An Expanded Peripheral T Cell Population to a Cytotoxic T Lymphocyte (CTL)-defined, Melanocyte-specific Antigen in Metastatic Melanoma Patients Impacts on Generation of Peptide-specific CTLs but Does Not Overcome Tumor Escape from Immune Surveillance in Metastatic Lesions

By Andrea Anichini,* Alessandra Molla,* Roberta Mortarini,*
Gabrina Tragni,§ Ilaria Bersani,* Massimo Di Nicola,‖
Alessandro M. Gianni,‖ Silvana Pilotti,§ Rod Dunbar,¶
Vincenzo Cerundolo,¶ and Giorgio Parmiani‡

*From the Department of Experimental Oncology *Human Tumor Immunobiology Unit and ‡Human Tumor Immunotherapy Unit, the §Division of Pathology, and the ‖Division of Medical Oncology C, Istituto Nazionale per lo Studio e la Cura dei Tumori, 20133 Milan, Italy; and the ¶Institute of Molecular Medicine, Nuffield Department of Clinical Medicine, Oxford, OX3 9DS, United Kingdom*

Summary

It is not known if immune response to T cell–defined human histocompatibility leukocyte antigen (HLA) class I–restricted melanoma antigens leads to an expanded peripheral pool of T cells in all patients, affects cytotoxic T lymphocyte (CTL) generation, and correlates with antitumor response in metastatic lesions. To this end, a limiting dilution analysis technique was developed that allowed us to evaluate the same frequency of peptide-specific T cells as by staining T cells with HLA–peptide tetrameric complexes. In four out of nine patients, Melan-A/Mart-1_{27-35}–specific CTL precursors (CTLp) were $\geq 1/2{,}000$ peripheral blood lymphocytes and found mostly or only in the CD45RO$^+$ memory T cell subset. In the remaining five patients, a low ($<1/40{,}000$) peptide-specific CTLp frequency was measured, and the precursors were only in the CD45RA$^+$ naive T cell subset. Evaluation of CTL effector frequency after bulk culture indicated that peptide-specific CTLs could be activated in all patients by using professional antigen-presenting cells as dendritic cells, but CTLp frequency determined the kinetics of generation of specificity and the final number of effectors as evaluated by both limiting dilution analysis and staining with HLA-A*0201–Melan-A/Mart-1 tetrameric complexes. Immunohistochemical analysis of 26 neoplastic lesions from the nine patients indicated absence of tumor regression in most instances, even in patients with an expanded peripheral T cell pool to Melan-A/Mart-1 and whose neoplastic lesions contained a high frequency of tetramer-positive Melan-A/Mart-1–specific T cells. Furthermore, frequent lack of a "brisk" or "nonbrisk" CD3$^+$CD8$^+$ T cell infiltrate or reduced/absent Melan-A/Mart-1 expression in several lesions and lack of HLA class I antigens were found in some instances. Thus, expansion of peripheral immune repertoire to Melan-A/Mart-1 takes place in some metastatic patients and leads to enhanced CTL induction after antigen-presenting cell–mediated selection, but, in most metastatic lesions, it does not overcome tumor escape from immune surveillance.

Key words: melanoma • cytotoxic T lymphocytes • Melan-A/Mart-1 • peptide-specific CTL precursors • tumor escape

As shown initially by Clark et al. (1), the presence of tumor-infiltrating lymphocytes (TILs)[1] is an independent prognostic factor in stage I vertical growth phase (VGP) melanoma. The best probability of survival is associated with the "brisk" pattern (i.e., TILs are present throughout the substance of the VGP or infiltrate the entire base of the VGP tumor). A lower survival rate is observed in "nonbrisk" cases (when TILs are present in one or more foci of the VGP), and the lowest probability of survival is found when TILs are coded as "absent" (1). An

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[1]*Abbreviations used in this paper:* DCs, dendritic cells; LDA, limiting dilution analysis; p, precursor; TILs, tumor-infiltrating lymphocytes; VGP, vertical growth phase.

Table I. *Characteristics of Metastatic Melanoma Patients*

Patient	Sex/age*	Primary lesion‡	Time from surgical exeresis of primary tumor to CTLp evaluation	Current status§	Therapy at time of or before isolation of PBLs for CTLp analysis	Progression of disease (site)	Time from CTLp evaluation to exitus
			mo				*mo*
1	M/62	3.1/IV	2	DOD	None	¶Liver, s.c., bone	16
2	M/51	1.49/III	14	DOD	None	Brain	1
3	F/19	3.9/IV	6	DOD	None	Lung	4
4	F/57	>4/V	18	DOD	None	s.c.	3.5
5	M/50	2/IV	26	AWD	‖TP5	LN, s.c.	
6	F/63	NA	134	DOD	None	S.T., LN, peritoneum	5
7	M/64	2.94/IV	54	NED	None	—	
8	F/39	3.97/IV	53	NED	None	—	
9	F/65	>4/V	6	DOD	None	s.c., lung	3

*Age of the patients at the time PBLs for CTLp evaluation were isolated.
‡Primary lesion thickness (mm)/Clark level (1). NA, not available.
§DOD, dead of disease; NED, no evidence of disease; AWD, alive with disease.
‖This patient received TP5 (thymopentin, a peptide from thymic hormone) intravenously for 7 wk.
¶Site of metastic lesions developing during progression of disease, in addition to lymph node (LN) metastases already diagnosed in all patients at time of CTLp analysis. LN, lymph node; s.c., subcutaneous; S.T., soft tissue.

association between TIL patterns and disease evolution has been observed even when observing TILs in lymph node metastases (in American Joint Committee on Cancer [AJCC] stage III patients) (2). A possible interpretation of these data is that brisk and, to a lesser extent, nonbrisk patterns of TILs reflect a protective immune response to melanoma antigens that results in control of tumor growth not only in primary but even in metastatic lesions. This possibility is in agreement with the large set of data indicating clonal expansion of tumor-specific T cells in melanoma lesions (for review see reference 3). Furthermore, in at least some melanoma patients, CTL clones and lines have been derived even from peripheral blood after activation with autologous tumor or tumor-derived peptides (4–6). In these instances, differential kinetics of antigen-specific T cell activation in vitro in patients versus healthy donors has been described (7), suggesting tumor immunogenicity.

In spite of the data for involvement of the immune system in controlling tumor growth, prognosis in metastatic melanoma remains poor, with a median survival of 6 mo in stage IV patients (8). Thus, in metastatic disease, tumor progression occurs despite evidence consistent with tumor immunogenicity, a paradox whose solution can now be approached. In fact, the molecular characterization of melanoma antigens and the identification of their CTL epitopes (9, 10) allows us to evaluate whether the expansion of the immune repertoire to a specific tumor antigen occurs in melanoma patients and correlates with response at the tumor site.

Among several antigens expressed by human melanoma, Melan-A/Mart-1 (11) has attracted the interest of several investigators due to its possible immunogenicity. This antigen is frequently recognized by PBLs and TILs of melanoma patients (4, 7) that express the HLA restricting element (HLA-A*0201) for its immunodominant peptide, AAGIGILTV (6, 11).

In this study, we evaluated frequency in peripheral blood of CTL precursors (CTLp) to Melan-A/Mart-1$_{27-35}$ peptide in metastatic melanoma patients and the possible relationship with immune response in metastatic lesions. To this end, we dissected the relative contribution of memory versus naive peptide–specific T cell subsets to the overall PBL precursor frequency against Melan-A/Mart-1$_{27-35}$ in HLA-A*0201$^+$ patients. The results indicated that in approximately half of patients, an expanded T cell population to Melan-A/Mart-1 can be found in peripheral blood and is characterized by peptide-specific T cells with a memory phenotype. The CTLp frequency in blood explained both kinetics of CTL induction in vitro and the number of effectors that could be generated by selection with peptide-loaded professional APCs. However, evidence of tumor regression was missing in most tumor lesions, including those isolated from the patients with an expanded T cell population to Melan-A/Mart-1. These results contribute to an explanation of tumor progression in human metastatic melanoma in spite of evidence for peripheral immune response to a tumor antigen.

Materials and Methods

Patients. Nine metastatic melanoma patients, admitted to our Institute (Istituto Nazionale per lo Studio e la Cura dei Tumori)

for surgery and chosen for expression of the HLA-A*0201[+] allele as determined by single-stranded oligonucleotide probe–PCR typing (6), were selected for this study. Characteristics of the patients are described in Table I. At the time the PBLs were isolated for CTLp frequency determination, all patients had already developed lymph node metastases (stage III, AJCC). Further progression of disease occurred in seven out of nine patients after CTLp analysis (Table I). Six out of nine patients died of disease between 1 and 16 mo after CTLp evaluation; the remaining three patients are still alive at 41 mo (patient 5, alive with disease) and 38 mo (patients 7 and 8, both without evidence of disease) after CTLp evaluation (Table I). None of the patients enrolled in this study had been subjected to chemotherapy or any other therapy with immunosuppressive activity before isolation of the PBLs used for limiting dilution analysis (LDA; Table I).

Phenotype of Melanoma Cells and Lines. Tumor lines used in this study were isolated as previously described (6) from HLA-A*0201[+] patients admitted to our Institute for surgical treatment of either primary or metastatic melanoma. Expression of Melan-A/Mart-1 antigen in these lines, as well as in fresh tumor cells isolated from some surgical specimens, was determined by intracellular fluorescence analysis on saponin-permeabilized cells followed by FACS™ analysis (Becton Dickinson) with the Melan-A/Mart-1–specific mAb M27C10 (12), a gift of Dr. F. Marincola (National Cancer Institute, Bethesda, MD). In addition, all lines were found positive by flow cytometry for HLA-A2, CD54, and lymphocyte function associate (LFA)-3, whereas none expressed CD80. Fresh tumor cells from surgical specimens were also characterized for HLA-A2 expression by flow cytometry after staining with mAb CR11.351 (anti–HLA-A2) (13).

Peptides and APC Loading. Melan-A/Mart-1$_{27-35}$ (AAGIGILTV), influenza A (Flu) matrix$_{58-66}$ (GILGFVFTL), and tyrosinase$_{366-378}$ (YMNGTMSQV) peptides were used in this study (11, 14, 15). All synthetic peptides were ≥95% pure (PRIMM srl; San Raffaele Biomedical Science Park, Milan, Italy). Stock solutions of peptides were set up in DMSO and kept at −20°C. The concentrations of Melan-A/Mart-1$_{27-35}$ (10 μg/ml) and Flu matrix$_{58-66}$ (5 μg/ml) peptides to load the TAP-deficient T2 cell line to be used in LDA assays were determined by the binding assay based on HLA-A2 stabilization and resulting in the same fluorescence ratio of 3.2 as previously described (16). These peptide concentrations were also used to load other APCs used in the study.

Isolation of CD45RO^{+} and CD45RA^{+} T Cells by Negative Immunomagnetic Sorting. 5×10^6 T lymphocytes purified by nylon wool column after Ficoll separation were resuspended in 100 μl of PBS containing 0.5% autologous human serum and 0.6% acid citrate dextrose (Baxter Healthcare Ltd.). To two identical aliquots of this cell suspension, 20 μL of MACS® CD45RA or MACS® CD45RO Microbeads (Miltenyi Biotec) were added, mixed, and allowed to incubate at 4°C for 15 min. After incubation, the cells were washed by adding 5 ml of PBS followed by centrifugation at 800 rpm for 10 min at 4°C. The supernatant was discarded and the cell pellet resuspended in 1 ml of cold PBS. The separation column (MS type; Miltenyi Biotec) was primed by washing with 1 ml of cold PBA and placed in a magnetic field. The washed cell pellet, pretreated with either MACS® CD45RA or MACS® CD45RO microbeads, was applied to the prefilled columns. Cells expressing either CD45RA or CD45RO were retained in the columns, whereas the negative fraction (i.e., CD45RO^{+} in columns loaded with CD45RA^{+}-stained cells or vice versa) was eluted and used for the LDA assays. Purity of the two T cell subsets was assessed by flow cytometry and resulted in ≥98% in all instances.

Determination of Melan-A/Mart-1$_{27-35}$–specific and Flu Matrix$_{58-66}$–specific CTLp Frequency in Peripheral Blood. The HLA-A*0201[+] TAP-deficient T2 cell line, an effective APC for the activation of even naive T cells and the generation of CTLs against peptides from self- and non-self proteins (17), was used as APC for peptide presentation in all LDA assays. Lymphocytes isolated from peripheral blood of melanoma patients or HLA-A*0201[+] healthy donors by Ficoll gradient centrifugation were used for determination of peptide-specific CTLp frequency after monocyte depletion. The LDA technique for determination of peptide-specific CTLp frequency was performed as described (6, 16). After 4 wk of culture, each of the replicate wells of all LDA cultures was split into two aliquots and tested against an empty or peptide-loaded HLA-A*0201[+] LCL (9742 LCL). Melan-A/Mart-1$_{27-35}$ or Flu matrix$_{58-66}$ peptides were used depending on the LDA sets. To evaluate the frequency of HLA-A2–restricted precursors, in some experiments the split well analysis was performed by testing lysis of an HLA-A*0201[+]Melan-A/Mart-1^{+} melanoma that was or was not preincubated with an anti–HLA-A2 A28 mAb (CR11.351). The cytolytic assay was performed as described (6, 16). To increase the efficiency of the assay, all cytolytic tests, involving either 9742 LCL or melanoma cells as targets, were performed in the presence of 3×10^2 targets per well. The threshold of significant lysis, criteria to score a well as containing a peptide-specific CTLp, and data analysis for CTLp frequency determination have been described elsewhere (6, 16). As developed, this LDA assay cannot detect CTLp frequencies <1/200,000. This LDA technique was also used to evaluate frequency of CTL effectors in bulk T cell cultures. In these instances, T cells from bulk cultures were seeded in LDA sets and tested for specificity by the split well technique after 1 wk.

T Cell Cultures. After monocyte depletion, PBLs were cultured in 24-well plates (Costar Corp.) at 10^6 cells/ml in 2 ml of RPMI 1640 supplemented with 10% heat-inactivated human serum in the presence of 0.5×10^6/ml irradiated, peptide-loaded T2 cells. Independent cultures were set up using T2, either empty or loaded with Melan-A/Mart-1$_{27-35}$ or tyrosinase$_{366-378}$ peptides. Low dose (10 U/ml) IL-2 was added on day 2 to all cultures. All cultures were then restimulated weekly with peptide-loaded or empty T2 cells. The resulting T cell lines were tested weekly from days 21–70 for specificity (by ^{51}Cr-release assays) on peptide-loaded or empty 9742 LCL cells. Specificity of these T cell lines was also tested on HLA-A*0201[+] melanomas that did or did not express Melan-A/Mart-1. HLA-A2 restriction of melanoma lysis was checked by comparing lysis of melanomas that were or were not preincubated with mAb CR11.351. Induction of Melan-A/Mart-1$_{27-35}$–specific CTLs was also carried out in two patients (patients 7 and 8) by coculture with peptide-loaded autologous dendritic cells (DCs) derived from CD34^{+} progenitors or monocytes. DCs were differentiated from purified CD34^{+} progenitors or monocytes as recently described (16). CD34^{+} progenitors were initially mobilized by G-CSF treatment (a written informed consent was signed by patients for this treatment). Phenotype of DCs and monocytes was evaluated by flow cytometry with mAbs to CD1a (Coulter Immunology), CD14, HLA-DR, HLA-DQ (Becton Dickinson), CD40, CD80 (Serotec Ltd.), CD86 (Ancell), and CD54 (Immunotech). Control cultures from the same two patients were also set up with autologous fresh monocytes loaded with either Melan-A/Mart-1$_{27-35}$ or Flu matrix$_{58-66}$ peptides. All cultures, set up with autologous DCs or monocytes as APCs, were restimulated weekly with fresh, peptide-loaded monocytes.

Flow Cytometry Analysis after T Cell Staining with HLA–Peptide Tetrameric Complexes. HLA–peptide tetrameric complexes were

Table II. *Frequency of Peptide-specific CTLp Directed to Melan-A/Mart-1$_{27-35}$ Peptide in Peripheral Blood of Melanoma Patients*

	Frequency of CTLp to Melan-A/Mart-1$_{27-35}$ peptide in		
Patient	PBLs	CD45RO$^+$ T cell subset	CD45RA$^+$ T cell subset
1	1/1,404	ND	ND
2	1/1,708	1/1,118	1/56,787
6	1/1,399	1/879	<1/200,000
9	1/2,225	ND	ND
3	1/43,717	ND	ND
4	1/42,600	ND	ND
5	1/117,555	ND	ND
7	<1/200,000	<1/200,000	1/42,919
8	1/96,691	<1/200,000	1/22,430

CTLp frequency was evaluated in peripheral blood by LDA as described in Materials and Methods by using peptide-loaded T2 cells as APCs. Results are expressed as peptide-specific CTL precursors/PBLs. In patients 6 and 9, frequency of Melan-A/Mart-1–specific T cells in PBLs was 1/1,456 and 1/1,843, respectively, by staining with HLA-A*0201–Melan-A/Mart-1$_{26-35}$ tetrameric complexes.

synthesized as described (18). Staining of PBLs, TILs, and T cell lines was performed by incubating for 30 min in ice with PE-conjugated HLA-A*0201–Melan-A/Mart-1$_{26-35}$ tetramers. The modified Melan-A/Mart-1$_{26-35}$ peptide (ELAGIGILTV) used for refolding the HLA–peptide complex has been shown to increase the binding affinity for HLA-A*0201 without affecting CTL recognition (19). Negative controls for tetramer staining included PBLs from HLA-A*0201–negative healthy donors and HLA-A2–restricted CTL clones directed to tyrosinase peptides (6); a Melan-A/Mart-1$_{27-35}$–specific CTL clone, A83 (16), was used as positive control. Two-color fluorescence analysis was performed in some instances by staining T cell lines with FITC-conjugated anti-CD8 and PE-conjugated tetramers. At least 10^5 cells were analyzed for staining with PE-conjugated tetramers.

Immunohistochemical Analysis of Primary and Metastatic Melanoma Lesions. Immunohistochemical analysis was performed on routinely formalin- or Bouin's-fixed and paraffin-embedded specimens. 4-μm-thick tissue sections were deparaffinized through graded series of ethanol passages and rehydrated in distilled water. Endogenous peroxidase was inhibited by a 30-min incubation in methanol containing 0.3% H_2O_2. To optimize immunodetection of Melan-A/Mart-1, gp100, and CD8, nonenzymatic antigen unmasking was performed by heating tissue sections at 95°C for 6 min in an autoclave in 5 mM citrate buffer, pH 6 (20). After cooling, sections were incubated with normal goat serum (1:50; DAKO Corp.) diluted in PBS containing 1% BSA for 30 min. Primary antibody incubation was performed overnight at 4°C with the following antibodies: M27C10 (12), anti–Melan-A/Mart-1 (1:50), HMB45, anti-gp100 (1:25; DAKO Corp.), and CD8 (1:20; DAKO Corp.). Staining with polyclonal antibody CD3 (DAKO Corp.) was performed after 0.1% trypsin treatment for 5 min as described for antigen unmasking. Sections were subsequently rinsed three times in PBS plus Triton X-100 and

treated with biotinylated goat anti–mouse Ig (1:100; DAKO Corp.) or with biotinylated goat anti–rabbit Ig (1:200; DAKO Corp.) for CD3 staining. The slides were then covered with streptavidin–horseradish peroxidase (1:300; DAKO Corp.) for 30 min and finally visualized with the use of red 3-amino-9-ethyl-carbazole (Sigma Chemical Co.) in 0.05 M acetate buffer containing 0.015% H_2O_2. Sections were then counterstained with hematoxylin and mounted with glycerine-gelatin 4. Tissue sections subjected to the same treatment but without incubation with primary antibody were used as negative controls. Positive controls were a reactive lymph node for CD3 and CD8 and a Melan-A/Mart-1$^+$ human melanoma cell line grown in nude mice that was subsequently formalin fixed and paraffin embedded for Melan-A/Mart-1.

Results

*An Expanded Pool of Melan-A/Mart-1$_{27-35}$–specific Memory T Cells Can Be Found in Peripheral Blood of a Fraction of HLA-A*0201$^+$ Metastatic Melanoma Patients.* Using LDA, we evaluated the CTLp frequency against a peptide (AAGIGILTV in the context of HLA-A*0201) from the melanocyte lineage–specific antigen Melan-A/Mart-1 in peripheral blood of nine HLA-A*0201$^+$ metastatic melanoma patients. Two CTLp frequency groups were found (Table II): a group (patients 1, 2, 6, and 9) with high frequency of CTLp to Melan-A/Mart-1$_{27-35}$ (between 1/1,400 and 1/2,225) and a group (patients 3, 4, 5, 7, and 8) with low frequency of CTLp (between 1/40,000 and <1/200,000) to the same antigen. In agreement with our previous results (6, 21), independent LDA assays performed using PBLs isolated from the same patient a few months apart indicated that the CTLp frequency remained in the same range in each of the two groups of patients (data not shown). Furthermore, staining with Melan-A/Mart-1–HLA-A*0201 tetrameric complexes indicated a frequency of peptide-specific T cells of 1/1,456 and 1/1,843 in PBLs of patients 6 and 9, respectively, in good agreement with CTLp frequency by LDA. By using lymphocytes from the same blood sample, CTLp analysis was reassessed in sorted memory (CD45RO$^+$) and naive (CD45RA$^+$) T cell subsets. In two patients from the high CTLp frequency group, Melan-A/Mart-1$_{27-35}$–specific CTLp were mostly (patient 2) or only (patient 6) found in the CD45RO$^+$ memory T cell subset (Table II). By contrast, in two patients from the low CTLp frequency group (patients 7 and 8), Melan-A/Mart-1$_{27-35}$–specific CTLp were detected only in the CD45RA$^+$ naive T cell subset.

Low frequency of CTLp to Melan-A/Mart-1$_{27-35}$, detected in patients 3, 4, 5, 7, and 8, was not the result of nonspecific immune suppression preventing precursor growth or development of cytolytic function in LDA cultures. In fact, in these patients, the CTLp frequency of a different HLA-A*0201–restricted T cell epitope (the immunodominant Flu matrix$_{58-66}$ peptide) was in the same range, or even higher, than that found in a panel of HLA-A*0201$^+$ healthy donors (Table III). Taken together, these data indicate that expansion of the Melan-A/Mart-1$_{27-35}$–specific T cell population occurs in a fraction of metastatic

Table III. *Frequency of Peptide-specific CTLp Directed to Flu Matrix$_{58-66}$ in the Context of HLA-A*0201 in Melanoma Patients and Healthy Donors*

Subject	Frequency of CTLp to Flu matrix$_{58-66}$ peptide
Patient 3	1/4,195
Patient 4	1/7,426
Patient 5	1/6,967
Patient 7	1/5,471
Patient 8	1/4,435
Healthy donor 1	1/10,506
Healthy donor 2	1/5,591
Healthy donor 3	1/13,177
Healthy donor 4	1/15,246
Healthy donor 5	1/7,588
Healthy donor 6	1/13,440

CTLp frequency was evaluated in peripheral blood by LDA as described in Materials and Methods using peptide-loaded T2 cells as APCs. Results are expressed as peptide-specific CTLp/PBLs.

melanoma patients, as indicated by a high-frequency peripheral pool of antigen-specific T cells with a memory phenotype.

A Low Frequency of CTLp to Melan-A/Mart-1$_{27-35}$ Reflects a Naive Immune Repertoire Requiring Professional APCs for CTL Activation. In patients of the low CTLp frequency group, Melan-A/Mart-1–specific CTLs were activated in bulk culture with professional APCs (DCs and T2) versus nonprofessional APCs (monocytes), and then the frequency of CTL effectors was quantitated by LDA. As shown in Table IV, after 42 d of bulk culture, Melan-A/Mart-1$_{27-35}$–specific effectors were expanded in patient 7 to a final fre-

quency ranging from 1/63 to 1/142 after activation with peptide-loaded T2 or autologous DCs. No peptide-specific CTLs were found by LDA in cultures activated with peptide-loaded monocytes. Similar results were obtained with another patient in the low-frequency group (data not shown). In contrast, nonprofessional APCs (monocytes) could reactivate and expand Flu matrix$_{58-66}$–specific T cells (a memory response that depends on previous viral exposure and does not require professional APCs for reactivation). In fact, a Flu matrix–specific CTL effector frequency of 1/1,190 was found in patient 7 after 21 d of bulk culture (Table IV), in comparison to a precursor frequency of 1/5,471 in fresh PBLs (Table III). These data indicate that patients with low CTLp frequency of Melan-A/Mart-1$_{27-35}$ are endowed with only a naive immune repertoire, made up of rare precursors that require professional APCs for priming and proliferation.

Impact of CTLp Frequency on Kinetics of Melan-A/Mart-1–specific CTL Generation in Bulk Culture. Kinetics of activation of Melan-A/Mart-1$_{27-35}$–specific CTLs in bulk culture was compared in patients with low and high CTLp frequency. T2 cells (shown in Table IV to have the same APC efficiency as DCs) were used as stimulators after loading with Melan-A/Mart-1$_{27-35}$ or control tyrosinase$_{366-378}$ peptide. In T cell cultures from patients 1 and 2 (two patients with high CTLp frequency), Melan-A/Mart-1$_{27-35}$ specificity was already evident on day 21 (data not shown) and was confirmed on days 28 and 36 of culture (Table V). Peptide specificity was documented by recognition of peptide-loaded 9742 LCL, HLA-A2–restricted lysis of HLA-A*0201$^+$Melan-A/Mart-1$^+$ tumors (INT-MEL-8 and INT-MEL-9), and absence of HLA-restricted lysis on an HLA-A*0201$^+$Melan-A/Mart-1$^-$ melanoma (INT-MEL-10) (Table V). By contrast, T cell cultures from patients 3, 4, and 5 (all with low CTLp frequency) lacked peptide specificity against 9742 LCL targets, either on day 28 or 36. T cell cultures from patient 3 showed HLA-A2–restricted lysis of two Melan-

Table IV. *Frequency of Melan-A/Mart-1$_{27-35}$–specific CTL Effectors on Day 42 of Bulk Culture after T Cell Activation with Different APCs*

APCs	Peptide used to load APCs in bulk culture	Frequency, after bulk culture, of peptide-specific CTL effectors directed to	
		Melan-A/Mart-1$_{27-35}$ peptide	Influenza matrix$_{58-66}$ peptide
T2 cells	Melan-A/Mart-1$_{27-35}$	1/69	ND
CD34$^+$-derived autologous DCs	Melan-A/Mart-1$_{27-35}$	1/63	ND
Monocyte-derived autologous DCs	Melan-A/Mart-1$_{27-35}$	1/142	ND
Fresh autologous monocytes	Melan-A/Mart-1$_{27-35}$	<1/200,000	ND
Fresh autologous monocytes	Flu matrix$_{58-66}$		1/1,190

Frequency of Melan-A/Mart-1$_{27-35}$- and Flu matrix$_{58-66}$–specific effectors was determined respectively on days 42 and 21 of bulk T cell culture. Responder lymphocytes were from one of the patients (patient 7) in the low CTLp frequency subset. Phenotype of all APCs was checked by flow cytometry. CD34$^+$ and monocyte-derived DCs expressed CD1a, CD40, CD80, CD86, CD54, HLA-DR, and HLA-DQ antigens. T2 cells expressed CD40, CD80, CD86, and CD54. CD14 was expressed only by monocytes, which were also positive for HLA-DR and CD54 but lacked CD40, CD80, CD86, and HLA-DQ antigens.

PBLs from patient	*T2 cells loaded with	‡Test day	9742 LCL			‖INT-MEL-8		‖INT-MEL-9		‖INT-MEL-10	
			—	+Melan-A/Mart-1$_{27-35}$	+Tyrosinase$_{366-378}$	−mAb	+mAb	−mAb	+mAb	−mAb	+mAb
1	—	28	2¶	0	3	2	1	19	17	2	1
	Melan-A/Mart-1$_{27-35}$	28	6	**39**	4	**70**	6	**47**	15	8	11
	Tyrosinase$_{366-378}$	28	2	0	3	4	4	12	8	5	4
1	—	36	7	12	7	3	7	22	26	1	0
	Melan-A/Mart-1$_{27-35}$	36	4	**80**	6	**55**	6	**51**	**3**	1	0
	Tyrosinase$_{366-378}$	36	9	9	12	2	5	5	3	1	0
2	—	28	2	1	3	10	7	10	11	7	5
	Melan-A/Mart-1$_{27-35}$	28	6	**17**	8	**69**	33	**57**	19	13	10
	Tyrosinase$_{366-378}$	28	2	2	6	45	34	30	15	13	16
2	—	36	9	12	10	9	11	48	41	6	5
	Melan-A/Mart-1$_{27-35}$	36	29	**95**	24	**81**	47	**86**	23	13	17
	Tyrosinase$_{366-378}$	36	12	17	**78**	55	17	82	17	8	8
3	—	28	3	2	3	18	9	14	16	7	11
	Melan-A/Mart-1$_{27-35}$	28	14	16	15	**28**	12	**36**	25	15	16
	Tyrosinase$_{366-378}$	28	0	0	1	10	2	9	3	2	1
3	—	36	5	1	2	9	12	48	49	8	16
	Melan-A/Mart-1$_{27-35}$	36	10	6	8	**36**	21	**88**	68	15	14
	Tyrosinase$_{366-378}$	36	3	0	0	6	11	28	30	2	2
3	Melan-A/Mart-1$_{27-35}$	49	2	**55**	5	**80**	15	**79**	50	ND	ND
4	—	28	0	0	0	2	1	0	2	0	0
	Melan-A/Mart-1$_{27-35}$	28	0	0	0	34	36	17	29	26	31
	Tyrosinase$_{366-378}$	28	4	2	7	28	19	28	23	25	17
4	—	36	3	3	2	2	4	8	9	0	0
	Melan-A/Mart-1$_{27-35}$	36	4	7	3	42	44	52	60	38	38
	Tyrosinase$_{366-378}$	36	3	1	2	24	21	38	31	12	13
5	—	28	1	0	2	4	3	37	46	5	7
	Melan-A/Mart-1$_{27-35}$	28	2	0	2	11	3	28	35	2	6
	Tyrosinase$_{366-378}$	28	16	13	22	75	73	31	44	12	15
5	—	36	3	2	1	7	8	52	67	0	1
	Melan-A/Mart-1$_{27-35}$	36	1	1	0	2	4	47	56	0	0
	Tyrosinase$_{366-378}$	36	61	46	49	66	71	60	60	18	26

*T cell cultures were set up by coculture of patients' PBLs with T2 cells either unloaded or loaded with peptides Melan-A/Mart-1$_{27-35}$ or tyrosinase$_{366-378}$.

‡T cell lines were tested for specificity after the indicated time of culture.

§Targets were HLA-A*0201$^+$ 9742 LCL (either unloaded or loaded with Melan-A/Mart-1$_{27-35}$ or tyrosinase$_{366-378}$ peptides), two HLA-A*0201$^+$ Melan-A/Mart-1$^+$ melanomas (INT-MEL-8 and INT-MEL-9), and one HLA-A*0201$^+$Melan-A/Mart-1$^-$ melanoma (INT-MEL-10).

‖Lysis of melanoma cells was tested after target preincubation (+mAb) or no preincubation (−mAb) with anti–HLA-A2 A28 mAb CR11.351.

¶Results expressed as percent lysis in a 4-h ^{51}Cr-release assay. E/T ratio was 10:1. Values in bold represent significant lysis of peptide-loaded 9742 LCL in comparison to empty targets or lysis of melanoma cells significantly inhibited by mAb CR11.351 (student-Newman-Kevls [SNK] test, $P = 0.01$).

A/Mart-1$^+$ melanomas on days 28 and 36, but evidence of peptide specificity on peptide-loaded 9742 LCL was obtained only on day 49 of culture (Table V). In other patients of the low CTLp frequency group, peptide specificity on 9742 LCL and HLA-A2–restricted recognition of tumors required up to 70 d of bulk culture to be obtained (data not shown). These data indicate that a high frequency of peptide-specific CTLp in blood of patients correlates with faster kinetics of generation of antigen specificity after in-vitro T cell activation with peptide-loaded APCs.

Impact of CTLp Frequency on CTL Effector Frequency.
CTL effector frequencies in bulk T cell cultures were compared in patients with low or high precursor frequency by LDA and T cell staining with Melan-A/Mart-1–HLA-A*0201 tetrameric complexes (Table VI). In day 28 bulk cultures of three patients of the high CTLp group, peptide-specific CTL effectors were between 1/10 and 1/24. By contrast, in day 28 bulk cultures from patients of the low CTLp group, the peptide-specific effectors were between 1/576 and 1/4,531. In two patients (patients 2 and 6), evaluation of CTL effector frequency by LDA was performed by two distinct readout systems: differential lysis of peptide-loaded or nonloaded 9742 LCL and differential recognition of HLA-A*0201+Melan-A/Mart-1+ melanoma cells that were or were not preincubated with an anti–HLA-A2 mAb. Effector frequencies were similar by both readout systems (Table VI). Control LDA assays, performed on T cell cultures activated by empty T2 (Table VI), gave no detectable peptide-specific effector frequency (patients 1, 4, 5, 7, and 8). Furthermore, frequency evaluation by LDA and Melan-A/Mart-1–HLA-A*0201 tetrameric complexes gave similar results (Table VI). This indicated that essentially all T cells expressing a Melan-A/Mart-1–specific TCR, as identified by tetrameric complexes, could also be functionally identified by our LDA approach. In addition, T cell staining with tetrameric complexes allowed us to follow the evolution in culture of Melan-A/Mart-1–specific T cells during selection with peptide-loaded APCs. As shown in Fig. 1, the number of peptide-specific T cells rose from 1/599 (Fig. 1 E, day 14) to 1/11 (Fig. 1 G, day 56) in

a T cell culture from patient 6 and from 1/76 (Fig. 1 H, day 14) to 1/1.36 (Fig. 1 J, day 42) in the T cell culture of patient 2. This indicates that in the patients with high precursor frequency, T cell activation with peptide-loaded T2 cells leads to early and progressive expansion of peptide-specific T cells. Taken together, these data suggest that a high CTL effector frequency, after APC-mediated T cell selection, can be achieved only in patients with a high CTLp frequency in blood. Thus, presence of an expanded peripheral pool of T cells to a tumor antigen is an important requisite for efficient in vitro selection of antitumor T cells from peripheral blood of patients.

Melan-A/Mart-1$_{27-35}$–specific CTLs Can Be Isolated from Metastatic Lesions of Patients with High Frequency of CTLp to the Same Antigen. T cells isolated from metastases of patients 1 and 2 (two patients of the high CTLp frequency group) and patient 3 (with low CTLp frequency) were tested for specificity after 3 wk of selection in bulk culture with Melan-A/Mart-1$_{27-35}$–loaded T2 cells as APCs. The T cell lines from lesions of patients 1 and 2 specifically recognized 9742 LCL loaded with Melan-A/Mart-1$_{27-35}$ peptide and lysed the two Melan-A/Mart-1+ tumors INT-MEL-8 and INT-MEL-9 in an HLA-A2–restricted fashion (Table VII). By contrast, only nonspecific lysis on all targets by T cells isolated from a lymph node metastasis of patient 3 was observed. These findings were confirmed even after 36 d of culture (data not shown). Staining of TILs from the subcutaneous lesion of patient 1 with Melan-A/Mart-1–HLA-A*0201 tetrameric complexes revealed a frequency of 1/17.6 peptide-specific T cells (Fig. 1 D), thus indicat-

Table VI. *Frequency of Melan-A/Mart-1$_{27-35}$–specific CTL Effectors in Day 28 Bulk Cultures after T Cell Activation with Peptide-loaded T2 Cells: Comparison of LDA and T Cell Staining with Melan-A/Mart-1–HLA-A*0201 Tetrameric Complexes*

Patient	*Evaluation by LDA		§HLA-A2–restricted CTL effector frequency after lymphocyte activation with peptide-loaded T2 cells	‖Evaluation by HLA-A*0201– Melan-A/Mart-1$_{26-35}$ tetrameric complexes
	‡Peptide-specific CTL effector frequency after lymphocyte activation with			Peptide-specific effector frequency after lymphocyte activation with peptide-loaded T2 cells
	Peptide-loaded T2	Empty T2		
1	1/9.8	<1/200,000	ND	1/10.3
2	1/15.8	ND	1/14.2	1/10.4
6	1/24	ND	1/42	1/23.1
3	1/4,531	ND	ND	ND
4	1/576	<1/200,000	ND	ND
5	1/1,093	<1/200,000	ND	1/961
7	1/1,509	<1/200,000	ND	ND
8	1/831	<1/200,000	ND	1/685

Patients' PBLs were cultured for 28 d in bulk culture in the presence of T2 cells either empty or loaded with Melan-A/Mart-1$_{27-35}$ peptide. Frequency of peptide-specific CTL effectors in bulk cultures was evaluated by LDA or by HLA-A*0201–Melan-A/Mart-1 tetramer staining. *Split well analysis was performed by ‡comparing lysis of empty and Melan-A/Mart-1$_{27-35}$–loaded 9742 LCL or §comparing lysis of the HLA-A*0201+Melan-A/Mart-1+ melanoma INT-MEL-8 that was or was not preincubated with anti–HLA-A2 A28 mAb CR11.351. ‖Frequency of peptide-specific T cells was evaluated by staining with PE-conjugated HLA-A*0201–Melan-A/Mart-1$_{26-35}$ tetrameric complexes. All T cell lines were >99% CD3+ at time of analysis.
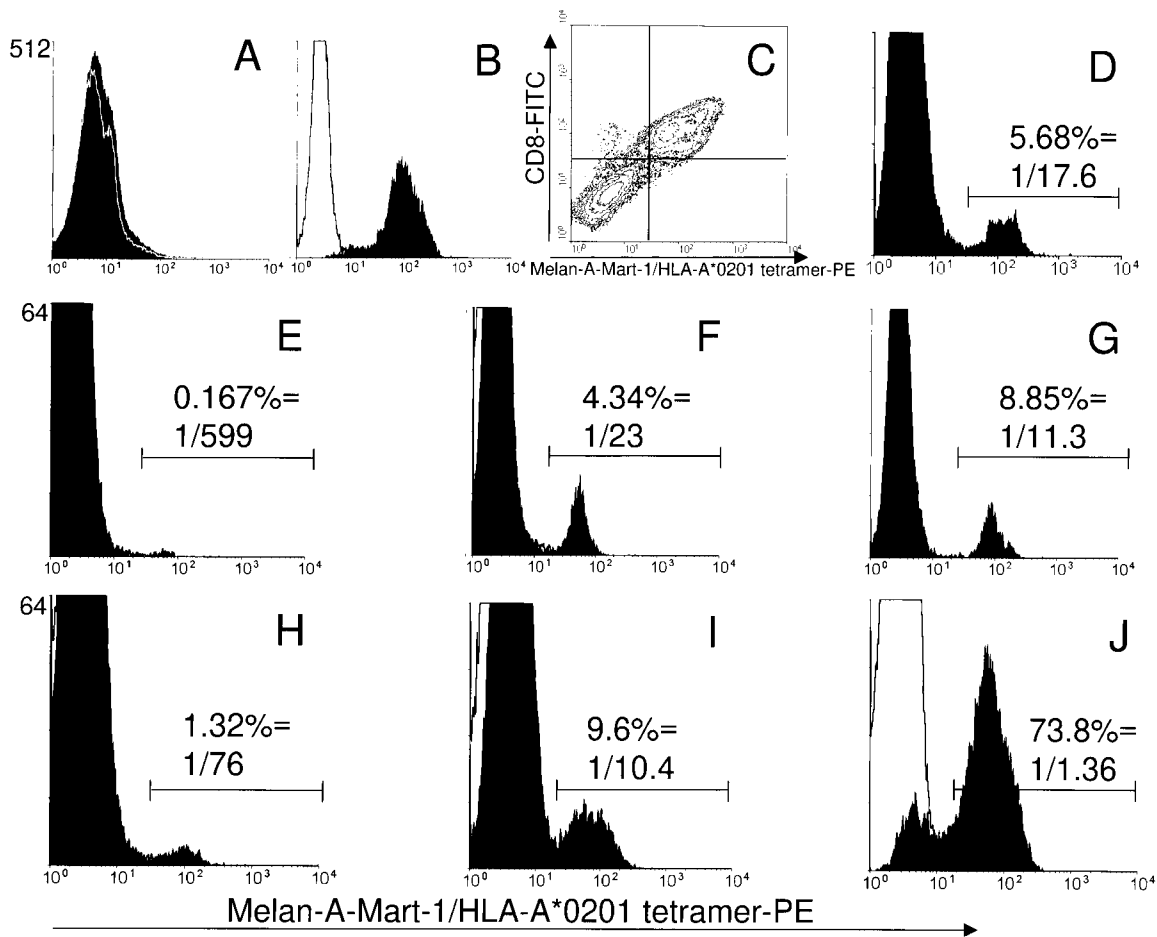
Figure 1. Identification of Melan-A/Mart-1–specific T cells in T cell lines from melanoma patients by Melan-A/Mart-1$_{26-35}$–HLA-A*0201 tetrameric complexes. Lymphocytes from peripheral blood of an HLA-A*0201$^-$ healthy donor (A) did not stain with the tetramer–PE, whereas 100% of cells from a Melan-A/Mart-1$_{27-35}$–specific CTL clone A83 (B) were tetramer positive. In a T cell line selected in vitro for Melan-A/Mart-1$_{27-35}$ specificity (from patient 2, week 5 of culture), all tetramer-positive cells were within the CD8$^+$ subset (C). Fresh TILs from a subcutaneous metastatic lesion (lesion 3) of patient 1 contained 5.68% of tetramer-positive T cells (D). T cell lines from two melanoma patients were selected in vitro with Melan-A/Mart-1$_{27-35}$–loaded T2 line as APCs and analyzed for tetramer staining at different times during culture (E–J). A T cell line from patient 6 was analyzed on weeks 2 (E), 4 (F), and 8 (G) of culture, and a T cell line from patient 2 was analyzed on weeks 2 (H), 4 (I), and 6 (J) of culture. The proportion of Mart-1$_{26-35}$–HLA-A*0201 tetramer–PE-positive T cells in each line is shown together with conversion to frequency. At least 10^5 cells were analyzed in each panel. The proportion of CD3$^+$ T cells was 97% in fresh TILs from patient 1 (D) and >99% in all T cell lines from the two patients (C, E–J), with the exception of the line shown in H (CD3$^+$ = 78.4%).

ing a very high enrichment in comparison to frequency of CTLp to the same antigen detected in peripheral blood of the same patient (1/1,404; Table II). Thus, patients with an expanded peripheral pool of Melan-A/Mart-1$_{27-35}$–specific T cells do have peptide-specific T cells in their metastatic lesions. Furthermore, the CTLp present in metastatic lesions could be readily activated by appropriate antigen presentation to acquire effector function with a fast kinetics of proliferation, suggesting absence of any irreversible functional block.

Immunohistochemical Analysis of Metastatic Lesions. To evaluate the relationship between the expanded peripheral pool of T cells to Melan-A/Mart-1 and in vivo response to tumor lesions, all available primary and metastatic lesions isolated during tumor progression from the nine patients were

analyzed by immunohistochemistry. To this end, the brisk/nonbrisk/absent code for defining patterns of infiltrating T cells was adopted (1, 2). A common pattern emerged: in all patients, including those with high frequency of CTLp to Melan-A/Mart-1, evidence of tumor regression/necrosis was often completely lacking or, with few exceptions, appeared to involve only a minor portion of the area containing neoplastic cells in each lesion (Table VIII). Moreover, tumor regression, when present, often appeared as areas of coagulative necrosis, sometimes admixed with hemorrhage, that were never infiltrated or immediately surrounded by CD3$^+$ lymphocytes, even in the lesions containing brisk or nonbrisk CD3$^+$CD8$^+$ T cells. Furthermore, with the exception of all lesions from patient 9, which lacked HLA class I antigens, including HLA-A2, all other lesions that

Table VII. *Melan-A/Mart-1$_{27-35}$–specific CTLs Can Be Isolated from Metastatic Lesions of Patients with High Frequency of CTLp to the Same Antigen*

					‖Lysis of								
					9742 LCL loaded with			INT-MEL-8		INT-MEL-9		INT-MEL-10	
Patient	*T cells isolated from	‡Lesion number	Classification of infiltrating T cells	§T cells cocultured with	—	P1	P2	−mAb	+mAb	−mAb	+mAb	−mAb	+mAb
1	s.c. met.	3	nonbrisk	Empty T2	25¶	24	28	49	50	49	37	23	28
				T2 loaded with Melan-A/Mart-1$_{27-35}$	8	**75**	7	65	**6**	70	**5**	2	0
2	LN met.	9	nonbrisk	Empty T2	58	66	53	76	71	82	61	44	54
				T2 loaded with Melan-A/Mart-1$_{27-35}$	15	**93**	13	82	**39**	100	**15**	11	18
3	LN met.	16	absent	Empty T2	54	72	59	81	83	62	57	44	64
				T2 loaded with Melan-A/Mart-1$_{27-35}$	41	59	44	79	84	60	51	43	63

*The subcutaneous metastasis (s.c. met.) from patient 1 was removed 8 d before CTLp frequency evaluation in peripheral blood. The lymph node metastases (LN met.) from patients 2 and 3 were removed 6 d after and the same day of, respectively, CTLp frequency evaluation in peripheral blood.

‡See Table VIII for characteristics of these lesions.

§T cell cultures were set up by coculture of patients' PBLs with T2 cells either unloaded or loaded with peptide Melan-A/Mart-1$_{27-35}$. T cell lines were tested on day 21 of culture.

‖Targets were HLA-A*0201$^+$ 9742 LCL, either unloaded (—) or loaded with Melan-A/Mart-1$_{27-35}$ (P1) or tyrosinase$_{366-378}$ (P2) peptides, two HLA-A*0201$^+$Melan-A/Mart-1$^+$ melanomas (INT-MEL-8 and INT-MEL-9), and one HLA-A*0201$^+$Melan-A/Mart-1$^-$ melanoma (INT-MEL-10). Lysis of melanoma cells was tested after target preincubation (+mAb) or no preincubation (−mAb) with anti–HLA-A2 A28 mAb CR11.351.

¶Results expressed as percent lysis in a 4-h ^{51}Cr-release assay. E/T ratio was 10:1. Values in bold represent significant lysis of peptide-loaded 9742 LCL, in comparison to empty targets or lysis of melanoma cells significantly inhibited by mAb CR11.351 (SNK test, $P = 0.01$).

could be analyzed expressed HLA-A2, suggesting that in these instances T cell epitope presentation was not impaired in tumor cells (Table VIII).

In patient 1, the primary lesion (lesion 1) and a satellitosis (lesion 2) were removed 2 mo before CTLp evaluation (Table VIII). The first lesion had an absent pattern of CD3$^+$ T cells and Melan-A/Mart-1 antigen expressed on 20% of the neoplastic cells. The satellitosis was nonbrisk for CD3$^+$ T cells, but CD8$^+$ T cells represented only 30% of them and Melan-A/Mart-1 was not expressed. A subcutaneous lesion (lesion 3) isolated 1 wk before CTLp evaluation was Melan-A/Mart-1$^+$ and expressed HLA-A2 on the tumor and 30% of CD8$^+$ cells among the nonbrisk CD3$^+$ infiltrate. However, no evidence of tumor destruction was observed, even though this same lesion contained a high frequency of Melan-A/Mart-1–specific CTLp (Table VII, Fig. 1). A lymph node metastasis (lesion 4) was almost completely negative for Melan-A/Mart-1 and absent for CD3$^+$ T cells. In the same patient, in spite of an expanded T cell population to Melan-A/Mart-1 in peripheral blood, three additional subcutaneous metastases developed within 6 mo of CTLp analysis. Two of these lesions expressed Melan-A/Mart-1, but no evidence of tumor regression or destruction was found, although all lesions contained a

brisk CD3$^+$CD8$^+$ infiltrate (Table VIII, lesions 5–7; Fig. 2, A–D).

In patient 2, a lymph node metastasis removed 6 d after CTLp analysis (Table VIII, lesion 9) showed tumor regression affecting 50% of the neoplastic tissue. Although this lesion contained Melan-A/Mart-1–specific CTLp (Table VII), the necrotic area appeared to be the result of an ischemic lesion and not of an immune response (Fig. 2 H). In fact, the nonbrisk CD3$^+$CD8$^+$ infiltrate (Fig. 2, E and F) did not surround nor infiltrate the necrotic area, which was instead surrounded by scattered granulocytes (Fig. 2 H). The tumor cells were HLA-A2$^+$, and some areas showed a weak staining for Melan-A/Mart-1 (Table VIII and Fig. 2 G), suggesting a possible tumor escape mechanism. In patient 6, an absent pattern of CD3$^+$ T cells was found in an HLA-A2$^+$ soft tissue metastasis lacking Melan-A/Mart-1 and removed 4 d after CTLp analysis (Table VIII, lesion 11), as well as almost no tumor regression but a 10% sclerosis. Again, the lack of Melan-A/Mart-1 suggests a possible tumor escape mechanism. In patient 9, three synchronous lesions were removed, including the primary tumor (lesion 12) and two metastases (lesions 13 and 14). All of these lesions were Melan-A/Mart-1$^+$ and lacked HLA class I, including HLA-A2, suggesting another mechanism of tumor

Table VIII.   *Immunohistochemical Analysis of Melanoma Lesions*

Patient	*Date	‡CTLp	Lesion number	§Tumor sample	‖Evidence of tumor regression	¶HLA class I on fresh tumor	¶HLA-A2 on fresh tumor	**CD3+ lymphocyte infiltrate	‡‡CD8+ lymphocyte infiltrate	§§Positive cells	‖‖Staining intensity
	d/m/y								%	%	
1	04/02/91		1	PR	Necr. (5%)			Absent		20	+++ (50%)
											+ (50%)
	04/02/91		2	PR¶¶	No			Nonbrisk	30	0	
	27/03/91		3	s.c. met.	No	95/371	91/225	Nonbrisk	30	90	
	29/03/91		4	LN met.	Necr. (1%)			Absent		<1	+
	04/04/91	High									
	24/10/91		5	s.c. met.	No			Brisk	100	50	+ (20%)
											+/− (80%)
	24/10/91		6	s.c. met.	No			Brisk	95	0	
	24/10/91		7	s.c. met.	No			Brisk	95	40	+ (20%)
											+/− (80%)
2	29/05/90		8	PR				NA		NA	NA
	04/07/91	High									
	10/07/91		9	LN met.	Necr. (50%)	100/312	92/170	Nonbrisk	100	15	+++ (20%)
											+ (80%)
6	11/80		10	PR				NA		NA	NA
	23/01/92	High									
	27/01/92		11	S.T. met.	Scler. (10%)	100/69	89/56	Absent		0	
9	28/10/87		12	PR	Scler. (10%)	0	0	Absent		70	+++ (60%)
											++ (40%)
	28/10/87		13	s.c. met.	No	0	0	Absent		50	+++ (30%)
											++ (70%)
	28/10/87		14	LN met.	No	0	0	Absent		80	+++ (70%)
	21/04/88	High									++ (30%)
3	23/04/91		15	PR	No			Absent		5	+ (100%)
	14/10/91	Low									
	14/10/91		16	LN met.	Necr. (30%)	94/80	95/32	Absent		20	+++ (70%)
											++ (30%)
	14/10/91		17	LN met.	Necr. (1%)			Absent		5	+++ (100%)
4	16/12/85		18	PR	No	100/169	82/83	Nonbrisk	50	<1	+
	16/12/85		19	LN met.	H.Necr. (1%)			Nonbrisk	20	0	

(continues)

Table VIII.   *(continued)*

Patient	*Date	‡CTLp	Lesion number	§Tumor sample	‖Evidence of tumor regression	¶HLA class I on fresh tumor	¶HLA-A2 on fresh tumor	**CD3+ lymphocyte infiltrate	‡‡CD8+ lymphocyte infiltrate	Melan-A/Mart-1 expression	
										§§Positive cells	‖Staining intensity
	19/01/87		20	S.T. met.	H.Necr. (1%)			Absent		0	
	19/01/87		21	S.T. met.	No			Absent		0	
	29/06/87	Low									
5	09/93		22	PR***	No	ND	80	Absent		0	
	08/11/95		23	s.c. met.	Scler. (30%)	100/98	95/118	Absent		20	+++ (90%) ++ (10%)
	10/11/95	Low									
	17/10/96		24	LN met.	H.Necr. (5%) Scler. (5%)			Absent		60	++ (40%) + (60%)
7	24/09/91		25	PR	No			Absent		20	+++
	27/01/93		26	LN met.	H.Necr. (25%) Scler. (15%)			Brisk	100	<1	+
	04/03/96	Low									
8	31/08/91		27	PR	No			Nonbrisk	100	70	++ (30%) + (70%)
	11/12/91		28	LN met.	Scler. (20%)			Brisk	50	30	++ (95%) +++ (5%)
	19/02/96	Low									

*Date of the surgical isolation of the different neoplastic lesions from each patient or of the isolation of PBLs for CTLp frequency analysis.

‡Patients were classified according to the two subsets of Melan-A/Mart-1$_{27-35}$–specific CTLp frequency: patients in the 1/2,000 range are coded as high, whereas patients in the ≤1/40,000 range are coded as low.

§Tumor specimens were from primitive lesions (PR) rather than from subcutaneous (s.c. met.), lymph node (L.N. met.), soft tissue (S.T. met.) metastases.

‖Evidence of tumor regression in each tissue section was coded as follows: absent (No), sclerosis (Scler.), necrosis (Necr.), or hemorrhagic necrosis (H.Necr.). The numbers in parentheses refer to the percentage of the area of neoplastic tissue showing the indicated type of regression. In all instances, with no exceptions, areas of tumor regression were never associated with infiltrating leukocytes but were often characterized as ischemic lesions.

¶Analysis was performed in the indicated cases on freshly isolated tumor cells from the same surgical specimen used for histological analysis. Expression of HLA class I antigens (as evaluated by staining with w6/32 mAb directed to a monomorphic determinant on all class I alleles) and HLA-A2 antigens (the restricting element for Melan-A/Mart-1$_{27-35}$ peptide) on tumor cells was evaluated by flow cytometry, as all available anibodies cannot detect HLA-A2 on paraffin-embedded sections. Results expressed as percent positive cells/mean fluorescence intensity on a four-decade log scale.

**CD3+ lymphocytes infiltrating the neoplastic tissue were identified by staining sections with anti-CD3 antibody and coded using the brisk/nonbrisk/absent classification (reference 2). NA, not available.

‡‡CD8+ lymphocytes infiltrating the neoplastic tissue were analyzed only in serial sections of lesions showing a brisk or nonbrisk CD3+ infiltrate. Results are expressed as percentages of the brisk or nonbrisk CD3+ lymphocytes that expressed CD8.

§§Percent positive cells staining with mAb M27C10 (anti–Melan-A/Mart-1).

‖Intensity of staining for Melan-A/Mart-1 was coded as: +/−, very weak; +, weak; ++, moderate; and +++, strong in comparison to negative and positive controls. Percentages of cells showing each of the previous three patterns are reported in parentheses.

¶¶A satellitosis from the same primitive lesion was also obtained.

***Frozen sections from this lesion were available, thus allowing staining for HLA-A2 antigens by CR11.351 mAb.
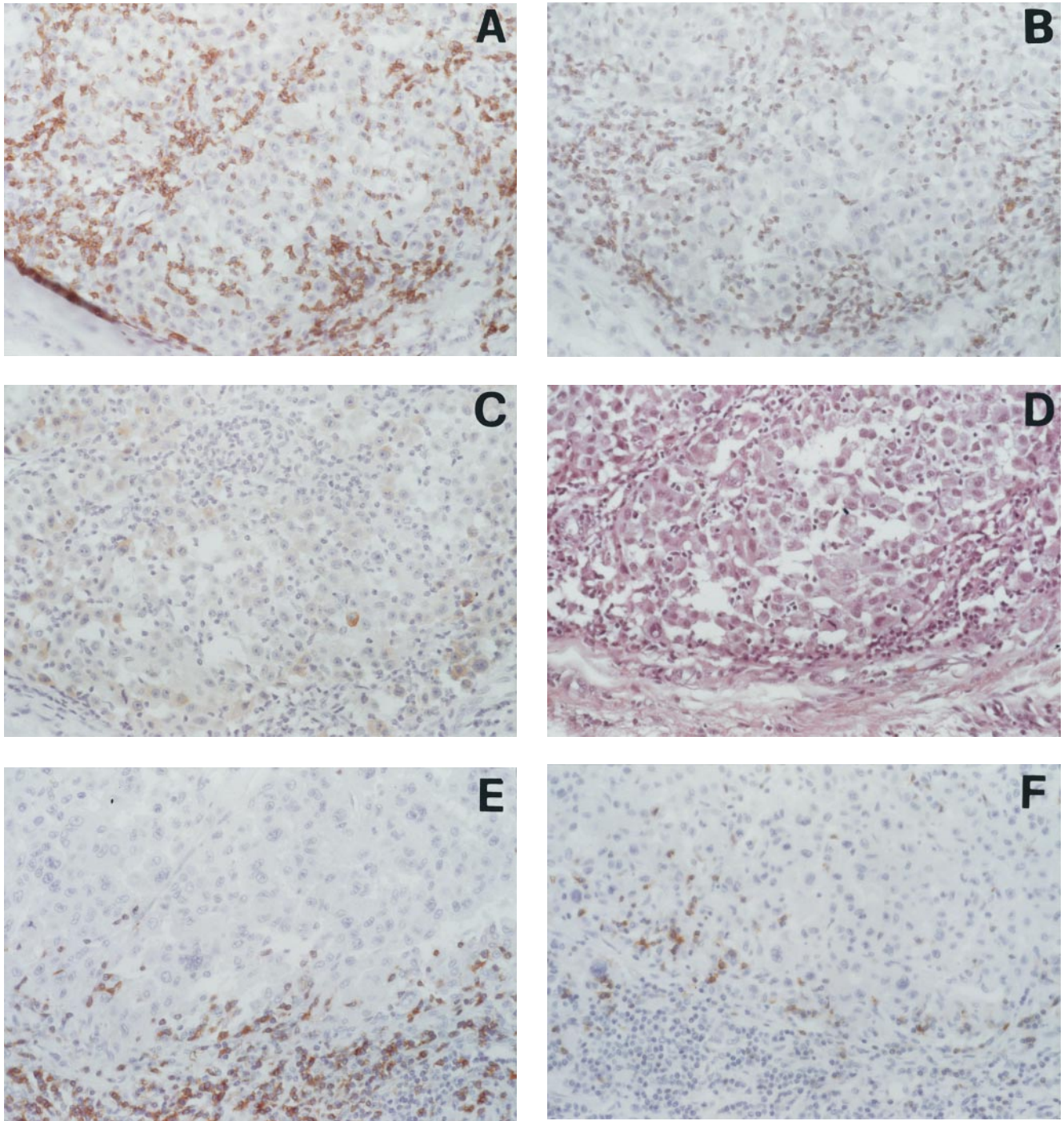
Figure 2 (continues on facing page).

escape from immune surveillance. All of these lesions expressed an absent pattern of CD3^{+} T cells; no evidence of tumor regression was observed in the two metastatic lesions, and only 10% sclerosis was documented in the primary lesion.

Furthermore, in patients 1, 2, 6, and 9, in spite of an expanded pool of Melan-A/Mart-1–specific T cells in peripheral blood, further disease progression occurred due to inoperable metastases at visceral organs or the brain. All of these patients died within 16 (patient 1), 1 (patient 2), 5 (patient 6), and 3 mo (patient 9) after CTLp evaluation, as summarized in Table I. In addition, in 12/14 neoplastic lesions from the group of patients with low CTLp frequency, infiltrating T cells, Melan-A/Mart-1, or both were missing (Table VIII, lesions 15–26 and Fig. 2 I–L).

Taken together, these data strongly suggest that an ex-
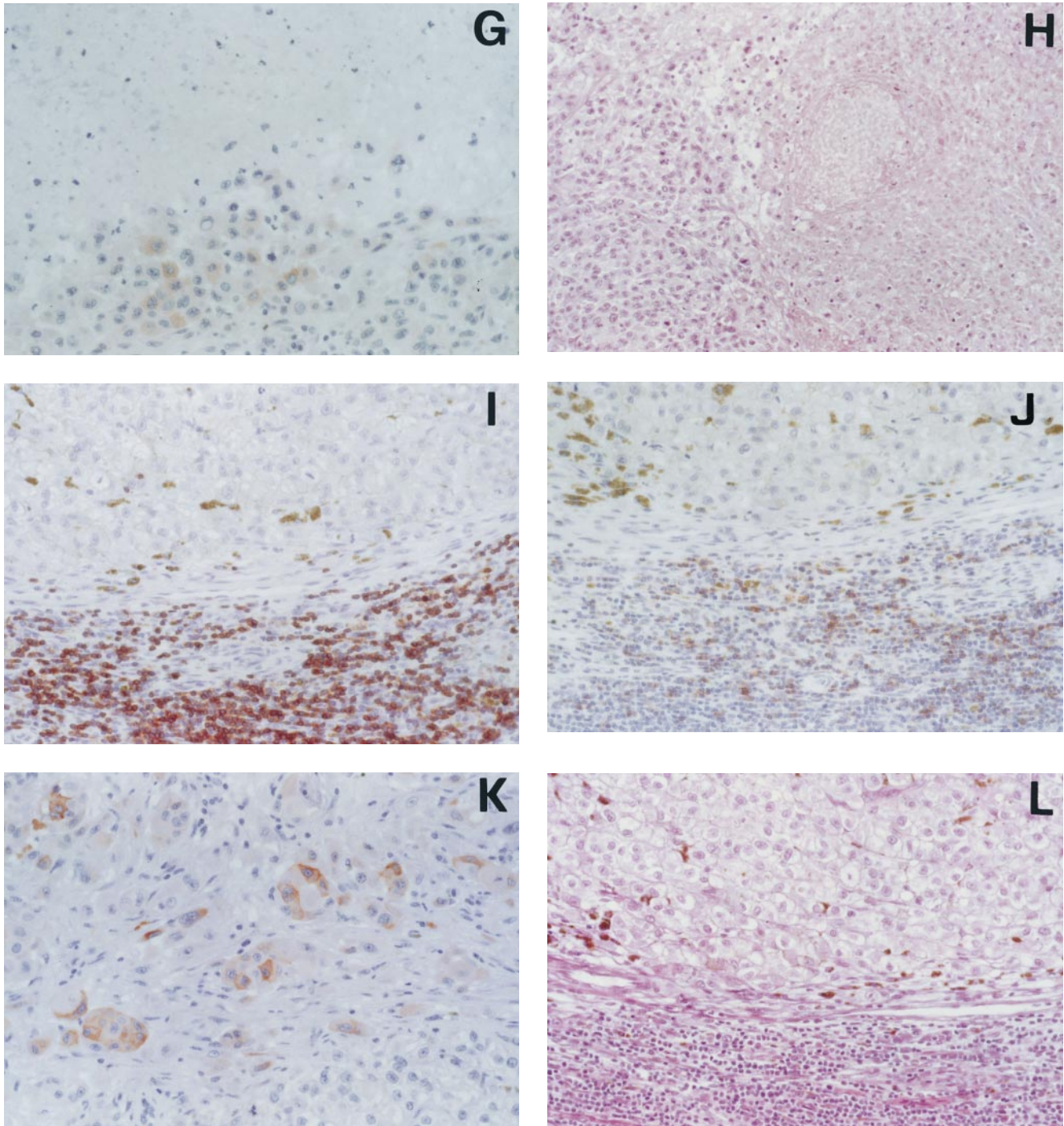
Figure 2. Immunohistochemical analysis of neoplastic lesions. Three examples of immunohistochemical analysis of neoplastic lesions from three patients are reported. Consecutive sections of paraffin-embedded tumor fragments were subjected to immunohistochemical staining with anti-CD3 (A, E, and I), anti-CD8 (B, F, and J) and anti–Melan-A/Mart-1 mAbs (C, G, and K) or conventionally stained with hematoxylin and eosin (D, H, and L). Patient 1 (Table VIII, lesion 7), A–D: a subcutaneous lesion showed no evidence of tumor regression or necrosis but did show an intense intratumoral lymphocytic infiltrate (D) characterized by CD3^{+} (A) and CD8^{+} (B) cells in the presence of heterogeneous cytoplasmatic reactivity for Melan-A/Mart-1 in tumor cells (C). Patient 2 (lesion 9), E–H: a nodal metastasis with extended coagulative necrosis with strong cytoplasmic eosinophilia of cell shadows lacking nuclei and scattered granulocytes infiltrating the border of the necrotic area (H, right side). The left side of the lesion, containing vital tumor cells, showed a nonbrisk lymphocytic infiltrate (H) mostly characterized by CD3^{+} T cells (E) but with a few CD8^{+} lymphocytes (F). In this lesion, heterogeneous cytoplasmatic positivity for Melan-A/Mart-1 in neoplastic cells was confined to a small area (G). Patient 3 (lesion 16), I–L: a nodal metastasis without evidence of tumor regression and lacking intratumoral lymphocytes (L), either CD3^{+} (I) or CD8^{+} (J), showed intense cytoplasmatic reactivity for Melan-A/Mart-1 limited to a few neoplastic cells (K). Original magnification was 400 for all panels except for H, magnification 250).

panded pool of antigen-specific T cells in peripheral blood cannot overcome tumor escape mechanisms in neoplastic lesions, even when peptide-specific T cells are present in the neoplastic tissue, as shown for patients 1 and 2.

Discussion

By coupling a high efficiency LDA assay to dissection of memory versus naive T cell subsets, we obtained evidence that the Melan-A/Mart-1$_{27-35}$ peptide is immunogenic in vivo in a fraction of metastatic melanoma patients, as documented by the presence of an expanded peripheral pool of antigen-specific CD45RO$^+$ memory T cells. In the patients with an expanded T cell population to Melan-A/Mart-1$_{27-35}$, the high CTLp frequency correlated with faster kinetics of CTL development and a higher number of effectors obtained in vitro after activation with peptide-loaded professional APCs in comparison to patients with low CTLp frequency. The first implication of our findings for immune intervention strategies is that activation of tumor-specific T cells by professional APCs will be much more efficient, in quantitative terms (total number of effectors that can be generated), in patients with an expanded peripheral pool of memory T cells than in patients with a low-frequency naive repertoire.

The results obtained in the patients with high CTLp frequency are in agreement with data on memory phenotype of circulating CTLp to Melan-A/Mart-1 recently reported by D'Souza et al. (22) and with studies that have examined the response to viral antigens like those encoded by hepatitis C virus, herpes simplex virus, and Epstein-Barr virus (23–25). In such studies, viral peptide–specific precursor frequency in infected individuals was 10–100-fold higher than in noninfected controls, and antigen-specific precursors were mostly in the CD45RO$^+$ subset. Furthermore, our results corroborate the findings indicating accelerated kinetics of Melan-A/Mart–specific CTL development in patients versus healthy donors (7).

In patients with low CTLp frequency, Melan-A/Mart-1$_{27-35}$–specific precursors were found only in the CD45RA$^+$ naive T cell subset. No evidence of immunosuppression was found in these patients, as shown by analysis of frequency of Flu matrix$_{58-66}$–specific CTLp in comparison to healthy donors. In addition, activation and expansion of Melan-A/Mart-1$_{27-35}$ CTLs could be obtained only by using professional APCs. These data indicate that these patients have a naive immune repertoire against Melan-A/Mart-1$_{27-35}$, and expansion of Melan-A/Mart-1$_{27-35}$–specific precursors did not occur during tumor growth or was transient and unable to generate memory T cells.

Differences in the extent and mechanism of tumor antigen release (26) in tumor lesions may impact on antigen uptake and presentation by APCs, thus leading to priming of peptide-specific T cells only in some patients. In addition, in some but not in all patients, tumor cells may produce factors, such as vascular endothelial cell growth factor (27), that inhibit APC differentiation and/or function. Further-

more, Melan-A/Mart-1$_{27-35}$–specific precursors could be primed, rather than tolerized, by naturally occurring epitope mimics of Melan-A/Mart-1$_{27-35}$ in some but not all patients (28, 29). These mechanisms may hamper tumor immunogenicity, even in the presence of an antigenic tumor.

Several reports have recently suggested that LDA may underestimate the frequency of antigen-specific T cells in comparison to techniques such as the ELISPOT (enzyme-linked immunospot assay) or staining antigen-specific T cells with MHC–peptide tetrameric complexes (for review see reference 30). In contrast with these concerns, in this study, evaluation of frequency of Melan-A/Mart-1–specific T cells in peripheral blood by LDA and tetramer staining provided similar values. In addition, we obtained a frequency range of Flu matrix–specific CTLp as high as that found by either ELISPOT or tetramer staining in previous studies (31, 32). The range of ~1/5,000 for Flu matrix$_{58-66}$–specific CTLp detected by our LDA assay in patients is at least 10-fold higher than that found by conventional LDA by other groups (33, 34). Those studies used an LDA technique based on 8–18 d culture time (instead of 28 d as in our study), PBMCs or B cells as APCs (instead of T2), and up to 4×10^3 targets in the split well assay (instead of 3×10^2 as in this study). Moreover, direct comparison of our LDA technique with tetramer staining on the same T cell cultures provided overlapping values in the frequency of Melan-A/Mart-1–specific effectors, both in high- and low-frequency cultures. This suggests that our modified LDA has improved sensitivity in detecting both high-frequency and low-frequency precursors. Furthermore, in agreement with a previous report (35), comparison between LDA and tetramer staining provided direct evidence that all antigen-specific T cells (on the basis of tetramer staining) were indeed functional cytotoxic T cells able to recognize the relevant peptide (as determined by LDA), either when exogenously added to an LCL or when endogenously expressed in melanoma cells.

In at least two patients of the high CTLp frequency subset, peptide-specific T cells were found in TILs from a subcutaneous and a lymph node metastasis. This indicated that in such patients, Melan-A/Mart-1$_{27-35}$–specific T cells could home to neoplastic tissue. Activation of these TILs with peptide-loaded T2 cells in bulk culture resulted in Melan-A/Mart-1 specificity after only 3 wk of selection, a finding consistent with absence of any irreversible functional block of these cells and a high precursor frequency in these lesions. Tetramer staining of TILs from subcutaneous lesions showed that Melan-A/Mart-1$_{27-35}$–specific T cells were 1/17.6 in comparison to 1/1,404 in peripheral blood of the same patient. This observation is in agreement with a recent report describing an expanded pool of Melan-A/Mart-1–specific T cells in metastatic tissue by tetramer staining (35). Our findings also suggest that appropriate in vitro T cell activation can rescue antitumor function of peptide-specific T cells that infiltrate neoplastic lesions but that apparently do not exert antitumor activity in vivo. The observation that T cell activation with professional APCs could activate Melan-A/Mart-1–specific CTLs from both peripheral

blood and tumor site suggests that antigen-specific vaccination approaches may reactivate and expand antitumor T cells in vivo. This is in agreement with the significant antitumor responses obtained by initial clinical studies of vaccination of melanoma patients with synthetic peptides plus adjuvants or with tumor antigen–loaded DCs (36, 37). Furthermore, our results indicate that high frequency of CTLp to a tumor antigen impacts on CTL generation. Thus, a possible relationship between an expanded pool of T cells to a tumor antigen (defined as high frequency of antigen-specific T cell precursors with a memory phenotype) before vaccination and clinical response to immune intervention should be evaluated in future studies.

In spite of the presence of peptide-specific T cells in the tumor lesions and peripheral immunity to Melan-A/Mart-1_{27-35}, the potential for immune response at the tumor site appeared impaired in most lesions of all patients tested. In fact, reduced/absent evidence of tumor regression was observed in the majority of the lesions available for investigation. In addition, even when areas of tumor regression were present, these areas were never associated with or surrounded by infiltrating $CD3^+$ lymphocytes. In many instances, areas of regression were identified as coagulative necrosis characterized by nuclear loss and marked cytoplasmic eosinophilia in the absence of inflammatory infiltrate. This is a typical aspect of ischemic lesions suggesting vascular damage or inadequate blood supply as the initial mechanism leading to regression, rather than an immune-mediated mechanism. Several mechanisms may impair T cell response at the tumor site. Lack of epitope expression (due to lack of either Melan-A/Mart-1 or HLA-A*0201) is a possibility supported by our findings and by a large set of reports (for review see reference 38), but several other mechanisms could be involved. For example, loss/defective function of TCR signaling molecules has been described in melanoma patients (39). Activation of the defective T cells in the presence of IL-2 can rescue TCR signaling molecule expression and T cell function (40). Similar mechanisms, based on defective TCR signal transduction, might explain why peptide-specific T cells infiltrating the tumor tissue in immunized patients may fail to destroy tumor cells in vivo while remaining responsive to in vitro activation.

Taken together, these results suggest that in most metastatic lesions tumor escape mechanisms can hamper T cell–mediated immune response, even in lesions containing Melan-A/Mart-1 CTLp and in patients with an expanded peripheral T cell pool to the same antigen. The implication of these findings for immune intervention approaches is that means to overcome tumor escape mechanisms in neoplastic lesions may be as relevant as the attempts to induce/boost systemic and local T cell–mediated immunity to tumor antigens.

Address correspondence to Andrea Anichini, Human Tumor Immunobiology Unit, Dept. of Experimental Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-02-2390817; Fax: 39-02-2390630; E-mail: Anichini@istitutotumori.mi.it

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