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# Effector Function of Human Tumor-Specific CD8 T Cells in Melanoma Lesions: A State of Local Functional Tolerance

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## ABSTRACT

**Although tumor-specific CD8 T-cell responses often develop in cancer patients, they rarely result in tumor eradication. We aimed at studying directly the functional efficacy of tumor-specific CD8 T cells at the site of immune attack. Tumor lesions in lymphoid and nonlymphoid tissues (metastatic lymph nodes and soft tissue/visceral metastases, respectively) were collected from stage III/IV melanoma patients and investigated for the presence and function of CD8 T cells specific for the tumor differentiation antigen Melan-A/MART-1. Comparative analysis was conducted with peripheral blood T cells. We provide evidence that *in vivo*-priming selects, within the available naive Melan-A/MART-1-specific CD8 T-cell repertoire, cells with high T-cell receptor avidity that can efficiently kill melanoma cells *in vitro*. *In vivo*, primed Melan-A/MART-1-specific CD8 T cells accumulate at high frequency in both lymphoid and nonlymphoid tumor lesions. Unexpectedly, however, whereas primed Melan-A/MART-1-specific CD8 T cells that circulate in the blood display robust inflammatory and cytotoxic functions, those that reside in tumor lesions (particularly in metastatic lymph nodes) are functionally tolerant. We show that both the lymph node and the tumor environments blunt T-cell effector functions and offer a rationale for the failure of tumor-specific responses to effectively counter tumor progression.**

## INTRODUCTION

Naive CD8 T cells constantly travel from the blood to secondary lymphoid organs whereas dendritic cells capture antigens in nonlymphoid peripheral tissues, migrate via afferent lymphatics to lymphoid organs, and present processed antigenic peptides to the naive T cells. After appropriate activation, antigen-primed T cells undergo proliferation and differentiation (1, 2). Their progeny includes effector T cells, which gain the ability to migrate to peripheral tissues and display immediate effector function to contain invasive pathogens or cancer cells, as well as memory cells, which travel through secondary lymphoid organs and can generate a new wave of effector cells after re-encounter with antigen. At the site of immune attack, the functions exerted by effector CD8 T cells include the release of cytokines to mediate local inflammation (3) and deposition of cytotoxic granules at the vicinity of target-cell membranes to induce target-cell apoptosis (4, 5). In human cancer, a state of immune tolerance has been documented for both T-cell activation and function (6–8). However, current knowledge of the *in vivo* functions of human tumor antigen-specific T cells is mainly restricted to peripheral blood T cells and is largely inferred from the expression of phenotypic markers (9–13). Studies on the functional activities of tumor antigen-specific T cells derived from human tumor lesions have been rarely performed and are in their infancy. Intriguingly, despite the coexistence of potentially tumor-reactive T cells and growing tumor cells, previous work on the efficacy of antitumor responses suggests adequate effector functions of T cells *ex vivo* and after *in vitro* stimulation in metastatic lymph nodes (LNs; Refs. 11, 14, 15).

In this study, we present a comprehensive analysis of the function of CD8 T cells directed against the tumor differentiation antigen Melan-A/MART-1 (hereafter, Melan-A) in the following three distinct body compartments: peripheral blood, metastatic LNs, and soft tissue/visceral metastases (referred to as nonlymphoid tissue metastases). Tissue samples were collected from a series of 61 stage III/IV HLA-A2 melanoma patients. We have chosen Melan-A as a model antigen because (*a*) Melan-A is expressed in the majority of HLA-A2 melanoma patients (16, 17), (*b*) immunological ignorance to Melan-A is overcome in the majority of these patients (14), and (*c*) high frequencies of Melan-A-specific T cells are readily detectable *ex vivo* (18, 19). We analyzed inflammatory and cytotoxic responses of Melan-A-specific CD8 T cells and compared them with those of T cells that control spreading of cytomegalovirus (CMV) infection in immune competent individuals. We provide evidence that Melan-A-specific CD8 T cells with high T-cell receptor (TCR) avidity are triggered *in vivo* and that they efficiently accumulate in tumor lesions (both within the lymphoid and nonlymphoid compartments) in the majority of melanoma patients. We show, however, that the tumor antigen-specific T cells in tumor lesions lack major T-cell effector functions (*i.e.*, are functionally tolerant). Our findings highlight the importance of the microenvironment in shielding tumor cells from T-cell immune attack.

## MATERIALS AND METHODS

**Tissues and Cells.** Peripheral blood, metastatic LNs, control LNs as diagnosed tumor free by pathological examination, and nonlymphoid tissue metastases were obtained from a total of 61 HLA-A2 melanoma patients; clinical characteristics appear in Table 1. Informed consent was obtained from all patients. The study and treatment of patients were approved by the ethical committee of the Medical Faculty, University of Lausanne, and the Ludwig Institute for Cancer Research. Thirty-eight HLA-A2 healthy subjects were blood donors at the blood transfusion center in Lausanne, Switzerland, or in Greifswald, Germany. Mononuclear cells were purified and immediately frozen as described previously (14).

**Major Histocompatibility Complex/Peptide Multimers and Monoclonal Antibodies (mAbs).** Phycoerythrin- or allophycocyanin-labeled HLA-A2/peptide multimers (14) were synthesized around Melan-$A_{26–35}$ A27L analog

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Table 1 *Clinical characteristics of melanoma patients*

| | Patient | Sex | Age | Years since primary[a] | Stage[b] | Site of metastasis | Prior therapy | Specificity of T-cell response analysed |
|---|---|---|---|---|---|---|---|---|
| 1 | BRU 1 | F | 57 | 23 | III | LN[c] | Surgery, vaccination | CMV |
| 2 | BRU 3 | F | 55 | 6 | IV | LN, lung, spleen | Surgery, vaccination | CMV |
| 3 | BRU 4 | M | 31 | 3 | III | LN | Surgery, chemotherapy, vaccination | CMV |
| 4 | BRU 6 | M | 17 | 4 | IV | LN, liver, lung | Surgery, IFN-$\alpha$, vaccination | CMV |
| 5 | GE 3 | M | 57 | 1 | IV | LN, liver, lung | Surgery, vaccination | CMV |
| 6 | GE 4 | M | 55 | 0.5 | IV | LN, lung, skin | Surgery, vaccination | CMV |
| 7 | GE 5 | M | 55 | 0.5 | IV | LN, intestine | Surgery | CMV |
| 8 | LAU 18 | F | 73 | 3 | III | LN | Surgery, isolated limb perfusion | Melan-A |
| 9 | LAU 50 | M | 65 | 2 | III | LN | Surgery, chemotherapy, isolated limb perfusion | Melan-A |
| 10 | LAU 56 | M | 41 | 10 | III | LN | Surgery, chemotherapy, isolated limb perfusion | Melan-A |
| 11 | LAU 97 | M | 59 | 5 | III | LN | Surgery, chemotherapy, IFN-$\alpha$ | Melan-A,CMV |
| 12 | LAU 119 | F | 73 | 5 | III | LN | Surgery | Melan-A |
| 13 | LAU 132 | M | 33 | 4 | IV | LN, skin | Surgery, IFN-$\alpha$, vaccination | Melan-A,CMV |
| 14 | LAU 155 | M | 56 | 5 | IV | LN, liver | Surgery, chemotherapy, IFN-$\alpha$ | Melan-A,CMV |
| 15 | LAU 156 | M | 46 | 17 | IV | LN, liver, lung | Surgery | Melan-A,CMV |
| 16 | LAU 169 | M | 52 | 0.5 | IV | LN, skin | Surgery, chemotherapy, IFN-$\alpha$ | Melan-A |
| 17 | LAU 181 | F | 41 | 0.5 | IV | LN | Surgery | Melan-A |
| 18 | LAU 198 | F | 70 | 3 | III | LN | Surgery, chemotherapy | Melan-A |
| 19 | LAU 203 | F | 67 | 5 | III | LN | Surgery | Melan-A,CMV |
| 20 | LAU 211 | M | 80 | 0.5 | IV | LN, skin | Surgery | Melan-A,CMV |
| 21 | LAU 212 | F | 69 | 10 | III | LN | Surgery | Melan-A |
| 22 | LAU 233 | F | 76 | 1 | III | LN | Surgery | Melan-A |
| 23 | LAU 240 | M | 65 | 1 | III | LN | Surgery | Melan-A |
| 24 | LAU 241 | M | 49 | 1 | I/II | | Surgery | CMV |
| 25 | LAU 253 | M | 63 | 2 | III | LN | Surgery | Melan-A |
| 26 | LAU 258 | F | 65 | 7 | III | LN | Surgery | CMV |
| 27 | LAU 267 | M | 45 | 1 | III | LN | Surgery, IFN-$\alpha$ | Melan-A |
| 28 | LAU 269 | M | 23 | 2 | III | LN | Surgery, isolated limb perfusion | Melan-A |
| 29 | LAU 306 | M | 58 | 0.5 | III | LN | Surgery | Melan-A |
| 30 | LAU 321 | M | 64 | 5 | IV | LN, lung | Surgery | Melan-A |
| 31 | LAU 337 | F | 25 | 3 | IV | LN, lung, skin | Surgery, IFN-$\alpha$, vaccination | Melan-A |
| 32 | LAU 343 | M | 78 | 1 | III | LN | Surgery | Melan-A |
| 33 | LAU 350 | M | 63 | 0.5 | III | LN | Surgery | Melan-A |
| 34 | LAU 352 | M | 77 | 2 | III | LN | Surgery, vaccination | Melan-A |
| 35 | LAU 359 | M | 61 | 1 | III | LN | Surgery, vaccination | Melan-A |
| 36 | LAU 362 | F | 59 | 2 | IV | LN, skin | Surgery | Melan-A,CMV |
| 37 | LAU 372 | M | 59 | 0 | III | LN | Surgery | Melan-A |
| 38 | LAU 387 | F | 59 | 3 | IV | LN, skin | Surgery, IFN-$\alpha$ | CMV |
| 39 | LAU 390 | F | 38 | 1 | III | LN | Surgery, chemotherapy, IFN-$\alpha$ | CMV |
| 40 | LAU 392 | F | 33 | 4 | III | LN | Surgery | Melan-A |
| 41 | LAU 435 | M | 67 | 4 | III | LN | Surgery | Melan-A |
| 42 | LAU 441 | M | 64 | 1 | III | LN | Surgery | CMV |
| 43 | LAU 444 | F | 31 | 3 | IV | LN, skin | Surgery | Melan-A |
| 44 | LAU 450 | F | 72 | 2 | IV | LN, skin, brain | Surgery, vaccination | Melan-A |
| 45 | LAU 455 | M | 56 | 20 | IV | LN, lung | Surgery, chemotherapy | Melan-A |
| 46 | LAU 460 | F | 61 | 7 | IV | LN, skin | Surgery, chemotherapy, vaccination | CMV |
| 47 | LAU 465 | M | 48 | 6 | III | LN | Surgery | Melan-A |
| 48 | LAU 498 | F | 60 | 1 | III | LN | Surgery, vaccination | CMV |
| 49 | LAU 529 | M | 63 | 3 | IV | LN, lung | Surgery, chemotherapy, vaccination | CMV |
| 50 | LAU 552 | F | 35 | 1 | IV | LN, skin | Surgery, IFN-$\alpha$ | CMV |
| 51 | LAU 562 | M | 55 | 5 | IV | LN, skin | Surgery, IFN-$\alpha$ | CMV |
| 52 | LAU 567 | F | 54 | 0.5 | III | LN | Surgery, vaccination | Melan-A,CMV |
| 53 | LAU 576 | M | 57 | 0 | III | LN | Surgery | Melan-A |
| 54 | LAU 608 | F | 66 | 5 | IV | LN, skin | Surgery, isolated limb perfusion | CMV |
| 55 | LAU 622 | M | 66 | 2 | IV | LN, lung, skin | Surgery | Melan-A |
| 56 | LAU 640 | F | 58 | 0.5 | IV | LN, skin | Surgery | Melan-A |
| 57 | LAU 651 | F | 45 | 0.5 | IV | LN, skin | Surgery | Melan-A |
| 58 | LAU 653 | M | 56 | 3 | IV | LN, skin | Surgery, isolated limb perfusion | Melan-A |
| 59 | LAU 671 | F | 57 | 0 | III | LN | Surgery | CMV |
| 60 | LAU 709 | F | 43 | 7 | III | LN | Surgery, vaccination | CMV |
| 61 | MEL 162 | M | 60 | 2 | III | LN | Surgery, chemotherapy, IFN-$\alpha$ | Melan-A |

[a] Years elapsed between excision of primary lesion and sample studied.
[b] American Joint Committee on Cancer staging system.
[c] LN, lymph node; CMV, cytomegalovirus.

(ELAGIGILTV; Ref. 20) or CMV $pp65_{495–503}$ (NLPMVATV). mAbs were from Becton Dickinson (San Jose, CA), except anti-CD28-FITC (Immunotech, Marseille, France), antiperforin-FITC (Ancell, Bayport, MN), antigranzyme B-FITC (Hoelzel Diagnostika, Cologne, Germany), goat antirat IgG FITC (Southern Biotechnology Associates, Birmingham, AL), anti-CD8-ECD (Coulter Corp., Miami, FL), and goat antirat IgG allophycocyanin (Caltag, Burlingame, CA). Anti-CCR7 rat IgG mAb 3D12 was kindly provided by Dr. M. Lipp, Max Delbrück Institute (Berlin, Germany). The lower limit of detection with HLA-A2/peptide multimers was determined by staining HLA-$A2^-$ CD8 T cells with multimers incorporating the relevant peptides or HLA-$A2^+$ CD8 T cells from HIV-seronegative individuals with multimers incorporating an HIV-1 peptide (mean $+3 \times$ SD $= 0.01\%$ of CD8 T cells, data not shown).

**Phenotype Analysis.** CD8 T lymphocytes were positively enriched using CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), stained with multimers for 1 h at room temperature and incubated with appropriate mAbs for 20 min at 4°C. Cells were either immediately analyzed or sorted into defined populations on a FACSCalibur or a FACSVantage SE, respectively, using CellQuest software (Becton Dickinson). For intracellular staining, cells were stained with cell surface mAbs, fixed and permeabilized in PBS/1% formaldehyde/2% glucose/5 mM Na-Azid for 20 min at room temperature and incubated with mAbs in PBS/0.1% saponin for 20 min at 4°C.

**IFN-$\gamma$ Cytospot Assay.** Measurement of intracellular IFN-$\gamma$ production was combined with multimer labeling. Purified CD8 T cells were incubated for 4 h with T2 cells at a 1:1 ratio with either irrelevant HIV-1 $Pol_{476–484}$ (ILKEPVHGV) peptide, 1 $\mu$g/ml cognate peptide, or 1 $\mu$g/ml phorbol 12-

myristate 13-acetate (PMA)/0.25 μg/ml ionomycin, respectively. After 1 h, 10 μg/ml brefeldin A (Sigma, St. Louis, MO) was added. After 3 additional h, cells were stained with multimers and mAbs, fixed and permeabilized, and incubated with anti-IFN-γ-FITC in PBS/0.1% saponin for 20 min at 4°C. Cells to be activated were stained with multimers before activation.

**Quantitative PCR of TCR Excision Circles (TRECs).** Quantification of signal joint TRECs was performed by real-time quantitative PCR with the 5′ nuclease (TaqMan) assay and an ABI7700 system (Applied Biosystems, Foster City, CA; Ref. 18). The internal standard was kindly provided by Dr. D. Douek, Human Immunology Section, Vaccine Research Center, NIH. The lower limit of quantification was $10^1$ copies of TRECs (18).

**Cytolytic Activity.** Single A2/Melan-A$^+$ T cells were sorted *ex vivo* and stimulated with 1 μg/ml phytohemagglutinin-leukoagglutinin, 100 IU/ml interleukin (IL)-2, and $10^6$/ml allogeneic-irradiated peripheral blood mononuclear cells (PBMCs). Lytic activity was measured against peptide-pulsed T2 cells (HLA-A2$^+$, TAP$^{-/-}$), and melanoma cells NA8-MEL (HLA-A2$^+$, Melan-A$^-$) or Me 275 (HLA-A2$^+$, Melan-A$^+$) in 4 h $^{51}$Cr-release assays (18).

**Lymphocyte Stimulation Assay.** Metastatic LN cells were cultured in Iscove Dulbecco's medium/0.55 mM Arg/0.24 mM Asn/1.5 mM Gln/8% human serum, supplemented with 30 IU/ml IL-2 and 10 ng/ml IL-7.

## RESULTS

**Efficient Homing of *in Vivo* Primed Melan-A-Specific CD8 T Cells to Tumor Lesions of HLA-A2 Melanoma Patients.** Using fluorescent HLA-A2/peptide multimers for *ex vivo* analysis, we quantified Melan-A-specific CD8 T cells in PBMCs from 21 HLA-A2 healthy individuals and in PBMCs, metastatic LNs, and nonlymphoid tissue metastases from 20, 28, and 8 HLA-A2 melanoma patients, respectively (Table 1). Naive and antigen-experienced T cells were defined as CCR7$^+$CD45RA$^{high}$ and non-CCR7$^+$CD45RA$^{high}$, respectively (21). In accordance with previous findings (18, 22), PBMCs from all healthy individuals contained detectable frequencies of

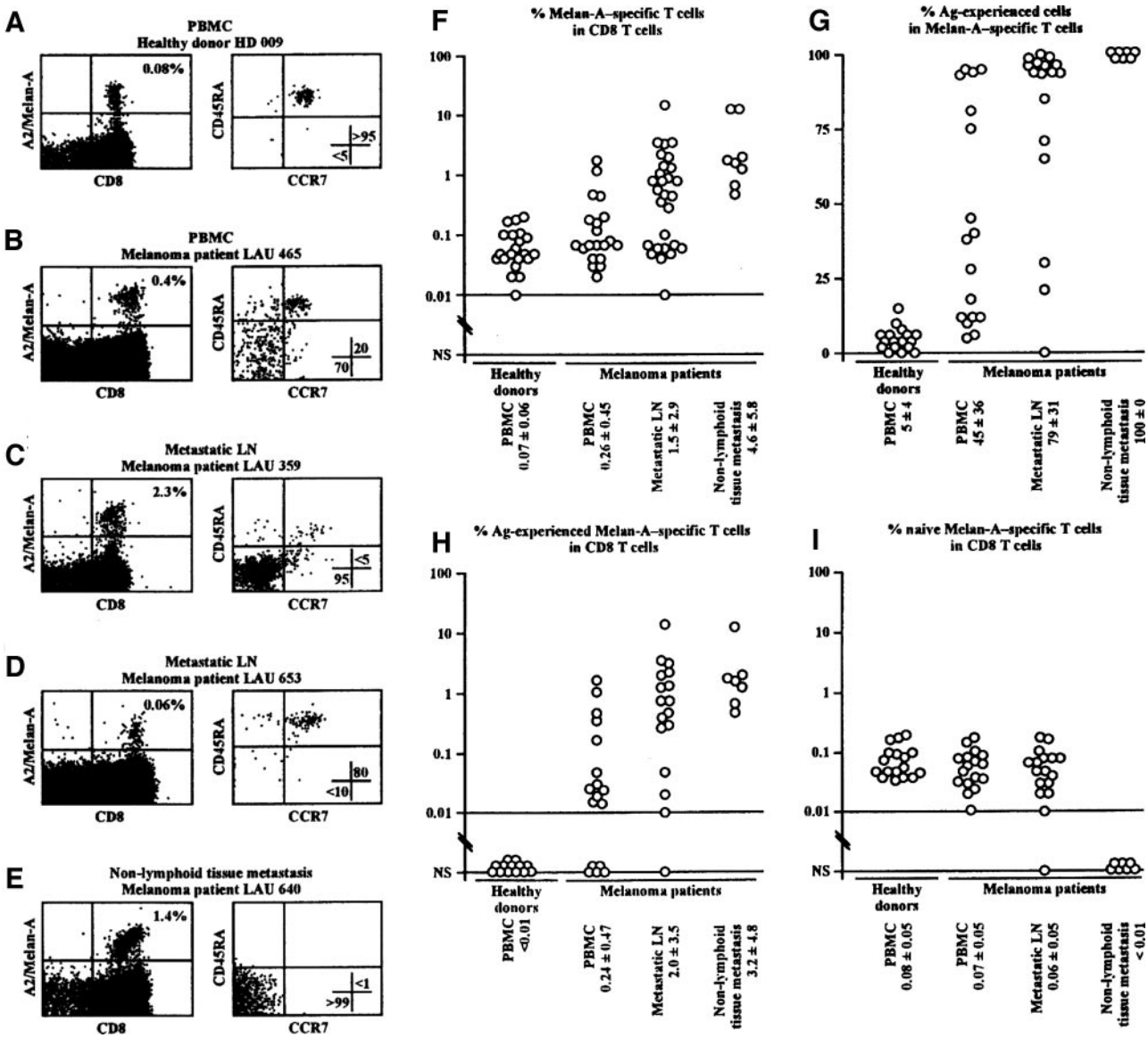


Fig. 1. Differentiation and homing of Melan-A-specific CD8 T cells in melanoma patients. *A-E*, percentage of Melan-A-specific T cells in CD8 T cells (*left*) and their CCR7/CD45RA phenotype (*right*) in peripheral blood mononuclear cells (PBMCs) (*A*) of healthy individuals, and in PBMCs (*B*), metastatic lymph nodes (LNs; *C* and *D*), and nonlymphoid tissue metastases (*E*) of melanoma patients. *F-I*, summary of phenotypic data of Melan-A-specific T cells. Percentage of Melan-A-specific T cells in CD8 T cells (*E*), percentage of antigen-experienced cells in Melan-A-specific T cells (*F*), percentage of antigen-experienced Melan-A-specific T cells in CD8 T cells (*G*), and percentage of naive Melan-A-specific T cells in CD8 T cells (*H*). Mean percentages ± SDs are shown. The *horizontal bars* represent the lower limit of detection with fluorescent HLA-A2/peptide multimers. The expression of CCR7 and CD45RA on Melan-A-specific T cells was analyzed when enough material was available.

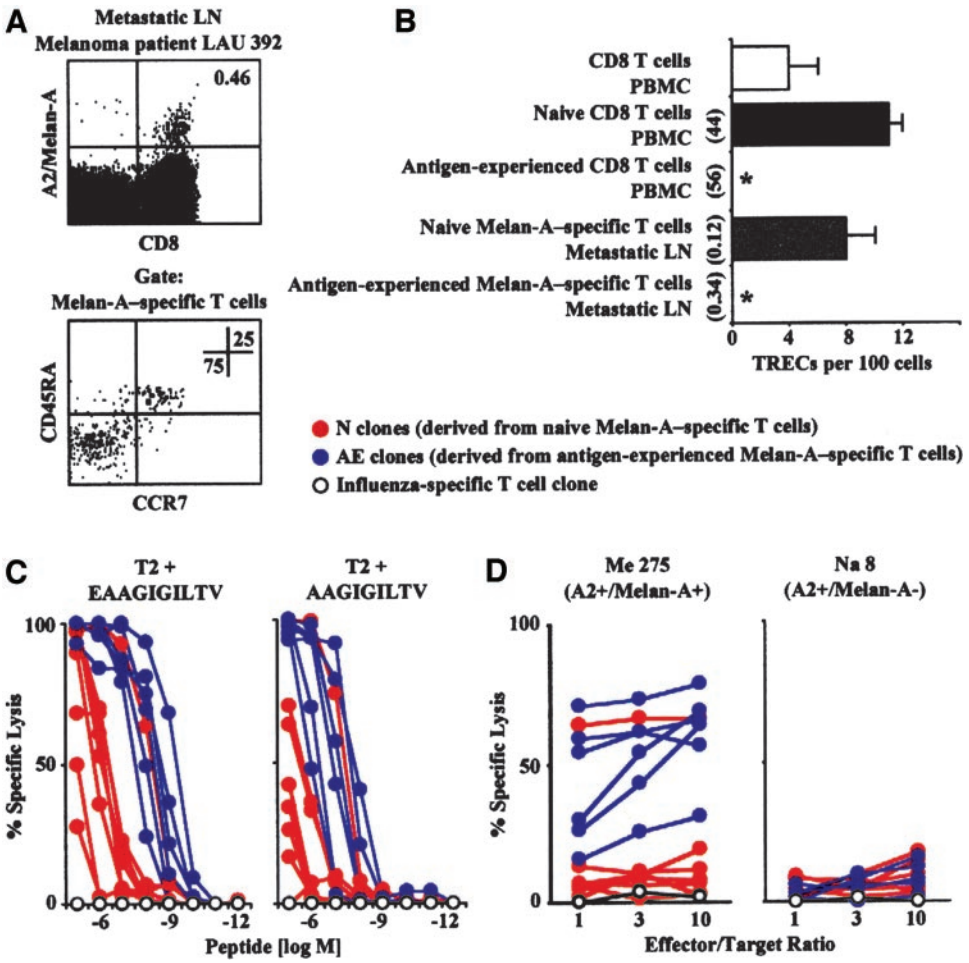


Fig. 2. Selective expansion *in vivo* of Melan-A-specific CD8 T cells with high T-cell receptor (TCR) avidity. *A*, percentage of Melan-A-specific T cells in CD8 T cells (*top*) and their CCR7/CD45RA phenotype (*bottom*) in a metastatic lymph node (LN; melanoma patient LAU 392). *B*, real-time PCR quantification of TCR excision circles (TRECs) for total CD8 T cells, naive and antigen-experienced CD8 T cells in peripheral blood mononuclear cells (PBMCs), and naive and antigen-experienced Melan-A-specific T cells in a metastatic LN. *, TREC levels under the lower limit of quantification. *C* and *D*, cytolytic activity of N clones (derived from naive Melan-A-specific T cells, *red circles*), AE clones (derived from antigen-experienced Melan-A-specific T cells, *blue circles*) and an irrelevant Influenza-specific T-cell clone (*white circles*), against $T_2$ target cells ($A2^+$/$TAP^{-/-}$) in the presence of serial dilutions of the Melan-A decapeptide EAAGIGILTV or nonapeptide AAGIGILTV (*C*), or against melanoma cell lines Me 275 ($A2^+$/Melan-$A^+$) or Na 8 ($A2^+$/Melan-$A^-$) (*D*).

Melan-A-specific T cells (mean ± SD, 0.07 ± 0.06% of CD8 T cells; Fig. 1, *A* and *F*) that were naive (Fig. 1, *A* and *G*). Melan-A-specific T cells were also found in PBMCs from all melanoma patients (0.26 ± 0.45% of CD8 T cells; Fig. 1, *B* and *F*). However, a significant fraction of these cells was antigen-experienced (45 ± 36% of Melan-A-specific T cells; Fig. 1, *B* and *G*). The vast majority of antigen-experienced Melan-A-specific T cells displayed a $CCR7^-CD45RA^{low}$ phenotype (data not shown). Metastatic LNs contained on average higher frequencies of Melan-A-specific T cells (1.5 ± 2.9% of CD8 T cells; Fig. 1, *C* and *F*) that were mostly antigen-experienced (79 ± 31% of Melan-A-specific T cells; Fig. 1, *C* and *G*). It is noteworthy that about one-third of metastatic LNs contained frequencies of Melan-A-specific T cells that were not higher than in PBMCs of healthy individuals and were mostly naive (Fig. 1*D*), confirming the absence of Melan-A-specific T-cell immune responses in some melanoma patients (11). All nonlymphoid tissue metastases contained high frequencies of Melan-A-specific T cells (4.6 ± 5.8% of CD8 T cells; Fig. 1, *E* and *F*) that were uniformly antigen-experienced (100 ± 0% of Melan-A-specific T cells; Fig. 1, *E* and *G*). Thus, this analysis of a large series of patients confirms and extends previous observations that natural CD8 T-cell immune responses directed against Melan-A often occur *in vivo* (10–12, 14, 15, 19, 23, 24). In particular, detectable fractions of antigen-experienced Melan-A-specific T cells accumulate in metastatic LNs and especially in nonlymphoid tissue metastases (Fig. 1*H*). Furthermore, our data clearly reveal that naive Melan-A-specific T cells are not detectable in nonlymphoid tissue metastases but are confined to peripheral blood and secondary lymphoid organs (Fig. 1*I*).

**Melan-A-Specific CD8 T Cells of High TCR Avidity Are Preferentially Primed *in Vivo*.** The naive Melan-A-specific T-cell repertoire is composed of cells with predominantly low TCR avidity (25). By contrast, increased TCR avidity has been observed in postvaccination Melan-A-specific T-cell populations (26, 27). To directly assess whether TCR avidity might play a role during the induction of tumor-driven immune responses *in vivo*, we compared naive and antigen-experienced Melan-A-specific T cells that coexisted in the same metastatic LN from a nonvaccinated melanoma patient (LAU 392; Fig. 2*A*). We first determined the relationship between the *ex vivo* CCR7/CD45RA phenotype and *in vivo*-replicative history of individual T-cell subsets by real-time PCR quantification of TRECs (18). Relatively high levels of TRECs were found in the naive Melan-A-specific T-cell subset, which were comparable with those found in naive peripheral CD8 T cells. In marked contrast, TREC levels were below detection in the antigen-experienced Melan-A-specific T-cell subset (Fig. 2*B*). Thus, antigen-experienced Melan-A-specific T cells in the metastatic LN had completed on average ≥5 more rounds of division than their naive counterpart, in agreement with our previous findings in peripheral blood (18). Eight clones, referred to as N clones (derived from naive $CCR7^+CD45RA^{high}$ A2/Melan-$A^+$ cells), and six clones, referred to as AE clones (derived from antigen-experienced $CCR7^-CD45RA^{low}$ A2/Melan-$A^+$ cells), were generated from the same metastatic LN. After *in vitro* expansion with cytokines, both N and AE clones displayed a similar and homogenous $CCR7^-$ $CD45RA^{low}$ $CD45RO^+$ $CD27^{int}$ $CD28^-$ $CD62L^-$ $CD69^{int}$ HLA-$DR^+$ $perforin^+$ granzyme $B^+$ phenotype (data not shown). The relative TCR avidity of N and AE clones was compared

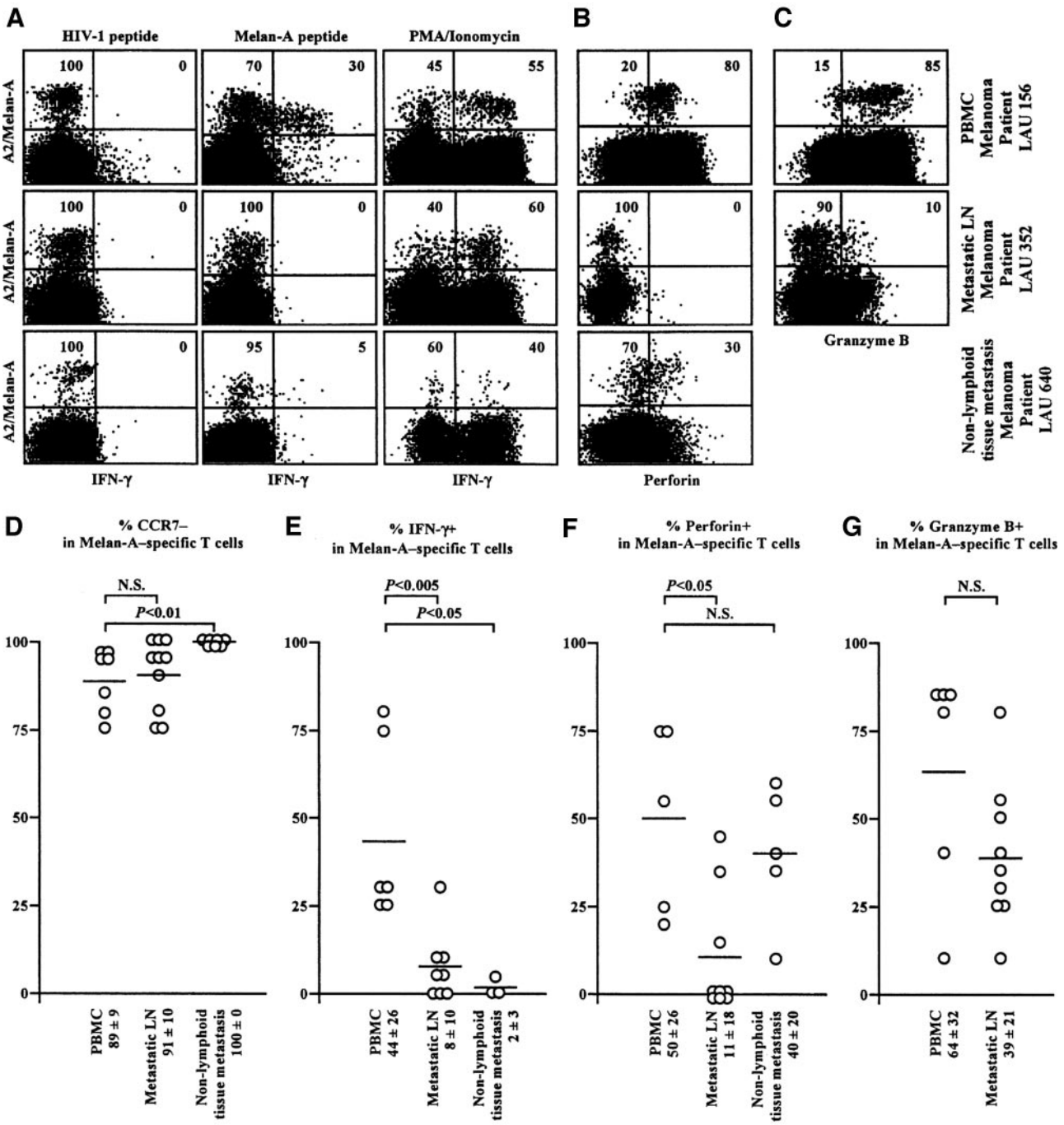


Fig. 3. Functionally tolerant Melan-A-specific CD8 T cells in metastatic lymph nodes (LNs) and nonlymphoid tissue metastases. *A*, IFN-γ production of Melan-A-specific T cells in gated CD8 T cells obtained from peripheral blood mononuclear cells (PBMCs), metastatic LNs, and nonlymphoid tissue metastases. CD8 T cells were stimulated either with irrelevant HIV-1 peptide (*left*), cognate peptide (*middle*), or phorbol 12-myristate 13-acetate (PMA)/Ionomycin (*right*). Representative examples are shown. Figures in the *upper quadrants* are the percentages of Melan-A-specific T cells with the corresponding phenotype. *B* and *C*, perforin (*B*) and granzyme B (*C*) expression of Melan-A-specific T cells in gated CD8 T cells. *D*, *in vivo*-primed CCR7$^-$ phenotype for Melan-A-specific T cells in the selected patients. *E-G*, summary of effector functions of Melan-A-specific T cells. Percentage of IFN-γ$^+$ cells in Melan-A-specific T cells stimulated with cognate peptide (*E*) and percentage of perforin$^+$ (*F*) and granzyme B$^+$ (*G*) Melan-A-specific T cells. Correlation analyses were performed using the Wilcoxon test [not significant (N.S.), $P \geq 0.05$].

using T2 target cells (HLA-A2$^+$/TAP$^{-/-}$) pulsed with serial dilutions of peptides corresponding to residues 26–35 (EAAGIGILTV) and 27–35 (AAGIGILTV), which mimic the naturally processed Melan-A peptides (20). All AE clones recognized the Melan-A$_{26-35}$ peptide with relatively high avidity (IC$_{50}$ ranging between $10^{-10}$ and $10^{-8}$ M; median, $10^{-9}$ M). In contrast, seven of eight N clones needed on average $10^3$ times more peptide to achieve similar activity (IC$_{50}$ ranging between $10^{-7}$ and $>10^{-6}$ M, median, $10^{-6}$ M). As an exception, one of eight N clones (N11) displayed relatively high avidity for the Melan-A$_{26-35}$ peptide (IC$_{50}$, $10^{-8}$ M; Fig. 2*C*). Similar differences in functional avidity between N and AE clones were observed when the Melan-A$_{27-35}$ peptide was used. IC$_{50}$ for all AE clones ranged between $10^{-8}$ and $10^{-7}$ M, whereas seven of eight N clones needed $>10^{-6}$ M. Again, clone N11 selectively displayed higher functional avidity (IC$_{50}$, $10^{-7}$ M; Fig. 2*C*). The comparative functional avidity of N and AE clones was further analyzed by determining the stability of peptide/major histocompatibility complex class I multimer interaction with the TCR (28). TCR/multimer off-rates, measured in dissociation

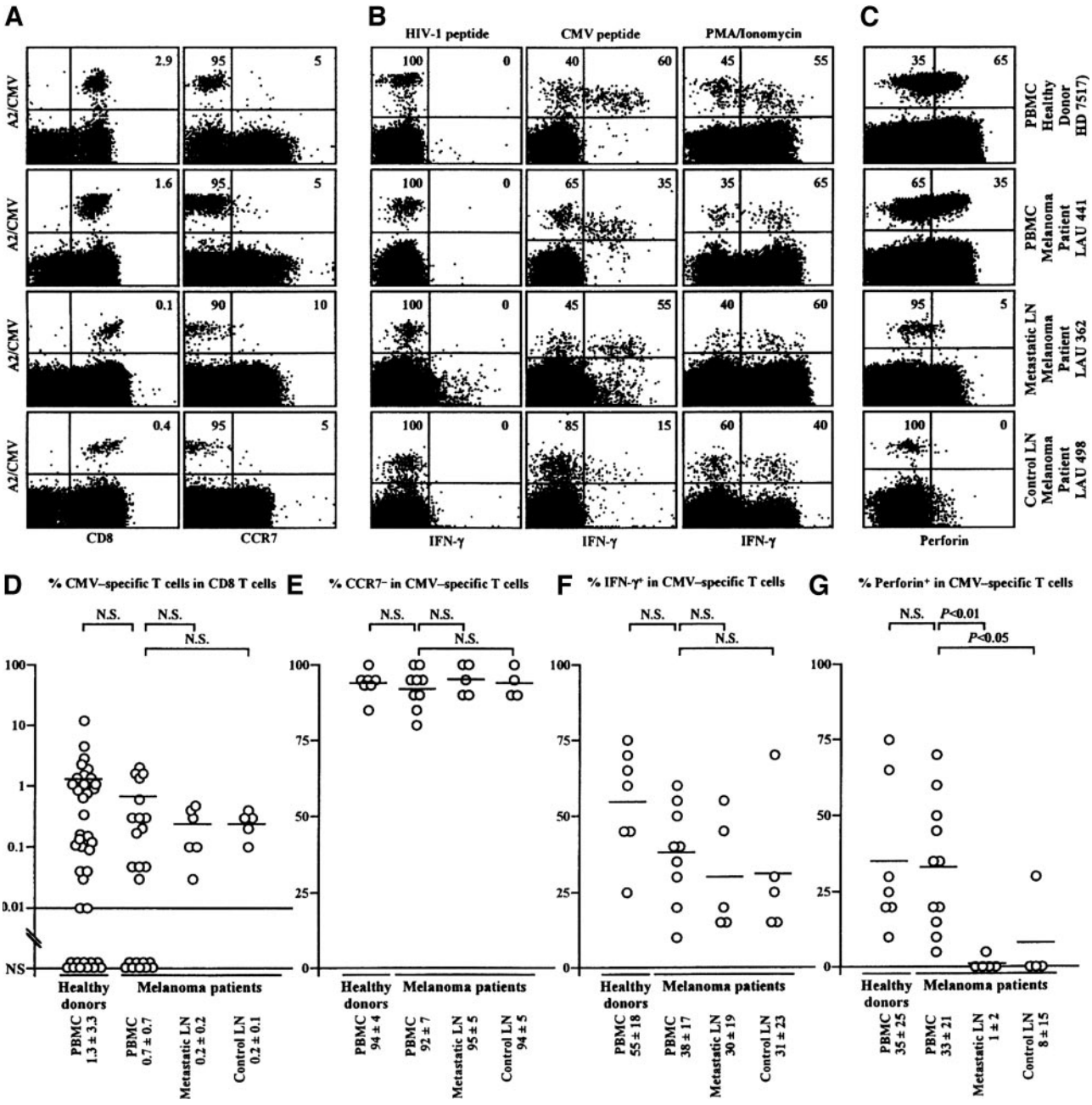


Fig. 4. Cytomegalovirus (CMV)-specific CD8 T cells selectively lack expression of perforin in the lumph node (LN) environment. *A*, percentage of CMV-specific T cells in CD8 T cells (*left*) and their CCR7 phenotype (*right*) in peripheral blood mononuclear cells (PBMCs) of healthy individuals and in PBMCs, metastatic LNs, and control LNs of melanoma patients. Representative examples are shown. Figures in the *upper quadrants* are the percentages of CMV-specific T cells with the corresponding phenotype. *B*, IFN-γ production of CMV-specific T cells in gated CD8 T cells. CD8 T cells were stimulated either with irrelevant HIV-1 peptide (*left*), cognate peptide (*middle*), or phorbol 12-myristate 13-acetate (PMA)/Ionomycin (*right*). *C*, perforin expression of CMV-specific T cells in gated CD8 T cells. *D-G*, summary of phenotypic data and effector functions of CMV-specific T cells. Percentage of CMV-specific T cells in CD8 T cells (*D*), percentage of CCR7$^-$ cells (*E*), percentage of IFN-γ$^+$ cells after stimulation with cognate peptide (*F*), and percentage of perforin$^+$ cells (*G*) in CMV-specific T cells. Correlation analyses were performed using the Wilcoxon test [not significant (N.S.); $P \geq 0.05$].

kinetic experiments, directly correlated with functional avidity, *i.e.*, were faster for N clones as compared with AE clones (data not shown). Importantly, all of the AE clones specifically and efficiently killed the Melan-A-expressing tumor cell line Me 275, whereas the N clones (with the exception of clone N11) did not (Fig. 2*D*). Identical observations were made with Melan-A-specific T-cell clones derived from two other melanoma patients (data not shown). These observations indicate that Melan-A-specific T cells with high TCR avidity and antitumor activity were preferentially triggered and expanded *in vivo*.

**Melan-A-Specific CD8 T Cells Are Functionally Tolerant in Tumor Lesions.** We selected 22 patients with detectable frequency of *in vivo*-primed CCR7$^-$ Melan-A-specific T cells (Fig. 3*D*) in peripheral blood, metastatic LNs, or nonlymphoid tissue metastases, and assessed the ability of these cells to produce IFN-γ, an important effector cytokine in antitumor immunity (3). In all blood samples tested, a high proportion (44 ± 26%; Fig. 3, *A* and *E*) of Melan-A-specific T cells produced IFN-γ after short-term antigenic challenge, which was comparable with that observed after nonspecific PMA/Ionomycin stimulation (Fig. 3*A*). In sharp contrast, only 2–8% of

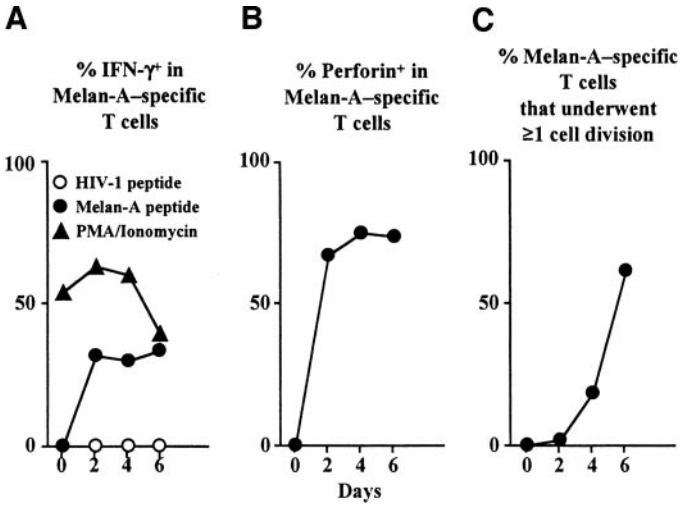


Fig. 5. Rapid acquisition of effector functions of Melan-A-specific CD8 T cells. Metastatic lymph node (LN) cells of melanoma patient LAU 465 were cultured in the presence of low-dose interleukin (IL)-2 and IL-7. *A*, percentage of IFN-$\gamma^+$ cells in Melan-A-specific T cells after restimulation with the irrelevant HIV-1 peptide, the cognate peptide, and phorbol 12-myristate 13-acetate (PMA)/Ionomycin at days 0, 2, 4, and 6. *B*, percentage of perforin$^+$ cells in Melan-A-specific T cells. *C*, *in vitro* replicative history of Melan-A-specific T cells followed after 5,6-carboxylfluorescein diacetate succinimidyl ester labeling at day 0. The percentage of Melan-A-specific T cells that underwent ≥1 cell division are shown.

Melan-A-specific T cells retrieved from tumor lesions (metastatic LNs and nonlymphoid tissue metastases) produced IFN-γ after antigenic challenge (Fig. 3, *A* and *E*). Importantly, the potential of these cells to produce the cytokine was intact, as shown after nonspecific PMA/Ionomycin stimulation (Fig. 3*A*). Furthermore, Melan-A-specific T cells retrieved from two metastatic LNs produced neither IL-4 nor IL-10 after antigenic or PMA/Ionomycin stimulation (data not shown). We also performed an *ex vivo* analysis of the intracellular content of perforin and granzyme B, two major effector molecules involved in cytotoxicity (4). High proportions of Melan-A-specific T cells in both PBMCs and nonlymphoid tissue metastases expressed perforin (on average 40–50%; Fig. 3, *B* and *F*). In marked contrast, perforin was not detectable in Melan-A-specific T cells from six of nine metastatic LNs (Fig. 3, *B* and *F*). Similar proportions of Melan-A-specific T cells in both PBMCs and metastatic LNs expressed granzyme B in the same series of samples (Fig. 3, *C* and *G*). Because of limited number of cells, expression of granzyme B in nonlymphoid metastases could not be analyzed. Our results indicate that the tumor-specific T cells residing in metastatic LNs lacked the capacity to mount perforin-mediated cytotoxic responses as well as to secrete IFN-γ upon antigenic contact and hence were impaired with regards to both major effector pathways.

**Both the LN and the Tumor Environment Independently Affect T Cell Effector Functions.** Because Melan-A-specific T cells selectively lacked perforin expression in metastatic LNs, we aimed at assessing whether the LN environment may preclude the development of cytotoxic effector function. Furthermore, because Melan-A-specific T cells from both metastatic LNs and nonlymphoid tissue metastases lacked the capacity to secrete IFN-γ after antigenic challenge, we wondered whether the tumor environment could also modulate their functional status. To address these issues, we analyzed the tissue distribution and effector functions of T cells that were specific for a HLA-A2-restricted nontumor antigen, such as the CMV antigen $pp65_{495–503}$. CMV-specific T cells were detected in PBMCs from 27 of 38 healthy donors and 14 of 26 melanoma patients (with frequencies reaching on average 1.3% and 0.7% of CD8 T cells, respectively; Fig. 4, *A* and *D*; Table 1). Six metastatic LNs and five control LNs from patients who had detectable CMV-specific T cells in peripheral blood also contained detectable numbers of CMV-specific T cells, although to a lower level (on average 0.2% of CD8 T cells; Fig. 4, *A* and *D*). In all compartments, CMV-specific T cells were mostly CCR7$^-$ (on average 92–95%; Fig. 4, *A* and *E*) and produced IFN-γ after antigenic challenge or PMA/Ionomycin stimulation (on average 30–55% of CMV-specific T cells; Fig. 4, *B* and *F*). Significant proportions of circulating CMV-specific T cells in healthy individuals and melanoma patients expressed detectable levels of perforin (on average 33–35%; Fig. 4, *C* and *G*). In agreement with these results, fluorescence-activated cell sorter-sorted A2/CMV$^+$ PBMCs from two healthy individuals exhibited direct cytotoxicity against T2 cells pulsed with the CMV peptide (data not shown). In marked contrast, very few CMV-specific T cells from metastatic and control LNs expressed perforin (on average 1–8%; Fig. 4, *C* and *G*). The same phenotype analysis extended to total CD8 T cells also revealed that significant fractions expressed perforin in the peripheral blood of healthy individuals and melanoma patients (on average 25–30%, data not shown), whereas perforin expression was strongly reduced in metastatic and control LNs (on average 1–5%, data not shown). These findings indicate that cytotoxic perforin$^+$ T cells do not reside in LNs and thus support the notion that the perforin deficit observed in tumor-specific T cells from metastatic LNs is a consequence of the LN environment itself and not the tumor. In contrast, the capacity to produce IFN-γ in response to antigen was selectively preserved by LN-derived virus-specific T cells. Impaired IFN-γ production by LN-derived tumor-specific T cells is likely attributable to the tumor and not to the LN environment.

**Effector Functions of Melan-A-Specific CD8 T Cells from Metastatic LNs Are Rapidly Acquired in the Presence of Low-Dose IL-2/IL-7 Cytokines *in Vitro*.** Lymphocytes obtained from a metastatic LN (patient LAU 465) were cultured in the presence of 30 units/ml IL-2 and 10 ng/ml IL-7, as described previously (14). Significant proportions of Melan-A-specific T cells acquired the capacity to produce IFN-γ after antigenic challenge (Fig. 5*A*), and expressed intracellular perforin (Fig. 5*B*) already after 2 days of culture. Production of IFN-γ by Melan-A-specific T cells was also observed in metastatic LN cultures from two other patients (data not shown). Because proliferation of Melan-A-specific T cells was only observed after 4–6 days of culture (as assessed by 5,6-carboxylfluorescein diacetate succinimidyl ester labeling; Fig. 5*C*), both effector functions were acquired before cell division. The acquisition of potent effector functions by *in vitro*-cultured tumor-specific T cells is in line with previous observations (11, 14). Altogether, these findings underscore the necessity of analyzing T cells directly *ex vivo* for a correct assessment of their effector functions.

## DISCUSSION

Cancer patients can naturally develop antitumor CD8 T-cell immune responses (29–31). However, it is still an unsolved question why these responses may fail to effectively prevent tumor progression. Here we present a comprehensive study on the magnitude and functional potential of CD8 T-cell responses directed against Melan-A, a well-known tumor antigen, in various body compartments of a large series of melanoma patients. We demonstrate that Melan-A-specific T cells with high TCR avidity are selectively primed *in vivo* and accumulate in tumor lesions. However, although primed Melan-A-specific T cells that circulate in peripheral blood display intact effector functions, the same cells that reside within tumor lesions are functionally tolerant. These findings contribute to our understanding why CD8 T-cell immune responses eventually fail to prevent tumor progression.

As a result of direct thymic output, naive ($CCR7^+CD45RA^{high}$) Melan-A-specific T cells are detectable by specific multimer staining in peripheral blood of HLA-A2 healthy individuals and melanoma patients (18). As shown here, the presence of naive Melan-A-specific T cells in LNs of melanoma patients suggests a continuous trafficking from the blood to secondary lymphoid organs, in line with the pivotal role of CCR7 in lymphocyte migration to LNs (32). This is further supported by our finding that naive Melan-A-specific T cells are not detected in nonlymphoid tissue metastases. Melan-A-specific T cells remain naive in healthy individuals despite the presence of antigen in melanocytes, a phenomenon akin to immunological ignorance. In contrast, a significant fraction of these cells becomes activated in some melanoma patients (10–12, 14, 19, 23, 24). Here, we found that immunological ignorance to Melan-A was overcome at least in 26 of 40 (65%) patients. As indicated by our analysis of TREC levels, the high frequency of antigen-experienced Melan-A-specific T cells is the result of extensive *in vivo* proliferation. Importantly, the naturally occurring immune responses observed in this study with a large series of melanoma patients were able to generate tumor-specific T cells that were "fit" in peripheral blood (*i.e.*, antigen-experienced circulating Melan-A-specific T cells displayed robust cytotoxic and inflammatory activities). Hence, we extend previous observations that circulating tumor-specific T cells exhibit the two major effector functions involved in tumor protection and suggest that anergy, as reported previously for one melanoma patient (12), is not a common feature of T cells directed against tumor-differentiation antigens.

The coexistence of both naive and antigen-experienced Melan-A-specific T cells within the same LN allowed us to investigate whether T cells with high TCR avidity were selected during natural antitumor immune responses. Indeed, whereas previous work showed that the naive Melan-A-specific T-cell pool is composed of cells that exhibit a broad range of functional avidities (25), we found here that the antigen-experienced T-cell repertoire consisted only of cells with high TCR avidity (33). Limiting amounts of antigenic peptides available *in vivo* may account for this selection process. In addition, because high avidity T cells represent only a minority of the naive Melan-A-specific T-cell pool, our data may explain why the size of the latter remained stable (on average 0.07% of CD8 T cells) in LNs, irrespective of the presence or absence of antigen-experienced T cells. Alternatively, independent homeostatic regulation of naive and antigen-experienced T-cell populations (34) and repopulation of the naive T-cell pool with recent thymic emigrants (35) may also explain the stable size of the naive Melan-A-specific T-cell pool observed in LNs from patients with documented natural responses.

A tolerant state of potentially tumor-reactive T cells has been documented both in mouse models and humans (6–8). In this study, we report on the direct functional characterization of single tumor-specific T cells, by measuring their cytotoxic and inflammatory effector activities, and we illustrate that these cells are selectively tolerant within tumor lesions. Melan-A-specific T cells residing in metastatic LNs were functionally tolerant with regard to both effector pathways. On one hand, the tumor-specific T cells did not display perforin-mediated cytotoxic activities. Virus-specific T cells present in metastatic and control LNs also contained undetectable or low levels of perforin. This observation was further confirmed by the analysis of total CD8 LN-derived T cells, potentially including tumor-specific T cells with specificity for antigens other than Melan-A. Absence of perforin in LN-derived T cells was associated with CD27 expression (data not shown), a feature of circulating virus-specific T cells with incomplete functional maturation (36). Granzyme B was expressed by a significant proportion of tumor-specific T cells. However, because of the essential role of perforin for appropriate delivery of granzyme B, these cells were presumably unable to induce target-cell apoptosis (4). These findings may be explained by the migratory properties of fully activated effector CD8 T cells, which leave the LN and home to peripheral tissues. Accordingly, Melan-A-specific T cells with cytotoxic effector activities were found in the peripheral blood and nonlymphoid tissues of some melanoma patients. It is worth noting that Melan-$A^+$ metastatic tumor cells, in some instances present at elevated numbers in the LNs, were not able to revert the homing properties of the cytotoxic T cells. On the other hand, the tumor-specific T cells present in metastatic LNs of the majority of melanoma patients also lacked the capacity to secrete IFN-$\gamma$ after antigenic challenge. Because this function was preserved for virus-specific T cells in both metastatic and control LNs, we propose that the tumor (and not the LN) environment is responsible for the IFN-$\gamma$ deficit exhibited by the tumor-specific T cells. Interestingly, lack of IFN-$\gamma$ production by enterocyte antigen-specific T cells that have homed to the intestine was also described as a mechanism for maintenance of tolerance to self in a transgenic mouse model (37, 38). Altogether, our findings suggest that both the homing properties of antigen-primed T cells as well as the tumor environment affect the two major CD8 T-cell effector functions. Overall, the absence of functionally active tumor-specific T cells in metastatic LNs likely explains the frequent failure of the immune system to eliminate LN tumor metastases. In contrast to a previous report describing anergic tyrosinase-specific T cells in one patient (12), the functional tolerance identified for tumor-specific T cells in this study were rapidly reversible *in vitro*. Interestingly, a recent study showed that the functionally tolerant state of self/tumor antigen-specific T cells could be reversed in mice after therapy with T cells, altered peptide, and IL-2 (39). Coadministration of the cytokine was required to induce the destruction of poorly immunogenic established melanoma tumors.

The effector functions observed for Melan-A-specific T cells in nonlymphoid tissue metastases were also considerably reduced. However, in contrast to those residing in metastatic LNs, these cells expressed detectable levels of perforin and thus were apparently able to mediate cytotoxic activity. This finding supports the notion mentioned above that paucity of perforin expression is a feature of T cells residing in lymphoid tissues. Nevertheless, Melan-A-specific T cells failed to produce IFN-$\gamma$ in both metastatic LNs and nonlymphoid tissue metastases. Thus, IFN-$\gamma$ secretion was turned off when the tumor-specific T cells were in the presence of melanoma cells *in vivo*. Although several mechanisms of tumor-induced immune defects have been proposed, including alterations in signal transduction (40, 41), functional unresponsiveness (42), and immunosuppressive environment (8, 43), the robust production of IFN-$\gamma$ by tumor-specific cells observed after stimulation with PMA and ionomycin suggests disruption of signaling events proximal to the TCR. Given the key role of IFN-$\gamma$ in the newly rekindled concept of cancer immunosurveillance (3), it is conceivable that the inability of tumor antigen-specific T cells to produce this cytokine critically reduces their protective potential. Understanding the mechanisms involved in the generation and/or maintenance of the functional tolerance of tumor-specific T cells in tumor lesions described in this study may provide the framework for improving the efficiency of cancer immunotherapy.

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# Effector Function of Human Tumor-Specific CD8 T Cells in Melanoma Lesions: A State of Local Functional Tolerance

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