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Lack of Terminally Differentiated Tumor-specific CD8$^+$ T Cells at Tumor Site in Spite of Antitumor Immunity to Self-Antigens in Human Metastatic Melanoma[1]

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ABSTRACT

Activation of CTL-mediated antitumor immunity to self-epitopes expressed by neoplastic cells is thought to be prevented, at any stage of tumor progression, by tolerance mechanisms. In contrast, in 74 American Joint Committee on Cancer stages I-IV melanoma patients, we found that development of lymph node metastases is a key event triggering CD8$^+$ T-cell-mediated immunity to self-epitopes encoded by melanocyte differentiation antigens. This was shown by the increased peripheral precursor frequency to Melan-A/Mart-1, gp100, and tyrosinase epitopes in stage III and IV compared with stage I and II patients, and by accumulation of functional memory T cells directed to Melan-A/Mart-1$_{26-35}$ in tumor-invaded lymph nodes. However, in tumor-invaded lymph nodes of most patients, CD8$^+$ T cells directed to melanocyte differentiation antigens or to tumor-restricted antigens (MAGE-3 and NY-ESO-1 epitopes), showed a CCR7$^+$ CD45RA$^+$ CD27$^+$ CD28$^+$ perforin$^-$ "precursor" phenotype. Only in 7 of 23 cases antigen-specific CD8$^+$ T cells in invaded lymph nodes showed a predominant CCR7$^-$ CD45RA$^-$ CD27$^+$ CD28$^-$ perforin$^+$ "preterminally differentiated" phenotype. In the latter subset of patients, by immunohistochemistry in lymph node lesions, we found that CD8$^+$ T lymphocytes intermingling with the neoplastic tissue expressed a CCR7$^-$ CD45RO$^+$/RA$^-$ phenotype, whereas CD4$^+$ lymphocytes did not infiltrate the tumor. Furthermore, perforin and granzyme B were expressed on a higher fraction of the CD8$^+$ cells surrounding the invading tumor compared with the lymphocytes infiltrating the neoplastic tissue. In addition, no evidence for tumor regression was found in such metastatic lesions, as documented by absence of neoplastic cell necrosis or apoptosis. These data indicate that neoplastic cells in the lymph nodes and/or increased tumor burden in metastatic disease activate CD8$^+$ T-cell-mediated antitumor immunity to self-epitopes. However, the paucity of terminally differentiated CD8$^+$ T cells at tumor site suggests that immunotherapy strategies may require not only the boosting of tumor immunity, but also effective means to promote CD8$^+$ T-cell differentiation in the neoplastic tissue.

INTRODUCTION

The disappointing clinical evolution of advanced disease collides with the evidence for immunogenicity of human melanoma, obtained over the past 2 decades by molecular and immunological analysis of peripheral blood and tumor-infiltrating lymphocytes (1, 2). This evidence has suggested a possible role for the immune system in controlling tumor growth, although with limited efficacy. The potential role of the immune system is also supported by the seminal observations that tumor-infiltrating lymphocytes represent a significant prognostic indicator of survival in stage I "thin" vertical growth phase melanomas (3) and are associated with better disease-free survival when present as a "brisk" infiltrate in stage III lymph node metastases (4). However, if the immune system is expected to play a role in controlling tumor growth, then immunity to tumor antigens should evolve as a function of tumor progression, possibly despite concurrent development of tumor escape mechanisms. This model would be in agreement with the dynamic concept of "cancer immunoediting" (5) that predicts a continuous influence of the immune response during tumor development, and not only in the initial stages of neoplastic transformation, as in the original cancer immunosurveillance theory (6). This progression-related activation of immunity has not yet been demonstrated to occur in human tumors. However, in support of such a hypothesis, recent results in murine models have indicated that activation of T-cell-mediated antitumor response is correlated with the ability of tumors to migrate into secondary lymphoid organs and to intermingle with T cells in such tissues (7). Interestingly, early metastasis to regional lymph nodes and frequent presence of a "brisk" or "nonbrisk" infiltrate of T cells in the neoplastic tissue, within the invaded lymph node, are two hallmarks associated with tumor progression in human melanoma (3, 4). Thus, as melanoma progresses to metastatic disease, T-cell-mediated immune response to tumor antigens could be promoted. This response may be directed against the different classes of epitopes that have been identified, including tumor-specific antigens, cancer-testis antigens, and self-epitopes, as the MDA[3] (1, 2). Presence of expanded pools of autoreactive T cells to MDAs has been described in tumor-infiltrating lymphocytes of melanoma patients (8), but without evidence of regression (9). One interpretation of these findings is that MDA-specific T cells are made tolerant by self-antigen expression on normal tissues and tumor (2), or after self-antigen presentation by immature dendritic cells (10). Alternatively, activation of immunity to self-antigens expressed on tumors might be prevented, at tumor site, by lack of proinflammatory signals required to break tolerance (11) or by lack of CD4$^+$ T-cell help. In addition, as melanoma cells invade the regional lymph nodes, the development of an effective T-cell-mediated immune response against any of the known antigen classes should require not only T-cell infiltration of the neoplastic tissue but, more importantly, maturation of antigen-specific CD8$^+$ T lymphocytes from precursors to terminally differentiated cytotoxic effector cells (12, 13) able to mediate tumor cell death by either the granule exocytosis pathway or by cross-linking of death receptors on the target cell (14).

In this study we investigated whether the natural evolution of human melanoma may promote the development of T-cell-mediated antitumor immunity to self-epitopes expressed on neoplastic cells. The results indicated that progression from primary lesions (stages I and II) to metastatic disease is associated with activation of CD8$^+$ T-cell-mediated immunity to self-MDAs at the systemic and, in a fraction of patients, at tumor levels. However, in tumor-invaded, but

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[3] The abbreviations used are: MDA, melanocyte differentiation antigen; AJCC, American Joint Committee on Cancer; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; PBL, peripheral blood lymphocyte; TFLN, tumor-free lymph node; TILN, tumor-invaded lymph node; PE, phycoerythrin; IL, interleukin; TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling.

not in TFLNs from the same nodal basin, $CD8^+$ T cells showed a precursor phenotype, or their maturation was skewed toward a preterminally differentiated state. Furthermore, $CD8^+$ T lymphocytes intermingling with neoplastic cells were defective for expression of key proteins for CTL activity mediated by the granule exocytosis pathway (14). These results have implications for understanding the natural evolution of human melanoma and for the design of immunotherapy protocols, and suggest a new mechanism of tumor escape.

MATERIALS AND METHODS

Patients and Donors. Informed consent was obtained from the patients. Lymphocytes were isolated from PBLs of HLA-A∗0201$^+$ healthy donors ($n = 16$) and HLA-A∗0201$^+$ melanoma patients ($n = 74$) in AJCC stages I-IV (15), and from TILNs ($n = 44$) or TFLNs ($n = 14$) as described (9). By clinical staging, performed at time of lymphocyte isolation from PBL or TILN, 21 patients were in AJCC stage I, 2 in stage II, 43 in stage III, and 8 in stage IV. HLA-A∗0201 subtyping was performed by PCR sequence-specific oligonucleotide probe typing as described (9). None of the patients enrolled in this study had been subjected to chemotherapy or any other therapy with immunosuppressive activity before isolation of lymphocytes.

Tetramers and Flow Cytometry. PE-labeled tetramers of HLA-A∗0201 containing peptides from Melan-A/Mart-1 (modified sequence 26–35: ELAGIGILTV; Refs. 16, 17), gp100 (154–162: KTWGQYWQV; 209–217: ITDQVPFSV; Ref. 18), Tyrosinase (368–377: YMDGTMSQV; Ref. 19), and Influenza Matrix (58–66: GILGFVFTL; Ref. 20) were provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility and NIH AIDS Research and Reference Reagent Program (Bethesda, MD). Tetramers of HLA-A∗0201 containing MAGE-$3_{271-279}$ (21) and NY-ESO-$1_{157-165}$ (22) peptides were purchased from ProImmune, Ltd. (Oxford, United Kingdom). Two × 10^6 cells were stained for 15 min with PE-labeled tetramers at 37°C,

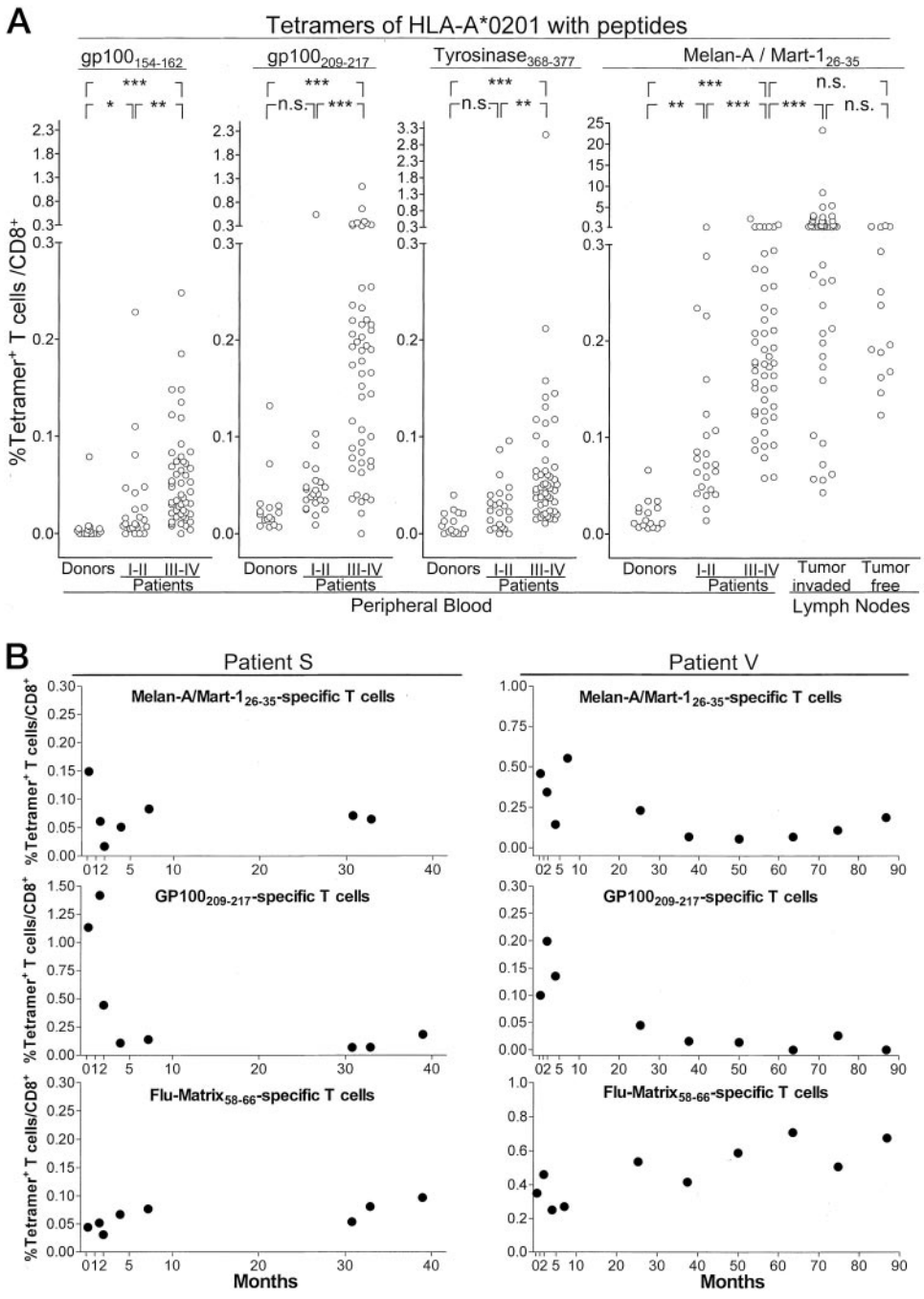

Fig. 1. *Ex vivo* analysis, by flow cytometry, of $CD8^+$ T cells with tetramers of HLA-A∗0201 containing peptides from gp100, tyrosinase, and Melan-A/Mart-1. *A*, frequency of $CD8^+$ T cells directed to 4 MDAs in PBL of healthy donors ($n = 16$), and PBL ($n = 74$), TILN ($n = 44$), and TFLNs ($n = 14$) removed at the same time from melanoma patients. AJCC stage I and II patients ($n = 23$) were analyzed at time of melanoma diagnosis, whereas stage III ($n = 43$) and IV ($n = 8$) patients at time of surgical removal of TILN. Statistical analysis of data sets was annotated as follows: ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$; n.s., not significant. *B*, frequency of circulating Melan-A/Mart-1_{26-35}-, gp$100_{209-217}$- and Flu-Matrix$_{58-66}$-specific $CD8^+$ T cells in PBL of two AJCC stage III melanoma patients remaining disease-free after removal of TILN. The first blood sample (labeled as month 0) was obtained at time of surgical removal of TILN.

then stained for 30 min on ice with other cell surface antibodies. Different combinations of the following mouse antihuman monoclonal antibodies were used: anti-CD8 coupled to PerCp or APC, FITC- or PE-anti-CD45RA or anti-CD45RO, PerCp- or PE-anti-CD3, FITC- or APC-anti-CD27 and anti-CD28 (Becton Dickinson, Milan, Italy). To detect CCR7, cells were stained with IgM anti-CCR7 (BD PharMingen, San Diego, CA), followed by biotin-conjugated rat-antimouse IgM and then by Cy-chrome-conjugated streptavidin (BD PharMingen). To detect intracellular perforin or granzyme B, cells were permeabilized with Cytofix/Cytoperm (BD PharMingen) and then stained with FITC anti-Perforin (BD PharMingen), or with PE-antigranzyme B (CLB, Amsterdam, the Netherlands) in the presence of Perm/Wash solution (BD PharMingen). Acquisition and analysis by four- or three-color flow cytometry was carried out by a dual-laser FACScalibur cytofluorimeter (Becton Dickinson) using the Cellquest software. Negative controls for tetramer staining included PBL from HLA-A*0201$^-$ healthy controls.

T-Cell Activation. Lymphocytes from peripheral blood or TILN were cultured for 2 weeks with HLA-A*0201$^+$ T2 cells either empty or loaded with 0.1, 1, or 10 μg/ml of Melan-A/Mart-1_{27-35} peptide as described (9). Frequency of tetramer$^+$ T cells in the CD8$^+$ fraction was analyzed before and at the end of culture. The frequency of tetramer$^+$ T cells found at the end of culture was plotted against the peptide concentration (in μM). The dose of antigen required to achieve a 2-fold increase in frequency, compared with the initial frequency of peptide-specific T cells, was then estimated by linear regression analysis.

Intracellular IFN-γ Detection. Lymphocytes from peripheral blood, TILN, or short-term T-cell cultures activated with peptide-loaded T2 cells were stained with PE-tetramers for 15 min at 37°C and then were stimulated in the presence of 2 μM Melan-A/Mart-1_{26-35} or gp$100_{209-217}$ peptides for 4–6 h. After the first hour GolgiStop (BD PharMingen) was added. Cell surface staining was then carried out with PerCp-anti-CD8, followed by cell permeabilization with Cytofix/Cytoperm and then by staining with APC anti-IFN-γ monoclonal antibody in the presence of Perm/Wash solution. Expression of IFN-γ was then analyzed by flow cytometry after imposing a double gating for CD8$^+$ and tetramer$^+$ T cells (12).

Immunohistochemistry. Immunohistochemical analysis of consecutive sections from routinely formalin or Bouin's fixed and paraffin-embedded specimens, or from frozen tissues, was performed as described (9). The following antibodies were used: anti Melan-A/Mart-1, (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), anti-gp-100 (Dako, Glostrup, Denmark), anti-CD3 (Novocastra), anti-CD8 (Dako), anti-CD45RO (Dako), anti-Perforin (BD PharMingen), anti-Granzyme B (Monosan, Uden, the Netherlands), anti-CCR7 (BD PharMingen), anti-GMP-17/TIA-1 (Beckman Coulter, Fullerton, CA), anti-Ki-67 (MIB-1 antibody; Immunotech, Marseille, France), anti-HLA-A2 (clone CR11.351; Ref. 23), and anti-NY-ESO-1 (D8.38 antibody; Ref. 24). Granzyme B$^+$ and perforin$^+$ lymphocytes in TILN were counted after acquiring digital images of 10 areas of tissue sections (at ×400 on a Zeiss Axiovert 100 microscope), each including both lymph node tissue and tumor tissue. Images of the corresponding fields stained with anti-CD8 mAb in serial sections were used to count intratumoral and extratumoral CD8$^+$ cells. Apoptosis was detected with the terminal deoxynucleotidyltransferase *in situ* apoptosis detection kit (Genzyme, Cambridge, MA) according to the manufacturer's instructions.

Statistical Analysis. The data sets of frequency of T cells recognized by each of the tetramers in PBL of donors and patients, as well as in TILN and TFLN of patients, were not distributed normally, as indicated by Kolmogorov-Smirnov statistics. A nonparametric statistical analysis (Kruskal-Wallis test) followed by Dunn's multiple comparison test was used.

RESULTS

MDA-specific T-Cell Precursor Frequency: Relationship with Clinical Stage in Peripheral Blood and Epitope-specific Accumulation at Tumor Site. By staining CD8$^+$ T cells from PBLs with tetramers of HLA-A*0201 containing gp$100_{154-162}$, gp$100_{209-217}$, Melan-A/Mart-1_{26-35}, and tyrosinase$_{368-377}$ peptides (16–19) we found a significant difference between healthy donors and AJCC stage I+II patients for the frequency of CD8$^+$ T cells directed to gp$100_{154-162}$ and Melan-A/Mart-1_{26-35} epitopes (Fig. 1*A*). The two

Table 1 *Flow cytometry analysis for the expression of CCR7 and CD45RA on CD8$^+$ T cells directed to gp$100_{209-217}$, Melan-A/Mart-1_{26-35} and Influenza-Matrix$_{58-66}$ epitopes in PBL, TILN, and TFLN of melanoma patients*

		[a]CCR7/CD45RA phenotype of gated CD8$^+$ tetramer$^+$ T cells directed to								[b]Frequency of tetramer$^+$ T cells directed to			
		gp$100_{209-217}$			Melan-A/Mart-1_{26-35}			Flu-Matrix$_{58-66}$					
Tissue	*n*	+/+	+/−	−/−	−/+	+/+	+/−	−/−	−/+	+/+	+/−	−/−	−/+	gp$100_{209-217}$	Melan-A/Mart-1_{26-35}	Flu-Matrix$_{58-66}$
PBL	11	21 (5–43)	13 (2–27)	25 (13–35)	40 (17–58)	37 (19–54)	15 (4–36)	18 (6–30)	30 (12–59)	26 (10–44)	12 (4–34)	25 (10–36)	38 (21–50)	0.347 (0.038–1.132)	0.194 (0.058–0.464)	0.203 (0.044–0.906)
[c]TILN	7	6 (1–11)	19 (7–42)	71 (50–83)	4 (1–8)	2 (0–5)	9 (2–17)	87 (76–98)	1 (0–3)	8 (3–20)	7 (1–12)	72 (40–86)	14 (6–30)	0.499 (0.082–2.332)	7.063 (1.502–23.285)	0.179 (0.021–0.528)
[c]TILN	16	65 (26–93)	18 (7–51)	8 (0–28)	9 (0–35)	69 (28–96)	16 (4–33)	9 (0–44)	6 (0–37)	70 (37–97)	19 (3–40)	6 (0–20)	5 (0–25)	0.144 (0.012–0.667)	0.250 (0.043–0.810)	0.109 (0.023–0.289)
TFLN	8	28 (13–45)	27 (4–47)	22 (4–38)	23 (4–45)	51 (32–66)	23 (14–34)	15 (0–35)	12 (0–17)	31 (13–45)	26 (3–47)	21 (9–35)	22 (2–45)	0.260 (0.073–0.606)	0.362 (0.123–0.892)	0.443 (0.086–1.280)

[a] Percentage of positive cells (mean value, with range in brackets) for each of the four possible CCR7/CD45RA phenotypes is reported. nd, not done.
[b] Frequency of tetramer$^+$ T cells (mean value, with range in brackets) is expressed as % of positive cells/CD8$^+$ lymphocytes.
[c] The two groups of TILN (7 patients *versus* 16 patients) identified by high *versus* low frequency to Melan-A/Mart-1 epitope differ for the proportion of tetramer$^+$ T cells expressing a CCR7$^+$/CD45RA$^+$ and a CCR7$^-$ CD45RA$^-$ phenotype ($P < 0.001$).

Table 2 *Flow cytometry analysis for expression of CCR7 and CD45RA on CD8+ T cells directed to MAGE-3$_{271–279}$ and NY-ESO-1$_{157–165}$ in TILN and TFLN of melanoma patients*

		[a]CCR7/CD45RA phenotype of gated CD8+ tetramer+ T cells directed to								[b]Frequency of tetramer+ T cells directed to	
		MAGE-3$_{271–279}$				NY-ESO-1$_{157–165}$					
Tissue	*n*	+/+	+/−	−/−	−/+	+/+	+/−	−/−	−/+	MAGE-3$_{271–279}$	NY-ESO-1$_{157–165}$
TILN	19	79 (28–99)	13 (1–44)	6 (0–41)	2 (0–18)	81 (28–98)	14 (2–36)	3 (0–26)	2 (0–15)	0.256 (0.027–0.632)	0.178 (0.021–0.571)
TFLN	6	81 (75–99)	17 (11–22)	2 (0–7)	1 (0–6)	88 (76–97)	11 (3–24)	0 (0–2)	0 (0–1)	0.192 (0.075–0.433)	0.177 (0.042–0.476)

[a] Percentage of positive cells (mean value, with range in brackets) for each of the four possible CCR7/CD45RA phenotypes is reported.
[b] Frequency of tetramer+ T cells (mean value, with range in brackets) is expressed as % of positive cells/CD8+ lymphocytes.

subsets of patients (stage I+II *versus* III+IV) differed for the proportion of CD8+ T cells directed against all four of the MDA epitopes. gp100$_{209–217}$- and Melan-A/Mart-1$_{26–35}$-specific T cells showed the most marked and significant increase in metastatic patients compared with stage I+II patients (Fig. 1*A*). In addition, we found a significant accumulation of MDA-specific T cells in 44 TILNs compared with PBLs of the same patients, but only for Melan-A/Mart-1$_{26–35}$-specific T cells (Fig. 1*A*), and not for gp100-, tyrosinase-, or influenza-matrix-specific T cells (data not shown). Frequency of T cells directed to all four of the MDA epitopes and to Influenza-Matrix$_{58–66}$ peptide (20) did not differ in TILN compared with TFLNs removed at the same time from the same nodal basin (Fig. 1*A*; for Melan-A/Mart-1-specific T cells; data not shown).

Analysis of Melan-A/Mart-1$_{26–35}$- and gp100$_{209–217}$-specific T cells in PBLs of patients who remained disease-free after removal of TILN indicated a progressive reduction in the frequency of circulating peptide-specific T cells during a follow-up of 34 or 88 months after surgery (Fig. 1B), a trend not found in Flu-matrix-specific T cells (Fig. 1*B*), suggesting that Cytotoxic T lymphocyte precursor frequency in PBL is affected not only by clinical stage but even by actual tumor burden.

Antigen-specific CD8+ T Cells in TILN Show a Precursor or a Preterminally Differentiated Phenotype. To characterize the maturation stages of CD8+ T cells in patients, we evaluated gp100$_{209–217}$, Melan-A/Mart-1$_{26–35}$, and Flu-Matrix$_{58–66}$-specific T cells in PBL, TILN, and TFLN of stage III metastatic patients for expression of CCR7 and of CD45RA. In PBL and in TFLN a predominant phenotype was not found, although the CCR7+ CD45RA− subset was the least represented in PBL (mean: 12–14% of the tetramer+ T cells). However, in both PBL and TFLN, all of the maturation stages were observed, including terminally differentiated CCR7− CD45RA+ cells (Table 1). In contrast, TILN split into two subsets: in 7 of 23 patients, gp100, Melan-A/Mart-1, and Flu-matrix-specific T cells showed a predominant CCR7− CD45RA− phenotype, a "preterminal" stage of differentiation, according to the model put forward by Champagne *et al.* (12). Interestingly, these 7 TILNs included all of the lesions with a very high (>1.5% of all CD8+ T cells) frequency of Melan-A/Mart-1$_{26–35}$-specific T cells ($P < 0.001$ for the association of phenotype with tetramer frequency in the two groups of lesions; see legend to Table 1). In the remaining 16 TILNs, the tetramer+ T cells showed a predominant CCR7+ CD45RA+ precursor phenotype (Table 1). Furthermore, analysis of T cells directed to two tumor-restricted antigens (MAGE-3$_{271–279}$ and NY-ESO-1$_{157–165}$ peptides), in TILN and TFLN from the same panel of patients, indicated a predominant CCR7+ CD45RA+ precursor phenotype in nearly all instances (Table 2). Immunohistochemistry for NY-ESO-1 in TILN sections from the same patients indicated that 78% of the lesions expressed this antigen on <10% of the neoplastic tissue (data not shown), suggesting that the predominant precursor phenotype of NY-ESO-1-specific T cells may be related to defective tumor antigen expression in the neoplastic tissue.

Down-regulation of CCR7 in CD8+ T cells can be associated with up-regulation of cytotoxic factors as perforin (12, 13). By flow cytometry, we then looked in TILN and TFLN at the perforin phenotype of T cells stained by tetramers to a MDA (Melan-A/Mart-1$_{26–35}$) or to a tumor-restricted peptide (MAGE-3$_{271–279}$). In TILN, we found perforin expressed mainly in the subset of Melan-A/Mart-1-specific T cells with a CCR7− phenotype (group of 4 patients in Table 3) and less frequently in those with a predominant CCR7+ phenotype (group of 8 patients in Table 3). Melan-A/Mart-1-specific T cells in TFLN were mainly CCR7+ and perforin−(Table 3). A similar CCR7+ perforin− phenotype was expressed even by the MAGE-3-specific T cells in both TILN and TFLN (Table 3).

We then characterized CD3+ CD8+ cells and MDA-specific T cells in TILN for expression of CD27 and CD28 to evaluate CD8+ T-cell differentiation as defined by Appay *et al.* (13). In patients (as in patients #13, #14, and #15) belonging to the subset with a predominant precursor phenotype (CCR7+ CD45RA+), the gp100$_{209–217}$- and Melan-A/Mart-1$_{26–35}$-specific T cells were mostly CD27+ CD28+, an "early" stage along the differentiation pathway (Fig. 2). In TILNs where MDA-specific T lymphocytes showed a predominant CCR7− CD45RA− phenotype (as in patients #3, #4, and #5), these cells expressed mostly the early (CD27+ CD28+) and intermediate (CD27+, CD28−) stages of differentiation, but not the CD27− CD28− phenotype associated with the "late" maturation stage (Ref. 13; Fig. 2). The CD27/CD28 phenotype of tetramer+ T cells was in agreement with that shown by the bulk CD3+ CD8+ T-cell population in the two groups of patients (Fig. 2) and with the phenotype for these markers expressed by Flu-Matrix$_{58–66}$-specific T cells in TILN (data not shown).

Taken together these data suggest that partially differentiated CD8+ T cells directed to MDA epitopes can be found in TILN lesions

Table 3 *Flow cytometry analysis for the expression of perforin and CCR7 in CD8+ T cells directed to Melan-A/Mart-1$_{26–35}$ and MAGE-3$_{271–279}$ from TILN and TFLN of melanoma patients*

		[a]CCR7/Perforin phenotype of gated CD8+ tetramer+ T cells directed to							
		Melan-A/Mart-1$_{26–35}$				MAGE-3$_{271–279}$			
Tissue	N#	+/+	+/−	−/−	−/+	+/+	+/−	−/−	−/+
[b]TILN	4	8 (5–11)	8 (2–20)	17 (5–29)	67 (40–85)	8 (3–15)	90 (84–95)	1 (0–4)	1 (0–5)
[b]TILN	8	7 (2–15)	80 (40–97)	12 (0–53)	2 (0–10)	10 (5–16)	89 (83–95)	1 (0–3)	0 (0–1)
TFLN	5	6 (3–8)	86 (74–93)	6 (0–19)	2 (0–11)	11 (2–27)	87 (71–98)	1 (0–3)	1 (0–2)

[a] Percentage of positive cells (mean values with range in brackets) for each of the four possible CCR7/perforin phenotypes is reported.
[b] The two groups of TILN (4 patients *versus* 8 patients) were identified by high *versus* low frequency to Melan-A/Mart-1 epitope.

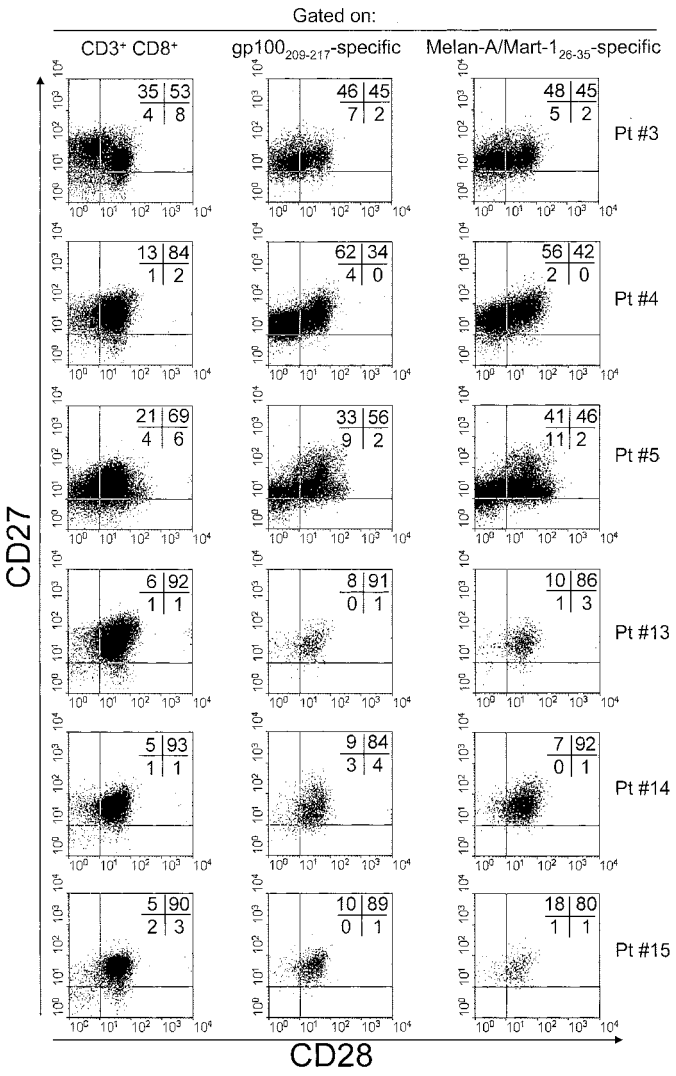

Fig. 2. Four-color flow cytometry analysis of lymphocytes from TILN for the expression of CD27 and CD28 costimulatory receptors. *Dot plots* were gated on the bulk $CD3^+$ $CD8^+$ T-cell population or on MDA-specific T cells identified by HLA-A*0201 tetramers with Melan-A/Mart-1 or gp100 peptides. *Numbers* represent percentages of positive cells in each quadrant.

of some metastatic melanoma patients. However, even when differentiation of $CD8^+$ T cells took place in TILN, the maturation of the antigen-specific T cells did not reach the terminal/late stages defined either by the $CCR7^-$ $CD45RA^+$ or $CD27^-$ $CD28^-$ phenotypes predicted by current models (12, 13).

Preterminally Differentiated Memory $CD8^+$ T Cells in TILN Are Not Functionally Impaired. To evaluate whether the preterminally differentiated T cells from TILN were functionally competent, $tetramer^+$ T cells directed to Melan-A/Mart-1_{26-35} or $gp100_{209-217}$ and showing a predominant (>80%) $CCR7^-$ $CD45RA^-$ phenotype were tested for IFN-γ production in response to the specific peptide. In such T cells, staining for intracellular IFN-γ, after peptide-stimulation, was detected not only after preactivation for 1 week with peptide-loaded APCs but even in freshly isolated lymphocytes (Fig. 3; representative of 6 patients investigated). The latter response was not detected when we tested TILN-derived Melan-A/Mart-1-specific T cells with a predominant $CCR7^+$ $CD45RA^+$ phenotype (data not shown). Similarly, Melan-A/Mart-1- or gp100-specific T cells containing <20% of $CCR7^-$ $CD45RA^-$ cells, and isolated from PBLs, stained for peptide-induced intracellular IFN-γ only after preactivation with peptide-loaded APCs (Fig. 3).

Additional evidence for functional competence of $CCR7^-$ $CD45RA^-$ $CD8^+$ T cells from TILN were obtained by evaluating antigen-specific proliferation. Melan-A/Mart-1_{26-35}-specific T cells from 12 TILN and 10 PBL samples did not differ for the highest expansion of $tetramer^+$ T cells (15.8-fold *versus* 15.1-fold increase in frequency in TILN *versus* PBL, respectively) achieved by culture with APCs loaded with a high dose (10 μM) of specific peptide. However, Melan-A/Mart-1_{26-35}-specific T cells (with a predominant $CCR7^-$ $CD45RA^-$ phenotype) from TILN showed enhanced proliferation at low antigen doses (0.1–1 μg/ml), compared with T cells with the same antigen specificity from PBLs (Fig. 4). Linear regression analysis of dose-response curves at peptide doses between 0.1 and 10 μM indicated that $CCR7^-$ $CD45RA^-$- Melan-A/Mart-1_{26-35}-specific T cells from TILN required a peptide dose ~46 times lower than Melan-A/Mart-1-specific T cells from PBLs ($P < 0.01$) to achieve the same outgrowth (2-fold increase compared with initial frequency; Fig. 5) in agreement with the concept that antigen-experienced T cells can express increased clonal expansion at low antigen doses compared with naive T cells (25).

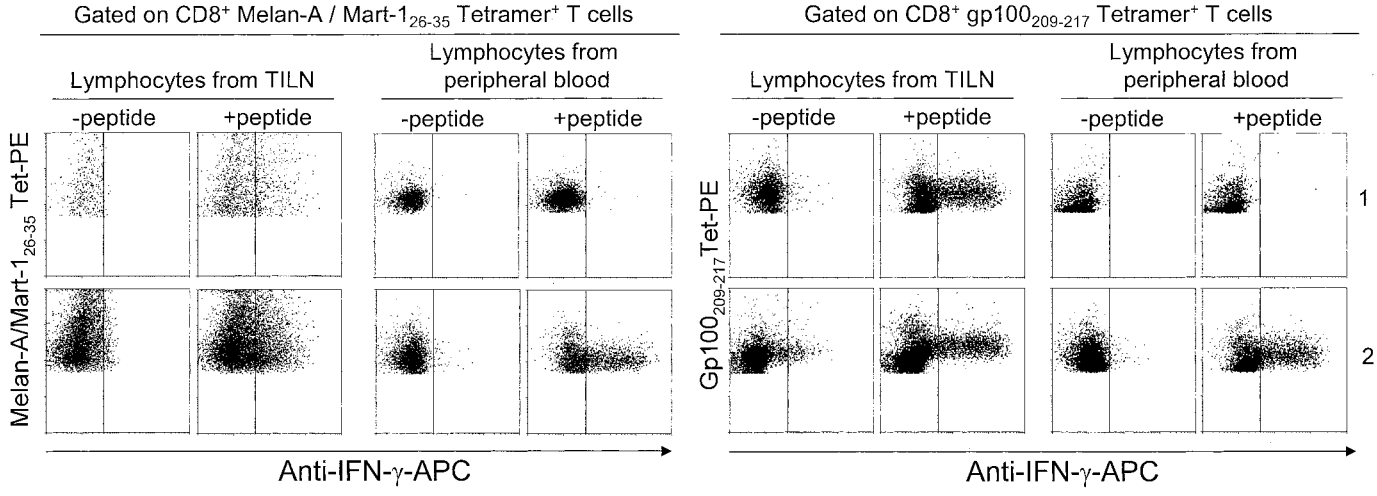

Fig. 3. Functional analysis of Melan-A/Mart-1- and gp100-specific T cells from TILN and PBL. Lymphocytes from TILN or PBL, either fresh (1) or preactivated for 1 week with peptide-loaded APCs (2), were stimulated with (+peptide) or without (−peptide) Melan-A/Mart-1_{27-35} or $gp100_{209-215}$ peptides, and then characterized by flow cytometry for staining by the corresponding tetramers and intracellular IFN-γ production. *Dot plots* have a double gating for the $CD8^+$ and the $tetramer^+$ fraction.

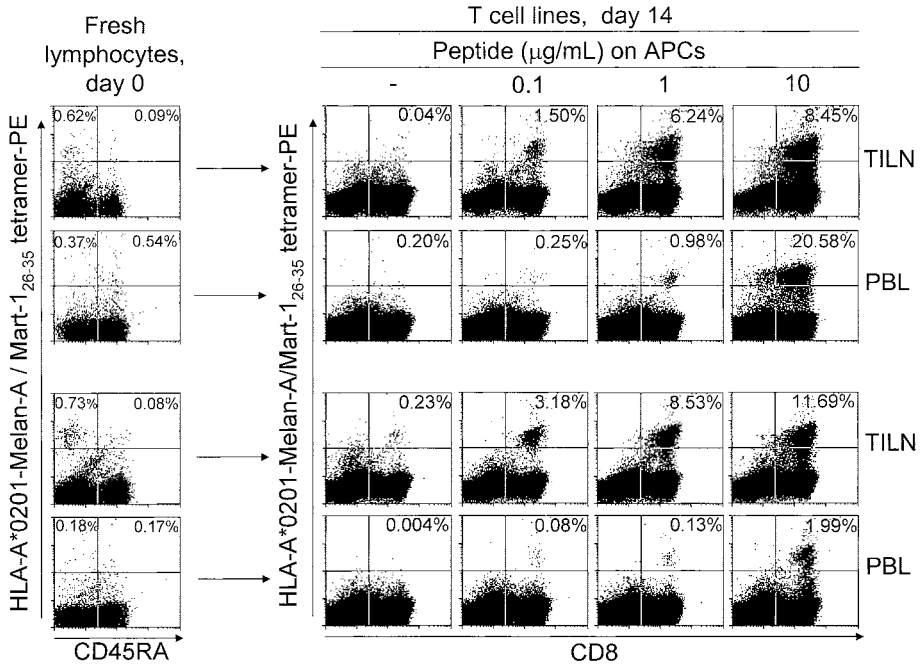

Fig. 4. Enhanced outgrowth of Melan-A/Mart-1-specific T cells from TILN compared with PBL, after stimulation with peptide-loaded APCs. Flow cytometry analysis for the frequency of Melan-A/Mart-1-specific T cells, by tetramer staining, in fresh (day 0) and activated (day +14) T cells from TILN and PBL of 2 patients. T cells from TILN and PBL were activated by coculture with T2 cells loaded with the indicated concentrations of Melan-A/Mart-1_{27-35} peptide. Percentage of tetramer$^+$ cells is shown in the *top left* and *right* quadrants (day 0), or in the *top right* quadrant (day +14) of each *dot plot*.

CD4$^+$ T Cells Do Not Infiltrate the Metastatic Lesions, and CD8$^+$ T Cells Intermingling with the Tumor Tissue Are Not Fully Differentiated, Even in the Patients with High Frequency of MDA-specific T Cells in TILN. To gain additional insight on the maturation stage of CD8$^+$ T cells *in vivo* and on the distribution of different T-cell subsets in the metastatic lesions, tissue samples of the same TILNs investigated previously *ex vivo* by tetramer analysis were characterized by immunohistochemistry. Analysis of 44 TILN lesions indicated that CD4$^+$ lymphocytes could be found in the residual lymph node tissue surrounding the tumor nodules, but such cells were either absent (45% of all TILN lesions investigated) or represented no more than 1% of the CD3$^+$ cells (55% of the lesions) into the neoplastic tissue (representative results shown in Fig. 6). In contrast, of 44 TILNs investigated, 33 (75%) contained infiltrating CD3$^+$ CD8$^+$ cells (Fig. 6; data not shown). These 33 lesions included the TILNs from the 7 patients with the highest frequency of T cells to Melan-A/Mart-1 and with a skewed CCR7$^-$ CD45RA$^-$ phenotype. In agreement, in the same lesions we found that CD8$^+$ cells infiltrating the neoplastic tissue of TILN lacked CCR7 and expressed CD45RO, the reciprocal isoform of CD45RA (Fig. 6). Thus, even by immunohistochemistry, we did not find evidence for terminally differentiated (*i.e.*, CCR7$^-$ CD45RO$^-$/RA$^+$) CD8$^+$ T cells infiltrating the metastatic lesions.

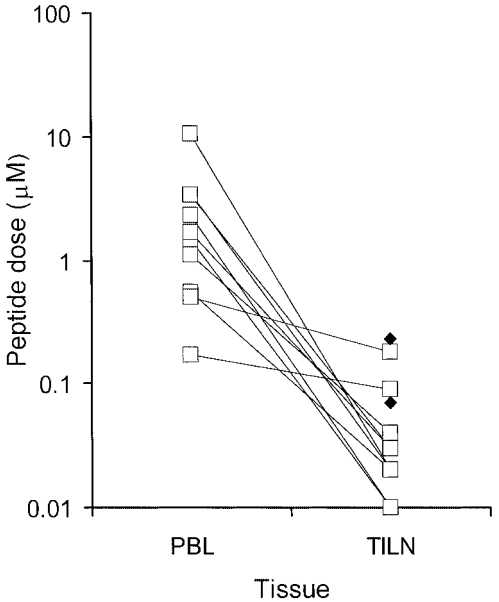

Fig. 5. Proliferative response of lymphocytes from PBL and TILN to antigen stimulation with peptide-loaded APCs. Lymphocytes from TILN of 12 patients and from PBL of 10 patients were cultured for 2 weeks with APCs loaded with increasing concentrations of Melan-A/Mart-1_{26-35} peptide. A CCR7$^-$CD45RA$^-$ phenotype was expressed by 83–98% of the Melan-A/Mart-1-specific T cells in these TILN samples and by 5–27% of the T cells with the same antigen specificity in the PBL samples. Results are shown as the peptide dose (μM) needed to achieve a 2-fold increase in peptide-specific T-cell frequency, compared with the initial frequency, as described in "Materials and Methods." □, matched PBL and TILN samples; ♦, unmatched TILN samples.

Reduced Expression of Perforin and Granzyme B in Lymphocytes That Infiltrate the Neoplastic Tissue and Lack of Tumor Cell Destruction in TILN. To obtain additional information on the differentiation of CD8$^+$ T cells in TILN, tissue sections of invaded lymph nodes were characterized for the expression of markers of cytolytic T-cell granules (GMP-17/TIA1; Ref. 26) and of cytotoxic factors (perforin and granzyme B). Most CD3$^+$ CD8$^+$ lymphocytes present in the tumor tissue of TILN showed a granular cytoplasmic staining with an antibody to GMP-17/TIA1 (data not shown). In contrast, we detected staining for perforin and granzyme B in the cytoplasm of only a fraction of the CD8$^+$ cells present in the neoplastic tissue. The reduced expression of these cytotoxic factors was observed even in the metastatic lesions from the 7 patients with high frequency of Melan-A/Mart-1-specific T cells in invaded lymph nodes (Fig. 7, *A* and *B*). In particular, in these patients, we found perforin and granzyme B expressed on a higher fraction of the CD8$^+$ lymphocytes located in the residual lymph node tissue surrounding the invading tumor, with remarkably fewer positive lymphocytes among those intermingling with the neoplastic tissue (Fig. 7 and Fig. 8). Such incomplete maturation and defective expression of cytotoxic factors in the infiltrating CD8$^+$ T cells was not associated with lack of HLA-A2 molecules or MDA on the neoplastic cells. In fact, in a panel of several TILNs the highest frequency of expression of HLA-A2, Melan-A/Mart-1, and gp100 on the neoplastic cells was found in the

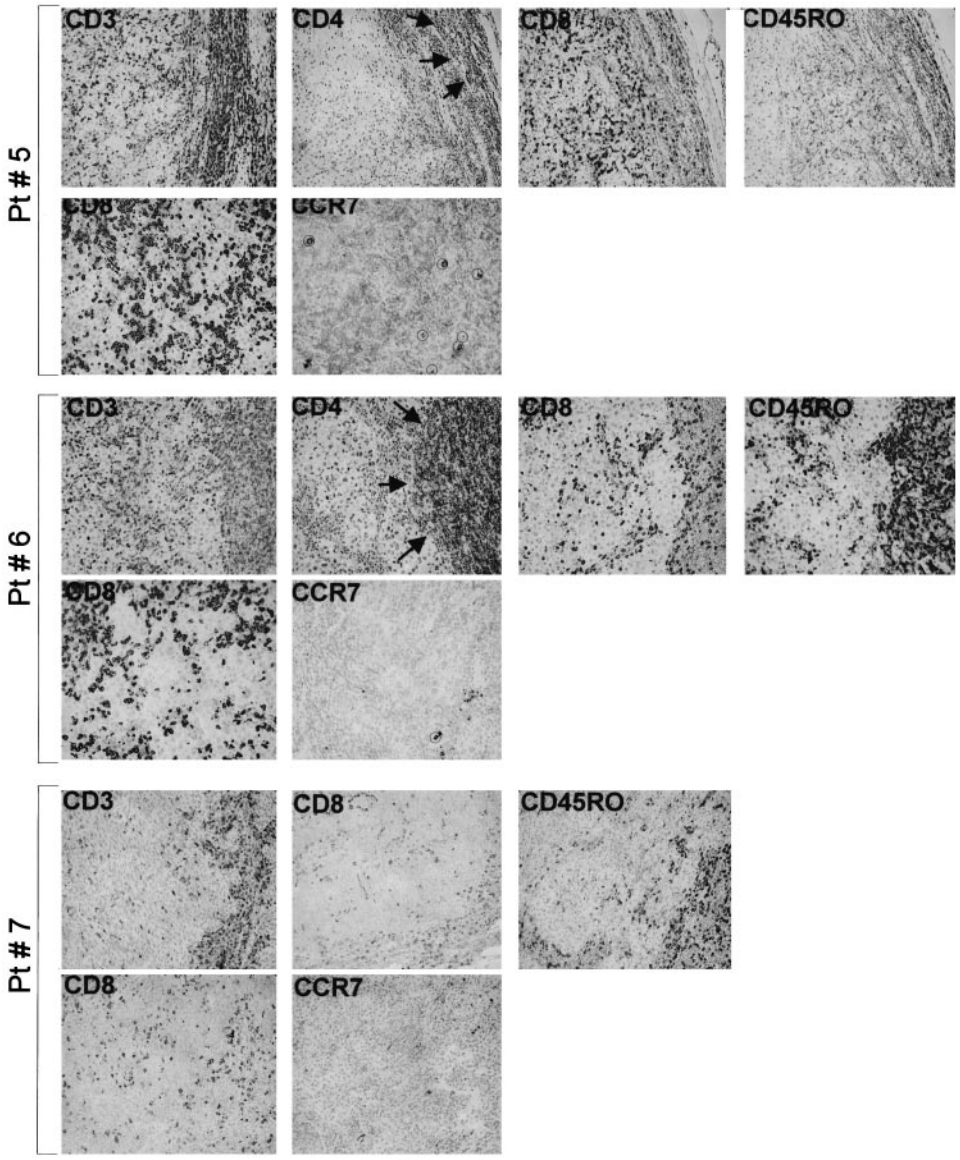

Fig. 6. Immunohistochemistry with mAbs to T-cell markers of TILN containing a brisk infiltrate of $CD3^+$ $CD8^+$ lymphocytes. Each *row* of *panels* shows consecutive sections stained with the indicated markers. $CD4^+$ lymphocytes (*arrows*) can be found only in residual lymph node tissue (*pt #5* and *#6*) surrounding the neoplastic tissue, but not among lymphocytes that infiltrate the tumor. Patients #5, #6, and #7 belong to a subset (of 7 patients) with the highest (>1.5% of $CD8^+$) T-cell frequency to Melan-A/Mart-1_{26-35} peptide in TILN and with a skewed $CCR7^-$ $CD45RA^-$ phenotype in $tetramer^+$ T cells. In such patients, CCR7 expression was found only on rare (*circled*) melanoma cells but not on infiltrating lymphocytes.

lesions from the 7 patients with the skewed $CCR7^-$ $CD45RA^-$ phenotype of $CD8^+$ T cells (Fig. 9). Finally, in all of the patients in the panel of TILNs, analysis of invaded lymph node sections for morphological evidence of tumor necrosis and/or regression, or apoptosis (by TUNEL staining), failed to show evidence for tumor destruction in all of the lesions (representative results from 3 patients shown in Fig. 10), suggesting that incomplete $CD8^+$ T-cell maturation and reduced expression of cytotoxic factors in $CD8^+$ T cells may impair tumor cell destruction in the invaded lymph nodes.

DISCUSSION

We found that progression to metastatic disease in human melanoma is associated with increased peripheral frequency of MDA-specific T cells and with accumulation of functional memory T cells in TILN. Furthermore, the comparison of healthy donors and stage I+II patients indicated that frequency of circulating $CD8^+$ T cells directed to $gp100_{154-162}$ and Melan-A/Mart-1_{26-35} epitopes is increased in a fraction of patients, suggesting that during melanoma progression, activation of antitumor immunity to MDA epitopes can be an earlier event than previously thought on the basis of studies where patients with advanced disease have been investigated (27, 28). On the other hand, comparison of TILN and TFLN did not provide evidence for increased MDA-specific T-cell frequency in the invaded lymph nodes. This may be because of the limited number of TFLNs that could be analyzed, but, at least for the Melan-A-Mart-1-specific T cells, it suggests that even TFLNs can harbor a significant fraction of tumor-specific T cells in agreement with the possibility of an immune response to tumor antigens that involves several lymph nodes from the same nodal basin.

Taken together our results suggest that the immune system of melanoma patients reacts to tumor growth by recognizing neoplastic cells in the invaded lymph nodes and/or changes in the level of MDA load, occurring at time of progression to metastatic disease. This possibility is in agreement with findings obtained in murine models (7), where the presence of T cells intermingling with the tumor nodules in TILN has been shown to represent a relevant factor for the activation of immunity at tumor site.

Differential epitope immunogenicity appears to play a significant role in T-cell accumulation at tumor site, even in the response to different epitopes of the MDA class. In fact, only Melan-A/Mart-

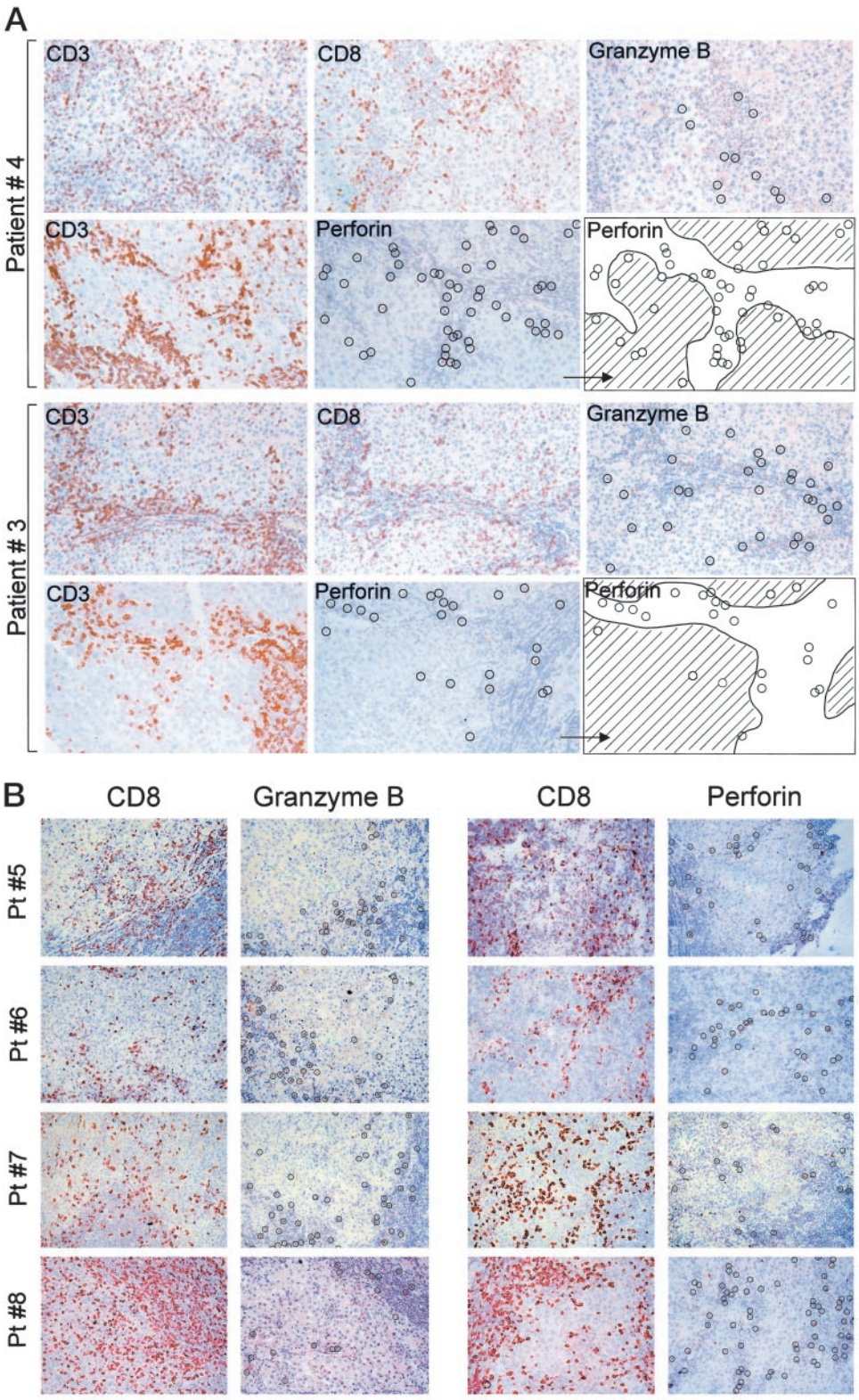

Fig. 7. Expression of cytotoxic factors in TILN. TILN sections from patients #3, #4, #5, #6, #7, #8, all characterized by a brisk $CD3^+$ $CD8^+$ T-cell infiltrate and a predominant $CCR7^-$ $CD45RA^-$ phenotype in MDA-specific T cells, were stained with mAbs to perforin or granzyme B. *A*, patients #3 and #4, the two drawings highlight distribution of $perforin^+$ lymphocytes in the residual lymph node tissue (*empty*) or in the neoplastic tissue (*shaded*). *B*, expression of perforin and granzyme B in lymphocytes from TILN sections of patients #5, #6, #7, #8. Each *row* of *panels* shows consecutive sections stained with mAbs to the indicated markers. Lymphocytes with a cytoplasmic staining for perforin or granzyme B are *circled*.

1_{26-35}-specific T cells, of the four MDAs investigated, showed a significant expansion in TILNs compared with PBLs of metastatic patients. This finding cannot be explained solely on the basis of the principle of degeneracy of antigen recognition, *i.e.*, the existence of largely cross-reactive subsets of naive $CD8^+$ T cells against this epitope (29). In fact, in patients with the highest frequency of T cells to this peptide, the T cells in TILN did not express a $CCR7^+$ $CD45RA^+$ "naive" phenotype and recognized the cognate antigen in a functional assay (IFN-γ production). Thus, we favor an alternative explanation based on the finding that Melan-A/Mart-1_{26-35} CTL epitope is poorly generated by the immunoproteasome of mature dendritic cells (30). On the basis of the proposed role of professional APCs in maintaining tolerance to self-antigens (10, 31), then epitopes poorly presented by such cells may not induce tolerance (32). There-

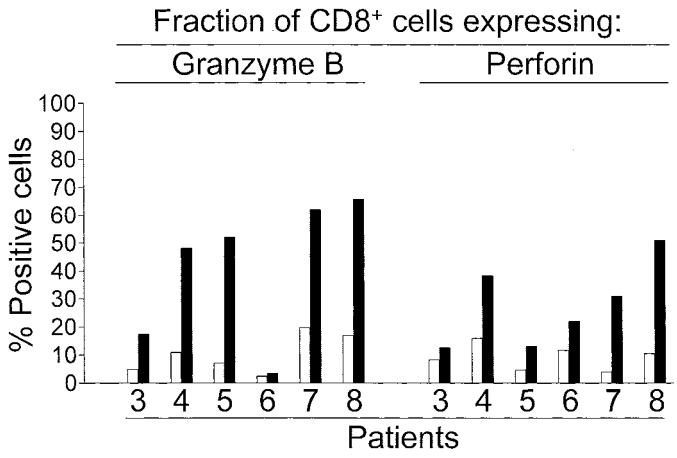

Fig. 8. Expression of perforin and granzyme B in TILN. Percentage of perforin$^+$ or granzyme B$^+$ lymphocytes, expressed as a fraction of CD8$^+$ cells, is lower inside (*empty*) than outside (*black*) the neoplastic tissue of TILN ($P = 0.002$, Mann Whitney t test) from patients #3, #4, #5, #6, #7, #8, as evaluated by immunohistochemistry in serial sections.

	Antigen expression on melanoma cells in TILN		
% Positive cells	HLA-A2	Melan-A/Mart-1	Gp100
<10%	○○○	○○○○○○○○○○	○○○○
10-70%	○○○○○ □□	○○○○○○○○ □	○○○○○○○○○○
>70%	○○○○○○○○○○ □□□□□	○○○○○○○○○○○○ ○○○○○○○○ □□□□□□	○○○○○○○○○○ ○○○○○○○○○○ ○○○○○○○○

Fig. 9. Summary of HLA-A2 and MDA expression, by immunohistochemistry, on neoplastic cells from TILN isolated from patients with >1.5% (□) or <1.5% (○) Melan-A/Mart-1-specific T cells in the metastatic lymph nodes.

fore, immunogenicity of Melan-A/Mart-1_{26-35} may at least in part depend on direct antigen presentation at tumor site, after Melan-A/Mart-1 protein processing through the constitutive proteasome of neoplastic cells.

The phenotypic and functional characteristics of the MDA-specific T-cell population found in TILNs of a group of metastatic patients are in agreement with a preterminally differentiated state, according to the model by Champagne *et al.* (12). However, this partial maturation of antitumor CD8$^+$ T cells took place only in a fraction of TILNs, as in the majority of lesions T cells directed to MDA or to tumor-restricted epitopes (such as MAGE-3 and NY-ESO-1 peptides) showed a predominant precursor phenotype (CCR7$^+$ CD45RA$^+$ CD27$^+$ CD28$^+$) and lacked of perforin. Furthermore, comparison of maturation phenotype of antigen-specific T cells in TILNs, by the CCR7/CD45RA and CD27/CD28 models, provided concordant evidence for the almost complete absence of T cells defined as "terminally differentiated" (*i.e.*, CCR7$^-$ CD45RA$^+$), or in "late stage" of differentiation (*i.e.*, CD27$^-$ CD28$^-$), even in those patients where a partial maturation of MDA-specific T cells took place.

We observed that tumor-infiltrating lymphocytes in TILN lacked CD4$^+$ cells, although these cells were present in the residual lymph node tissue not invaded by neoplastic cells. This suggests that lack of CD4 T-cell help in the tumor tissue may prevent terminal differentiation of the infiltrating memory CD8$^+$ T cells, as suggested by Champagne *et al.* (12). However, the impact of the cytokine "milieu" of the tumor microenvironment should also be considered. For example, inadequate production, at tumor site, of cytokines such as IL-15 and IL-7, which play a major role in generation and homeostasis of memory CD8$^+$ T cells (33), could prevent efficient T-cell maturation.

Furthermore, by immunohistochemistry, in TILNs, we found that CD8$^+$ cells expressed a marker of cytolytic granules (TIA-1/GMP-17;

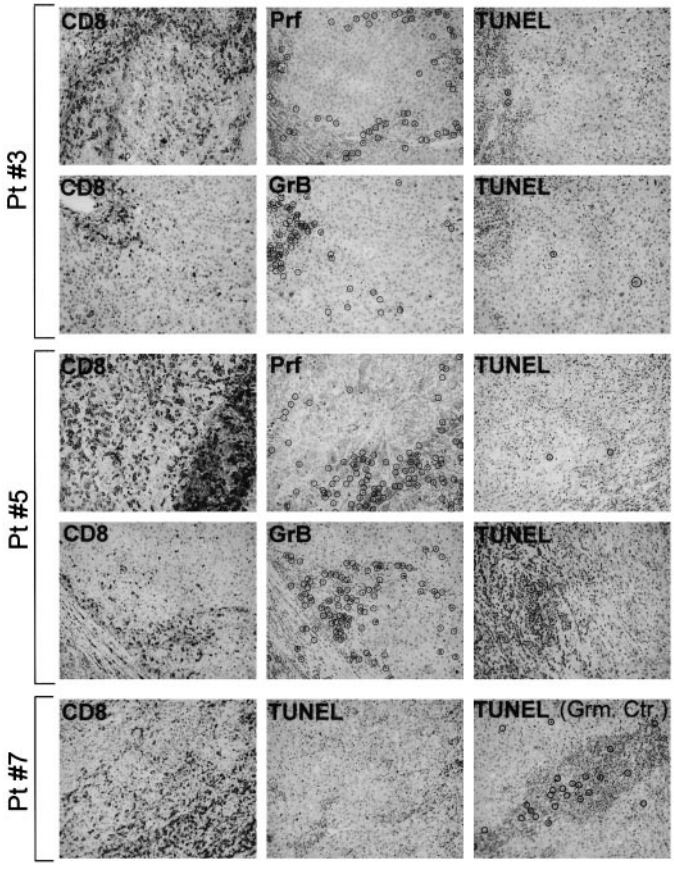

Fig. 10. Lack of tumor apoptosis in TILN containing a brisk infiltrate of CD3$^+$ CD8$^+$ lymphocytes. Lymphocytes with a cytoplasmic staining for perforin (*Prf*) or granzyme B (*GrB*), and tumor cells staining for TUNEL are *circled*. The sections lacked areas of tumor necrosis. Furthermore, neoplastic cells did not show evidence for apoptosis, as TUNEL$^+$ neoplastic cells were either extremely rare or absent in the lesions, although many TUNEL$^+$ lymphocytes could be found in germinal centers (*grm. ctr.*) of the same TILN (pt #7, *bottom rightmost panel*). Each *row* of *panels* shows consecutive sections stained for the indicated markers.

Ref. 26), whereas perforin and granzyme B, two key proteins for CTL activity mediated by the granule exocytosis pathway (14), were expressed only in a fraction of these lymphocytes, and mainly in cells that surrounded the tumor, rather than in those that infiltrated it. These results are consistent with the hypothesis of incomplete maturation of the $CD8^+$ T lymphocytes that infiltrate the tumor. Furthermore, the expression of HLA-A2 and MDA on most neoplastic cells, in the lesions of patients with a predominant $CCR7^-$ $CD45RA^-$ $CD27^+$ $CD28^-$ phenotype of the tetramer$^+$ T cells, suggests that lack of terminally differentiated $CD8^+$ T cells may not be attributable simply to loss of MHC and antigen expression on the tumor. On the other hand, the expression of HLA-A2 on the cell surface and the presence of MDA proteins in the cytoplasm, detected by immunohistochemistry, do not rule out the possibility that the neoplastic cells lack the specific HLA-A2-peptide complexes needed to promote differentiation of antigen-specific $CD8^+$ T cells. An alternative explanation for the low expression of cytotoxic factors in the $CD8^+$ cells in TILN is degranulation, but this is not supported by the results on TIA-1/GMP-17 staining. In fact, no membrane staining for TIA-1/GMP-17 was detected, as it occurs when degranulation takes place (26), as the staining for this marker was always granular and associated with the cytoplasm. In addition, we never observed perforin or granzyme B expression associated with neoplastic cells, as it has been described in nodal metastasis of breast and lung tumors (34), where it indicates degranulation from the infiltrating lymphocytes and transfer of cytotoxic factors to neoplastic cells.

The markedly reduced expression of perforin and granzyme B, among infiltrating $CD8^+$ T cells, suggests that tumor cell destruction by infiltrating T cells is markedly impaired even in those lesions where partial differentiation of $CD8^+$ T cells directed to MDA epitopes took place. In agreement with this possibility, by TUNEL staining for apoptosis, or by morphological analysis for tumor necrosis and/or regression, we did not find evidence for tumor cell destruction in the TILN. Taken together, these findings have implications not only for the understanding of the natural evolution of human melanoma, but even for the design of immune intervention approaches to therapy. The activation and/or boosting of T cell-mediated immune response to melanoma antigens, through vaccines, may need to be coupled with efficient means to promote efficient $CD8^+$ T-cell maturation in the tumor tissue. To this end the use of adjuvants that may promote CTL maturation, such as cytokines as IL-2 and IL-15, may lead to increased efficacy of melanoma vaccines.

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