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Correlation of effector function with phenotype and cell division after *in vitro* differentiation of naive MART-1-specific CD8$^+$ T cells

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Abstract

Adoptive transfer of antigen-specific CD8$^+$ T cells may represent an effective strategy for immunotherapy of tumors such as melanoma, but is limited by the number and functionality of *in vitro* expanded T cells. Here, we document that although ELAGIGILTV-specific CD8$^+$ T cells from different donors initially possessed a naive phenotype, after antigen-induced *in vitro* expansion two distinct phenotypes correlating with cell proliferation rate emerged in the different donors. Those cultures achieving fewer cumulative population doublings (CPDs) were cytotoxic and displayed a CD45RA$^+$CCR7$^-$ phenotype. In contrast, cultures reaching higher CPDs were non-cytotoxic T cells with a CD45RA$^-$CCR7$^-$ phenotype. Thus, the generation of larger numbers of ELAGIGILTV-specific CD8$^+$ T cells correlates negatively with the acquisition of a CD45RA$^+$CCR7$^-$ phenotype and cytotoxic capacity. A better understanding of the differentiation pathways of cytotoxic T cells to obtain optimally efficient cells for adoptive transfer will allow the development of new immunotherapy protocols.

Introduction

During the last decade, a considerable effort has been made to elucidate the cellular and molecular basis of T cell immune responses against melanoma in order to establish new immunotherapy strategies (1–3). The development of MHC-tetramer/pentamer technologies has made it feasible to monitor tumor-specific T cell responses generated after antigen priming. In addition, adoptive transfer of tumor-specific T cells has opened new avenues for successfully treating cancer patients.

Melanoma patients usually mount a spontaneous T cell response against tumor antigens and a positive correlation between the T cell responses and the tumor regression has been observed (reviewed in 4). Nevertheless, at some point, the responder T cells may become ineffective, probably because of a local immunosuppressive process occurring at the tumor sites. Similar results are found after vaccination of metastatic melanoma patients with tumor antigens that induce tumor regressions only in a small minority of patients (5–7).

Cancer immunotherapy exploits the power and specificity of the immune system for the treatment of tumors. Although cancer cells are less immunogenic than pathogens, the immune system is capable of recognizing and eliminating tumor cells. Adoptive immunotherapy with CD8$^+$ T cells permits the use of large numbers of effector T cell clones or lines of defined specificity and function, but has the disadvantage of requiring a significant effort to isolate and expand such cells *in vitro* to the numbers needed to establish an effective *in vivo* response and successfully control the tumor (8–10).

The end point of passive and active T cell-based immunotherapy is the assessment of an effective immune response to recognize and eliminate tumor cells expressing the appropriate antigen. Thus, several protocols have been developed to obtain large-scale T cell expansions useful for immunotherapy of cancer (3, 11–15). To assess the functional capacity of the expanded T cells, phenotyping different activation

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markers allows an indirect evaluation of their quality as effector cells and functional assays *in vitro* can confirm their specificity and efficacy (16).

Antigen-induced CD8$^+$ T cell maturation is tracked in terms of post-thymic differentiation pathways marked by changes in the expression of surface and intracellular molecules (17–19). In the model proposed by Lanzavecchia and Sallusto, four different stages have been defined within CD8$^+$ T cells according to their expression of the leukocyte common antigen isoform CD45RA and the chemokine receptor CCR7, namely: naive (CD45RA$^+$CCR7$^+$), central memory (CD45RA$^-$CCR7$^+$) and at least two subset of effector–memory (EM) cells (CD45RA$^-$CCR7$^-$ and CD45RA$^+$CCR7$^-$) (20–22). Appay *et al.* (23, 24) have analyzed the differentiation phenotype of virus-specific CD8$^+$ T cells based on the co-expression of the co-stimulatory receptors CD28 and CD27 defining three distinct memory T cell populations: early (CD28$^+$CD27$^+$), intermediate (CD28$^-$CD27$^+$) and late (CD28$^-$CD27$^-$). Recently, several intermediate stages within the EM T cell subset have been reported according to their CD28/CD27 phenotype (19, 25). Analysis of T cell differentiation in melanoma patients reveals T cells at different stages of maturation (26–32). Thus, melanoma-specific CD8$^+$ T cells from tumor-free lymph nodes showed a naive phenotype characterized mainly by the expression of CCR7 and CD45RA (26). In contrast, in tumor-invaded lymph nodes, CD45RA$^-$CCR7$^-$ CD27$^+$CD28$^-$, perforin-positive CTLs were observed in some melanoma patients (26, 33).

Melan-A/MART-1 (referred to as MART-1 hereafter) is a melanocyte lineage-specific antigen which appears to be most frequently recognized by tumor-specific T cells, at least in HLA-A*0201$^+$ melanoma patients. Thus, MART-1 is expressed by most fresh melanoma samples and by ~60% of melanoma cell lines. The analysis of MART-1-specific T cells by tetramers in melanoma patients revealed high frequencies of tumor-specific CTLs in tumor-infiltrating lymph nodes (17, 34–36). Furthermore, circulating tetramer-positive T cells were also detectable in peripheral blood of melanoma patients and healthy individuals (17, 37–39). Therefore, MART-1 can be considered as a good candidate for *in vitro* expansion and adoptive transfer of tumor-reactive CD8$^+$ T cells.

In the present study, we have analyzed the phenotype and function of peptide-specific CD8$^+$ T cells generated *in vitro* from CD8-purified T cells from different healthy individuals after stimulation with autologous peptide-loaded PBMCs. The protocol used was effective to generate large expansions of ELAGIGILTV-specific CD8$^+$ T cells. Peptide-specific CD8$^+$ T cells from healthy donors possessed a naive phenotype *ex vivo,* which changed on *in vitro* stimulation with the MART-1 antigen analogue ELAGIGILTV peptide. Here, we show that under the conditions used, different donors' CD8 cells acquired one of two distinct surface phenotypes on *in vitro* expansion, paralleling differences in their effector function. Cultures achieving fewer cumulative population doublings (CPDs; fewer cells expanded for adoptive transfer) were cytotoxic cells with a CD45RA$^+$CCR7$^-$ phenotype, whereas generation of larger numbers of cells was associated with low cytotoxicity and a CD45RA$^-$CCR7$^-$ phenotype. These results underline the importance of prior replicative history of CD8$^+$ T cells for development of cytotoxic capacity and potential use in adoptive immunotherapy.

Methods

Synthetic peptides

The ELAGIGILTV peptide (MART-1 26–35, A27L) is a MART-1 analogue in which substitution of alanine (A) at position 2 of the decapeptide with leucine (L) results in greater antigenicity than the natural peptide (40). The HLA-A*0201-binding peptide NLVPMVATV (CMV pp65, 495–503) was used as negative control.

These peptides were synthesized using an automated peptide synthesizer (431 Peptide Synthesizer; Applied Biosystems, Foster City, CA, USA), purified by preparative reverse-phase HPLC and shown to be >90% pure. Lyophilized peptides were dissolved in dimethyl sulfoxide, stored at −20°C and used for the different assays as indicated.

Blood samples

Peripheral blood from healthy donors, selected on the basis of HLA-A*0201 (hereafter, HLA-A2) expression, was obtained after informed consent under the auspices of the appropriate Research and Ethics Committees. PBMCs were obtained by centrifugation over Histopaque-1077 (Sigma, St Louis, MO, USA). The age range of the donors was between 25 and 35 years. The number of individuals in each experiment is indicated in the figure legends.

Cells

The melanoma cell lines Mel-624, MeWo and FM-93/2 (HLA-A*0201$^+$ MART-1$^+$) and ESTDAB-173 (HLA-A*0201$^+$ MART-1$^-$) were obtained from European Searchable Tumor Cell Line Data Base (ESTDAB; http://www.ebi.ac.uk/ipd/estdab/). Melanoma cell lines and T2 cells, a peptide transport-deficient cell line that effectively presents exogenously supplied peptides in the context of HLA-A*0201, were maintained in RPMI 1640 supplemented with 10% FCS, 1% glutamine and 1% penicillin–streptomycin (Lonza, Verviers, Belgium).

In vitro expansion of ELAGIGILTV-specific CD8$^+$ T cells

Peptide-specific CD8$^+$ T cell expansions were carried out as reported by Valmori *et al.* (40) with minor modifications. Briefly, CD8$^+$ T cells from healthy individuals were isolated from 40 to 50 $\times$ 10^6 PBMCs by positive selection using Miltenyi magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD8$^+$ T cells were used as responder cells for the expansion of MART-1-specific CD8$^+$ T cells. The negative fraction consisting of autologous CD8$^-$ cells was used as stimulator cells. The stimulators were pre-incubated with 10 μM of ELAGIGILTV peptide for 2 h in serum-free medium and then irradiated prior to use as antigen-presenting cells (APCs). The CD8$^+$ T cells and APCs were co-cultured in 24-well tissue culture plates and maintained in RPMI 1640 supplemented with 20% human serum, 1% glutamine, 1% penicillin–streptomycin and 1% non-essential amino acids from Lonza. IL-7 (10 ng ml^{-1}) from PeproTech EC (London, UK) and IL-2 (100 U ml^{-1}) (41) were included in the culture medium throughout the entire culture period. On day 7, cells

were re-stimulated with peptide-loaded autologous stimulator cells (CD8-negative fraction), IL-2 and IL-7. Where indicated, a second re-stimulation was performed on day 14.

Flow cytometry

For flow cytometry analysis, PBMCs and *in vitro* expanded cells were stained for 40 min at room temperature with allophycocyanin-labeled A*0201/ELAGIGILTV (A2/ELA) pentamer (Proimmune, Oxford, UK) according to the protocol provided by the manufacturer. The lower detection limit for A2/MART-1 pentamer was 0.01%. Cells were then washed and stained with the appropriate combination of mAbs. The following mAbs were used in this study: peridinin chlorophyll protein-conjugated anti-CD8 (SK1); PE-conjugated, anti-CD28 (L293), anti-CCR7 (3D12) and anti-CD45RA (L48); FITC-conjugated anti-CD45RA (L48), anti-CD27 (L128) and anti-perforin (δG9) from BD Biosciences (San Jose, CA, USA). For intracellular perforin staining, after surface marker labeling, cells were fixed with paraformaldehyde at a final concentration of 4% and permeabilized with 0.1% saponin before intracellular staining with anti-perforin–FITC. Isotype-matched negative control antibodies were used in all the experiments. Flow cytometric analysis was performed on a FACScalibur cytometer (BD Biosciences) after acquisition of 10^5–10^6 events. Viable cells were selected using forward and side scatter characteristics. Resulting profiles were analyzed using Cell Quest software (BD Biosciences).

IFN-γ secretion assay

IFN-γ secretion was tested by using the IFN-γ secretion assay (Miltenyi Biotec) according to the protocol provided by the manufacturer. Briefly, responder T cells, previously labeled with cytokine catch reagent, were mixed at a 1:1 ratio with T2 cells pulsed with 10 μg ml^{-1} of ELAGIGILTV peptide or with an irrelevant control peptide. Responder and target cells were incubated at 37°C, 5% CO_2 under slow continuous rotation for 3 h. Finally, cells were labeled with the cytokine detection antibody, A2/ELA pentamer and anti-CD8 (SK1) and analyzed by flow cytometry.

Cytotoxicity assay

Cytotoxic activity was measured by standard ^{51}Cr release assays. MART-1-specific cytotoxicity was determined using HLA-A*0201⁺ melanoma cell lines obtained from European Searchable Tumor Cell Line and Data Bank and T2 cells loaded either with 10 μg ELAGIGILTV peptide or an irrelevant peptide. Target cells were incubated for 90 min with 50 mCi of ^{51}Cr sodium chromate (CIS Biointernational, Cedex, France) before being mixed with effector cells. Assays were performed in triplicate in round-bottomed microtiter plates. After 4 h at 37°C, 25 μl of supernatant was collected from each well and spotted onto glass fiber Spot-on filter mats (Wallac, Turku, Finland). Filter mats were analyzed on a 1205 Betaplate counter (Wallac). The percentage-specific cytotoxicity was calculated as follows: [(c.p.m. experimental − c.p.m. spontaneous)/(c.p.m. maximum − c.p.m. spontaneous)] × 100, in which spontaneous release was that obtained from target cells incubated with medium alone and maximum release was that obtained from target cells incubated with 5% Triton X-100. Spontaneous lysis was always <10% of maximum release.

Analysis of ELAGIGILTV-specific CD8⁺ T cells proliferation

Carboxyfluorescein succinimidyl ester (CFSE) staining was performed on day 14 by labeling T cells with 2.5 μM CFSE (Sigma) in PBS for 10 min at 37°C, before quenching with ice-cold 10% FCS RPMI and washing. Cells were then re-stimulated as indicated above. After 5 days of culture with CFSE, cells were labeled with A2/MART-1 pentamer, anti-CD8, anti-CCR7 and anti-CD45RA and tested for CFSE dilution by flow cytometry.

The CPD of peptide-specific CD8⁺ T cells in culture was calculated as CPD = log(N14/N0)/log2, where N14 and N0 represent the absolute number of peptide-specific CD8⁺ T cells at day 14 and at day 0, respectively.

Statistical analysis

The Student's *t*-test for independent values and the Pearson correlation analysis were performed using SPSS software. A P value $\leqslant 0.05$ was considered significant.

Results

MART-1 analogue peptide, ELAGIGILTV, induces the expansion of peptide-specific CTLs

Using A2/ELA pentamers, we have analyzed *ex vivo* the frequency of circulating CD8⁺ T cells specific for ELAGIGILTV in healthy individuals. Table 1 shows the percentage of peptide-specific cells within the CD8⁺ T cell subset in HLA-A2 healthy donors. ELAGIGILTV peptide was used to induce the expansion of peptide-specific CD8⁺ T cells. As shown in Fig. 1(A), after two rounds of antigen stimulation, a large expansion of peptide-specific CD8⁺ T cells was obtained. Figure 1(B) shows the percentage of peptide-specific CD8⁺ T cells present in eight individual expansions at different time points.

Table 1. Frequencies of A*0201/ELAGIGILTV pentamer-positive CD8⁺ T cells

HLA-A2 donors	Day 0
C10	0.02
C11	0.02
C21	0.02
C33	0.02
C35	0.02
C12	0.03
C13	0.03
C27	0.03
C32	0.03
C34	0.03
C26	0.04
C28	0.04
C20	0.05
C29	0.05
C17	0.06
C23	0.08
C24	0.08
C25	0.09

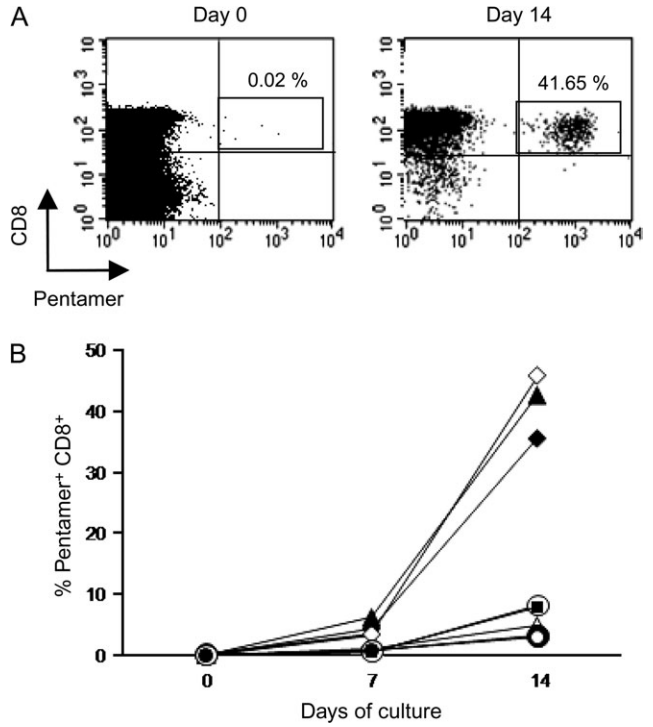

Fig. 1. *In vitro* expansion of ELAGIGILTV-specific CD8⁺ T cells. CD8⁺ T cells were incubated with ELAGIGILTV-pulsed PBMCs in the presence of IL-2 and IL-7 for 14 days. (A) Representative flow cytometric analysis of peptide-specific CD8⁺ T cells from donor C21. Values represent the percentage of A*0201/ELAGIGILTV (A2/ELA) pentamer-reactive cells within the CD8⁺ gate. (B) Percentage of peptide-specific cells referred to total CD8⁺ T cells at different time points (n = 8). Values for each individual expansion are represented.

The maximum expansion observed was a >600-fold increase in the percentage of peptide-specific cells within the CD8⁺ T cell subset after 2 weeks.

Functional analysis of cultured ELAGIGILTV-specific CD8⁺ T cells reveals expansions with different cytotoxic capacity

Effector function of the ELAGIGILTV-specific CD8⁺ T cells was tested by standard ^{51}Cr release assays on day 14 after two *in vitro* stimulations. Cytotoxicity assays showed that cultures could be distinguished according to their cytotoxic capacity. In five of eight donors, peptide-specific CD8⁺ T cells had high lytic activity against ELAGIGILTV-loaded T2 cells (Fig. 2A), whereas three donors showed no cytotoxicity even at different effector:target ratios (Fig. 2B).

In addition, the intracellular perforin content was analyzed in the peptide-specific CD8⁺ T cells derived from cytotoxic (Fig. 2C) and non-cytotoxic (Fig. 2D) expansions. Perforin analysis demonstrated a clear correlation with cytotoxicity. On the contrary, when the IFN-γ production was tested, both cytotoxic (Fig. 2E) and non-cytotoxic (Fig. 2F) peptide-specific CD8⁺ T cells were found to secrete IFN-γ in response to re-challenge with ELAGIGILTV-loaded T2 cells. The production of IFN-γ was observed in ELAGIGILTV-specific CD8⁺ T cells and was peptide specific. The IFN-γ production by pentamer-negative CD8 cells was always <2% (Fig. 2E and F).

The cytotoxic capacity of expanded ELAGIGILTV-specific CD8⁺ T cells is associated with the expansion of CD45RA⁺ CCR7⁻ cells

In order to understand why some donors generated cytotoxic cells, but other did not, a comparison of the surface expression of naive, EM type-associated markers between the cytotoxic and non-cytotoxic T cells was carried out by flow cytometry. As illustrated in Fig. 3(A), when tested *ex vivo*, most circulating peptide-specific CD8⁺ T cells from all tested healthy donors displayed a characteristic naive phenotype (CD45RA⁺CCR7⁺). No differences were noted before culture between those donors generating cytotoxic cultures and those yielding non-cytotoxic cells (black and white histograms, respectively), neither in the peptide-specific cells (Fig. 3A) nor in the whole CD8 T cell population (data not shown). After *in vitro* stimulation, peptide-specific CD8⁺ T cells from those expansions that failed to generate cytotoxicity displayed a phenotype characterized by loss of CCR7 and CD45RA (Fig. 3B, white histograms). On the other hand, peptide-specific CD8⁺ T cells from those expansions that did develop cytotoxic capacity had a CD45RA⁺CCR7⁻ phenotype (Fig. 3B, black histograms) and according to their high cytotoxic capacity against ELAGIGILTV-loaded T2, these cells were defined as cytotoxic effectors. Further characterization of *in vitro* stimulated peptide-specific CD8⁺ T cells according to CD28 and CD27 expression showed no significant differences in the expression of these markers (Fig. 3C and D) between cytotoxic and non-cytotoxic expanded T cells.

The cells were examined at various times to assess CD45RA and CCR7 phenotype. By day 4, half of the cells remained CD45RA⁺CCR7⁺ and no significant differences were observed between those expansions that at day 14 acquired a CD45RA⁺CCR7⁻ phenotype (Fig. 4A, upper row) and those that generated CD45RA⁻CCR7⁻ cells (Fig. 4B, upper row).

For the expansions that resulted in a CD45RA⁺CCR7⁻ phenotype, the majority of peptide-specific CD8⁺ T cells acquired that phenotype at day 7 and it was maintained during the rest of the culture period (Fig. 4A, upper row). Pentamer-negative CD8⁺ T cells from the same donor showed a CD45RA⁻CCR7⁻ phenotype (Fig. 4A, lower row).

In those expansions that resulted in CD45RA⁻CCR7⁻ cells, we can observe this phenotype in a significant percentage of peptide-specific cells at day 7, and at day 14 the majority of cells displayed this phenotype that was maintained at day 21 (Fig. 4B, upper row). From day 7 to day 21, the majority of pentamer-negative CD8⁺ T cells from the same donor had a CD45RA⁻CCR7⁻ phenotype (Fig. 4B, lower row).

Analysis of specific cytotoxicity was performed at days 14 and 21 against peptide-loaded T2 cell line. Whereas a significant cytotoxic capacity of CD45RA⁺CCR7⁻ expansions was observed at day 14 and day 21 (Fig. 4C), CD45RA⁻CCR7⁻ expansions were not cytotoxic (Fig. 4D).

ELAGIGILTV-specific CD8⁺ T cells with a CD45RA⁺CCR7⁻ phenotype show cytotoxic capacity against MART-1⁺ melanoma cell lines

Peptide-specific CD8⁺ T cells derived from CD45RA⁺CCR7⁻ expansions effectively killed the HLA-A2⁺ MART-1⁺ melanoma cell lines Mel-624, MeWo and FM-93/2 (Fig. 5A),

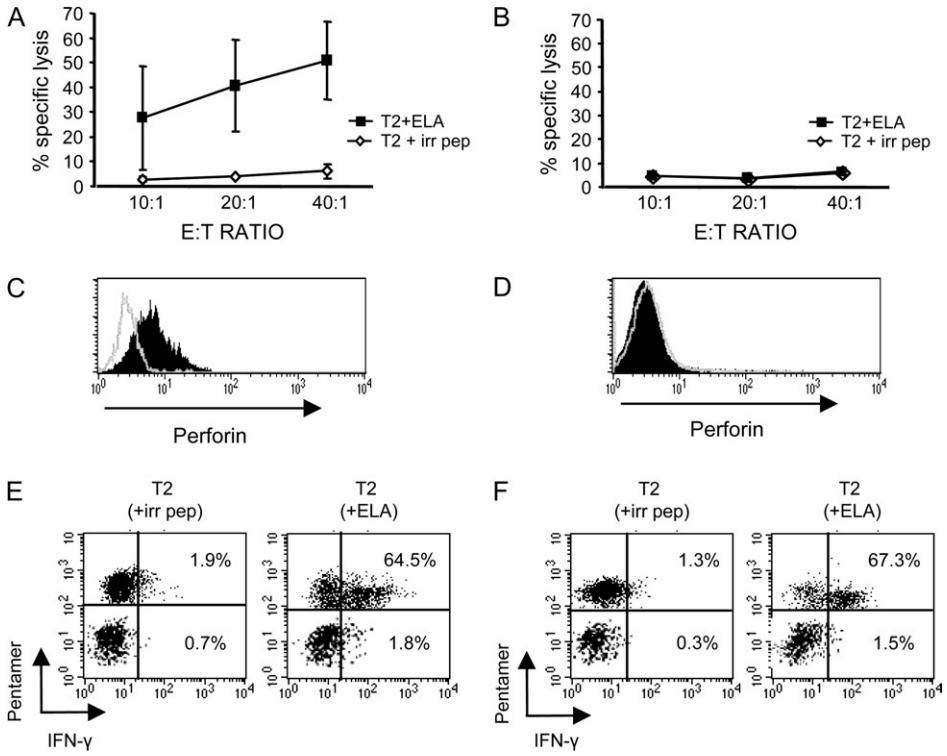

Fig. 2. Functional capacity of ELAGIGILTV-specific CD8⁺ T cells expanded *in vitro*. The cytotoxic capacity of T cell expansions was analyzed on day 14. Five expansions were cytotoxic against T2 cells loaded with ELAGIGILTV (ELA) peptide (A) and three did not lyse T2 cells loaded with ELA peptide (B). As negative control, T2 cells loaded with an irrelevant peptide (irr pep) were used. Representative histograms of intracellular perforin staining gated on peptide-specific CD8⁺ T cells from cytotoxic (C) or non-cytotoxic (D) T cell expansions. IFN-γ analysis on gated CD8⁺ T cells derived from cytotoxic (E) and non-cytotoxic (F) *in vitro* expansions against T2 cells loaded with ELAGIGILTV (ELA) or irrelevant peptide. Values represent the percentage of positive cells referred to the total of pentamer-positive or pentamer-negative CD8⁺ T cells.

demonstrating that ELAGIGILTV-specific CD8⁺ T cells were cross-reactive to natural peptide (EAAGIGILTV). ESTDAB-173 (HLA-A*0201⁺ MART-1⁻) melanoma cell line was not lysed. In contrast, CD45RA⁻CCR7⁻ expansions did not kill HLA-A2⁺ MART-1⁺ melanoma cell lines (Fig. 5B).

Proliferation of cultured ELAGIGILTV-specific CD8⁺ T cells inversely correlates with their CD45RA expression

Analysis of peptide-specific CD8⁺ T cell division using CFSE staining showed that cells from those cultures resulting in a predominant expansion of CD45RA⁺CCR7⁻ cells had lower proliferation than cells from cultures resulting in expansion of CD45RA⁻CCR7⁻ cells (Fig. 6A).

Analysis of CPD of peptide-specific CD8⁺ T cells indicated that, in the culture conditions described, these cells achieved a large number of population doublings indicating that they had a significant proliferation capacity. At the end of the culture period, when we analyzed the relationship of the CPD achieved by peptide-specific CD8⁺ T cells with their differentiation phenotype, a statistically significant inverse correlation (P = 0.002) was found between the number of CPD and the percentage of CD45RA⁺CCR7⁻ cells (Fig. 6B). Indeed, peptide-specific CD8⁺ T cells with a CD45RA⁺CCR7⁻ phenotype, shown to be cytotoxic against peptide-loaded T2 target cells, accomplished less CPD than non-cytotoxic CD45RA⁻CCR7⁻ cells.

Discussion

The identification of novel tumor antigens and advances in the technology to monitor the persistence of transferred cells have provided new impetus to research on immunotherapy against cancer using adoptive cell transfer. This approach relies on the *ex vivo* generation of high-affinity tumor-specific lymphocytes and their administration back to the patient in large numbers. The characterization of the most efficient tumor–antigen-specific CD8⁺ T cells for immunotherapy in different pre-clinical models has underscored the relevance of using large numbers of high-affinity cells (42–45).

Melanocyte differentiation antigens such as MART-1 are frequently recognized by T cells and can stimulate naive CD8⁺ T cells *in vivo* to proliferate and differentiate into EM cells (34, 46). As compared with natural tumor peptide sequences, selected analogue peptides may be more immunogenic and thus more suitable for vaccination. Thus, it has been shown that patient immunization with the analogue peptide ELAGIGILTV leads to *in vivo* activation of T cells that were specific for the natural tumor antigen. Although analogue peptides have an enhanced immunogenicity in terms of specific T cell frequencies (47), it has been recently published by Speiser *et al.* (48) that compared with analogue peptides, vaccination with natural peptides induced T cells with a stronger anti-tumor reactivity. Moreover, in ELAGIGILTV-vaccinated patients, Appay *et al.* (49) showed that

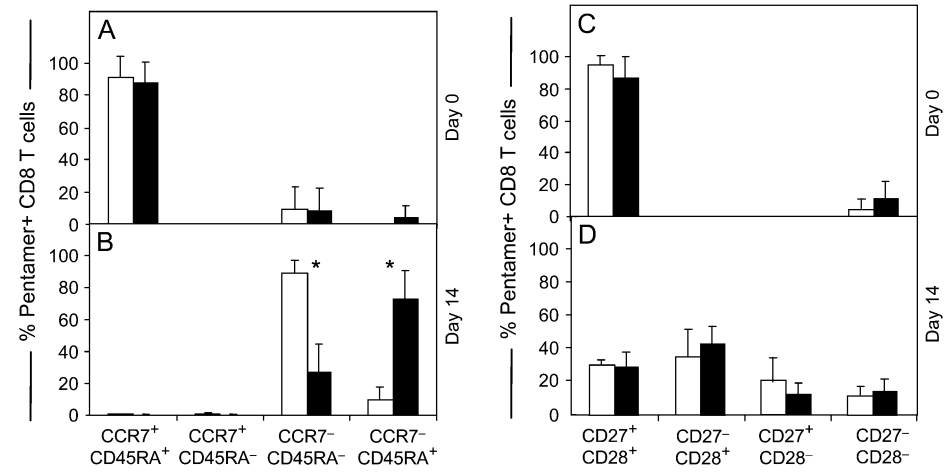

Fig. 3. Differentiation phenotype after *in vitro* expansion of ELAGIGILTV-specific CD8⁺ T cells. Analysis of CCR7/CD45RA differentiation phenotype of peptide-specific CD8⁺ T cells from healthy donors at day 0 (A) and at day 14 (B) and analysis of CD27/CD28 phenotype at day 0 (C) and at day 14 (D). The percentages of positive cells are referred to A*0201/ELAGIGILTV (A2/ELA) pentamer-positive cells. White bars represent mean percentage values of those expansions that led to non-cytotoxic CD8⁺ T cells (n = 5). *P = 0.0005.

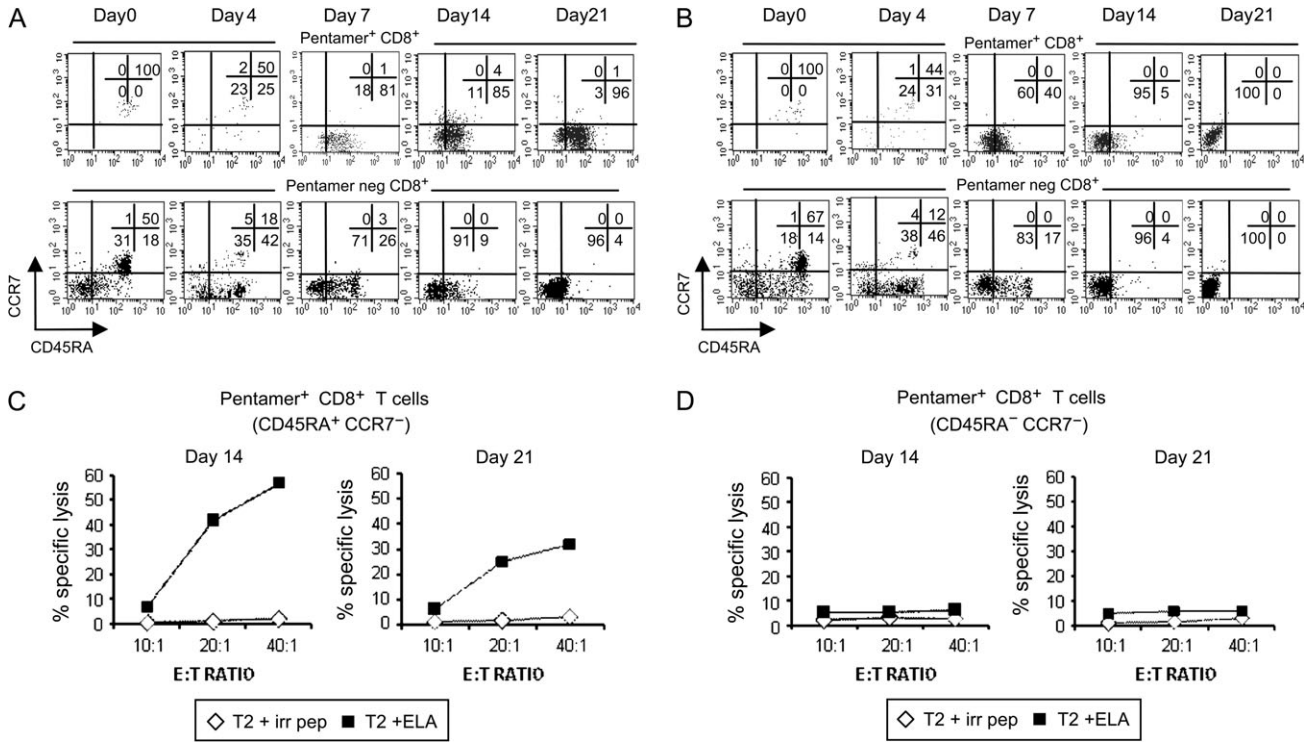

Fig. 4. Kinetics of ELAGIGILTV-specific CD8⁺ T cell differentiation in relation to their cytotoxic capacity. Representative dot plots of CCR7/CD45RA phenotype of those expansions that resulted in cytotoxic CD8⁺ T cells (A) and those that resulted in non-cytotoxic CD8⁺ T cells (B). Upper row represent pentamer-positive CD8⁺ T cells and lower row represent pentamer-negative CD8⁺ T cells. Specific lysis on days 14 and 21 of T2 cells loaded with ELAGIGILTV (ELA) peptide or an irrelevant peptide (irr pep) mediated by CD45RA⁺CCR7⁻ (C) and CD45RA⁻CCR7⁻ (D) CD8⁺ T cell expansions.

vaccination stimulates a broadly cross-reactive T cells with reactivity against MART-1⁺ melanoma cell lines. This is supported by our results showing that ELAGIGILTV-specific CD8 T cells expanded *in vitro* are able to kill not only ELAGIGILTV-loaded T2 but also melanoma cell lines expressing MART-1.

To date, the anti-tumoral response mediated by naturally or vaccine-induced melanoma-reactive T cells correlated poorly with clinical responses probably as a consequence of the development of immune evasion mechanisms by the tumor (4, 6, 32, 50–55). In contrast, T cell adoptive

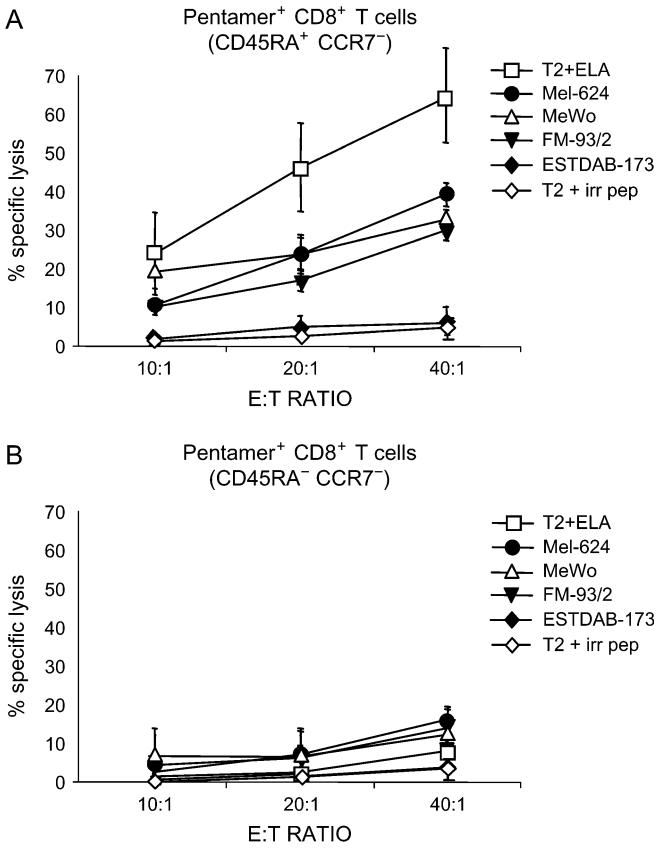

Fig. 5. CD45RA⁺CCR7⁻ ELAGIGILTV-specific CD8⁺ T cell expansions lyse MART-1⁺ melanoma cell lines. Specific lysis at day 14 of three HLA-A*0201⁺ MART-1⁺ and one HLA-A*0201⁺ MART-1⁻ melanoma cell lines by CD45RA⁺CCR7⁻ (A, n = 3) and CD45RA⁻CCR7⁻ (B, n = 2) CD8⁺ T cell expansions. T2 cells loaded with ELAGIGILTV (ELA) peptide or an irrelevant peptide (irr pep) were used as positive and negative controls, respectively.

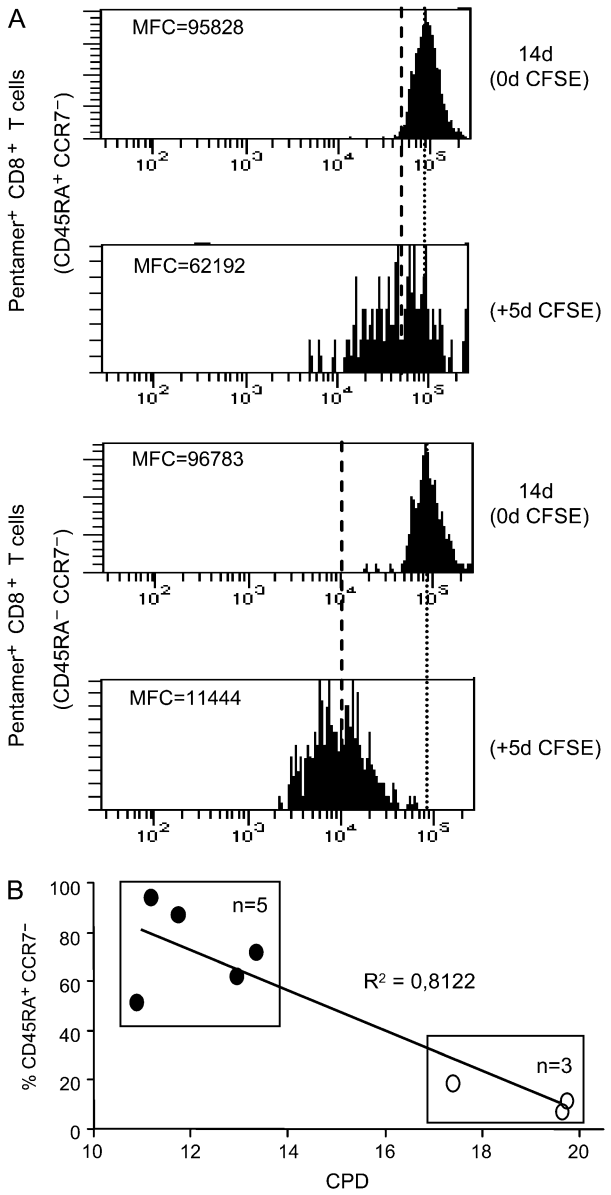

Fig. 6. Proliferative behavior of ELAGIGILTV-specific CD8⁺ T cell expanded *in vitro*. CD8⁺ T cell expansions were labeled with CFSE at day 14 and CFSE dilution was assayed 5 days after (A). Upper histograms represent those expansions that resulted in CD45RA⁺CCR7⁻ phenotype and lower histograms those showing a CD45RA⁻CCR7⁻ phenotype. Population doubling from day 0 to day 14 correlates with the percentage of CD45RA⁺CCR7⁻ peptide-specific CD8⁺ T cells at the end of culture (B). White dots represent those expansions that resulted in non-cytotoxic cells (n = 3) and black dots correspond to those showing cytotoxic capacity (n = 5).

therapies are showing promising results, although limited by the requirement for generation of large numbers of tumor-specific T cells with selected phenotypic and functional characteristics.

For the *in vitro* expansion of antigen-specific CTLs, we have applied, with minor modifications, a previously reported protocol (40). This is a simple and efficient method that easily permits expansions of T cells necessary for the development of adoptive transfer therapies in clinical laboratories using autologous non-professional APCs loaded with analogue peptide (42). Here, we demonstrate that expansions of specific T cells can be obtained from healthy donors even with initial pentamer-positive cell frequencies as low as 0.02% of CD8⁺ T cells using a simple procedure with non-professional APCs. Other authors have shown that dendritic cells (DCs) can be used as potent APCs to activate *in vitro* naive T cells and its use as a natural adjuvant has been proposed to be superior to conventional strategies of immunotherapy (56). However, the complexity of the system that involves not only DC maturation and antigen transfer but also the interactions of DCs with NK, Tregs and CD8⁺ T cells makes difficult to apply it as a standard method for expanding T cells with reproducible phenotype and function.

It has been described that several types of dysfunctional antigen-specific T cells can occur in the presence of high antigen load (57). Here, we show that some *in vitro* expansions of ELAGIGILTV-specific CD8⁺ T lymphocytes generated cells with a CD45RA⁺CCR7⁻ effector phenotype and cytotoxic capacity against ELAGIGILTV-loaded T2 cells and melanoma cell lines expressing MART-1. However, other expansions generated non-cytolytic cells with an EM phenotype (CD45RA⁻CCR7⁻).

According to CD27/CD28 expression, our results showed no statistically significant differences when compared those expansions resulting in CD45RA$^-$CCR7$^-$ cells with those that developed CD45RA$^+$CCR7$^-$ cells. Within the CD45RA$^-$CCR7$^-$ subset, the four distinct populations of EM cells delineated by Romero *et al.* (25) were found: EM_1 (CD27$^+$CD28$^+$), EM_2 (CD27$^+$CD28$^-$), EM_3 (CD27$^-$CD28$^-$) and EM_4 (CD27$^-$CD28$^+$). Interestingly, we found that ~40% of expanded cells had down-regulated CD27 expression while maintaining the expression of CD28 and therefore correspond to the recently described EM_4 subset. In a similar way, by analyzing CD27/CD28 expression within the CD45RA$^+$CCR7$^-$ subset, several subsets have been shown: CD27$^+$CD28$^+$, CD27$^+$CD28$^-$ and CD27$^-$CD28$^-$ that correspond to those defined as pE1, pE2 and E, respectively, by Romero *et al.* (25). We have found an additional subset CD27$^-$CD28$^+$ CD45RA$^+$CCR7$^-$ cells that may represent CD8$^+$ T cells with an intermediate differentiation stage. It is interesting to note that following adoptive transfer in patients with metastatic melanoma, a correlation was observed between tumor regression and CD28 expression, telomere length and T cell persistence (58, 59).

It has been recently reported that the loss of CD45RA on expanded CD8$^+$ T cells is a process that can be reverted and that a large fraction of CD45RA$^-$CCR7$^-$ CD8$^+$ T cells re-express CD45RA when these cells do not receive further antigenic stimulation for >1 month (60). We have not observed re-expression of CD45RA cells in those expansions that resulted into CD45RA$^-$CCR7$^-$ EM cells probably due to the differences in the experimental system as we performed weekly stimulations with the antigen. Our results show that both CD45RA$^-$CCR7$^-$ and CD45RA$^+$CCR7$^-$ peptide-specific CD8$^+$ T cells had proliferative capacity although the analyses of CPD and CFSE dilution indicate that CD45RA$^-$CCR7$^-$ cells proliferate more than their counterpart CD45RA$^+$CCR7$^-$ CD8$^+$ T cells.

It is clear that the generation of large numbers of specific T cells may not be sufficient to trigger tumor regression but that their quality is of paramount importance. In this sense, the inadequate or inconsistent function of *in vitro* peptide-induced T cells and the preservation of the replicative life span of transferred T cells are major problems concerning the adoptive transfer of tumor-specific T cells (11, 61–63).

Although ELAGIGILTV-specific CD8$^+$ T cells are frequently found in peripheral blood from melanoma patients and, in some patients, they are phenotypically identical with those found in healthy donors (17), we cannot exclude that melanoma patients may respond in a different manner to peptide stimulation. Studies in murine models have confirmed the superior immunotherapeutic function of less-differentiated CD8$^+$ T cells on adoptive transfer and have provided insight into the mechanisms that confer a functional advantage to these cells *in vivo* (64).

For optimal adoptive cell therapy, different cell cultures system have been developed using other cytokines together or not with IL-2. These cytokines are IL-7, IL-15, IL-12 or IL-21 and could have an impact on the efficacy of transferred T cells (65). In our experimental system, the addition of IL-15 to T cell cultures did not affect the differentiation process into CD45RA$^-$CCR7$^-$ or CD45RA$^+$CCR7$^-$ CD8$^+$ T cells (data not shown), suggesting that other factors are involved in the differentiation course.

Here, we show evidence for the generation of distinct phenotypes associated with effector function of peptide-specific T cells on *in vitro* expansions where the generation of tumor antigen-specific cells with a CD45RA$^+$ effector phenotype is linked to the number of CPD. *In vitro* stimulations showing higher CPD results in non-cytotoxic CD45RA$^-$CCR7$^-$ peptide-specific CD8$^+$ T cells; in contrast, lower CPD is associated with an effector phenotype characterized by the expression of CD45RA$^+$ and cytotoxic capacity against peptide-loaded T2 cells and MART-1$^+$ melanoma cell lines. Our *in vitro* results correlating differentiation stage and proliferative capacity of tumor antigen-reactive CD8$^+$ T cells together with previous observations using CD8$^+$ T cell adoptive transfers *in vivo* highlight the importance of examining the replicative history of expanded CD8$^+$ T cells prior to their adoptive transfer to patients.

In summary, the generation of effector CD45RA$^+$ CTLs can be correlated to CPD evidencing a link between replicative history and acquisition of effector function on *in vitro* T cell culture. Finally, it was difficult to establish the time point when CD8$^+$ T cells are committed to an EM or CD45RA$^+$ effector phenotype and additional analysis will be necessary to determine the mechanisms that contribute to the differentiation pathway of specific CD8$^+$ T cells and to learn how to manipulate this process.

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Abbreviations

A2/ELA	HLA-A*0201/ELAGIGILTV
APC	antigen-presenting cell
CFSE	carboxyfluorescein succinimidyl ester
CPD	cumulative population doubling
DCs	dentritic cells
EM	effector–memory
ESTDAB	European Searchable Tumor Cell Line Data Base

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