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Characterization of Melan-A reactive memory CD8+ T cells in a healthy donor

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Abstract

Melan-A specific CD8+ T cells are thought to play an important role against the development of melanoma. Their *in vivo* expansion is often observed with advanced disease. In recent years, low levels of Melan-A reactive CD8+ T cells have also been found in HLA-A2 healthy donors, but these cells harbor naive characteristics and are thought to be mostly cross-reactive for the Melan-A antigen. Here, we report on a large population of CD8+ T cells reactive for the Melan-A antigen, identified in one donor with no evidence of melanoma. Interestingly, this population is oligoclonal and displays a clear memory phenotype. However, a detailed study of these cells indicated that they are unlikely to be directly specific for melanoma, so that their *in vivo* expansion may have been driven by an exogenous antigen. Screening of a Melan-A cross-reactive peptide library suggested that these cells may be specific for an epitope derived from a *Mycobacterium* protein, which would provide a further example of CD8+ T cell cross-reactivity between a pathogen antigen and a tumor antigen. Finally, we discuss potential perspectives regarding the role of such cells in heterologous immunity, by influencing the balance between protective immunity and pathology, e.g. in the case of melanoma development.

Introduction

The discovery of genes encoding tumor antigens and the subsequent characterization of tumor-associated T-cell epitopes have drawn attention on the importance of human T cells in anti-tumor immunity. One human tumor antigen in particular has proven to be extremely informative, the melanocyte/melanoma differentiation antigen known as Melan-A (for melanoma antigen A) or melanoma antigen recognized by T cells 1 (MART-1), which was among the first human tumor antigens to be cloned (1). The Melan-A/MART-1 gene encodes a 118 amino acid transmembrane protein, which contains an immunodominant HLA-A2 restricted CTL epitope, mapped to residues 26–35 (EAAGIGILTV). Remarkably, the screening of tumor infiltrating lymphocyte populations revealed that a vast majority contained T cells reactive for the Melan-A peptide (2). The inclusion of a high affinity Melan-A peptide (i.e. with a Ala27Leu substitution) into fluorescently labeled HLA-A2–peptide tetrameric complexes enabled the identification and tracking of Melan-A-specific T cells directly in peripheral blood (3). It became soon apparent that a high proportion of HLA-A2-positive melanoma patients display circulating tetramer positive cells with features of antigen-experienced cells (4, 5).

The considerable analytical power of HLA-A2–ELA peptide complexes led to the surprising finding of measurable numbers (0.07–0.08% of CD8+ T cells) of tetramer+ lymphocytes in over 70% of HLA-A2 healthy individuals (4). Even more surprising was the finding that these cells belonged to the naive T-cell population, indicating that thymic selection generates a high number of precursors recognizing the self-HLA-A2/Melan-A antigen in humans (6). The ligands

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involved in the selection of this T-cell repertoire have not yet been identified. Although accumulating evidence indicates a central role for self-peptides in thymic selection, no Melan-A mRNA expression has been reported in the thymus. It is possible that this Melan-A-reactive T-cell pool may be generated through recognition of unknown Melan-A cross-reactive peptides expressed in the thymus. It is likely that the fine specificity of healthy individuals HLA-A2/Melan-A tetramer+ naive cells is not the Melan-A antigen and that these cells may be better defined as Melan-A cross-reactive (7).

We present here the unanticipated identification of a population of Melan-A reactive memory CD8+ T cells in a HLA-A2 healthy individual. Functional and phenotypic analysis and screening with Melan-A cross-reactive peptides derived from combinatorial peptide library were employed in order to investigate the nature of this population, which appeared to be highly cross-reactive, with reactivity for both pathogen and tumor antigens.

Methods

PBMC samples

Samples were obtained from HLA-A2+ healthy volunteers or Melan-A-vaccinated patients, as well as *Mycobacterium tuberculosis* (MTB) infected Gambian donors. The relevant local Institutional (in Lausanne, Switzerland or in Banjuls, The Gambia) Review Boards and Ethics Committees approved this study. Blood samples were generally used fresh within 4 h (for optimal functional assessment of the lymphocytes) or PBMCs were separated from heparinized blood and cryopreserved for subsequent studies.

Reagents and flow cytometry

HLA–peptide tetrameric complexes (tetramers) were produced as previously described (8) and included the following peptides: Melan-A ELAGIGILTV, flu matrix GILGFVFTL and *Mycobacterium* VVAGIGILAI. Anti-TCR-BV antibodies were purchased from Beckman Coulter (San Diego, CA, USA) and all other antibodies from Becton Dickinson Pharmingen (San Diego, CA, USA). Stainings were usually performed directly on whole blood or on thawed PBMC as previously described (9). Samples were analyzed on a Becton Dickinson FACSCalibur or CANTO, after compensation was checked using freshly stained PBMC. For intracellular IFNγ staining and CD107a mobilization assay, thawed cryopreserved PBMCs were stained with tetrameric complexes (15 min at 37°C) prior to activation. Cells were subsequently incubated with specific antigens at 10 μM final concentration and with anti-CD107a antibodies (7 μl) in RPMI 1640/10% FCS and left for 6 h at 37°C. Brefeldin A (Sigma, at 10 μg ml^{-1} final concentration) and Monensin (Sigma, at 2 μM final concentration) were added during the second hour of incubation. Non-activated PBMCs were stained with tetrameric complexes for 15 min at 37°C at the end of the incubation. Cells were washed in PBS, 0.5 mM EDTA, 1% BSA, fixed and permeabilized in FACS™ Permeabilization buffer (Becton Dickinson) for 10 min. After washing, staining was performed for 15 min at room temperature in the dark using a panel of PerCP- or APC-conjugated antibodies. Cells were then washed and stored in 5% formaldehyde at 4°C until analysis.

Molecular analysis of tetramer positive cells

TCR analysis was performed on tetramer positive cells sorted by flow cytometry as previously described (10). Briefly, CD8+ T cells were enriched with a MiniMACS device and stained with Melan-A tetramers. Five cell aliquots were sorted directly into wells of different 96-V-bottom plates using a FACSVantage SE (Becton Dickinson), followed by cDNA preparation, amplification and TCR BV22 spectratyping. PCRs were run using fluorescent probe labeled primers specific for the BV22 subfamily and for the BC chain. Fluorescent run-off products and fluorescent DNA weight markers were loaded on sequence gel in an automated sequencer (ABI Prism, Applied Biosystems). Sequencing of the PCR product including the CDR3 region was then performed using the same primers.

Peptide collection and Elispot assays

Decapeptides predicted to be stimulatory to Melan-A-specific CD8+ T cells are ranked using a scoring matrix generated from data previously obtained by screening an amidated C terminus decapeptide positional scanning synthetic combinatorial peptide library screening (PS-SCL) with one high avidity Melan-A-specific CTL clone (expanded *in vitro* from melanoma patient PBMC) in a functional chromium release assay (11). Briefly, a Z-scoring matrix was generated using the average and standard deviation of the percentage of specific lysis values of multiple experimental data obtained for each mixture defined with one of 18 natural L-amino acids in each of the 10 positions of the decamer library (12). Based on the assumption of independent and additive contribution of the individual amino acids at each position of a peptide to the peptide's activity, the score of each individual peptide was calculated by adding individual stimulatory values of the composing amino acids. A program was designed to use the matrix to score all overlapping decapeptides contained in the GenPept protein database (ftp://ftp.ncicfr.gov/pub/genpept) and thus identify sequences with the highest predicted stimulatory scores (12). Individual peptides were synthesized at Mixture Science Inc. (San Diego, CA, USA) by the simultaneous multiple peptide synthesis method. Purity and identity of each peptide were characterized using an electrospray mass spectrometer interfaced with a liquid chromatography system. Single or pooled synthetic peptides were used at a concentration of 10 μM in IFNγ enzyme-linked immunospot (Elispot) assays on PBMC (1.5 10^5 cells per well) to define CD8+ T-cell responses directly *ex vivo*, as previously described. IFNγ Elispot kits were purchased from Diaclone Biotest. PHA was always included as a positive control. Spots were counted with an automatic reader (Bioreader 2000; BioSys GmbH). Assays were performed in duplicates that showed good similarity.

Generation of Melan-A specific T-cell clones and cytotoxic assays

Tetramer+/BV22+ CD8+ T cells were sorted using a FACSVantage, cloned by limiting dilution and expanded with PHA

and allogenic irradiated feeder cells in medium containing 150 U ml^{-1} human recombinant IL-15 (hrIL-15). Subsequently, they were periodically (every 3–4 weeks) re-stimulated with PHA, irradiated feeder cells and hrIL-15. Clones were tested by tetramer and BV22 staining and then for CDR3 region length and for TCR sequence. Chromium release experiments were performed as previously described. Target cells (1000 cells per well) were T2 cells (A2+/Melan-A−) for peptide titration assays and the melanoma cell lines Me 275 (A2+/Melan-A+), Me 260 (A2−/Melan-A+) and NA8 (A2+/Melan-A−) for tumor cell recognition assays.

Structural predictions of the VVA-HLA-A2 epitope

An *ab initio* method has been designed to theoretically predict the structure of peptides bound to the MHC class I molecule from the amino acid sequences alone (13). This method is composed of two steps. First, the peptide conformational space in the MHC environment is sampled. Second, the conformers are evaluated by using a combination of clustering and energy/entropy calculations. The conformational sampling was performed using a simulated annealing protocol in which 1000 heating–cooling cycles were completed. The CHARMM molecular modeling program and the allatom CHARMM 22 protein parameter set were used to perform the simulated annealing protocol. At the end of each cycle, a conformation of the peptide in the MHC molecule was kept after energy minimization. The simulations were performed in vacuum, using a distance-dependent dielectric constant that accounts in part for the solvent screening of the electrostatic interactions. For long-range non-bonded interactions, an atom-based force switching was applied from 14 to 15 Å. During the entire simulation, the MHC molecule was kept fixed, but no constraints were applied to the peptide. For the evaluation of the conformers, clustering according to geometric similarities was performed. Using additional energy and entropy calculations, involving solvation-free energies computed with the Poisson–Boltzmann continuum models, the mean effective energy and the conformational free energy of clusters were computed. The final conformation, i.e. the prediction, was chosen as the center of the lowest conformational free energy cluster.

Results

High frequency of Melan-A tetramer+ CD8+ T cells in one healthy donor

During the screening of a large number of HLA-A2 healthy donors to study and expand Melan-A tetramer positive naive CD8+ T cells (14), we came across a single donor (BC26) who displayed a remarkable Melan-A tetramer-positive CD8+ T-cell population, representing 1.35% of the whole CD8+ T cells. The frequency of this population was 20 times superior to the mean frequency (0.078% of CD8 T cells, $n = 76$) measured in the majority of healthy donors (Fig. 1A). In addition, tetramer staining of these cells yielded a well-defined population (in contrast with smeary stains from common healthy donor populations), resembling memory cell populations, like Flu-specific CD8+ T cells, as seen in the same donor (Fig. 1B).

BC26 Melan-A tetramer+ CD8+ T cells display a memory phenotype

Phenotypic analysis revealed that the vast majority of these cells were antigen experienced (CCR7−/CD45RA−), unlike populations found in HLA-A2 healthy donors, but alike Flu-specific CD8+ T cells found in the same donor (Fig. 1C). To confirm that BC26 Melan-A tetramer-positive cells were indeed antigen-experienced cells, TCR repertoire analysis of this population was performed. Using a panel of anti-TCR antibodies, we observed that this population was highly oligoclonal with the great majority of the cells displaying BV22 and AV2.2 expression (Fig. 1D). This is similar to Melan-A-specific CD8+ T cells that usually express AV2.1 and are stained with the anti-AV2.2 specific mAb [PR, unpublished observations and (15)]. Interestingly, BV22 spectratyping analysis performed on Melan-A tetramer-sorted BC26 cells and subsequent sequencing of the CDR3 region revealed that this BV22+ population represented a single clonotype (Fig. 1E and F) and therefore that the majority of BC26 Melan-A tetramer-positive CD8+ T-cell population was one expanded clone. CD27/CD28 expression showed that these cells were still at an early stage of differentiation (i.e. mostly CD27+/CD28+) (16) (Fig. 1C). They appeared to be armed cytotoxic T cells, expressing low levels of perforin as well as granzymes A and B, again in clear contrast with Melan-A tetramer-positive CD8+ T cells from common healthy donors (Fig. 1C).

BC26 Melan-A tetramer+ CD8+ T cells are poorly responsive to Melan-A

The occurrence of Melan-A tetramer-positive CD8+ T cells with such characteristics is usually observed in tumor infiltrated lymph nodes and associated with advanced melanoma disease. The presence of an unidentified melanoma tumor in this donor was considered but excluded after thorough clinical evaluation. Moreover, the donor presented no sign of vitiligo or of cancer development over a follow-up period of 4 years. Although unreported to date, one possibility for the presence of this Melan-A-reactive CD8+ T-cell population in the absence of clinical melanoma evidence may actually be related to a particular ability of these cells to control effectively melanoma growth following initial cancer development and T-cell priming. This would imply in part that this CD8+ T-cell population displays robust functional capacity in order to halt tumor growth. Functional responsiveness of these cells was assessed through IFNγ secretion and CD107a mobilization (as a marker of lytic degranulation), following short-term stimulation with the Melan-A peptide. However, to our disappointment, BC26 Melan-A tetramer-positive cells showed a sub-optimal responsiveness compared with Flu-specific CD8+ T cells from the same donor as well as to Melan-A-specific CD8+ T cells from a Melan-A-vaccinated melanoma patient, for same peptide concentrations (Fig. 2). This observation was repeated at distinct times. This result, together with the prolonged lack of melanoma evidence, eventually raised doubts as regards the fine specificity of this population. We started to consider a second possibility: that these cells may actually be specific for another antigen than Melan-A (consistent with their presence in the absence of melanoma).

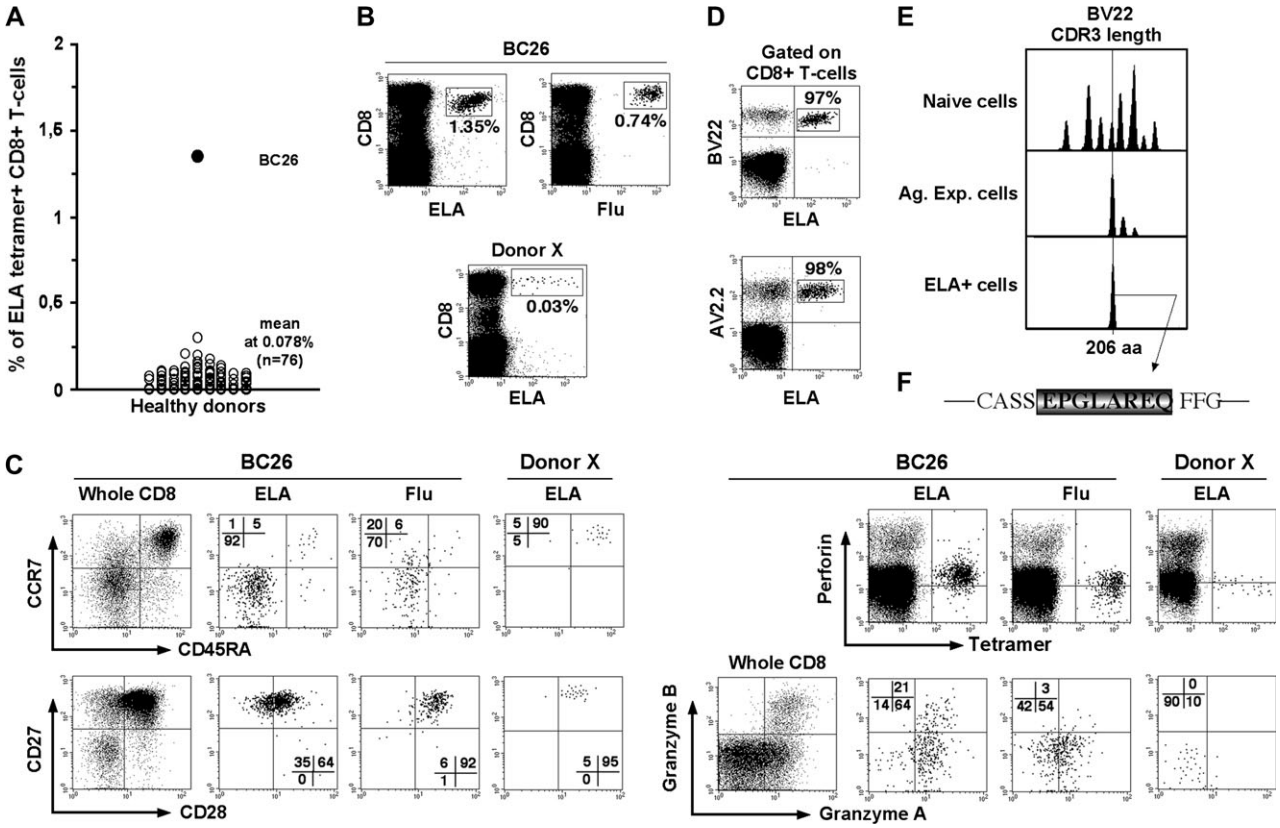

Fig. 1. Identification of a Melan-A tetramer+ memory CD8+ T-cell population in one healthy donor. (A) Proportion of Melan-A tetramer+ T cells within the CD8+ population of BC26 compared with other healthy donors. (B) Representative stainings of Melan-A tetramer+ CD8+ T cells for BC26 and one healthy donor. Percentages of tetramer+ cells within the CD8+ T cells are shown. (C) Expression of differentiation markers (CD45RA/CCR7/CD28/CD27) or cytotoxic factors (perforin, garnzymes A and B) on BC26 Melan-A tetramer+ CD8+ T cells compared with BC26 Flu-specific CD8+ T cells and Melan-A tetramer+ CD8+ T cells from one representative healthy donor. Percentages of cells present in quadrants are shown. This phenotype was consistent in three different blood samples obtained over 3 years. (D) Expression of BV22 and AV2.2 by BC26 Melan-A tetramer+ CD8+ T cells. (E) BV22 spectratyping analysis performed on naive, whole antigen experienced and Melan-A tetramer+ CD8+ T cells FACS sorted from BC26 PBMC. (F) CDR3 sequence of the BV22+ Melan-A tetramer+ CD8+ T-cell clonal population.

Preferential recognition of a Mycobacterium peptide by BC26 CD8+ T cells

To identify the antigen of interest, we decided to use a collection of Melan-A cross-reactive peptides derived from a PS-SCL. The PS-SCL approach has previously led to the successful study of T-cell specificity and the identification of biologically active peptides (17–21). A collection of 100 peptides, retrieved from self or pathogen protein databases by means of biometric score matrix analysis of a PS-SCL (composed of trillions of decapeptides covering all possibilities of amino acids at each position of nonapeptides or decapeptides, tested in pools for their ability to stimulate a cytolytic response from a high avidity Melan-A-specific CTL clone in a functional assay), with ranked reactivity for Melan-A-specific TCR (11, 22, 23), was used to screen BC26 PBMC by IFNγ Elispot directly *ex vivo*. Four out of the 100 selected peptides elicited a response, but only one peptide (i.e. 91) induced a response that was significantly higher than the optimized Melan-A peptide ELA (Fig. 3A). BC26 Melan-A tetramer-positive cells presented a stronger recognition efficacy for this peptide than for ELA as assessed in peptide titration assays (Fig. 3B), as well as a good functional responsiveness following stimulation with this peptide (Fig. 3C) (in contrast to the ELA response, Fig. 2A). In addition, we were able to generate peptide–HLA-A2 tetrameric complexes with peptide 91. These tetramers yielded a robust staining of BC26 PBMC, brighter than with classic Melan-A tetramers, resembling more, in terms of MFI, Melan-A-specific CD8+ T cells from a Melan-A peptide-vaccinated patient (Fig. 3D). In the same line, while double Melan-A tetramer staining (PE/APC) led to co-staining of the BC26 population, Melan-A tetramer staining was lost when performed concurrently with tetramer 91 staining (Fig. 3E). Overall, this suggests that the avidity of BC26 Melan-A tetramer-positive population TCR is much higher for peptide 91 than for ELA, making this peptide a good candidate for the specificity of BC26 population.

BC26 responsiveness for purified protein derivative

Peptide 91 (VVA) is derived from a probable export or membrane protein of 165 amino acids from Mycobacteria according to genomic databases (ORF Rv1382). It is found at least in MTB and *Mycobacterium bovis*, but may also be present in other Mycobacteria (that have not been sequenced yet). Ensuing these results, a chest X-ray scan and a Mantoux tuberculin skin test were performed on BC26, but they did not

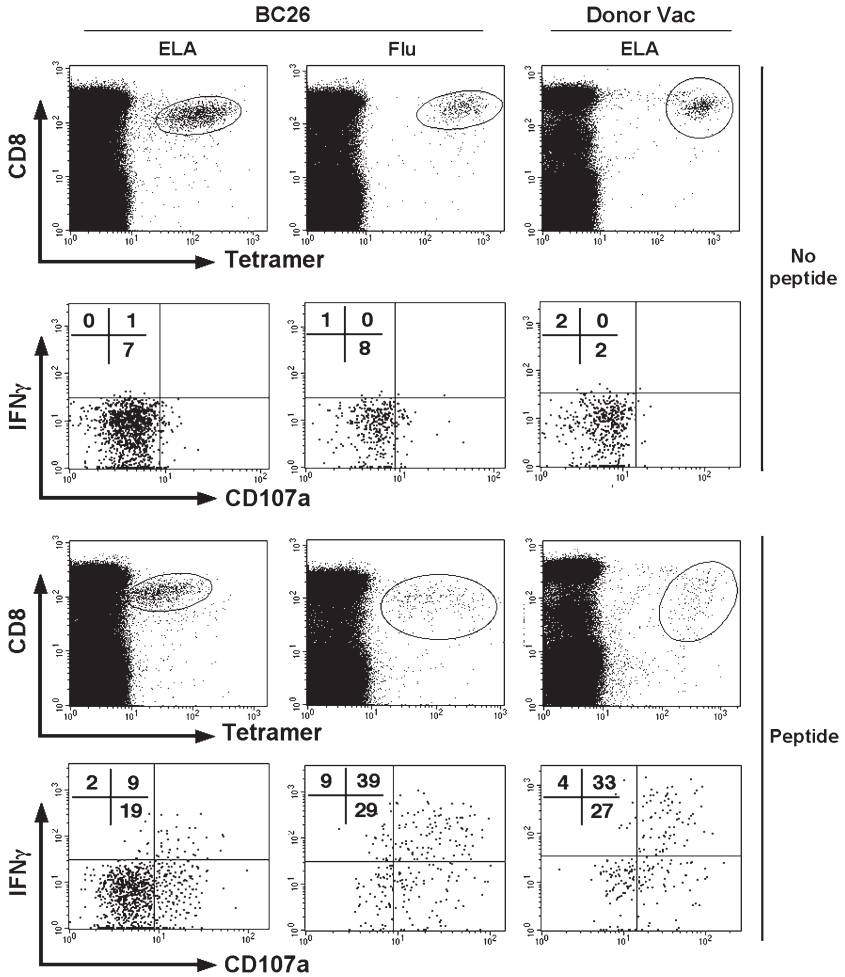

Fig. 2. Low recognition efficacy of BC26 Melan-A tetramer+ CD8+ T cells for the Melan-A peptide. Functional responsiveness (IFNγ secretion and degranulation) of Melan-A tetramer+ or Flu-specific CD8+ T cells from BC26 and Melan-A-specific CD8+ T cells from one Melan-A peptide-vaccinated melanoma patient upon stimulation with cognate peptides (at 5 μM). IFNγ production and CD107a up-regulation are shown on tetramer-gated cells. Percentages of cells present in quadrants are shown.

permit to conclude that this donor was infected with MTB. Interestingly, stimulation of BC26 PBMC with MTB protein extracts [purified protein derivative (PPD)] yielded a clear positive response (Fig. 4). Similar results were observed both using intracellular cytokine and Elispot assays at two distinct time points. However, since reactivity to PPD can be observed not only in case of MTB infection but also in case of infections with other Mycobacteria, BC26 PBMC reactivity to MTB-specific proteins CFP-10 and ESAP-6 was tested (24). Stimulation with these antigens did not lead to a clear response (compared with responses obtained with a known active MTB control), preventing us to conclude on MTB infection with certainty. Overall, BC26 reactivity for PPD indicates that BC26 has been infected with a pathogen belonging to the *Mycobacterium* genus (i.e. MTB or environmental Mycobacteria).

Cross-reactivity between Melan-A and Mycobacterium peptides

To assess the antigen recognition efficacy of BC26 mostly clonal population in more details, we generated and expanded a clone *in vitro* expressing the TCR BV22-CDR3-EPGLAREQ clonotypic sequence. Chromium release assays were performed using this clone in comparison with a clone derived from a Melan-A peptide-vaccinated patient (25, 26), presenting a high avidity for the ELA peptide. Peptide titration assays confirmed the high recognition efficacy for peptide 91 (as high as the recognition efficacy of the vaccinated patient's clone for ELA, with IC_{50} values in the range of 10^{-11} M) (Fig. 5A). Remarkably, its recognition efficacy for the natural Melan-A antigen EAA was still relatively good (and similar to the one of the vaccinated patient's clone, range of 10^{-9} M) (Fig. 5A). Most importantly, this clone was able to recognize a melanoma tumor cell line that expressed naturally the Melan-A antigen, in the same range as the vaccinated patient's clone (Fig. 5B), indicating that the cross-reactivity of BC26 population for the Melan-A antigen is significant. The homology of sequence between the Melan-A antigen and peptide 91 is mostly located in the core of the peptide (Fig. 5C). Modeling of the Mycobacteria peptide and both the optimized and the natural Melan-A peptides within the groove of the HLA-A2 molecule revealed

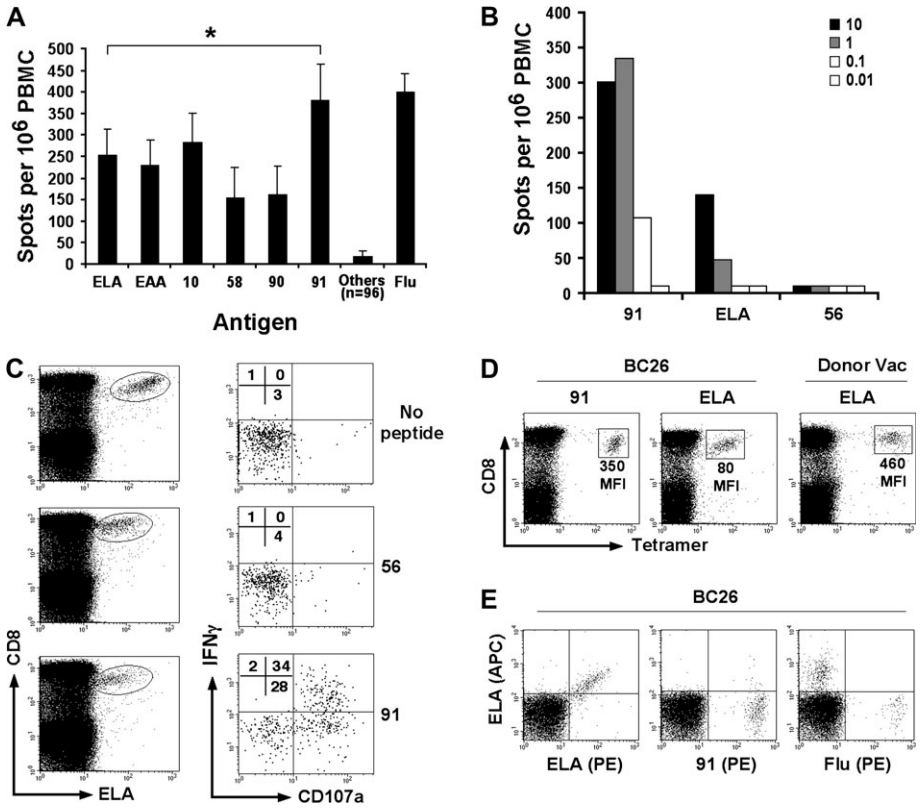

Fig. 3. Identification of BC26 Melan-A tetramer+ CD8+ T-cell fine specificity. (A) Screening of peptides derived from a Melan-A-based combinatorial peptide library analysis using BC26 PBMC. IFNγ Elispot assays were performed to examine the reactivity of BC26 PBMC to the ELA and EAA peptides as well as to the PS-SCL-derived peptide collection. Results are expressed in spot-forming units (background subtracted, ±standard deviation). *$P = 0.013$ with the non-parametric Mann–Whitney test. (B) Peptide titrations in IFNγ Elispot assays (spot forming units/10^6 PBMC) to assess recognition efficacy of BC26 PBMC for peptide 91 (VVA) versus ELA (peptide 56 was used as a negative control). (C) Functional responsiveness (IFNγ secretion and degranulation) of BC26 CD8+ T cells upon stimulation with VVA (no stimulation or stimulation with peptide 56 was used as controls). IFNγ production and CD107a up-regulation are shown on tetramer-gated cells. Percentages of cells present in quadrants are shown. (D) Staining of BC26 PBMC with VVA tetramers compared with ELA tetramer stainings on BC26 or one Melan-A peptide-vaccinated melanoma patient PBMC. (E) Co-staining of BC26 cells with ELA/VVA, ELA/ELA and ELA/Flu tetramers.

a remarkable homology of structure between these epitopes, which explains this degree of cross-reactivity.

Discussion

The study of a large number of HLA-A2 healthy individuals led to the identification of a remarkable population of Melan-A reactive CD8+ T cells in one healthy donor (BC26). In order to comprehend the nature of this population, we performed a thorough investigation, which included the analysis of its phenotype, clonality, functional responsiveness and the use of Melan-A cross-reactive peptides to assess antigenic reactivity. The unexpected memory phenotype displayed by the population could suggest that the expansion of this population may have been driven by a tumor, and let us speculate that these cells may be able to drive control of tumor development. However, this population eventually presented a poor functional responsiveness to the Melan-A antigen, making unlikely that it had a particular efficacy against the tumor, but rather indicating that it may be cross-reactive for Melan-A.

In order to investigate further the potential specificity of this population, we used a selection of Melan-A cross-reactive peptides derived from a combinatorial peptide library: screening of these peptides indicated that BC26 Melan-A tetramer+ cells were highly reactive for a peptide derived from a putative export or membrane protein from a *Mycobacterium*, which presented a strong structural homology with the Melan-A peptide. Although, it is tempting to think that this approach led to the identification of BC26 Melan-A tetramer+ cell cognate antigen, one cannot rule out the possibility that the identified peptide may simply be another cross-reactive mimotope. For instance, this would also be the case for three other peptides from our library which could elicit responses (although these were of lower magnitude). We therefore look for evidence of *Mycobacterium* infection in the donor to support the possibility that this Melan-A tetramer-positive population may have been generated as a result of such an infection. Since the clinical diagnosis of latent *Mycobacterium* infection is difficult, the detection of an immunological memory (i.e. cytokine producing PBMC upon stimulation with *Mycobacterium*-related antigens) is considered as the most sensitive test available (27, 28). BC26 PBMC reactivity to PPD indicates that this donor must have been infected with a *Mycobacterium* at some point in her life. PPD-positive

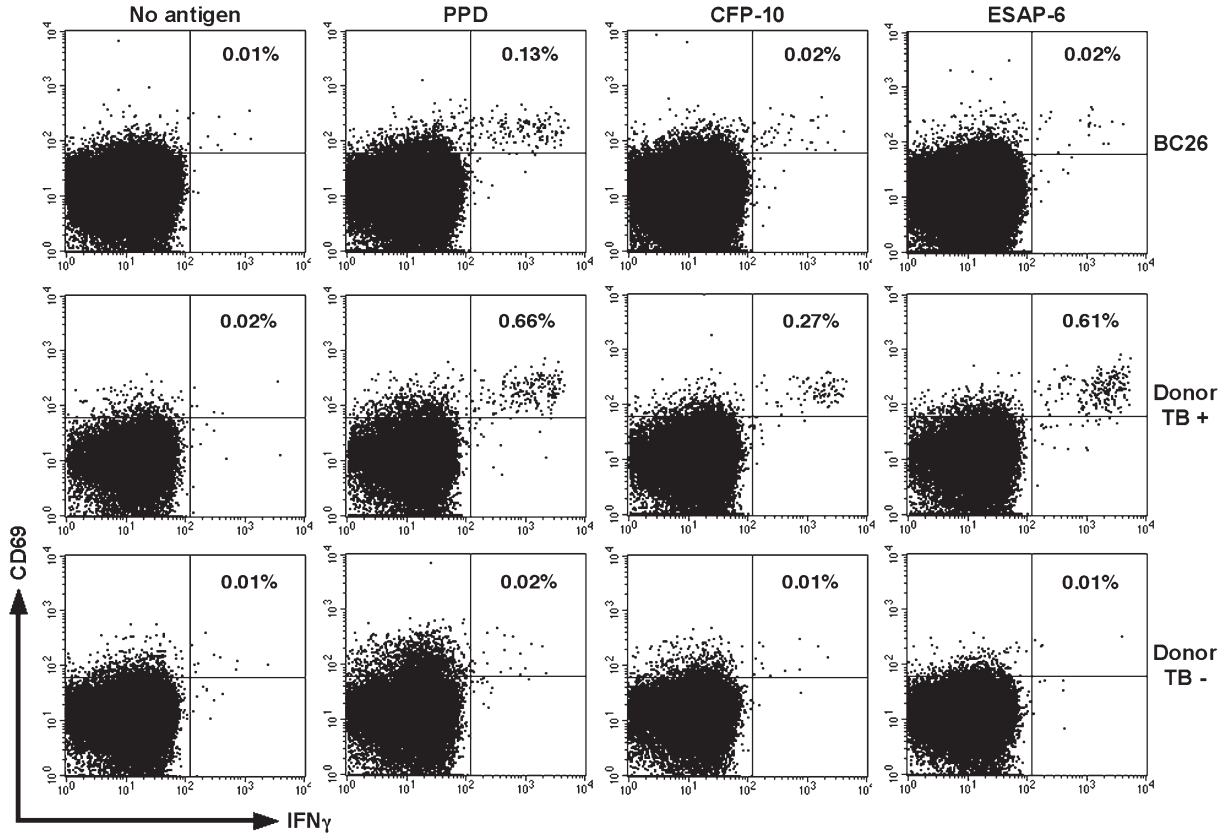

Fig. 4. BC26 reactivity for *Mycobacterium* antigens. Representative CD69 expression and IFNγ secretion upon stimulation with PPD and two MTB-specific proteins by CD4+ T cells from BC26; one patient with known active MTB disease and one healthy donor control. Percentages of CD69+/IFNγ+ cells are shown.

response can also be detected in individuals vaccinated with the 'bacille de Calmette–Guérin' (BCG). However, no record of BCG vaccination was found for this donor. Moreover, if BCG vaccination had led to the induction of such Melan-A cross-reactive cells, these cells would have been detected in more HLA-A2 donors during the screening. Despite the low prevalence of *Mycobacterium* infection, exposure of BC26 to such pathogen may have been increased since BC26 works as a nurse in an hospital. Responses to the MTB-specific antigens, CFP-10 and ESAP-6, remained too weak (borderline significance) to enable a definitive conclusion regarding MTB infection. Seventy Gambian patients with active MTB were screened with peptide 91 using IFNγ Elispot, but no significant response to this peptide was found (data not shown), providing no further evidence of a link with MTB infection. However, additional screening in different settings may be required since this experimental setting may not be optimal (i.e. different ethnic group, active MTB, unknown HLA). Taken together, BC26 reactivity for PPD and for a *Mycobacterium*-derived peptide (i.e. 91) suggests that BC26 has been infected with a pathogen belonging to the *Mycobacterium* genus (i.e. MTB or environmental Mycobacteria, with which infections are rare and poorly studied), and that its Melan-A tetramer-positive population might indeed be specific for this pathogen. However, evidence (e.g. clear association between 91 reactivity and Mycobacteria infection in a large cohort) is lacking to support unequivocally this assumption, so one cannot rule out the possibility that these observations may only be coincidental and that the cognate antigen that primed these cells still remains unknown.

TCR recognition of MHC–peptide complexes is central in T-cell development, including thymic selection, survival of naive T cells and differentiation into antigen-experienced T cells. For long, it was commonly believed that TCR recognition was highly specific, with an individual T cell being capable of only recognizing a particular peptide. Hence, TCR cross-reactivity has often been considered as a rare event, and its biological relevance questioned by scientists. However, increasing examples of pathogen-specific T cells recognizing cross-reactive epitopes within different proteins of the same pathogen, proteins from closely related or totally unrelated pathogens or even self-proteins have now been documented in both human or murine settings (29–36). The case presented in this study may illustrate further the possibility of CD8+ T-cell cross-reactivity between pathogen and tumor antigens.

Cross-reactive ability is thought to be valuable to the host, considering the limited immunological space and T-cell number versus the large number of pathogenic antigens to which one may be exposed over a lifetime (37). For instance, Mason (38) calculated that a single T cell would need to recognize many different peptides (e.g. up to 10^9)

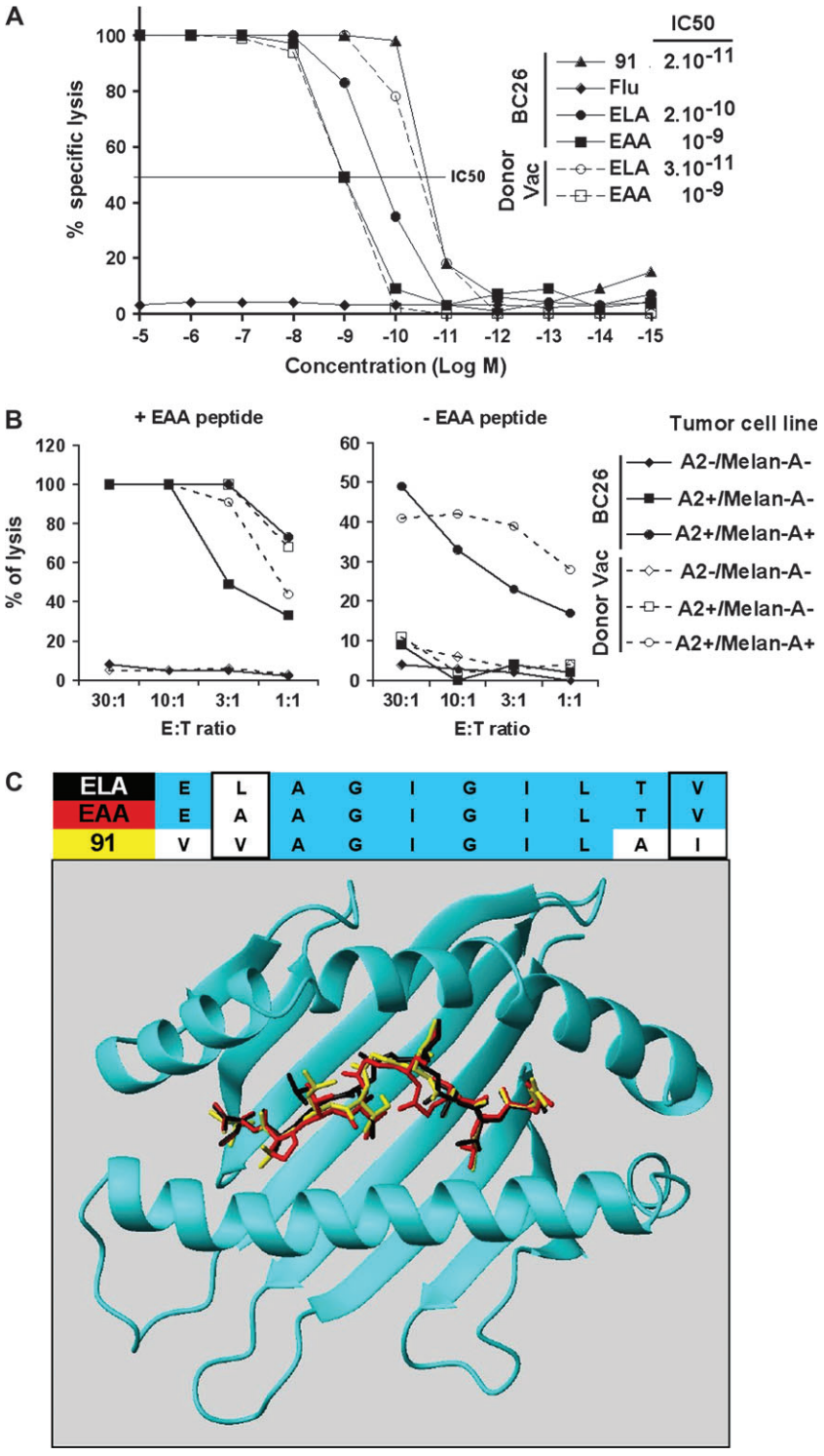

Fig. 5. Recognition efficacy and tumor recognition of BC26 CD8+ T-cell clone. (A) Recognition efficacy for peptide 91 as well as ELA or EAA was tested in chromium release assays by peptide titrations. Peptide-pulsed T2 cells were used as targets for one CTL clone generated by FACS sorting of tetramer+ T cells and subsequent *in vitro* expansion in the presence of IL-15, in comparison with one high avidity Melan-A-specific CTL clone generated from a Melan-A-vaccinated donor [10:1 effector:target (E:T) ratio]. (B) Tumor recognition was tested for the EPGLAREQ clone by cytotoxicity against Melan-A-expressing HLA-A2+ melanoma cell lines at different E:T ratios and in comparison with a high avidity Melan-A-specific CTL clone. Melan-A-/HLA-A2+ or Melan-A+/HLA-A2− melanoma cell lines as well as EAA-pulsed cell lines were used as controls. (C) Sequence alignment and structural prediction of the peptide 91 (yellow), ELA (black) and EAA (red) bound to the HLA-A2 molecule.

in order for the T-cell repertoire to recognize the vast array of possible different peptide–MHC antigens (e.g. up to 10^{17}). Cross-reactive T cells may thus be employed in immune responses and mediate protective immunity. While T-cell clones characterized by a high affinity TCR interaction with a given peptide–MHC complex are usually less cross-reactive than lower avidity T cells (39), we identified here a CD8+ T-cell clone presenting a high avidity for a Mycobacterial antigen as well as a relatively good avidity for a melanoma antigen. Although the functional capacity of this clone *ex vivo* was weak in response to the Melan-A peptide, the level of cross-reactivity for this antigen was sufficient to enable the recognition *in vitro* of melanoma antigen naturally expressed by tumor cells. The potential role that these cross-reactive memory T cells could play in heterologous immunity is obviously fascinating, and would deserve further investigation to see if these cells could mediate effector functions in case of melanoma development and influence the balance between protective immunity and disease progression. Since the phenomenon of cross-reactivity occurs here at the level of a memory T-cell population (in contrast to naive T-cell populations in other HLA-A2 healthy donors), their influence might be particularly relevant as memory cells are present at a high frequency and are easier to activate than naive cells (40). The balance between specificity and degeneracy of T-cell antigen recognition may represent a compromise that permits the host to respond to a multitude of pathogens as well as accommodating these numerous large memory pools in a finite immune system.

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Abbreviations

BCG	bacille de Calmette–Guérin
hrIL-15	human recombinant IL-15
MART	melanoma antigen recognized by T cells
MTB	*Mycobacterium tuberculosis*
PPD	purified protein derivative
PS-SCL	positional scanning synthetic combinatorial peptide library screening

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