the reactivity of the generated CD4 T-cell lines against naturally processed telomerase.

Generation of tumor antigen–specific CD4 T-cell libraries from patients with melanoma

Next, tumor antigen–specific CD4 T-cell responses were measured in malignant melanoma patients' peripheral blood and within tumor infiltrated tissues (Supplementary Table SIII for clinical characteristics of the patients), either pre- or postpeptide-based immunotherapy, consisting in consecutive cycles of immunization with a long synthetic peptide from the TAA NY-ESO-1 together with CpG-ODNs, TLR-9 agonists, as immune

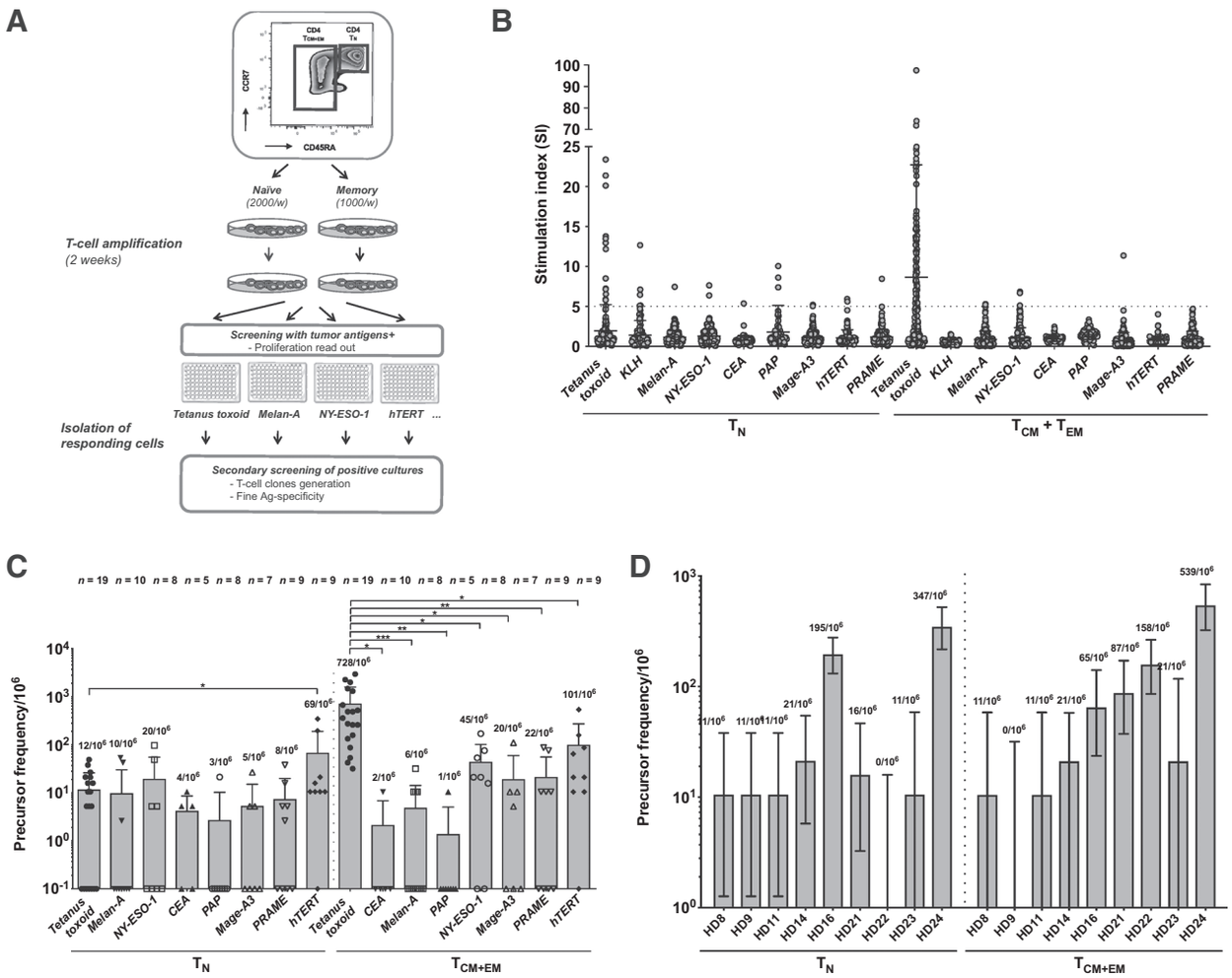

Figure 1.

Quantification of TAA-specific CD4 T cells using amplified CD4 T-cell libraries. **A,** Schematic representation of the CD4 T-cell library screening method. Sorted naïve and memory CD4 T cells are clonally expanded from microcultures. Reactivity is tested upon coculture with autologous monocyte loaded with whole antigenic proteins and reactive cultures are selected. On the basis of the number of responding over the total tested cultures, the frequency of specific CD4 T cells can be estimated. Positively responding cultures can be further tested using peptide pools for fine specificities and affinity of the response, and to isolate clonal T cells. **B,** Representative example of a CD4 T-cell library from a healthy donor using peripheral blood cells screened for reactivity to various TAAs. Purified CD4 T cells were sorted according to the naïve phenotypic markers ($CD45RA^+/CCR7^+/CD45RO^-$) or memory markers ($CD45RA^-/CCR7^{+/-}$) and were polyclonally stimulated with PHA, IL2, and allogeneic feeder cells during 2 weeks. Thereafter, each cell culture was stimulated with autologous monocytes pulsed with the indicated antigens at the concentration of 5 μg/mL. Proliferation was assessed after 3 days by H^3-incorporation. Frequency calculations were based on a positivity threshold of stimulation index (SI) >5. Dotted lines represent the cut off. TT and KLH are used as controls. **C,** Estimated TAA-specific CD4 T-cell frequencies in peripheral blood from healthy donors. Precursor frequencies were measured in a total of 19 healthy individuals for the different antigenic specificities [positivity threshold: stimulation index (SI) >5]. The number of healthy donors tested for each individual TAA is indicated at the top of each bar. **D,** hTERT-specific CD4 T-cell–estimated precursor frequencies in the naive and memory populations in the periphery of healthy donors. On the basis of the number of nonresponding wells precursor frequencies were calculated following a Poisson distribution (15, 17).

adjuvant (16). At baseline, patients with melanoma showed frequencies of CD4 T cells specific for the 2 TAAs tested, that is, Melan-A and NY-ESO-1, comparable with those observed in T-cell libraries from healthy donors, regardless whether they were derived from the naïve or the memory T-cell compartments (Fig. 2A). Upon peptide vaccination, antitumor-specific CD4 T-cell frequencies for both antigens showed some modulation albeit the differences with baseline were not statistically significant (Fig. 2B). To address the possibility of a contribution of the stem cell memory (T_{SCM}) compartment to the variation in frequency of antitumor NY-ESO-1 responsiveness within naïve CD4 T cells, libraries from naïve CD4 T cells specifically depleted of T_{SCM} were interrogated. No difference was observed when comparing total naïve CD4 T cells to naïve CD4 T cells depleted of T_{SCM} (Supplementary Fig. S4). This was not unexpected, as the T_{SCM} were a minor fraction of the T naïve subset (up to 1.3% of total naïve CD4 T cells).

Finally, we generated T-cell libraries from CD4 T cells isolated by flow cytometry–based sorting from tumor-infiltrated lymph nodes (TILNs) for 3 patients with melanoma. As

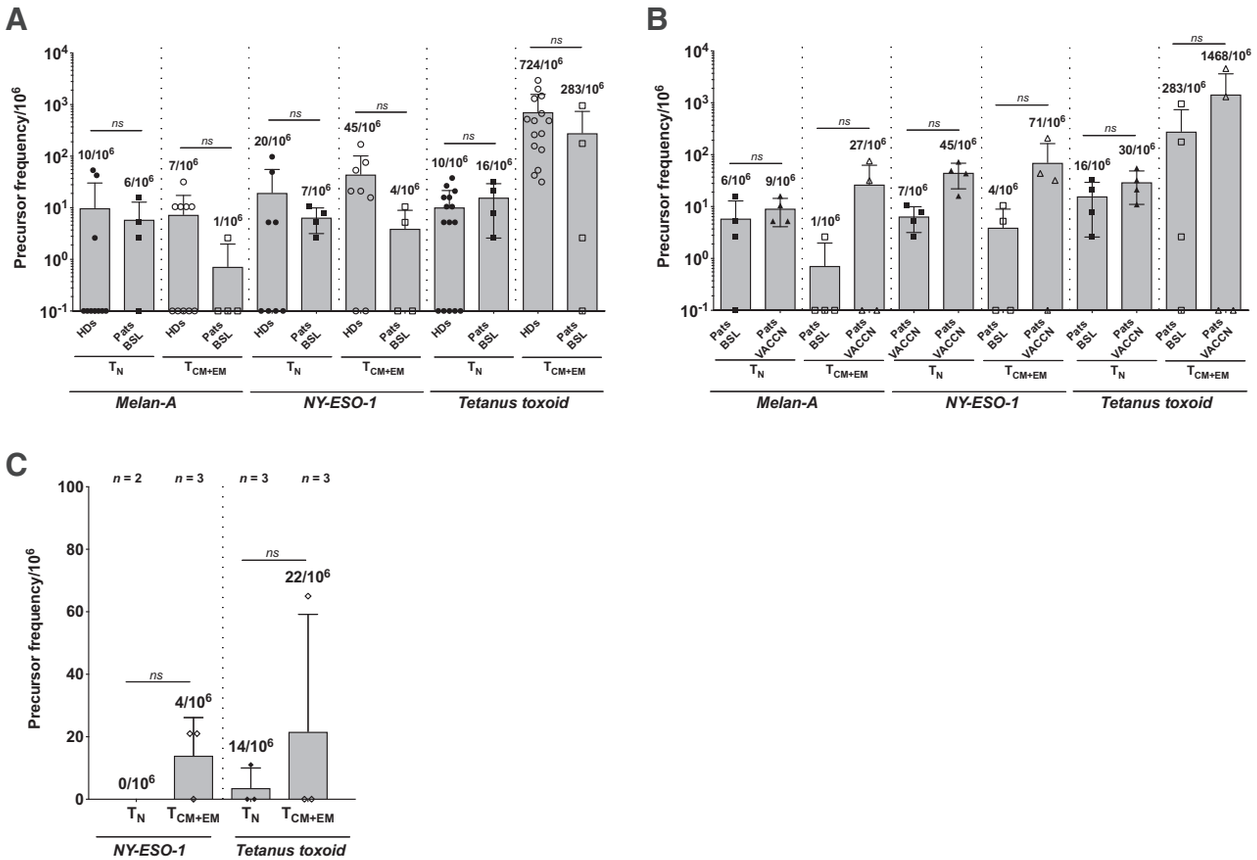

Figure 2.

TAA-specific CD4 T cells can be identified in patients with cancer using amplified CD4 T-cell libraries. **A,** Comparison of estimated TAA-specific CD4 T-cell frequencies in the naïve and memory compartment of peripheral blood T cells of healthy donors and patients with melanoma. TAA are Melan-A/MART-1 differentiation antigen and NY-ESO1 cancer/testis antigen. TT is used as control antigen. **B,** Estimated frequencies of TAA-specific CD4 T cells in peripheral blood of patients with melanoma before (BSL = baseline) and after NY-ESO-1 peptide–based immunotherapy (VACCN). **C,** Estimated frequencies of TAA-specific CD4 T cells isolated from tumor-infiltrated lymph nodes (TILN) of patients with melanoma.

expected, a higher proportion of bulk CD4 T cells within the TILNs were differentiated in effector-memory than in the periphery, with very low numbers of naïve CD4 T cells (representative example in Supplementary Fig. S5). Interestingly, no Melan-A–specific T cells were expanded in the three libraries and only NY-ESO-1–specific CD4 T-cell responses were detected selectively in the memory subset (Fig. 2C; summary in Supplementary Table SIV).

Functional profiling of expanded tumor-specific CD4 T-cell libraries

The microcultures showing peptide antigen responses in the expanded CD4 T-cell libraries were then functionally tested by cytokine secretion profiling upon short-term antigen rechallenge. A Th1 functional profile (mainly IFNγ secretion) was apparent for TT-derived antigen-specific responses in the microcultures generated from the naïve CD4 T cells from peripheral blood from both healthy donors and patients with melanoma (Fig. 3A). In contrast, those derived from the memory subset in melanoma showed an increased Th2 polarization if compared with the healthy donors, with increased IL13 secretion in clones derived from patients' libraries. It was also noteworthy that TT-derived antigen-reactive CD4 T cells could also be recovered from both the naïve and memory subset CD4 TILN-created libraries. Relatively high proportions of these specific microcultures released IL13 and IL5 compared with those derived from healthy donor peripheral blood and, to a lesser extent, to those from the blood of patients with melanoma (Fig. 3A, bottom left pie charts).

For TAA antigens, reactive lines originating from naïve CD4 T-cell libraries displayed a comparable polarization in healthy donors and patients, with predominant secretion of IFNγ, concomitant with discrete amounts of TNFα and IL13. However, TAA-specific lines showed different polarization in healthy donors compared with patients with cancer. Similar to TT-specific memory, CD4 T-cell library–derived T-cell lines, TAA-specific cells were mainly Th1 biased in healthy controls. In contrast, TAA-specific T-cell lines issued either from PBMCs or TILN-derived libraries from patients showed a clear polyfunctionality: diverse Th1 (TNFα and IFNγ) and Th2 cytokines (IL13, IL5, and IL4 at low levels), and even IL10 were abundantly secreted (Fig. 3B; Supplementary Fig. S6A and S6B).

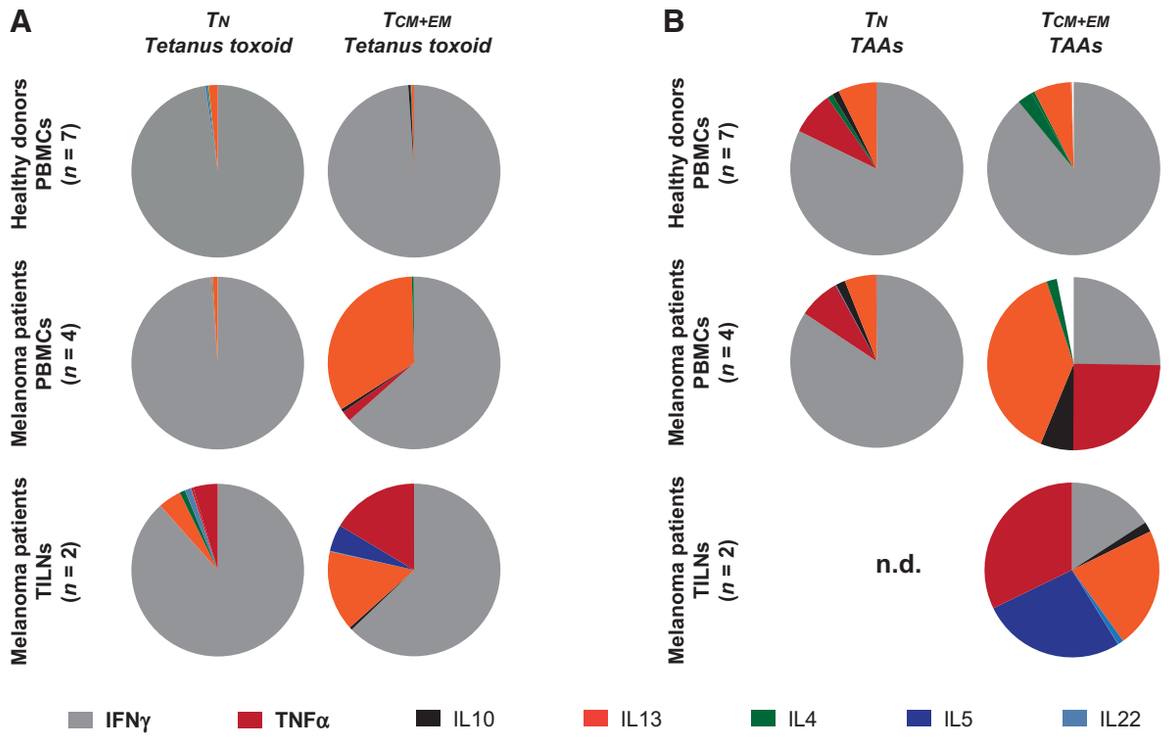

Figure 3.

TAA-specific CD4 T cells in patients are polyfunctional. Cytokine profiles of TAA-specific CD4 T cells from healthy donors and patients with melanoma for recall (TT; **A**) and TAAs (**B**) in the naïve (T_N) or memory (T_{CM+EM}) compartments. Responding CD4 T-cell expanded microcultures were assessed for cytokine patterns to characterize the responses. Results from responding CD4 T cells against individual tested tumor antigens were assembled (TAAs).

Deconvolution of the fine antigen specificity of the reactive CD4 T-cell lines and comparison of TCR Vβ usage

Antigen-specific reactive microcultures from tested TAAs were further screened to assess fine antigenic specificity of antigen recognition using overlapping peptide pools (30), HLA-matched tumor cell lines and to define TCR Vβ usage. To that purpose, responding cultures were sorted on the basis of the expression of the activation markers ICOS, CD25, and OX40 (Fig. 4A), as described previously (17). After further expansion *in vitro*, growing clones were screened for their reactivity with overlapping peptides (20-mer overlapping by 10 amino acids) spanning the entire sequence of Melan-A (118 aa long) and NY-ESO-1 (180 aa long) proteins. By the quantification of IFNγ in the culture supernatants, we were able to identify individual epitopes accounting for the detected responses (examples in Fig. 4B). Finally, we assessed the reactivity of the NY-ESO-1 and Melan-A T-cell clones to HLA-matched tumor cell lines. To that purpose, we selected microcultures showing recognition of the NY-ESO-$1_{87\text{-}99}$ epitope and the Melan-$A_{25\text{-}36}$ epitope, previously reported to be immunogenic in a HLA-DR7$^+$ and HLA-DQ6$^+$ manner, respectively (16, 31; Fig. 4C). Peptide-reactive clones were able to recongize HLA-matched tumor cells electroporated with mRNAs so as to express the cognate antigen, as assessed by IFNγ quantification in the coculture supernatants (Fig. 4D). In addition, by the use of HLA-DR7$^+$ tumor cells endogenously expressing NY-ESO-1, either untreated or pretreated with IFNγ to upregulate MHC class II molecules, we measured direct antitumor T-cell reactivity. As shown in Fig. 4E, only NY-ESO-1–specific CD4 T-cell clones, but not viral-specific HLA-DR7–restricted clones ($HA_{307\text{-}318}$/DR7) significantly secreted IFNγ after the coculture with the tumor cell line endogenously expressing NY-ESO-1 (Fig. 4E). In parallel, we analyzed TCR Vβ usage of some of the clones, confirming monoclonality and distinct usage by different clones (Table 1).

Identification of neoantigen-specific CD4 T cells using amplified T-cell libraries

CD4 T cells were recently shown both in murine tumor models and in patients with cancer to recognize peptides encoded by mutated genes within the tumors, the so called neoepitopes (8–10, 32). Therefore, we investigated whether the T-cell library method is suitable to screen for neoantigen-specific CD4 T-cell responses by a combination of tumor exome sequencing and peptide screening. We interrogated naïve and memory CD4 T cells isolated from PBMCs of a patient with ovarian cancer, for which we previously identified CD4 T-cell responses specific for a neoantigen from the *SLCRA1*$_{F308C}$ gene (neoantigen peptide sequence MDQYSTEPGRYRCMGTEAYAE-SIDR). After the T-cell expansion period, we challenged each individual culture with the peptide and measured specific responses by IFNγ ELISA (Fig. 5A). We could successfully detect reactive wells and estimate specific precursor frequencies (Fig. 5B) confirming the presence of such reactive cells in the memory CD4 T-cell compartment, although we cannot exclude cross-reactivity to the wild-type peptide.

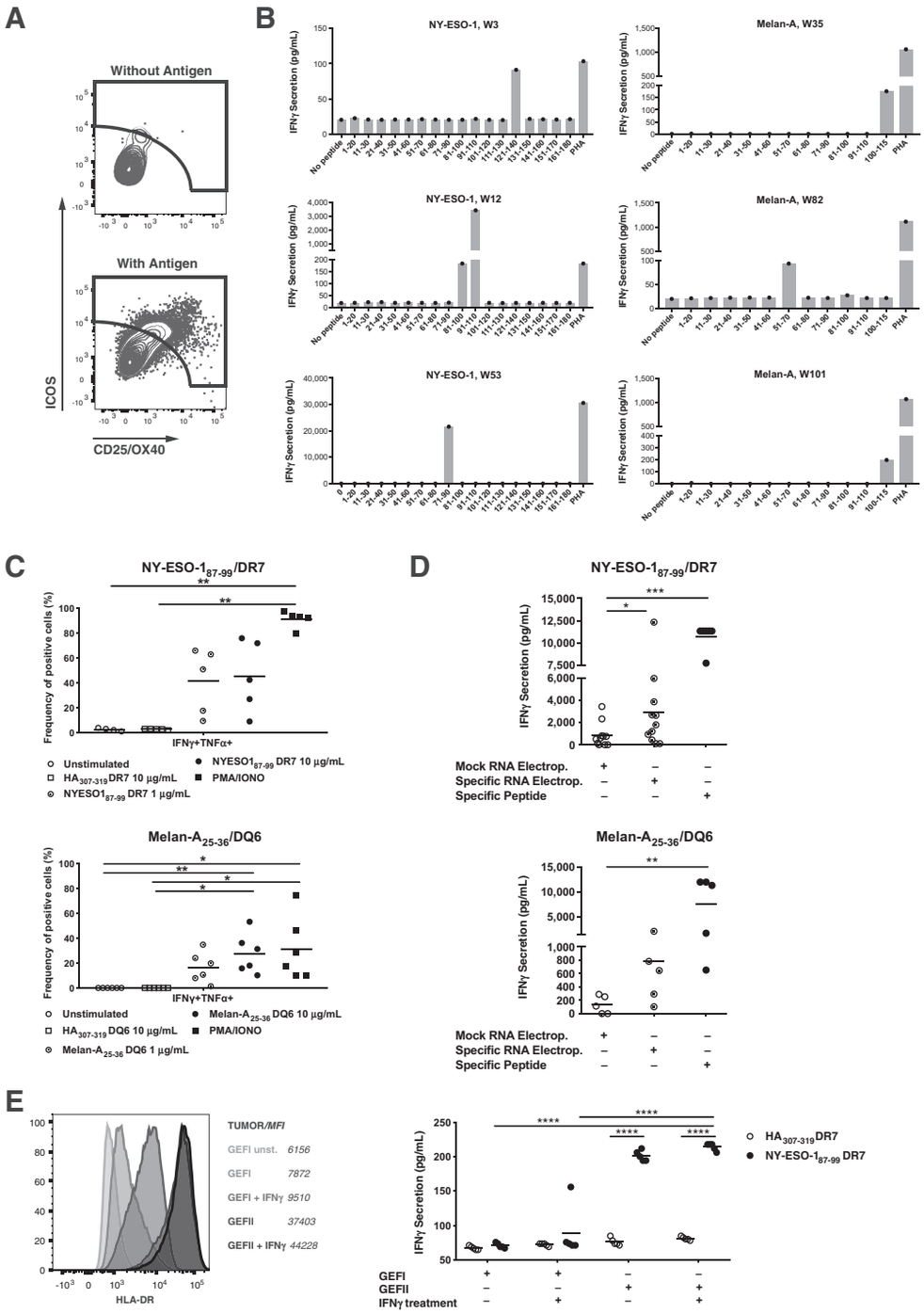

Figure 4.

Interrogation of responding TAA-specific microcultures. **A,** TAA-specific CD4 T cells were isolated from responding microcultures according to the expression of the activation markers (CD25, ICOS, OX40). **B,** TAA-specific CD4 T-cell lines (NY-ESO-1 and Melan-A–specific lines) were tested for fine antigen specificity with overlapping peptide pools covering the entire TAA protein and cytokine secretion (IFNγ) was quantified after 24 hours of activation. **C,** Reactivity of NY-ESO-1 and Melan-A–specific lines issued form HLA-DR7$^+$ and HLA-DQ6$^+$ donors were tested against the cognate peptides at 2 different concentrations, as well as against an irrelevant peptide. **D,** Reactivity of NY-ESO-1 and Melan-A peptide–specific lines isolated form HLA-DR7$^+$ and HLA-DQ6$^+$ donors to HLA-matched tumor cell lines mock electroporated, electroporated with a mRNA encoding the corresponding TAA or stimulated with the specific peptide. **E,** Reactivity of NY-ESO-1–specific and HA-specific lines (irrelevant control) to HLA-DR7$^+$ tumor cell lines endogenously expressing the tumor antigen NY-ESO-1. Reactivity was assessed against the untreated tumor cells, or IFNγ pretreated tumors, to induce MHC class II upregulation (left histograms). Cytokine secretion (IFNγ) was quantified after 24 hours of T-cell–tumor cell coculture (right graph).

Table 1. Representative examples of TCRVβ CDR3B sequencing of Melan-A and NY-ESO-1–specific CD4 T-cell clones obtained from positive wells in the CD4 T-cell libraries

Antigen specificity	TCRVbeta	CD3RB sequence	TRBJ
Melan-A 25-36/DQ6	hTRBV12-3	CASSGQGSSYNSPLHFG	hTRBJ01-6
Melan-A 25-36/DQ6	hTRBV05-1	CASSLGNPSTDTQYFG	hTRBJ02-3
Melan-A 25-36/DQ6	hTRBV28	CASPILRQPQHFG	hTRBJ01-5
Melan-A 25-36/DQ6	hTRBV03-1	CASSQSGTLYGYTFG	hTRBJ01-2
Melan-A 25-36/DQ6	hTRBV28	CASPILRQPQHFG	hTRBJ01-5
NY-ESO-1 87-99/DR7	hTRBV11-1	CASSPGQGNPEAFFG	hTRBJ01-1
NY-ESO-1 87-99/DR7	hTRBV09	CASSPGGGAYEQYFG	hTRBJ02-7
NY-ESO-1 87-99/DR7	hTRBV09	CASSVVPTGNTEAFFG	hTRBJ01-1

Discussion

Here we show the application of T-cell libraries to detect in a simple, high-throughput procedure multiple rare tumor antigen-specific CD4 T cells from healthy individuals and patients with cancer, including neoantigen-specific CD4 T cells. This approach lends itself to determine the frequency of specific T cells for a single antigen thus enabling a quantitative monitoring of specific T-cell responses. Furthermore, it also enables the parallel generation and functional characterization of human tumor antigen-specific CD4 T-cell clones starting with little biologic material and circumventing the need for peptide–MHC multimers, or autologous DC isolation and production. The resulting monoclonal T-cell populations can be used for T-cell epitope selection for optimization of immunotherapeutic vaccines, for TCR sequencing, and cloning in the context of adoptive cell transfer, and in combination with tumor exome sequencing it allows to select optimal human neoantigen-reactive CD4 T cells. Thus, this approach allows for the rapid and consistent building of TCR warehouses for feeding personalized immunotherapy intervention pipelines.

Because of the low frequency of antigen-specific T cells in the naïve repertoire, detailed information on circulating TAA-specific CD4 T cells in humans is lacking. We observed that precursor frequencies for TAAs in healthy cancer-free individuals ranged from less than 1 up to 97 TAA-specific T cells per million of naïve CD4 T cells. These frequencies are similar to previous reports on precursor frequencies of naïve pathogen-specific CD4 T cells (17), arguing for a similar thymic output of TAA-specific and pathogen-specific naïve CD4 T cells. These results might help in guiding the antigen selection for immunization of patients with cancer and in shedding light on the extent of thymic selection of self-specific TAA T cells (33). Among TAA, the highest precursor frequencies were observed for NY-ESO-1 and hTERT-specific CD4 T cells; however, variability was seen depending on the donor and the final proof of the hTERT-specific response would imply further testing against naturally expressing hTERT targets. In that regard, recent evidence showed unusual high frequencies of Melan-A–specific CD8 T cells in HLA-A2^{+} healthy donors, that is, at least in part the consequence of an inefficient induction of central tolerance to the immunodominant region of Melan-A (34). It was also reported that the presence of cytotoxic CD8 T cells against hTERT were equivalently induced upon *ex vivo* expansion of cells isolated from patients with melanoma and healthy donors, despite evidence of immunologic ignorance (35). These hTERT-specific CTLs killed telomerase-positive tumor cells in an MHC-restricted fashion showing that the hTERT-specific CTLs are not deleted from the T-cell repertoire (35). As shown for the lack of Melan-A$_{26\text{-}35}$ expression in the medullary thymic epithelial cells (mTEC), it might be speculated that the hTERT protein, or stretches of it, are not expressed in mTECs (35), leading to incomplete central tolerance in the context of defined HLA class II molecules. Regarding NY-ESO-1, mRNA of this cancer-testis (CT) antigen was detected in mTECs (36); however, expression of NY-ESO-1 protein in normal thymus remains controversial (37).

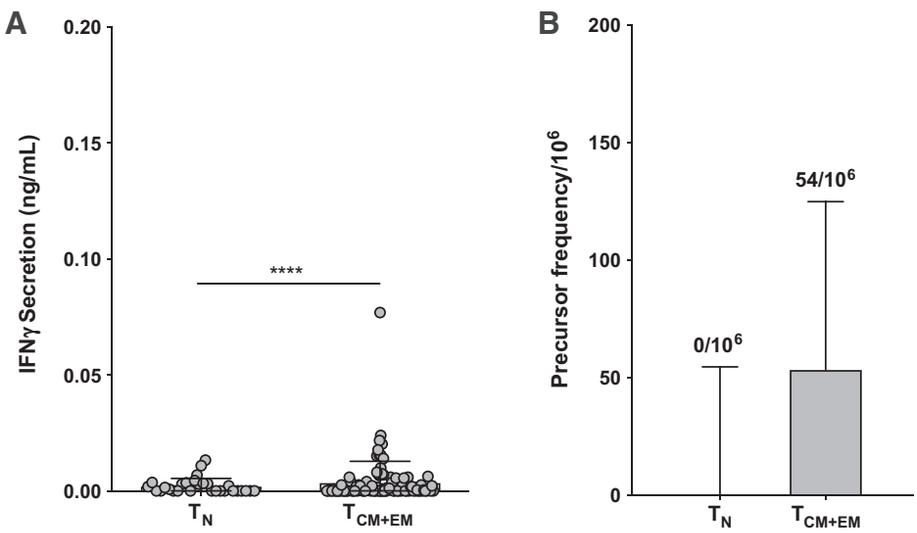

Figure 5.

Quantification of neoepitope-specific CD4 T cells using amplified CD4 T-cell libraries based on IFNγ secretion. **A,** Sorted naïve and memory CD4 T cells from a patient with ovarian cancer were clonally expanded and microcultures tested for reactivity against a previously identified neoantigen (MDQYSTEPGRYRCMGTEAYAESIDR). Cytokine secretion from the unstimulated condition was excluded. **B,** On the basis of the number of responding over the total tested cultures, the frequency of specific CD4 T cells is estimated assuming a positivity threshold based on the average of the unstimulated conditions, accordingly to a recent work from Lullo and colleagues (15).

Overall, knowledge on the frequencies of TAA-specific naïve precursors in combination with the evaluation of TAA expression in the thymus might be highly relevant for antigen selection in the context of immunotherapy, because preexisting immunity might influence the TAA-specific CD4 T-cell repertoire upon disease development and is central to define immunogenicity of vaccines.

Interestingly, we identified variable, but detectable TAA-specific CD4 T cells within the memory compartment in healthy donors, for nearly all the tested TAA (Melan-A, NY-ESO-1, CEA, PAP, MAGE-A3, and PRAME). The estimated frequencies for memory TAA-specific CD4 T cells are significantly lower when compared with previous reports regarding viral specificities (38–41). For some of the tested antigens, priming could have occurred by the encounter of the specific TAAs, which are known to be expressed at low levels in healthy tissues (e.g., Melan-A, CEA, PRAME). In that regard, we previously reported on the *ex vivo* detection of Melan-A–specific CD4 T cells in HLA-DQ6$^+$ HDs, using pMHC class II multimers (27). Others showed elevation of CEA-specific CD4 T cells in the blood of patients with breast and ovarian cancer, being measurable in 40% of patients compared with only 7%–8% of healthy donors (42). PRAME-specific CD4 T cells are measurable in healthy donors; however, only rarely, and following multiple rounds of *in vitro* stimulation (43). The presence of memory CT-specific CD4 T cells is more intriguing, because these antigens have a restricted expression pattern in immune privileged tissues. Thus, their existence in healthy donors might result from rare cross-reactivity events to environmental antigens or human microbiota, as previously reported for memory virus-specific CD4 T cells in unexposed healthy donors (28, 41). In line with our observations, memory NY-ESO-1–specific CD4 T cells were reported in healthy donors without spontaneous immunity to NY-ESO-1 (44). These cells showed high avidity for NY-ESO-1 and were under tight control by CD4$^+$CD25$^+$ regulatory T cells.

Memory TAA-specific CD4 T cells are expected to be higher in patients with melanoma, particularly after immunotherapy (16, 22, 27, 43, 45). Indeed, using the library approach, we observed a tendency for increased frequencies of TAA-specific CD4 T cells in patients vaccinated with long synthetic peptide emulsified in mineral oil together with the TLR-9 agonist CpG-ODN (16). A possible underestimation of TAA-specific T cells using expanded T-cell libraries might result from the loss of some rare clonotypes with inherently limited proliferation potential, more terminally differentiated phenotype or by the presence of T cells with regulatory attributes and limited proliferative potential in patients with cancer. However, TCR Vβ screening during the expansion process suggested that this may not be the case as it confirmed faithful expansion of the input CD4 T cells during the culture (data not shown).

In melanoma, tumor-infiltrating lymphocytes are known to comprise CD4 T cells specific for class II restricted epitopes from tumor antigens (46). Using the library screening strategy on dissociated TILN, we detected memory NY-ESO-1–specific CD4 T cells within CD4 T cells in tumor-infiltrated lymph nodes, in an average of 14 specific CD4 T cells per million. Interestingly, in patient samples, both from blood and tissue origin, we observed an augmented degree of polyfunctionality of TAA-specific CD4 T-cell lines, arguing for an influence of the tumor microenvironment in defining T-cell polarization (47). TAA-specific cells in patients secreted prototypic Type 1, but also Type 2 cytokines and IL10, when exposed to cognate antigens, suggesting that suppressive molecules present in the tumor bed include a Treg phenotype. In line with this, we observed increased Treg frequencies by *ex vivo* staining of PBMCs and TILN of patients as compared with controls (data not shown). Library from purified Tregs compared with Th subsets could be established to assess the responsiveness to tumor-associated antigens of individual CD4 T-cell subsets.

Because the library approach is suitable for readouts using synthetic peptides as challenge reagents (18, 20) instead of recombinant proteins, we further aimed at providing proof-of-principle for the use of this strategy to screen for the presence and isolate neoantigen-specific CD4 T cells. We were able to define precursor frequency and generate a clonal population of T cells specifically recognizing a neoantigen that we previously characterized in a patient with ovarian cancer indicating that this methodology is perfectly adapted for high-throughput neoantigen T-cell screening. Further applications include the use of autologous tumor lysates pulsed on APCs to scan for the presence of tumor antigen–reactive CD4 T cells among the expanded libraries, as well as the concomitant screening for CD4 and CD8 T-cell responses, as recently evaluated by others (18).

Finally, given that the isolated T-cell lines from individual wells generally correspond to monoclonal populations of TAA-specific cells, this method will also accelerate TCR-based applications.

In conclusion, we show that the amplified library method can be applied for isolation, identification, and characterization of rare human tumor-specific T cells allowing parallel functional analysis, fine-specificity screening, and potential TCR engineering for a therapeutic usage.

Disclosure of Potential Conflicts of Interest

P.M. Sousa Alves holds ownership interest (including patents) in GlaxoSmithKline. G. Coukos reports receiving commercial research grants from Celgene, Boehringer Ingelheim, Bristol-Myers Squibb, Roche, Iovance, and Kite; reports receiving speakers bureau honoraria from Roche and Genentech; holds ownership interest (including patents) in University of Pennsylvania; and is a consultant/advisory board member for Genentech, Roche, AstraZeneca, Bristol-Myers Squibb, Sanofi-Aventis, Nextcure, and Geneos Tx. P. Romero reports receiving commercial research grants from Roche pRED-Zurich; is a consultant/advisory board member for Immatics Biotechnologies; and reports receiving other remuneration from the Journal for Immunotherapy of Cancer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: C. Jandus, G. Coukos, P. Romero
Development of methodology: C. Jandus, F. Sandoval, A. Cachot, P. Romero
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Jandus, C. Costa-Nunes, O. Adotévi, A. Harari, A. Cachot, W. Reith
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Jandus, C. Costa-Nunes, P.M. Sousa Alves, A. Harari, A. Cachot, R. Genolet, M.P. Protti, P. Romero
Writing, review, and/or revision of the manuscript: C. Jandus, C. Costa-Nunes, D.E. Speiser, F. Sandoval, O. Adotévi, A. Cachot, W. Reith, M.P. Protti, P. Romero
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Costa-Nunes, S. Bobisse, P. Baumgaertner, D.E. Speiser, P.M. Sousa Alves, F. Sandoval, G. Coukos, A. Cachot, M. Arnaud, M.P. Protti
Study supervision: C. Jandus, G. Coukos, P. Romero

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References

1. Romero P, Banchereau J, Bhardwaj N, Cockett M, Disis ML, Dranoff G, et al. The Human Vaccines Project: A roadmap for cancer vaccine development. Sci Transl Med 2016;8:334ps9.
2. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell 2015;27: 450–61.
3. Gill S, June CH. Going viral: chimeric antigen receptor T-cell therapy for hematological malignancies. Immunol Rev 2015;263:68–89.
4. Rehman H, Silk AW, Kane MP, Kaufman HL. Into the clinic: Talimogene laherparepvec (T-VEC), a first-in-class intratumoral oncolytic viral therapy. J Immunother Cancer 2016;4:53.
5. Spitzer MH, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhireddy D, Martins MM, et al. Systemic immunity is required for effective cancer immunotherapy. Cell 2017;168:487–502.
6. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 2012;12: 298–306.
7. Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R, et al. Treatment of Metastatic Melanoma with Autologous CD4+ T Cells against NY-ESO-1. N Engl J Med 2008;358:2698–703.
8. Linnemann C, van Buuren MM, Bies L, Verdegaal EME, Schotte R, Calis JJA, et al. High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. Nat Med 2014;21: 81–5.
9. Tran E, Turcotte S, Gros A, Robbins PF, Lu Y-C, Dudley ME, et al. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. Science 2014;344:641–5.
10. Kreiter S, Vormehr M, van de Roemer N, Diken M, Löwer M, Diekmann J, et al. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. Nature 2015;520:692–6.
11. Ott PA, Hu Z, Keskin DB, Shukla SA, Sun J, Bozym DJ, et al. An immunogenic personal neoantigen vaccine for patients with melanoma. Nature 2017;547:217–21.
12. Kwok WW, Tan V, Gillette L, Littell CT, Soltis MA, LaFond RB, et al. Frequency of epitope-specific naive CD4(+) T cells correlates with immunodominance in the human memory repertoire. J Immunol 2012;188: 2537–44.
13. Moon JJ, Chu HH, Hataye J, Pagán AJ, Pepper M, McLachlan JB, et al. Tracking epitope-specific T cells. Nat Protoc 2009;4:565–81.
14. Davis MM, Altman JD, Newell EW. Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis. Nat Rev Immunol 2011;11: 551–8.
15. Di Lullo G, Ieva F, Longhi R, Paganoni AM, Protti MP. Estimating point and interval frequency of antigen-specific CD4+ T cells based on short in vitro expansion and improved poisson distribution analysis. PLoS One 2012;7: e42340.
16. Baumgaertner P, Costa Nunes C, Cachot A, Maby-El Hajjami H, Cagnon L, Braun M, et al. Vaccination of stage III/IV melanoma patients with long NY-ESO-1 peptide and CpG-B elicits robust CD8+ and CD4+ T-cell responses with multiple specificities including a novel DR7-restricted epitope. Oncoimmunology 2016;5:e1216290.
17. Geiger R, Duhen T, Lanzavecchia A, Sallusto F. Human naive and memory CD4+ T cell repertoires specific for naturally processed antigens analyzed using libraries of amplified T cells. J Exp Med 2009;206:1525–34.
18. Theaker SM, Rius C, Greenshields-Watson A, Lloyd A, Trimby A, Fuller A, et al. T-cell libraries allow simple parallel generation of multiple peptide-specific human T-cell clones. J Immunol Methods 2016;430:43–50.
19. Uchtenhagen H, Rims C, Blahnik G, Chow I-T, Kwok WW, Buckner JH, et al. Efficient *ex vivo* analysis of CD4+ T-cell responses using combinatorial HLA class II tetramer staining. Nat Commun 2016;7:12614.
20. Lindestam Arlehamn CS, Gerasimova A, Mele F, Henderson R, Swann J, Greenbaum JA, et al. Memory T cells in latent Mycobacterium tuberculosis infection are directed against three antigenic islands and largely contained in a CXCR3+CCR6+ Th1 subset. PLoS Pathog 2013;9:e1003130.
21. Ayyoub M, Dojcinovic D, Pignon P, Raimbaud I, Schmidt J, Luescher I, et al. Monitoring of NY-ESO-1 specific CD4+ T cells using molecularly defined MHC class II/His-tag-peptide tetramers. Proc Natl Acad Sci U S A 2010;107: 7437–42.
22. Redjimi N, Duperrier-Amouriaux K, Raimbaud I, Luescher I, Dojcinovic D, Classe J-M, et al. NY-ESO-1-specific circulating CD4+ T cells in ovarian cancer patients are prevalently T(H)1 type cells undetectable in the CD25+ FOXP3+ Treg compartment. PLoS One 2011;6:e22845.
23. François V, Ottaviani S, Renkvist N, Stockis J, Schuler G, Thielemans K, et al. The CD4(+) T-cell response of melanoma patients to a MAGE-A3 peptide vaccine involves potential regulatory T cells. Cancer Res 2009;69:4335–45.
24. Zhang Y, Sun Z, Nicolay H, Meyer RG, Renkvist N, Stroobant V, et al. Monitoring of anti-vaccine CD4 T cell frequencies in melanoma patients vaccinated with a MAGE-3 protein. J Immunol 2005;174:2404–11.
25. Inderberg-Suso E-M, Trachsel S, Lislerud K, Rasmussen A-M, Gaudernack G. Widespread CD4+ T-cell reactivity to novel hTERT epitopes following vaccination of cancer patients with a single hTERT peptide GV1001. Oncoimmunology 2012;1:670–86.
26. Kitano S, Tsuji T, Liu C, Hirschhorn-Cymerman D, Kyi C, Mu Z, et al. Enhancement of tumor-reactive cytotoxic CD4+ T cell responses after ipilimumab treatment in four advanced melanoma patients. Cancer Immunol Res 2013;1:235–44.
27. Jandus C, Bioley G, Dojcinovic D, Derré L, Baitsch L, Wieckowski S, et al. Tumor antigen-specific FOXP3+ CD4 T cells identified in human metastatic melanoma: peptide vaccination results in selective expansion of Th1-like counterparts. Cancer Res 2009;69:8085–93.
28. Campion SL, Brodie TM, Fischer W, Korber BT, Rossetti A, Goonetilleke N, et al. Proteome-wide analysis of HIV-specific naive and memory CD4(+) T cells in unexposed blood donors. J Exp Med 2014; 211:1273–80.
29. Godet Y, Fabre E, Dosset M, Lamuraglia M, Levionnois E, Ravel P, et al. Analysis of spontaneous tumor-specific CD4 T-cell immunity in lung cancer using promiscuous HLA-DR telomerase-derived epitopes: potential synergistic effect with chemotherapy response. Clin Cancer Res 2012;18: 2943–53.
30. Roederer M, Koup RA. Optimized determination of T cell epitope responses. J Immunol Methods 2003;274:221–8.
31. Bioley G, Jandus C, Tuyaerts S, Rimoldi D, Kwok WW, Speiser DE, et al. Melan-A/MART-1-specific CD4 T cells in melanoma patients: identification of new epitopes and ex vivo visualization of specific T cells by MHC class II tetramers. J Immunol 2006;177:6769–79.
32. Tran E, Ahmadzadeh M, Lu Y-C, Gros A, Turcotte S, Robbins PF, et al. Immunogenicity of somatic mutations in human gastrointestinal cancers. Science 2015;350:1387–90.
33. Legoux FP, Lim J-B, Cauley AW, Dikiy S, Ertelt J, Mariani TJ, et al. CD4+ T cell tolerance to tissue-restricted self antigens is mediated by antigen-specific regulatory T cells rather than deletion. Immunity 2015;43: 896–908.
34. Pinto S, Sommermeyer D, Michel C, Wilde S, Schendel D, Uckert W, et al. Misinitiation of intrathymic MART-1 transcription and biased TCR usage explain the high frequency of MART-1-specific T cells. Eur J Immunol 2014; 44:2811–21.
35. Vonderheide RH, Schultze JL, Anderson KS, Maecker B, Butler MO, Xia Z, et al. Equivalent induction of telomerase-specific cytotoxic T lymphocytes from tumor-bearing patients and healthy individuals. Cancer Res 2001;61: 8366–70.

36. Gotter J, Brors B, Hergenhahn M, Kyewski B. Medullary epithelial cells of the human thymus express a highly diverse selection of tissue-specific genes colocalized in chromosomal clusters. J Exp Med 2004;199:155–66.
37. Jungbluth AA, Chen YT, Stockert E, Busam KJ, Kolb D, Iversen K, et al. Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. Int J Cancer 2001;92:856–60.
38. 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.
39. Judkowski V, Bunying A, Ge F, Appel JR, Law K, Sharma A, et al. GM-CSF production allows the identification of immunoprevalent antigens recognized by human CD4+ T cells following smallpox vaccination. PLoS One 2011;6:e24091.
40. Nishikawa H, Tsuji T, Jäger E, Briones G, Ritter G, Old LJ, et al. Induction of regulatory T cell-resistant helper CD4+ T cells by bacterial vector. Blood 2008;111:1404–12.
41. Su LF, Kidd BA, Han A, Kotzin JJ, Davis MM. Virus-specific CD4(+) memory-phenotype T cells are abundant in unexposed adults. Immunity 2013;38:373–83.
42. Karyampudi L, Krco CJ, Kalli KR, Erskine CL, Hartmann LC, Goodman K, et al. Identification of a broad coverage HLA-DR degenerate epitope pool derived from carcinoembryonic antigen. Cancer Immunol Immunother 2010;59:161–71.
43. McNeel DG, Becker JT, Eickhoff JC, Johnson LE, Bradley E, Pohlkamp I, et al. Real-time immune monitoring to guide plasmid DNA vaccination schedule targeting prostatic acid phosphatase in patients with castration-resistant prostate cancer. Clin Cancer Res 2014;20:3692–704.
44. Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW. Autoreactive T cells in healthy individuals. J Immunol 2004;172:5967–72.
45. Janosky M, Sabado RL, Cruz C, Vengco I, Hasan F, Winer A, et al. MAGE-specific T cells detected directly *ex-vivo* correlate with complete remission in metastatic breast cancer patients after sequential immune-endocrine therapy. J Immunother Cancer 2014;2:32.
46. Hadrup S, Donia M, Thor Straten P. Effector CD4 and CD8 T cells and their role in the tumor microenvironment. Cancer Microenviron 2013;6:123–33.
47. Dobrzanski MJ. Expanding roles for CD4 T cells and their subpopulations in tumor immunity and therapy. Front Oncol 2013;3:63.