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New Generation Vaccine Induces Effective Melanoma-Specific CD8$^+$ T Cells in the Circulation but Not in the Tumor Site[1]

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Although increasing evidence suggests that CTL are important to fight the development of some cancers, the frequency of detectable tumor-specific T cells is low in cancer patients, and these cells have generally poor functional capacities, compared with virus-specific CD8$^+$ T cells. The generation with a vaccine of potent CTL responses against tumor Ags therefore remains a major challenge. In the present study, ex vivo analyses of Melan-A-specific CD8$^+$ T cells following vaccination with Melan-A peptide and CpG oligodeoxynucleotides revealed the successful induction in the circulation of effective melanoma-specific T cells, i.e., with phenotypic and functional characteristics similar to those of CTL specific for immunodominant viral Ags. Nonetheless, the eventual impact on tumor development in vaccinated melanoma donors remained limited. The comprehensive study of vaccinated patient metastasis shows that vaccine-driven tumor-infiltrating lymphocytes, although activated, still differed in functional capacities compared with blood counterparts. This coincided with a significant increase of FoxP3$^+$ regulatory T cell activity within the tumor. The consistent induction of effective tumor-specific CD8$^+$ T cells in the circulation with a vaccine represents a major achievement; however, clinical benefit may not be achieved unless the tumor environment can be altered to enable CD8$^+$ T cell efficacy. *The Journal of Immunology,* **2006, 177: 1670–1678.**

Virus infections trigger strong immune responses, which include the generation of potent cytotoxic CD8$^+$ T cell populations able to control viral replication. In contrast, tumors induce very few, if any, detectable tumor-specific T cells in cancer patients, and these cells usually show poor functional capacities (1, 2). Based on the assumption that large numbers of effective T cells may be necessary to control tumor development, considerable enthusiasm has been directed to the development of CTL vaccines to boost tumor-specific T cell response in cancer patients. However, poor efficacy to induce effective antitumor CTL, associated with limited clinical impact, have been the main outcomes of the different vaccine trials to date. Most attempts failed to elicit significant T cell numbers, which remained either undetectable (3–6) or in low frequencies (7–9) in ex vivo assays. One trial using repeated vaccination with high doses of peptide enabled the induction of large numbers of tumor-specific CTL (10), but this approach also seemed to result in quiescent cells lacking functionality (11). To perform their effector functions and mediate effective immune protection, T cells need to be in sufficient numbers and in the correct state of activation and differentiation. There is currently much uncertainty regarding the ideal differentiation status (12). Considering that most individuals display virus-specific CD8$^+$ T cells that are able to control their respective viruses, thus preventing clinical manifestations of the disease, one could speculate that an effective antitumor vaccine should induce CTL with features similar to those of virus-specific CTL.

We have recently reported that vaccination with low doses of the Melan-A/MART-1 peptide in combination with IFA and synthetic CpG oligodeoxynucleotides (VaxImmune/CPG 7909; stimulate DC activation through TLR9) is particularly efficient at driving the in vivo expansion of tumor-specific CD8$^+$ T cells in melanoma patients (13). To gain insight into the functional status of these vaccine-driven cells, we have performed ex vivo characterization of these cells in comparison with CTL specific for immunodominant viral Ags (from EBV, CMV, and influenza), in the same donors. Analysis were performed directly on whole blood, which enables an optimum assessment of the functional features of T cells (14). We report that the circulating Melan-A-specific CD8$^+$ T cells boosted with this new generation vaccine exhibit characteristics similar to those of virus-specific cells, being mostly functional. Nonetheless, this immunological achievement does not seem sufficient to induce a detectable reversion of progressive disease. To get insight into the discrepancies between immunological and clinical observations, detailed analysis of the tumor-infiltrated lymphocytes were performed on s.c. lesions obtained from two vaccinated patients. Melan-A-specific CD8$^+$ T lymphocytes infiltrated in the tumor appear to differ in functional capacities compared with the circulation, which goes along with a strong activity of regulatory T cells.

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Table I. *Clinical characteristics of the patients studied*

Patient	Age (years)	No. of Vaccinations	Time Elapsed from Last Vaccination (mo)	Months in the Study	Clinical Follow-Up and Disease Status at Time of Blood Sampling (mo)[a]
LAU 321	69	8	6	14	SD(5)⇒PD
LAU 371	33	6	4	10	PD
LAU 444	33	12	1	13	SD(12)⇒PD
LAU 618[b]	74	13	1.5	14.5	SD(6)⇒PD
LAU 672	38	4	5.5	9.5	PD
LAU 701[b]	72	9[c]	1	10	PD
LAU 944	28	9	1	10	NED

[a] Abbreviations: PD, progressive disease; SD, stable disease; NED, no evidence of disease.
[b] Patients with tumor samples studied.
[c] Last vaccination was without CpG.

Materials and Methods

Study subjects and samples

Seven patients who received various numbers (between 4 and 13) of peptide/IFA/CpG vaccinations as previously described (13) and responded well by displaying a notable Melan-A-specific T cell expansion were selected. Briefly, HLA-A2$^+$ patients with histologically proven metastatic melanoma of the skin expressing Melan-A/MART-1 (RT-PCR or immunohistochemistry) were enrolled in a phase I trial of the Ludwig Institute for Cancer Research to receive monthly vaccinations s.c. composed of 500 μg of CPG 7909, 100 μg of Melan-A analog peptide, and 300 μl of Montanide ISA-51. Samples from HLA-A2$^+$ nonvaccinated melanoma patients were obtained from volunteers attending the clinic. The relevant local Institutional Review Boards and Ethics Committees approved this study (LUD 00-018) sponsored by the Ludwig Institute of Cancer Research. Blood samples were generally used fresh within 4 h (for optimal functional assessment of the lymphocytes), or PBMC were separated from heparinized blood and cryopreserved for subsequent studies. Two nonlymphoid tissue metastasis were also obtained from patients LAU 618 and 701; tumor-infiltrating lymphocytes (TIL)[3] were mechanically extracted from the tissue and studied in parallel with the blood.

Reagents and flow cytometry

HLA-peptide tetrameric complexes ("tetramers") were produced as previously described (15) and included the following MHC peptides: A2 melan-A ELAGIGILTV, CMV pp65-NLVPMVATV, EBV BMLF1-GLCTLVAML and flu matrix-GILGFVFTL, B7 CMV pp65-TPRVTGG GAM and B8 EBV BZLF1-RAKFKQLL. Anti-FoxP3-FITC Abs were purchased from eBioscience and used according to the manufacturer's recommendations. All other Abs were purchased from BD Pharmingen. Analysis was usually performed directly from whole blood as previously described (14). Samples were analyzed on a BD Biosciences FACSCalibur or CANTO, after compensation was checked using freshly stained PBMC.

Intracellular IFN-γ staining and CD107a mobilization assay

Whole blood (400 μl) or thawed cryopreserved PBMC were stained before activation with tetrameric complexes for 15 min at 37°C. Cells were subsequently incubated with specific Ags at 10 μM final concentration, and with anti-CD107a Abs (7 μl) in RPMI 1640/10% FCS and left for 6 h at 37°C. Brefeldin A (Sigma-Aldrich; at 10 μg/ml final concentration) and Monensin (Sigma-Aldrich; at 2 μM final concentration) were added during the second hour of incubation. Nonactivated PBMC 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 (BD Biosciences) for 10 min. After washing, staining was performed for 15 min at room temperature in the dark using a panel of PerCP- or allophycocyanin-conjugated Abs. Cells were then washed and stored in 5% formaldehyde at 4°C until analysis. Comparative analysis of the optimized ELA (ELAGIGILTV)-natural EAA (EAAGIGILTV) peptides were performed without tetramers. Staphylococcal enterotoxin B (5 μg/ml) (Sigma-Aldrich) was used as positive control.

Molecular analysis of tetramer-positive cells

TCR analysis was performed on FACS-sorted tetramer-positive cells as previously described (16). Briefly, CD8$^+$ T cells from both blood and tumor samples 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 (BD Biosciences), followed by cDNA preparation and amplification (17). TCR BV17 spectratyping was performed on single five-cell aliquots or (10) pools of amplified cDNA: PCR were run using fluorescent-probe labeled primers specific for the BV17 subfamily and for the BC chain (18). 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.

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

Tetramer$^+$BV17$^+$CD8$^+$ T cells were sorted by flow cytometry, cloned by limiting dilution, and expanded with PHA and allogenic irradiated feeder cells in medium containing 150 U/ml human recombinant IL-2. Subsequently, they were periodically (every 3–4 wk) restimulated with PHA, irradiated feeder cells, and human recombinant IL-2. Clones were tested by tetramer and BV17 staining, and then for CDR3 region length and for TCR sequence. Chromium release experiments were performed as previously described. Target cells (1000 cells/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 (19).

Immunohistochemistry

For Melan-A staining, paraffin sections were stained with the anti-Melan-A Ab A103 (20) using an avidin-biotin peroxidase system following citrate buffer (pH 6.0) Ag retrieval. Diaminobenzidine was used as chromogen. Sections were counterstained with hematoxylin. For double-labeling CD3/FoxP3, sections were incubated with the goat polyclonal Ab against the C terminus of the FoxP3 protein (ab2481; dilution, 1/50; Abcam), followed by rabbit anti-goat Ab and the EnVision Peroxidase kit (DakoCytomation). The slides were then incubated with the second Ab against CD3 (clone UCHT1; 1/25; DakoCytomation), and the alkaline phosphatase anti-alkaline phosphatase method was used for detection.

Results

Similar frequency, phenotype, and functional competence of vaccine-driven Melan-A-specific CD8$^+$ T cells and virus-specific CD8$^+$ T cells

For the present study, blood samples from seven vaccinated patients were obtained at least 1 mo after the donor received his/her last vaccine (Table I). At that time, Melan-A-specific cells represented a relatively stable population, characterized by a resting "memory" phenotype (CD38^{low}Bcl-2^{high}) (data not shown) as observed with virus-specific CD8$^+$ T cells (21). In most vaccinated melanoma patients, the frequency of Melan-A-specific CD8$^+$ T cells attained relatively high levels, comparable with those of CD8$^+$ T cells specific for viruses, in particular CMV and EBV (Fig. 1*a*). Analysis of the cell surface differentiation markers CD28, CD27, and CD57 revealed that these cells had differentiated, and included highly differentiated cells (i.e., CD28$^-$

[3] Abbreviation used in this paper: TIL, tumor-infiltrating lymphocyte.

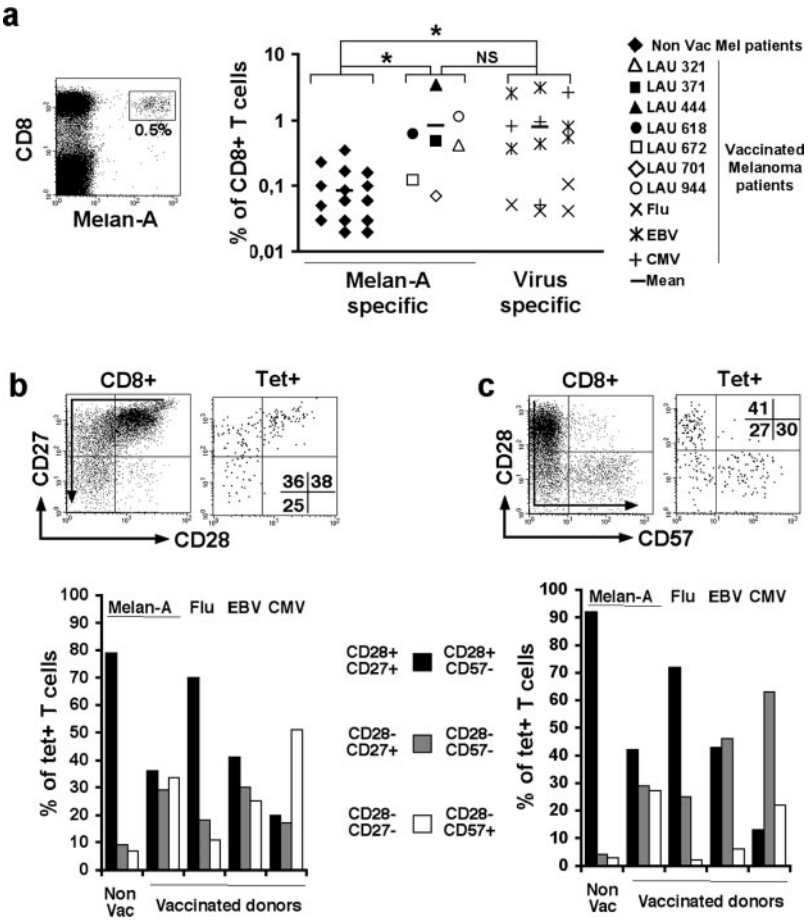

FIGURE 1. Similar frequency and phenotype of vaccine-driven Melan-A-specific and virus-specific CD8$^+$ T cells. *a*, Percentages of tetramer- (Melan-A, EBV, CMV, or Flu) positive cells in the CD8$^+$ T cell population identified ex vivo in vaccinated individuals. Frequencies of Melan-A tetramer staining cells in nonvaccinated melanoma patients ($n = 15$) are shown for comparison. One representative staining as well as data for each patient are shown. Statistics are calculated in between the different Ag-specific CD8$^+$ T cell populations. *, $p = 0.001$; NS, nonsignificant with the nonparametric Mann-Whitney *U* test. *b* and *c*, Distribution of tetramer-positive cells along CD8$^+$ T cell differentiation using the cell surface markers CD28/CD27 (*b*) and CD28/CD57 (*c*). One representative staining as well as mean values for Melan-A-specific CD8$^+$ T cells in nonvaccinated melanoma patients (Non Vac, $n = 15$), and Melan-A- ($n = 7$), EBV- ($n = 6$), CMV- ($n = 4$), or Flu- ($n = 4$) specific CD8$^+$ T cells in vaccinated melanoma patients are shown. The arrow in the whole CD8$^+$ T cell population dot plot illustrates the pathway of differentiation. Percentages of cells present in quadrants are shown. Virus-specific CD8$^+$ T cells displayed the usual patterns of differentiation relative to their viral specificity (12).

CD27$^-$CD57$^+$), in contrast to nonvaccinated melanoma patients (Fig. 1, *b* and *c*). Overall, the phenotype of these cells resembled that of virus-specific CD8$^+$ T cells, in particular those specific for CMV and EBV, rather than influenza. The immune control of EBV and CMV is highly dependent on cellular immunity, contrary to influenza, which is more dependent on humoral immunity. Therefore, the possibility for a cancer vaccine to drive tumor-specific T cells to display phenotype similar to that of cells specific for these two persistent viruses may be an encouraging sign of efficacy and suggests a strong level of stimulation (22, 23).

We next analyzed the lytic factors perforin, granzymes A and B, whose expression provides information regarding the cytotoxic potential of the cells. In contrast to Melan-A-specific CD8$^+$ T cells from melanoma patients that lack expression of these molecules (2), Melan-A-specific CD8$^+$ T cells from all vaccinated patients emerged as highly armed cytotoxic T cells, expressing at least as much cytolytic factors as virus-specific CD8$^+$ T cells from the same patients (Fig. 2*a*). Of note, Melan-A-specific CD8$^+$ T cells seemed to express even higher levels of perforin. Higher granzyme A expression than granzyme B is in line with the observation that granzyme A is produced earlier along the pathway of differentiation than granzyme B (12). To assess the capacity to release their cytotoxic factors, we analyzed the ex vivo appearance of CD107a on the cell surface, which occurs as CTL liberate the content of their lytic granules upon Ag-mediated activation (24). CD107a staining was combined with intracellular IFN-γ staining as another means to assess the functional capacity of the cells. Antigenic triggering of the Melan-A-specific CD8$^+$ T cells from vaccinated patients resulted in CD107a appearance, and a subsequent production of IFN-γ (Fig. 2*b*). Overall, vaccine-driven Melan-A-specific CD8$^+$ T cells displayed a functional pattern comparable with virus-specific CD8$^+$ T cells, with >50% of them being CD107a$^+$ functionally responsive, including two-thirds on average also staining positive for IFN-γ (data not shown). The vaccine-driven T cells presented good functional capacities when stimulated with the low-affinity natural melanoma peptide EAA (>50% responded upon stimulation with EAA), even though the responsiveness to the optimized ELA peptide was higher, as expected (Fig. 2*c*). Melan-A-specific CD8$^+$ T cells were therefore successfully boosted in the circulation using a peptide vaccine with CpG as adjuvant, to display frequencies, phenotypes, and functional capacities equivalent to those of protective virus-specific CD8$^+$ T cells.

Infiltration and activation of Melan-A-specific CD8$^+$ T cells in vaccinated patient metastasis

However, despite the strong immunological response and the similarity between vaccine-driven antitumor and natural antiviral CTL, tumor development and spreading did not seem to be successfully inhibited: in our small series, some patients exhibited stabilization of disease for some time but eventually most developed progressive disease (Table I). To get insight into the discrepancy between successful T cell boosting and clinical outcome, we attempted to study Melan-A-specific CD8$^+$ T cells from tumor samples. This represents a particularly difficult challenge, due to limitations associated with accessibility to growing tumors by surgery, and the presence of TIL to be studied in the retrieved material. Of the seven vaccinated patients, we could biopsy nonlymphoid s.c. metastasis from two patients (LAU 618 and 701), during the course of disease progression, from which Melan-A-specific

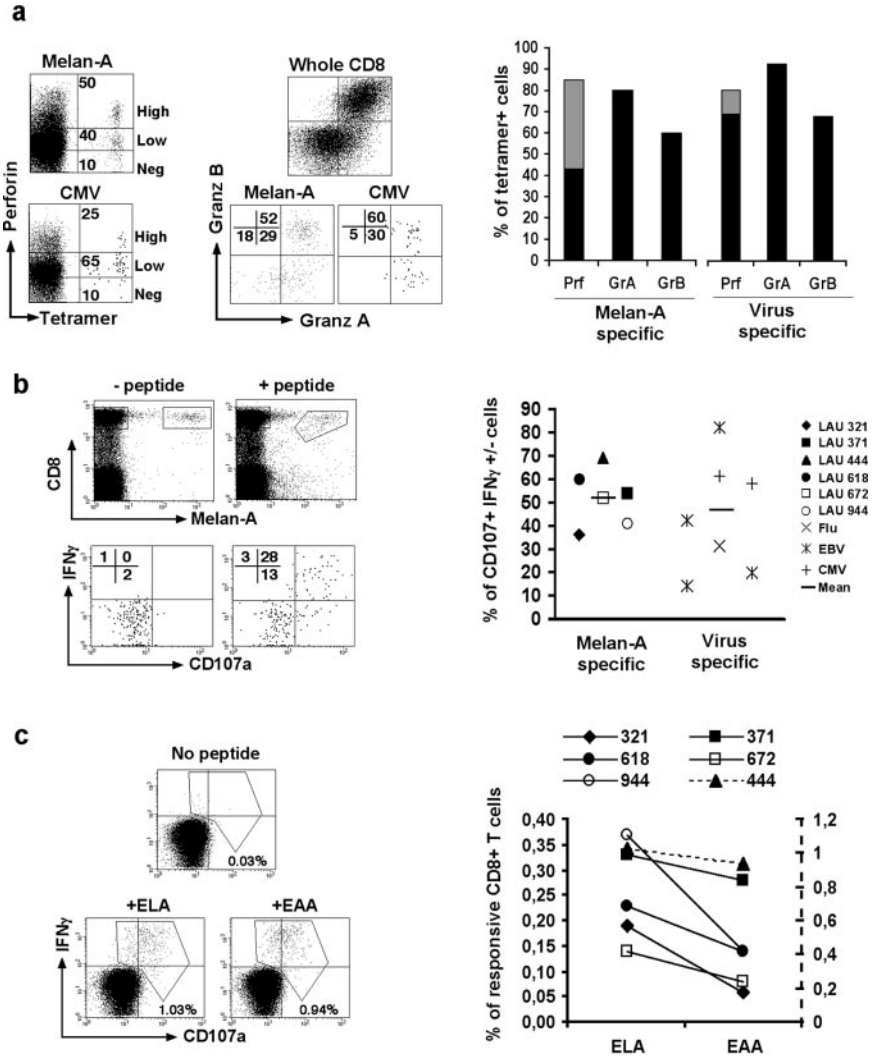

FIGURE 2. Vaccine-driven Melan-A-specific CTL display potent effector functions. *a*, Expression of the cytotoxic factors perforin, granzymes A and B by Melan-A-specific CD8$^+$ T cells compared with virus-specific CD8$^+$ T cells in vaccinated patients. One representative staining (patient LAU 618) as well as mean values for Melan-A-specific CD8$^+$ T cells ($n = 6$), and EBV- or CMV-specific CD8$^+$ T cells ($n = 5$) in vaccinated melanoma patients are shown. Cells are gated on the CD8$^+$ T cell population for perforin expression (separated in three intensities as previously described (25); in the histogram, the gray bars correspond to high expression). Expression of granzymes A and B is shown in tetramer-positive gated cells. Numbers indicate percentages of tetramer-positive cells present in the different areas. *b*, Representative staining of CD107a mobilization and IFN-γ secretion by vaccine-driven Melan-A-specific CD8$^+$ T cells following activation with the cognate peptide for 6 h (patient LAU 944), and comparison of responsiveness (CD107a mobilization with or without IFN-γ secretion) to activation (with specific peptide) between Melan-A-specific CD8$^+$ T cells and virus-specific CD8$^+$ T cells from vaccinated patients. *c*, Functional responsiveness (CD107a mobilization) to stimulation with the optimized peptide analog ELA or the natural peptide EAA. One representative staining for CD107a and IFN-γ (patient LAU 444) as well as values for all vaccinated patients are shown (values for LAU 444 are on the *right* *y*-axis to accommodate the scale). Functionality of Melan-A-specific CD8$^+$ T cells from patient LAU 701 was not assessed due to the too low frequency of this population to enable satisfactory readouts.

TIL populations could be detected ex vivo and analyzed in comparison with peripheral blood counterpart (Fig. 3*a*). These cells were all Ag-primed cells, based on their phenotype as CD45RA$^-$

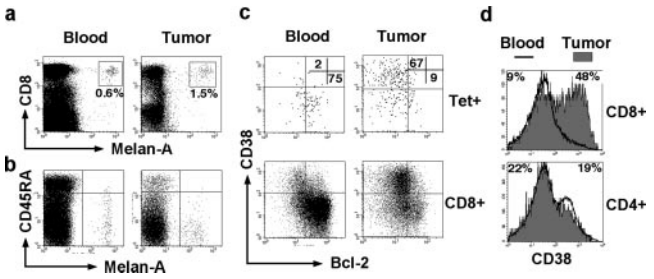

FIGURE 3. Ex vivo detection of activated tumor-infiltrating Melan-A-specific lymphocytes in a Melan-A-expressing s.c. metastasis. *a* and *b*, Identification of tumor-infiltrating Melan-A-specific CD8$^+$ T cells with tetramers (*a*), and expression of CD45RA on CD8$^+$ T cells (*b*) in patient LAU 618. Numbers show percentages of tetramer-positive cells in the CD8$^+$ T cells population. *c*, Expression of the activation marker CD38 and the antiapoptotic marker Bcl-2 on Melan-A-specific CD8$^+$ T cells as well as whole CD8$^+$ T cells. Percentages of cells present in the different areas are shown. *d*, Expression of CD38 on whole CD8$^+$ or CD4$^+$ T cells comparing blood and tumor. Percentages of CD38^{high} cells are indicated (on the *left side* for circulating cells, on the *right side* for tumor-infiltrating cells). Similar observations were done with LAU 701.

(Fig. 3*b*). Interestingly, in contrast to blood cells, these TIL displayed a phenotype of highly activated cells (CD38^{high}Bcl-2^{low}) (Fig. 3*c*), resembling virus-specific cells during strong viral replication periods (21, 25). This is the likely consequence of Ag-mediated stimulation related to the strong expression of Melan-A by the tumor cells, as revealed by immunohistochemistry staining on a section of the same metastatic lesion (data not shown). Of note, a large proportion of the whole CD8$^+$ T cells present in the tumor was actually highly activated, in contrast with the whole CD4$^+$ T cells, which did not display any significant differences in terms of activation between blood and tumor (Fig. 3*d*).

Using anti-TCR BV chain Abs, we identified in LAU 618 blood a significant Melan-A-specific CD8$^+$ T cell subpopulation that had particularly differentiated following vaccination, characterized by BV17 expression (Fig. 4*a*). Cell sorting of the Melan-A tetramer-positive CD8$^+$ T cell subpopulation and subsequent spectratyping analysis indicated that the BV17 subpopulation was oligoclonal, mostly constituted by cells harboring a TCR with a single CDR3 length (161 aa) (Fig. 4*b*). Sequencing of the CDR3 region revealed that this dominant population represented one single clonotype (i.e., characterized by a unique CDR3 sequence). This sequence was found in 17 of 20 samples (each of five-cell tetramer-positive sorted cells), confirming the immunodominance of this clonotype in the tetramer-positive population (data not shown). In addition, all CTL clones obtained by tetramer$^+$BV17$^+$ single-cell sorting

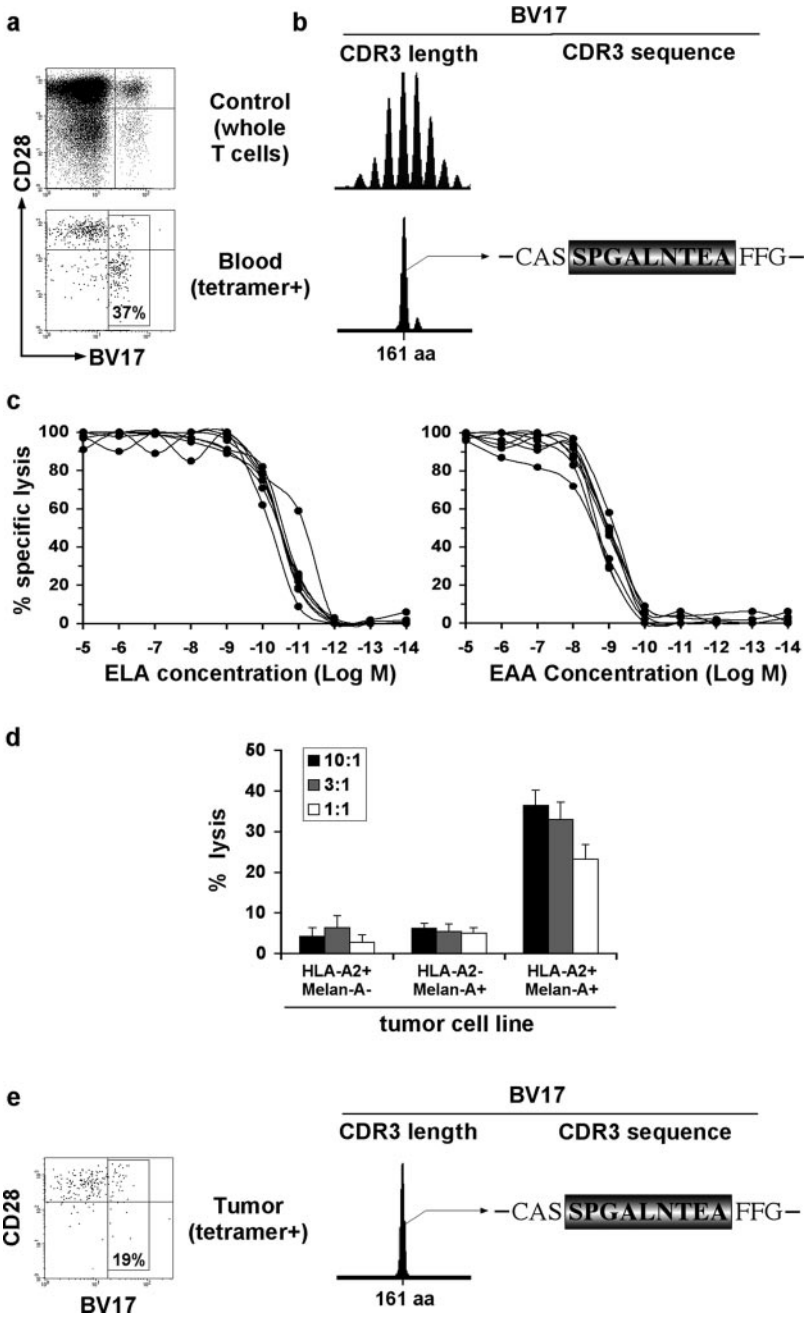

FIGURE 4. Infiltration of a vaccine-driven tumor-specific CD8$^+$ T cell clone in LAU 618 metastasis. *a*, Expression of BV17 and the differentiation marker CD28 on circulating Melan-A-specific CD8$^+$ T cells. Percentage of BV17$^+$ cells within the tetramer-positive population is shown. *b*, TCR BV17 spectratypic analysis of whole T lymphocytes (polyclonal population characterized by a Gaussian CDR3 length distribution) and tetramer-sorted circulating Melan-A-specific CD8$^+$ T cells (oligoclonal population), and CDR3 region sequence of the dominant clonotype. *c* and *d*, Melan-A-specific CTL clones with SPGALNTEA TCR were generated by FACS sorting of BV17$^+$tetramer$^+$ T cells and subsequent in vitro expansion. Recognition efficacy for the Melan-A peptides ELA or EAA was tested for eight SPGALNTEA clones by cytotoxicity against T2 cells in the presence of peptide titrations (*c*). Tumor recognition was tested for SPGALNTEA clones by cytotoxicity against Melan-A-expressing HLA-A2$^+$ melanoma cell lines at different E:T ratios. Melan-A$^-$HLA-A2$^+$ or Melan-A$^+$HLA-A2$^-$ melanoma cell lines were used as controls. Data are means with SDs for four clones (*d*). *e*, Expression of BV17 and CD28 on Melan-A-specific CD8$^+$ TIL, and TCR BV17 spectratypic analysis and CDR3 region sequence of tetramer-sorted Melan-A-specific CD8$^+$ TIL.

and subsequent T cell cloning (i.e., in vitro expansion) appeared to be the same clonotype (20 CTL clones were tested for CDR3 length and 10 for CDR3 sequence). One could speculate that this clonotype was selectively expanded from a pool of cells with large TCR diversity after vaccination due to its high avidity of Ag recognition. Avidity is likely to have a major influence in the selection of dominant clones as recently shown by Price et al. (26), with the highest avidity clonotypes becoming dominant in viral infection. Due to their high avidity, certain clonotypes may then be more effective in recognizing and destroying tumor cells. As assessed using a series of the cultured CTL clones in cytolytic assays with peptide-pulsed T2 cells as targets, this clonotype had a good recognition efficacy for both the low-affinity natural melanoma peptide EAA and the optimized ELA peptide (although better for ELA, in keeping with the ex vivo functional data) (Fig. 4*c*). In addition, it was able to recognize the tumor (i.e., naturally expressing melanoma cell lines) (Fig. 4*d*). Importantly, a significant BV17$^+$ Melan-A-specific CD8$^+$ T cell population, expressing the same TCR as the circulating dominant BV17$^+$ population, was also detected among the tumor-infiltrated T cells (Fig. 4*e*). Overall, these observations indicate that vaccination resulted in the expansion and differentiation of circulating Melan-A-specific CD8$^+$ T cells, able to recognize Melan-A-expressing tumor, which could infiltrate the metastasis where they became activated.

Infiltration of regulatory T cells in metastasis and alteration of Melan-A-specific TIL function

A potential reason for continuous tumor growth despite the detection of Melan-A-specific TIL may relate to the escape of tumors from CTL recognition (27). In LAU 618 metastasis, although ~80% of the tumor showed Melan-A Ag expression, we could indeed observe some tumor cells with undetectable Melan-A Ag (Fig. 5), which may therefore not be recognized by the vaccine-driven T cells.

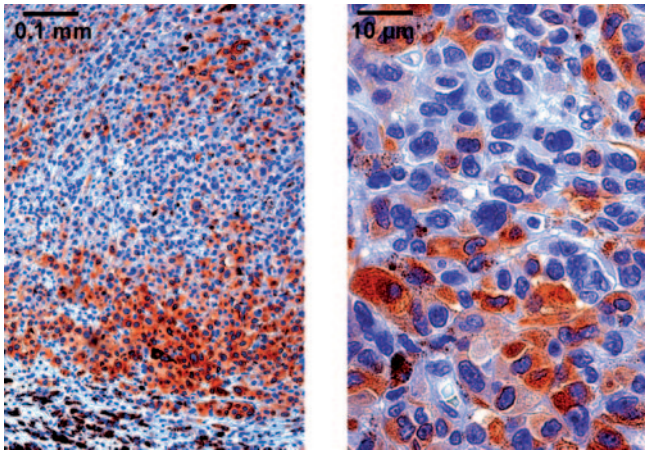

FIGURE 5. Loss of Melan-A Ag expression by tumor cells. Melan-A expression (in reddish brown) in a paraffin-embedded section of LAU 618 resected metastasis at two different magnifications. Example of region with undetectable Melan-A Ag expression by tumor cells.

In addition, ex vivo functional analysis, similar as those performed on whole blood, revealed noticeable differences in terms of functional status for the Melan-A-specific TIL compared with their blood counterparts. Melan-A-specific TIL showed a less differentiated phenotype, mostly $CD28^+CD57^-$ (Fig. 6*a*), and they expressed lower levels of the cytotoxic factors perforin, granzymes A and B (Fig. 6*b*). In addition, they failed to secrete IFN-γ upon activation (Fig. 6*c*). Even though tumor-infiltrating Melan-A-specific $CD8^+$ T cells were not completely tolerant because they could respond to Ag stimulation and degranulate (i.e., became $CD107a^+$), they showed overall a suboptimal functional status compared with the vaccine-driven circulating Melan-A-specific CTL, which may render them incompetent to destroy tumor cells, even when expressing Melan-A.

The tumor environment is thought to present conditions favoring tumor immune tolerance (28), and increasing evidence, in particular from mice studies, has drawn attention to the influence of $CD4^+$ regulatory T cells on $CD8^+$ T cell function in anticancer responses (29–31). We therefore examined the presence of regulatory T cells in the metastasis studied. For this purpose, we assessed the expression of the transcription factor FoxP3, currently considered as the most appropriated marker to define regulatory T cells (32–34). Intracellular FoxP3 expression was observed in T cell-infiltrated areas of lesion sections from vaccinated patients by immunohistochemistry (Fig. 7*a*). Furthermore, direct flow cytometry analysis revealed increased proportions of $CD25^+FoxP3^+$ $CD4^+$ T cells in the tumor compared with the blood (Fig. 7*b*). Interestingly, although there were no significant differences in terms of activation (i.e., CD38 expression) when comparing whole $CD4^+$ T cell populations in the tumor and in the blood (Fig. 3*e*), careful gating on the regulatory T cells subset showed that these cells were significantly activated in the tumor (Fig. 7*c*). Taken together, these data demonstrate an increased activity of the regulatory T cells within the s.c. metastasis, coinciding with the observation of suboptimal $CD8^+$ T cell function in this compartment.

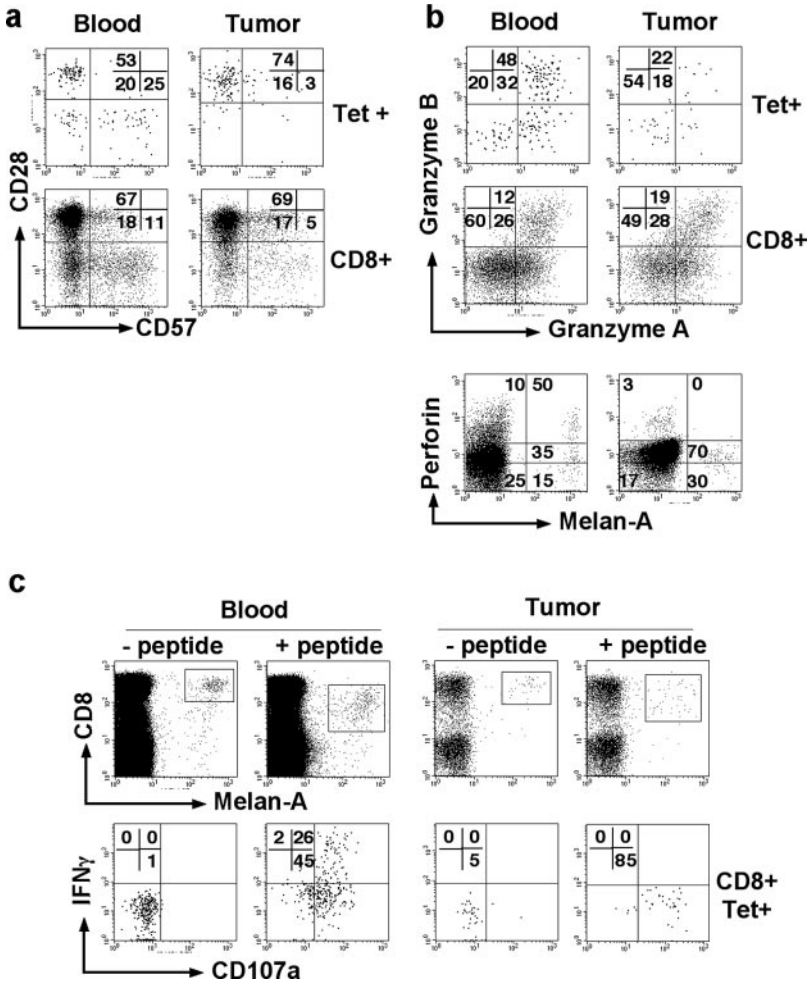

FIGURE 6. Tumor-infiltrating Melan-A-specific lymphocytes differ in functional status compared with circulating Melan-A-specific CTL. *a* and *b*, Expression of the differentiation markers CD57 and CD28 (*a*), and the cytotoxic factors perforin, granzymes A and B (*b*) in Melan-A-specific $CD8^+$ T cells from the tumor compared with the blood (patient LAU 618). Cells are gated on the $CD8^+$ T cell population or directly on tetramer-positive cells. Similar observations were done with LAU 701. *c*, CD107a mobilization and IFN-γ secretion by Melan-A tetramer-positive cells following activation with cognate peptide. Percentages of cells present in the different areas are shown.

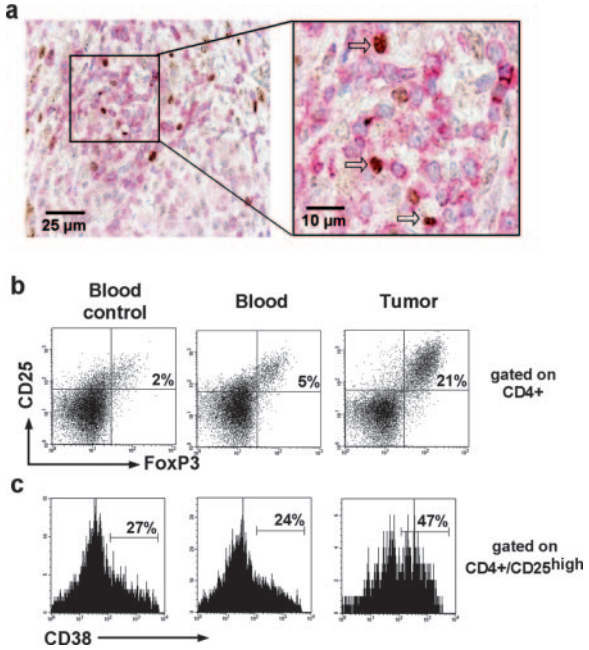

FIGURE 7. Increased regulatory T cell activity in the tumor. *a*, Costaining for CD3 (red, membranous) and FoxP3 (brown, nuclear) by immunohistochemistry on the lesion from LAU618. $CD3^+FoxP3^+$ cells are indicated with arrows. *b*, FoxP3 and CD25 costaining on $CD4^+$ T cells from the blood or the tumor of LAU 618. Blood control from a nonmelanoma donor is shown in comparison. Percentages of $FoxP3^+CD25^+$ cells are indicated. *c*, Expression of the activation marker CD38 on regulatory T cells. Percentages of $CD38^+$ cells are indicated. Similar observations were done with LAU 701.

Discussion

In the present study, we report the successful induction, in peptide/CpG-vaccinated melanoma patients, of circulating Melan-A-specific CTL with ex vivo phenotypic characteristics and functional capacities apparently similar to those of natural virus-specific CTL. This represents a significant immunological achievement, considering that boosting of potent Ag-specific $CD8^+$ T cells with a synthetic vaccine in the hope to control tumor growth or viral spreading is a long-sought objective. In addition, the study of two vaccinated patient metastases indicated that vaccine-driven Melan-A-specific $CD8^+$ T cells were able to infiltrate the tumor, where they appeared to be activated. Interestingly, a significant proportion of the whole $CD8^+$ T cell population found in the tumor was activated. Although bystander activation related to tumor environment cannot be totally ruled out, this seems unlikely because the whole $CD4^+$ T cell population did not exhibit a similar feature. Instead, it is possible that these activated $CD8^+$ T cells may all be Ag-stimulated tumor-reactive cells, which is in line with a recent study suggesting that tumor-specific cells could represent as much as 40% of $CD8^+$ T cells in a metastasis (35).

Nonetheless, despite the general assumption that induction of potent tumor-specific $CD8^+$ T cells with a vaccine should be sufficient to control tumor development, and the strong $CD8^+$ T cell activity observed in the tumor site, the clinical benefit in the small series we studied was rather limited. Additional parameters, besides the detection of functional T cells in the circulation, may have to be considered to generate effective T cell vaccines in this context. Even though we observed that at least a part of the vaccine-driven cells were able to recognize the natural Melan-A epitope and tumor cell lines, more in-depth studies of TCR avidity and tumor recognition may be needed; these factors are central for the efficacy of a response, because vaccines may fail to drive optimum high-avidity T cell response as recently suggested (36). Then, T cell numbers may be crucial: reaching levels equivalent to those of virus-specific T cells may still not be sufficient in the context of cancer. In addition, the escape of tumors from CTL recognition through the loss of Ag expression, as observed in one metastasis, may lessen even further the potential efficacy of tumor-specific $CD8^+$ T cells, despite successful vaccination. Induction of potent CTL responses recognizing a broad number of tumor Ags may therefore be necessary to tackle this problem; we are currently investigating the effects of CpG-based multiepitope vaccines. Last, advanced stage IIIB-IV disease may not be the ideal target for tumor vaccination, because bulky disease may actively inhibit the activity of TIL and is known to be difficult to eradicate by immune based therapy only.

In the present report, we have focused our investigation at the level of the tumor site in two vaccinated patients. Despite the encouraging observations that 1) Ag-specific $CD8^+$ T cells did migrate to tumor sites and 2) tumor-specific TIL could be responsive to antigenic stimulation, these cells still exhibited functional discrepancies compared with their peripheral blood counterpart. These observations are in line with previous findings made in nonvaccinated cancer patients, of less differentiated (37) and less functional TIL (38). These functional discrepancies may account for a lack of efficacy within the metastasis and failure to stop tumor growth. Inducing antitumor activity in the circulation may be insufficient, and it is necessary to boost T cell functionality directly at the tumor site. However, increasing evidence suggests that a strong phenomenon of immune tolerance takes place within the tumor (28). Among several possible tolerizing factors (e.g., suppressive dendritic cells, expression of indoleamine 2,3-dioxygenase (39)), the presence of regulatory T cells may have a major influence, because they are known to hamper effective T cell functionality. We have observed a significant infiltration and increased activity of $FoxP3^+CD25^+CD4^+$ T cells within the two s.c. metastasis studied. Practical limitations (i.e., access to sufficient biopsied material) did not allow us to examine directly the suppressive effect of these cells on the anti-Melan-A T cell response. However, the coexpression of FoxP3 and CD25 on $CD4^+$ T cells has been defined as the most appropriate marker of regulatory T cells currently available (40). Our observation is in keeping with the increased presence of these cells in metastatic melanoma lymph nodes as reported previously (41). Data from mouse models have shown that regulatory T cells can impair antitumor T cell responses (30), through a TGF-β-dependent mechanism (31), thereby promoting cancer growth. In another mouse model study, Zhou et al. (42) have recently reported the induction of T regulatory cells following recognition of tumor Ags, a phenomenon that may even be amplified by therapeutic vaccination. In humans, increased numbers of regulatory T cells within tumors have been associated with continuous tumor growth and reduced survival (43). The presence of tumor-induced regulatory T cells may therefore prevent the benefit of successful T cell vaccination, by rendering vaccine-induced TIL stunned or ineffective, thus apparently tolerant to the tumor. This is not dissimilar to observations made in hepatitis C virus infection, with reports of stunned hepatitis C virus-specific $CD8^+$ T cells associated with increased regulatory T cell activity (44).

In conclusion, we now hold powerful tools to efficiently boost functionally active Ag-specific CTL in the circulation. However, this does not seem to be sufficient to lead to potent T cells within tumors and to profoundly change the clinical course of advanced

stage melanoma patients. The assumption that the sole induction of potent circulating CTL with a vaccine would be the key of cancer immunotherapy may need revision. This is in line with observations from other fields of investigation, like HIV immunology, where CTL induction has had only a limited, if any, impact on the final clinical outcome (45, 46). Much work is still needed to dissect the mechanisms involved in the regulation of antitumor T cell responses and the prevention of tumor tolerance to establish protective immunity. The induction of an effective antitumor immunity will be based on a combinatorial strategy, depending not only on our ability to boost T cells but also on our capacity to subvert immune-tolerizing conditions within the tumor, in particular by blocking the activity of T regulatory subpopulations (e.g., using anti-CD25, CTLA-4, or CCL22 Abs, or Denileukin diftitox) (28). In the battle against cancer, most likely, the final fight will have to be won at the level of the tumor.

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Disclosures

A. M. Krieg is an employee of Coley Pharmaceutical Group and holds shares in the company.

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