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Clinical Cancer Research

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Cancer Therapy: Clinical

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T-Cell Immune Function in Tumor, Skin, and Peripheral Blood of Advanced Stage Melanoma Patients: Implications for Immunotherapy

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Abstract

Purpose: To predict the potential antitumor effect of antigen-specific T cells in melanoma patients, we investigated T-cell effector function in relation to tumor-escape mechanisms.

Experimental Design: $CD8^+$ T cells isolated from tumor, adjacent normal skin, and peripheral blood of 17 HLA-$A2^+$ patients with advanced-stage melanoma were analyzed for their antigen specificity and effector function against melanocyte differentiation antigens MART-1, gp100, and tyrosinase by using HLA-A2/peptide tetramers and functional assays. In addition, the presence of tumor-escape mechanisms PD-L1/PD-1 pathway, FoxP3 and loss of HLA or melanocyte differentiation antigens, both required for tumor cell recognition and killing, were studied.

Results: Higher percentages of melanocyte antigen-specific $CD8^+$ T cells were found in the melanoma tissues as compared with adjacent normal skin and peripheral blood. Functional analysis revealed 2 important findings: (i) in 5 of 17 patients, we found cytokine production after specific peptide stimulation by tumor-infiltrating lymphocytes (TIL), not by autologous peripheral blood lymphocytes (PBL); (ii) $CD8^+$ T cells from 7 of 17 patients did not produce cytokines after specific stimulation, which corresponded with significant loss of tumor HLA-A2 expression. The presence of other tumor-escape mechanisms did not correlate to T-cell function.

Conclusions: Our data show that functional T-cell responses could be missed when only PBL and not TIL are evaluated, emphasizing the importance of TIL analysis for immunomonitoring. Furthermore, loss of tumor HLA-A2 may explain the lack of T-cell functionality. These findings have important implications for selecting melanoma patients who may benefit from immunotherapy. *Clin Cancer Res; 17(17); 5736–47. ©2011 AACR.*

Introduction

Antitumor immunity can be induced in melanoma patients through several treatment strategies, including immunotherapy. Despite improved (immuno) therapeutic approaches to melanoma, significant numbers of patients with melanoma either respond poorly or show relapses. This may be caused by tumor resistance to T-cell–mediated lysis or impaired T-cell effector function, which can be because of lack of T-cell priming. Successful eradication of tumor cells by CTL depends on the number of antigen-specific $CD8^+$ tumor-infiltrating lymphocytes (TIL) within the tumor and their ability to recognize the tumor cells. Tumor cells can avoid T-cell–mediated attack by down-regulating their melanocyte antigen and/or HLA molecule expression (1–3). In addition, the intratumoral presence of activated, cytokine-producing TIL and preserved HLA class-I expression were found to be associated with favorable outcome and prolonged patient survival (3, 4).

T cells might also become anergic by immunosuppressive $FoxP3^+$ regulatory T cells (Treg; refs. 5–7). Additional regulatory pathways that hinder T-cell function include direct inhibition by inhibitory ligands such as programmed death ligand-1 (PD-L1)/PD-1 pathway. Several studies

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Translational Relevance

In this article, we provide an integrated analysis of the T-cell immune function in relation to tumor tissue-escape mechanisms in advanced melanoma patients. These aspects determine the efficacy of antitumor immunity to suppress melanoma growth and are predictive for the potential antitumor effect of immunotherapy. Given the current progress of applying immunotherapy in clinical trials, our study provides insight in the patient characteristics, which enable selection of those patients who may benefit from this approach.

described that PD-L1 can block T-cell (effector) function (8, 9) and promotes T-cell apoptosis (9–11). Furthermore, PD-L1 expression correlates with unfavorable prognosis for many human tumors (12). More recently, it has been shown that tumor antigen-specific $CD8^+$ T cells infiltrating melanoma express high levels of PD-1 and are functionally impaired (13, 14). However, it still remains elusive why some melanoma patients experience clinical benefit after (immuno) therapy, whereas others do not. In this study, we have carried out a detailed functional analysis of $CD8^+$ T cells from the tumor, adjacent normal skin and peripheral blood of 17 $HLA\text{-}A2^+$ patients with advanced melanoma in relation to immune-escape mechanisms, the level of intratumoral T-cell infiltration, and the survival of patients. Our study provides insights that may predict the potential success of antimelanoma immunotherapy in individual patients.

Material and Methods

Patient material

All blood and tissue biopsies of patients with advanced stage melanoma ($n = 24$) or congenital nevi ($n = 6$) were collected after written informed patient consent by using protocols approved by the medical ethical committees of the Netherlands Cancer Institute–Antoni van Leeuwenhoek Hospital and the Academic Medical Center in Amsterdam, according to the Declaration of Helsinki Principles. Biopsies of cutaneous metastatic melanoma with adjacent skin (<2 cm) were obtained at surgical resection. Part of the tumor/skin specimen was freshly frozen in liquid nitrogen or fixed in 4% formaldehyde after resection. The remaining part was used to isolate TIL or skin-infiltrating lymphocytes (SIL).

HLA typing

HLA typing of melanoma patients was done on peripheral blood lymphocytes (PBL) by flow cytometry using fluorescein isothiocynanate (FITC)-conjugated mouse anti-human HLA-A2–specific monoclonal antibody (mAb; BD Biosciences) and biotinylated HLA-A1, 36-specific antibody (BIH0331), HLA-A2–specific antibody (BIH0648), HLA-A3–specific antibody (BIH0269), all from One Lambda Inc.), followed by allophycocyanin (APC)-conjugated streptavidin (BD Biosciences) to detect biotin. In parallel, IgG1-FITC (BD Biosciences) and IgM-biotin isotype control antibodies (One Lambda) were included as negative controls.

Isolation of tumor-infiltrating lymphocytes and skin-infiltrating lymphocytes

Biopsies of melanoma lesions (<2 cm) or adjacent skin were cultured in a humidified atmosphere at 37°C and 5% CO_2 in 24-wells plates with 1 mL Iscove's modified Dulbecco's Medium (IMDM; Cambrex Bio Science), supplemented with 10% heat-inactivated human serum type AB (Cambrex Bio Science), 40 U/mL IL-2 (Proleukin; Novartis Pharma), 5 ng/mL IL-15 (Strathmann Biotec AG), 15 μg/mL gentamycin (Duchefa), 2 mmol/L L-glutamine (Gibco Invitrogen), 50 U/mL penicillin and 50 μg/mL streptomycin (Gibco Invitrogen), and 50 mmol/L 2-mercaptoethanol (Sigma-Aldrich). In addition, 1.25 μL/mL anti-CD3/CD28 mAb-coated T-cell expander beads (Dynal Biotech-Invitrogen) were added to promote T-cell outgrowth.

Peripheral blood mononuclear cells and CTL cell clones

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by Ficoll gradient centrifugation (Lymphoprep; Fresenius Kabi Norge AS) and resuspended in IMDM with supplements as mentioned above. PBMC were cultured by using equal conditions and time periods compared with isolated TIL prior to the tumor/skin explant assays.

The $HLA\text{-}A2^+$ CTL clones, CTL AKR103TP, and CTL INFA13 with established cytotoxic capability were obtained, cultured, and restimulated as described previously (15, 16) in the same medium as that used for the TIL.

Detection of melanocyte-specific T cells in peripheral blood lymphocytes and melanoma tissues

Flow cytometric analyses using HLA-A2/peptide tetramers for the melanocyte differentiation antigens tyrosinase, gp100, MART-1 and the control antigen, influenza virus, were done on T cells isolated from melanoma, adjacent skin, or peripheral blood, as previously described (17). Briefly, R-phycoerythrin (PE)- or APC-conjugated HLA-A2/peptide complex tetramers were synthesized for the antigens $tyrosinase_{369–377}$ (YMDGTMSQV), $gp100_{280–288}$ (YLEPGPVTA), $gp100_{209–217}$ (ITDQVPFSV), $MART\text{-}1_{26–35}$ (modified position 27 (A>L): ELAGIGILTV) and the control antigen $influenza\ virus_{58–66}$ (GILGFVFTL; tetramer synthesis by J.W.D. and K.F., Leiden, the Netherlands). T cells were incubated with HLA-A2/peptide tetramers in PBS, 1% bovine serum albumin (BSA) for 10 minutes at room temperature (RT). Subsequently, cells were counterstained with FITC-conjugated mouse anti-human CD8 mAb (CD8-FITC; BD Biosciences). Antibody and tetramer binding to T cells were analyzed by flow cytometry (FACS

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Canto II; Beckton Dickinson). Data were analyzed by using Flow Jo software (Tristar).

Melanocyte differentiation antigen-specific T-cell stimulation and flow cytometric analysis

T cells were stimulated in a 1:1 ratio with 10^5 JY cells loaded with a pool of melanocyte differentiation antigen-specific peptides (500 ng/mL each): tyrosinase$_{369-377}$, gp100$_{280-288}$, gp100$_{209-217}$, and MART-1$_{26-35}$. Incubation with Flu$_{58-66}$ peptide-loaded JY was used as a separate negative control, with PMA (phorbol 12-myristate 13-acetate)/ionomycin (1:500; Leukocyte Activation Cocktail; Becton Dickinson) as a separate positive control. Cells were incubated for 5 hours (PMA/ionomycin stimulation) or 14 hours (specific peptide stimulation) at 37°C and 5% CO_2 in 200 μL per 96-well IMDM, with supplements and culture conditions as stated above, in the presence of protein transport inhibitor brefeldin A (Golgiplug; Becton Dickinson). If required, CD107a mAb was present during the coculture (BD Pharmingen). Subsequently, the cells were stained on ice for 20 minutes with CD8-APC-Cy7 (348813; BD Bioscience). Cells were permeabilized, using the cytofix/cytoperm kit according to the manufacturer's instructions (BD), and subsequently stained for intracellular markers for 20 minutes on ice: IL-4-FITC (MP4-25D2; Biolegend), IL-17-PE (eBio64CAP17; eBioscience), IL-10-APC (BD Pharmingen), TNFα-PerCP-Cy5.5 (Mab11; Biolegend), and IFN-γ-Alexa 700 (4SB3; Biolegend). Antibody binding to T cells was subsequently analyzed by flow cytometry, measuring 6 fluorochromes simultaneously (FACS Canto II; Becton Dickinson).

CFSE labeling of T cells

To track T-cell infiltration *ex vivo*, T cells were labeled with carboxylfluorescein succinimidyl ester (CFSE; Molecular Probes-Invitrogen). T cells were centrifuged in a 15 mL tube to remove supernatant. The cell pellet was resuspended in 1 mL of PBS containing 5 μmol/mL of CFSE. Cells were incubated 10 minutes at 37°C in the dark, thereafter 14 mL of T-cell culture medium was added. Cells were washed in T-cell culture medium 3 times before use in tumor/skin explant assays.

Tumor/skin explant assay

Melanoma/skin biopsies (<4 mm) of HLA-A2$^+$ patients were cocultured in a 96-well round bottom plate (1 biopsy per well) with 0.5×10^6 to 1×10^6 autologous TIL (previously isolated from the first tumor tissue resection), PBL or CTL clones of interest, labeled with CFSE (Molecular Probes-Invitrogen), for 3 days in 200 μL per well in IMDM with supplements. Subsequently, the explants were washed and frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek). The tumor/skin explant cryosections were analyzed for the presence of CFSE labeled infiltrated T cells, their activation state (polarized granzyme B expression), and apoptosis of melanocytes/melanoma cells (active caspase-3 together with gp100) by immunofluorescence using Confocal Laser Scanning Microscopy (CLSM).

Immunohistochemistry and immunofluorescence staining

Immunohistochemical analysis was done on frozen as well paraffin-embedded tissue sections. Acetone fixed 5 μm cryosections of the tumor/skin explants were incubated with 0.25% hydrogen peroxide (Sigma) and 0.001% sodium azide in TBS for 20 minutes at RT to block endogenous peroxidase. Subsequently, the sections were preincubated with 10% normal goat serum (DAKO) in TBS for 15 minutes. Sections were stained with the following primary antibodies, incubated for 1 hour at RT in TBS in 1% BSA: mouse anti-gp100 (NKI-beteb, MONOSAN, SANBIO), mouse anti–melan-A (clone A103; DAKO); mouse antityrosinase (clone T311; Zymed); mouse anti-human CD8 mAb (clone 346310; BD Bioscience), mouse anti-human CD4 mAb (clone 346320; BD Bioscience); mouse anti-HLA class-I (HLA-ABC, clone W6/32; DAKO), mouse anti-human HLA-A2-FITC (clone BB7.2; BD Pharmingen), rabbit antiactive caspase-3 mAb (affinity-purified; BD Pharmingen); mouse anti-FoxP3 (Abcam); mouse anti-PD-1 (clone JII6; eBioscience), mouse anti–PD-L1 (clone M1H1; eBioscience), mouse anti-granzyme B-PE (clone GB11; Sanquin), mouse anti-CD69 (clone FN50), all from eBioscience. Bound antibodies, including fluorochrome-conjugated antibodies, were either detected by biotinylated polyclonal goat anti-mouse Ig (GaM-bio; DAKO) or goat anti-rabbit Ig (DAKO), followed by streptavidin–horseradish peroxidase (HRP; DAKO). Tissue sections were visualized with AEC substrate (Vector Laboratories) and counterstained with hematoxylin (Sigma-Fluka). Coverslips were mounted using Kaiser's glycerol gelatin (Sigma).

In case of TNFα/von Willebrand factor (vWF) double staining, acetone-fixed frozen sections were incubated with a mAb against human TNFα (MONOSAN), followed by goat anti-mouse biotin (DAKO) and HRP-labeled streptavidin (Perkin Elmer). Next, the sections were labeled with AP-conjugated streptavidin (DAKO) after using the tyramide signal amplification (TSA) system (Perkin Elmer). The color development was achieved with an AP staining kit (Vector; Brunschwig Chemie). After this staining, the sections were blocked with 10% normal mouse serum and incubated with HRP-labeled vWF (DAKO) to distinguish TNFα-positive vascular endothelial cells from other TNFα−positive cells. The color development was achieved with AEC substrate (Vector Laboratories).

For immunofluorescence staining, acton-fixed cryosections were double stained with mouse anti-gp100 (NKI-beteb; MONOSAN) and rabbit antiactive caspase-3 (affinity purified; BD Biosciences) diluted in TBS containing 1% BSA (Sigma-Aldrich). Subsequently, primary antibodies were detected by biotinylated polyclonal goat anti-mouse Ig (GaM-bio; DAKO), followed by Cy5-conjugated streptavidin (Jackson Immunoresearch Laboratories), and by Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories). The sections were mounted by using Vectashield mounting medium (Vector Laboratories LTD). Isotype-matched control antibodies were included in each assay and found to be negative. Scoring was done on

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at least 20 high-power field (original magnification ×400) for each staining of serial sections of each tissue sample. T-cell infiltration in the tumor (Table 1) was estimated as the relative density of infiltration (−, no infiltration; +, low; ++, intermediate; +++, high density infiltration), according to standard measures of pathologists. HLA and antigen expression by the tumor (Table 4) was estimated as the percentage of positive tumor cells relative to the total number of tumor cells. Percentages of PD-1- and Foxp3-positive cells (Table 4) were scored relative to the total number of CD3-positive T cells in the tumor area, detected in serial sections. Scoring was done by 2 observers. Images (magnification ×200) were acquired by using a Qwin based analysis system (Leica).

CLSM analysis

Immunofluorescence staining was analyzed by using a Leica TCS-SP2 CLSM system, equipped with argon/krypton and helium/neon lasers and by using a 40× 1.25 (oil UV-HCX PL APO CS) numerical aperture 1.25 objective (Leica Microsystems). Possible crosstalk between different fluorochromes, which could lead to false-positive colocalization, was avoided by sequential measurement of individual channels. Color images were taken from electronic overlays by using Leica LCS-Lite confocal software (v2.00; Leica Microsystems). To quantify T-cell infiltration and tumor cell apoptosis, whole cryosections of the tumor/explant were scanned by CLSM, resulting in approximately 35 nonoverlapping tile scans spanning the whole tissue

Table 1. Patient characteristics and analysis of intratumoral T-cell infiltration

Patient	Sex/Age[a]	Disease stage	Prior therapy before tissue resection[b]	Therapy received after tissue resection[b]	Survival (mon) after date of tissue resection[c]	T-cell infiltration in the tumor[d]	
						CD8	CD4
1	F/81	III	Surg	Surg	16	++	+
2	F/72	III	Surg	Surg	32+	++	+
3	M/88	III	Surg	Surg	43+	+	+
4	M/96	III	Surg	Surg	43+	+	+
5	F/75	III	Surg	Surg	43+	+	+
6	F/62	IV	Surg	Surg	4	++	++
7	F/94	IV	Surg	Surg	9+	+	+
8	M/75	IV	Surg	Surg	21+	++	+
9	F/78	IV	Surg	Surg	22+	+	+
10	F/71	IV	Surg	Surg	44+	+++	+++
11	F/71	III	DTIC, RT, HT	Surg	44+	+++	+++
12	M/69	IV	Perfusion	RT –> DTIC	9	+	+
13	F/60	IV	RT	DTIC	11+	++	+++
14	M/48	IV	Temozolomide	DTIC –> ipilimumab	40+	+	+
15	F/61	IV	Temozolomide/ IFN/IL2	DTIC– > HT/RT– > DNA vaccin	35	++	+
16	F/81	II	Surg	Perfusion	8+	+	+
17	F/69	IV	Surg	Perfusion	14+	++	+
18	F/80	IV	Surg	CT	41+	+	+
19	F/49	IV	Surg	CT	32+	+	+
20	M/67	IV	Surg	RT/CT/HT	22	+	+
21	M/62	IV	Surg	DTIC	9+	+	+
22	M/70	IV	Surg	DTIC	12+	+	+
23	F/83	IV	Surg	DTIC	9	+	++
24	F/38	IV	Surg	DTIC–>DNA vaccine	10	+++	++

Abbreviations: [a]F, female; M, male; [b]surg, surgery only; RT, radiotherapy; CT, chemotherapy; HT, hyperthermia; DTIC, dacarbazine; [c]survival in months: + = follow up ongoing; [d]T-cell infiltration in the tumor was estimated as the relative density of infiltration (-, no infiltration; +, low; ++, intermediate; +++, high-density infiltration).

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section. Images showing complete tumor areas were selected for quantification, excluding pictures only showing adjacent skin tissue. Of each explant, approximately 15 photomicrographs, including pictures with minor or dense infiltration of CFSE-labeled T cells in the tumor area, were counted (375 μm × 375 μm/photomicrograph). The T-cell–mediated killing of melanoma cells was quantified by counting the number of added TIL and apoptotic melanoma cells within the same tumor area, by CFSE-labeled cells and active caspase-3–positive gp100-expressing cells, respectively.

Results

Melanocyte differentiation antigen-specific T cells are present in metastatic melanoma

Intratumoral $CD8^+$ and $CD4^+$ T cells were found in tumor tissues of 24 advanced stage melanoma patients, regardless of prior therapy. The observed T-cell infiltration varied among patients from low to moderate, but comparable infiltration of $CD8^+$ and $CD4^+$ T cells was detected per identical tumor tissue (Table 1).

To investigate whether the $CD8^+$ T cells in the melanoma tissue can recognize melanoma cells, we tested the antigen specificity of the TIL from 17 HLA-$A2^+$ patients with advanced melanoma. In addition, T cells in the peripheral blood and normal skin adjacent to the tumor were analyzed. Isolated T cells were briefly expanded by using anti-CD3/CD28 mAb stimulation before cellular analysis. The antigen specificity was measured by flow cytometry by using HLA-A2/peptide tetramers for MART-$1_{26–35}$, A2/$gp100_{209–217}$, A2/$gp100_{280–288}$, A2/$tyrosinase_{369–377}$ and the control antigen influenza $virus_{58–66}$ ($Flu_{58–66}$). Tetramer-positive T cells were found in blood, tumor, and skin tissues of the HLA-$A2^+$ patients, with A2/gp100 as most frequent (Table 2). More importantly, significantly increased levels of antigen-specific $CD8^+$ T cells were found in the melanoma tissues as compared with peripheral blood. Surprisingly, the antigen specificity of the T-cell population infiltrating the melanoma differed from the population infiltrating the adjacent skin, called skin-infiltrating cells (SIL). No recognition of the A2/$flu_{58–66}$ control antigen was observed in TIL and SIL, indicating that A2/$flu_{58–66}$ T cells were not found in the skin (data not shown). The antigen specificity of the TIL corresponded with the antigens expressed by the melanoma. Taken together, our findings show that melanocyte differentiation antigen-specific T cells are present in the tumor and adjacent skin and are capable of recognizing the tumor.

TIL become activated and cytotoxic upon melanocyte differentiation antigen-specific stimulation *in vitro*

To gain insight in the activation status of the T cells present in the melanoma tissue, T-cell activation markers CD69 and proinflammatory cytokine TNF-α were used to detect activated T cells *in situ* by immunohistochemistry. Although few CD69-positive cells were observed in the tumor tissues of melanoma patients, hardly any TNF-α—positive cells were found (data not shown), suggesting lack of activated TIL in the tumor.

To further test the potential effector function of the T cells isolated from the tumor, skin, and blood, we stimulated these T cells with a specific pool of HLA-A2/melanocyte antigenic peptides, including MART-$1_{26–35}$, A2/$gp100_{209–217}$, A2/$gp100_{280–288}$, A2/$tyrosinase_{369–377}$, or the control antigen influenza $virus_{58–66}$ ($Flu_{58–66}$) loaded onto EBV-transformed B cells (JY) as target. Intracellular expression of IL-4, IL-10, and proinflammatory cytokines IL-17, IFN-γ, and TNF-α, and upregulation of activation marker CD107a, which is upregulated upon antigen recognition, were measured by flow cytometry (Supplementary Fig. S1). These functional analyses showed that $CD8^+$ T cells from 10 of 17 melanoma patients produced various cytokines after melanocyte antigenic peptide stimulation (i.e., ≥2-fold cytokine induction, Supplementary Fig. S1B and Table 3), with IFN-γ being the most predominant cytokine. Remarkably in the other 7 patients, none of the $CD8^+$ T cells isolated from the different tissue compartments (i.e., tumor, skin, or blood) produced cytokines following specific stimulation. Interestingly, TIL from 5 of 17 patients produced cytokines after specific stimulation, which was absent in the PBL. CD107a upregulation was observed in only 2 of 17 patients with cytokine-producing $CD8^+$ T cells (Table 3). Nonspecific stimulation with PMA/ionomycin resulted in considerable cytokine production by T cells from all 17 patients (Supplementary Fig. S1C and data not shown). No clear cytokine production or upregulation of CD107a was detected after control peptide stimulation (Supplementary Fig. S1C and data not shown), indicating that the cells are specifically activated by melanocyte antigens.

Inhibitory factors in tumor microenvironment

Nonresponsiveness of T cells toward tumors is frequently found in cancer patients. Several immune-escape mechanisms might underlie the lack of T-cell responsiveness or anergy. Whereas activation of CTLs requires recognition of specific antigenic peptides bound to HLA class-I molecules, melanoma cells frequently display loss of melanocyte differentiation antigens or HLA class-I molecules, which prevents immune recognition (1–3). To explore any antigenic expression profile responsible for the impaired T-cell effector function, we compared the expression of HLA class-I, HLA-A2, melanocyte differentiation antigens and T-cell inhibitory factors, including PD-1/PD-L1 pathway, FoxP3, in the tumor tissues of the HLA-$A2^+$ melanoma patients ($n = 17$) with congenital benign nevi ($n = 6$).

Interestingly, HLA class-I expression and in particular HLA-A2, was significantly lower in the melanoma of the HLA-$A2^+$ patients with $CD8^+$ T cells that did not produce (average HLA-A2 expression: 32%) than those that did produce cytokines after specific stimulation (average HLA-A2 expression: 82%; Supplementary Fig. S2 and Table 4). Expression of the melanocyte antigens MART-1,

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Table 2. Tetramer analysis of CD8+ T cells from the tumor, adjacent skin, and peripheral blood

HLA-A2+		% Tetramer-positive cells of CD8+ T cells[a]						
Patient	**T cells**	**A2/MART**	**A2/Gp100 (209)**	**A2/gp100 (280)**	**A2/TYR**	**Sum[b]**	**A2/Flu**	
5	TIL	0.00	1.50	0.00	0.00	1.50	0.00	
	PBL	0.42	0.03	0.18	0.04	0.67	0.12	
11	TIL	1.50	2.00	1.40	0.20	5.10	0.00	
	PBL	0.03	0.00	0.14	0.02	0.19	0.01	
15†	PBL	4.80	3.00	0.29	0.24	8.33	0.50	
24†	TIL	0.17	1.79	0.35	0.16	2.47	0.08	
	PBL	0.17	2.08	0.35	0.02	2.62	0.03	
1†	TIL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
	SIL	0.05	0.04	0.57	0.01	0.67	0.06	
	PBL	0.05	0.04	0.04	0.00	0.13	0.01	
7	TIL	0.15	0.01	0.95	0.02	1.13	0.16	
	SIL	0.16	0.05	3.09	0.08	3.38	0.06	
8	TIL	0.04	0.04	0.29	0.01	0.38	0.03	
	PBL	0.36	0.26	0.48	0.08	1.18	0.30	
16	TIL	0.46	0.54	0.34	0.12	1.46	0.15	
	SIL	0.17	0.02	0.33	0.07	0.59	0.42	
	PBL	0.07	0.76	0.15	0.09	1.07	0.03	
18	TIL	0.00	3.40	0.00	0.00	3.41	1.49	
	SIL	0.00	3.60	0.00	0.00	3.60	1.04	
	PBL	0.00	0.27	0.01	0.00	0.28	0.00	
20†	TIL	0.90	4.00	0.70	0.40	6.00	0.01	
	PBL	0.07	0.06	0.55	0.03	0.71	0.07	
2	TIL	0.15	0.06	0.13	0.00	0.34	0.17	
	SIL	0.00	0.00	0.00	0.17	0.17	0.15	
	PBL	0.30	0.00	0.00	0.00	0.30	0.09	
3	TIL	0.00	1.10	0.80	0.00	1.91	0.00	
	SIL	0.83	0.00	0.00	0.00	0.83	0.00	
	PBL	0.00	0.02	0.00	0.19	0.21	0.00	
4	TIL	0.90	7.10	0.90	6.50	15.40	0.03	
	PBL	0.65	0.03	0.00	0.00	0.68	0.10	
17	TIL	0.00	0.09	0.40	0.00	0.49	0.00	
	PBL	0.08	0.00	0.00	0.00	0.08	0.00	
19	TIL	0.27	0.04	0.16	0.01	0.49	0.02	
	PBL	0.70	2.00	0.70	0.00	3.40	0.23	
21	SIL	0.19	0.11	3.08	0.51	3.89	0.00	
	PBL	0.00	0.02	0.11	0.04	0.17	0.19	
23†	TIL	0.00	10.6	0.00	0.00	10.60	0.00	
	SIL	0.30	0.31	0.39	0.06	1.06	0.00	
	PBL	0.00	0.64	0.00	0.00	0.64	0.00	
Significance of TIL versus PBL[c]		$P > 1.00$	$\mathbf{P < 0.05}$	$P > 0.18$	$P > 0.66$	$\mathbf{P < 0.05}$	$P > 0.61$	$n = 13$[f]
Significance of TIL versus SIL[d]		$P > 0.36$	$P > 0.17$	$P > 0.80$	$P > 0.34$	$P > 0.39$	$P > 0.87$	$n = 6$
Significance of PBL versus SIL[e]		$P > 0.45$	$P > 0.84$	$P > 0.31$	$P > 0.42$	$P > 0.08$	$P > 0.54$	$n = 7$

TIL, SIL, and PBL were tested for their antigen specificity by using HLA-peptide tetramers composed of HLA-A2 molecules and melanocyte antigenic peptides MART-1, gp100, and tyrosinase or influenza virus peptide by flow cytometric analysis.

[a]The numbers represent the percentage of tetramer-positive T cells of the CD8+ T-cell population. [b]Sum of percentages of melanocyte antigen tetramer-positive CD8+ T cells. [c]TIL were compared with PBL, [d]TIL were compared with SIL, or [e]PBL were compared with SIL for the difference in melanocyte-specific T cells for each antigen analyzed or [b]for the total percentage of melanocyte antigen-specific T cells, using the nonparametric Mann–Whitney U-test (95% CI), the difference between compared samples was significant if $P < 0.05$. [f]The number of patients used for each comparison is depicted for each comparison. n.a., not available. †, patient died.

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Table 3. Functional analysis of CD8+ T cells from the tumor, adjacent skin and peripheral blood

		Cytokine production[a]					Upregulation of CD107a[b]
Patient	**T cells**	**IL-4**	**IL-10**	**IL-17**	**TNF-α**	**IFN-γ**	
5	TIL	**2.19**	1.41	1.63	**2.37**	**2.36**	0.93
	PBL	**2.33**	1.39	1.27	1.22	1.57	**15.67**
11	TIL	**5.20**	1.62	1.69	1.46	**6.81**	**3.45**
	PBL	0.00	1.08	1.00	0.67	1.22	1.82
15†	PBL	1.73	**3.18**	1.97	1.68	**2.32**	1.28
24†	TIL	1.80	1.45	1.88	1.77	**4.94**	0.79
	PBL	**3.26**	1.03	**3.93**	1.01	1.17	0.54
1†	TIL	0.67	1.14	0.33	1.19	**2.03**	n.a.
	SIL	1.00	1.16	0.75	0.79	1.18	n.a.
	PBL	0.00	1.56	0.00	1.31	1.44	1,00
7	TIL	1.80	1.90	1.14	1.45	1.18	0.94
	SIL	1.13	1.33	0.50	1.59	**3.13**	1.52
8	TIL	0.72	1.05	0.63	1.38	1.23	0.43
	PBL	1.74	1.09	0.06	0.10	**4.15**	0.65
16	TIL	1.08	1.63	1.11	1.67	**2.05**	0.84
	SIL	0.54	1.23	0.14	1.60	**2.14**	1.10
	PBL	1.10	1.12	1.03	0.00	1.53	0.88
18	TIL	0.92	1.44	1.40	**2.27**	1.86	1.06
	SIL	**2.18**	0.05	1.44	1.87	1.56	1.96
	PBL	1.33	1.29	1.25	1.43	1.43	1.16
20†	TIL	1.67	1.21	**10.83**	1.35	1.05	1.40
	PBL	0.00	0.00	0.00	1.81	1.08	0.87
2	TIL	1.00	1.13	1.71	1.14	1.03	1.10
	SIL	0.96	1.47	0.00	1.13	1.17	0.33
	PBL	0.98	1.33	0.80	1.03	1.22	0.93
3	TIL	1.85	0.93	1.66	1.63	1.03	0.60
	SIL	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	PBL	1.33	0.36	1.25	1.29	0.99	0.28
4	TIL	1.50	1.05	1.86	1.28	1.60	0.85
	PBL	0.00	0.46	0.00	0.72	1.15	1.14
17	TIL	0.00	0.79	0.00	1.04	1.45	0.73
	PBL	0.00	0.96	1.00	1.17	0.46	0.95
19	TIL	0.00	1.42	0.00	1.37	1.39	1.19
	PBL	0.00	1.93	0.00	1.41	1.68	n.a.
21	SIL	1.00	0.00	1.00	1.21	0.80	n.a.
	PBL	0.00	1.00	0.00	1.68	1.67	1.78
23†	TIL	0.73	1.89	1.61	0.47	0.81	n.a.
	SIL	1.00	1.07	1.00	1.26	1.50	1.24
	PBL	0.00	0.96	1.00	1.15	0.97	n.a.

TIL, SIL, and PBL of HLA-A2+ patients were stimulated *in vitro* with pooled HLA-A2/MART-1 26–35, gp100280–288, gp100209–217 and tyrosinase369–377 peptides loaded on EBV-transformed B cells (JY).

[a]The numbers indicate the fold induction of cytokine responses of $CD8^+$ T cells reactive to the peptides relative to stimulation with JY alone. A 2-fold or more induction of cytokine production was considered positive (bold).

[b]Upregulation of CD107a was analyzed likewise. n.a., not available. †, patient died.

gp100, and tyrosinase was detectable in melanoma tissues, but most patients displayed partial loss of the various melanocyte differentiation antigens in the tumor (Table 4), which did not correlate to T-cell function. Although, low expression of PD-L1 and FoxP3 was detected on T cells in the tumor tissue of both patient groups (Table 4) and in congenital nevi (data not shown), FoxP3 expression was more frequent in patients without cytokine-producing $CD8^+$ T cells. Furthermore, only few PD-1-positive cells were found in melanoma tissue,

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Table 4. Immunohistochemical analysis of melanoma

Patient	Expression in the tumor (%)[a]						Expression on lymphoid cells in Tumor (%)[b]	
	HLA class-I	HLA-A2	MART-1	gp100	Tyrosinase	PD-L1	PD-1	FoxP3
Cytokine-producing CD8$^+$ T cells (CP)								
5	100	100	90	100	100	0	0	1
11	100	100	100	100	100	0	0	0
15	100	100	80	80	20	0	0	0
24	100	100	90	30	80	10	10	10
1	90	50	30	30	30	10	1	1
7	100	100	100	40	5	0	1	0
8	90	0	80	100	90	10	0	1
16	100	100	90	80	60	0	0	0
18	100	100	40	100	30	1	1	0
20	70	70	80	100	100	0	0	0
Average ± SE ($n = 10$)[c]	95 ± 3	82 ± 11	78 ± 8	76 ± 10	62 ± 12	3 ± 2	1 ± 1	1 ± 1
No cytokine-producing CD8$^+$ T cells (NCP)								
2	90	40	80	60	60	1	1	5
3	100	5	100	100	100	0	0	1
4	20	20	80	80	80	5	5	1
17	90	0	80	80	90	20	20	5
19	100	50	60	30	90	0	0	1
21	10	10	5	20	30	1	1	1
23	100	100	40	5	30	5	10	5
Average ± SE ($n = 7$)[c]	73 ± 15	32 ± 13	64 ± 12	54 ± 13	69 ± 11	5 ± 3	5 ± 3	3 ± 1
Significance of CP versus NCP[d]	$P > 0.28$	***P* < 0.05**	$P > 0.25$	$P > 0.14$	$P > 0.84$	$P > 0.44$	$P > 0.14$	***P* < 0.05**

NOTE: Tumors of HLA-A2+ patients with (top) or without (bottom) cytokine-producing CD8+ T cells following specific stimulation were analyzed for [a]the expression of HLA class-I, HLA-A2, the melanocyte differentiation antigens MART-1, gp100, and tyrosinase, PD-L1 in the tumor and [b]PD-1 and FoxP3 on intratumoral T cells by immunohistochemistry. Numbers show the percentage of positive tumor cells relative to the total number of tumor cells (HLA and melanocyte differentiation antigen expression), or the percentage of PD-1- or Foxp3-positive cells relative to the total number of CD3-positive T cells present in the tumor tissue. [c]Expression of each antigen-/molecule- determined in average ± standard error (SE); n = number of patients analyzed; [d]Patients with cytokine-producing CD8+ T cells (CP) were compared with those with no cytokine-producing CD8+ T cells (NCP) for the difference in HLA class-I, HLA-A2, specific melanocyte differentiation antigens MART-1, gp100, tyrosinase, PD-L1/PD-1, and FoxP3 using the nonparametric Mann–Whitney U-test (95% CI). The difference between compared samples was significant if $P < 0.05$.

despite the clear presence of CD8$^+$ T cells in the tumor (Table 1).

T-cell function in tumor/skin explant assay

Because impaired effector function of CTL or tumor resistance to T-cell–mediated lysis are the most important causes of tumor growth and progression, we developed an *ex vivo* tumor explant assay to discriminate these 2 processes. With this assay, we tested the functional capacity of isolated, cultured melanocyte differentiation antigen-specific T cells to actively kill melanoma cells and compare this with killing of melanocytes within the adjacent skin environment. This assay was based on the well-characterized skin explant model, showing the reactivity of antigen-specific T cells in the skin during graft versus host disease (18, 19) or vitiligo (17). Because of limited patients' recurrence or tissue material, we carried out 6 tumors/skin explants (patients 1, 5, 15, 18, 19, and 24).

Coculture of autologous TIL from patient 5, HLA-A2/MART-1- or influenza-specific CTL clones with melanoma/skin biopsies resulted in infiltration of T cells in the tumor and skin tissues as detected by CFSE labeling (green), indicating active T-cell migration toward melanoma cells and melanocytes (Fig. 1, left and middle columns). However, less infiltration of added T cells in the tumor tissue occurred when compared with adjacent skin. The level of T-cell infiltration varied among tumor areas, as illustrated by the 2 pictures in the middle columns of representative tumor tissue areas showing either dense or low T-cell

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Table 5. Quantification of T cell-mediated apoptosis of melanoma cells

	TIL bulk	CTL MART clone	CTL INFA clone
T-cell infiltration index[a]	6 (48/866)	10 (99/977)	24 (230/965)
Tumor cell apoptosis index[b]	6 (49/866)	33 (318/977)	1 (10/965)
Infiltration/apoptosis index[c]	1:1	0.3:1	24:1

NOTE: Tumor explants of patient 5 from Figure 1 were quantified for the infiltration of added TIL, HLA-A2/MART, or influenza (INFA)-specific CTL clones as well as apoptotic tumor cells. [a]the T-cell infiltration index indicates the numbers of T cells (CFSE) per 100 tumor cells, [b]the tumor cell apoptosis index indicates the numbers of active caspase-3–positive tumor cells per 100 tumor cells, or [c]the infiltration/apoptosis index indicates the ratio of infiltrated T cells and apoptotic melanoma cells. Score between brackets indicates total cell numbers counted.

infiltration. This heterogeneity was predominantly found in explants incubated with TIL and, to a lesser extent, in explants incubated with the HLA-A2/influenza virus–specific CTL clone. Interestingly, substantial apoptosis of both melanoma cells and melanocytes was found by the added autologous TIL or the HLA-A2/MART-1 CTL clone (Fig. 1, top two panels) as detected by active caspase-3 (red) and gp100 (blue) staining. Apoptosis was not observed in explants with the HLA-A2/influenza virus–specific CTL clone or in the medium control without added T cells (Fig. 1, bottom two panels). Most of the CFSE-infiltrated T cells and CTL clones were activated as shown by polarized granzyme B expression (red; Fig. 1; right column), indicative for functional effector capacity.

Quantification of these data revealed that autologous TIL bulk populations induced apoptosis of melanoma cells in an infiltration:apoptosis ratio of 1 to 1, suggesting functional effector T-cell capacity. The HLA-A2/MART-1 CTL

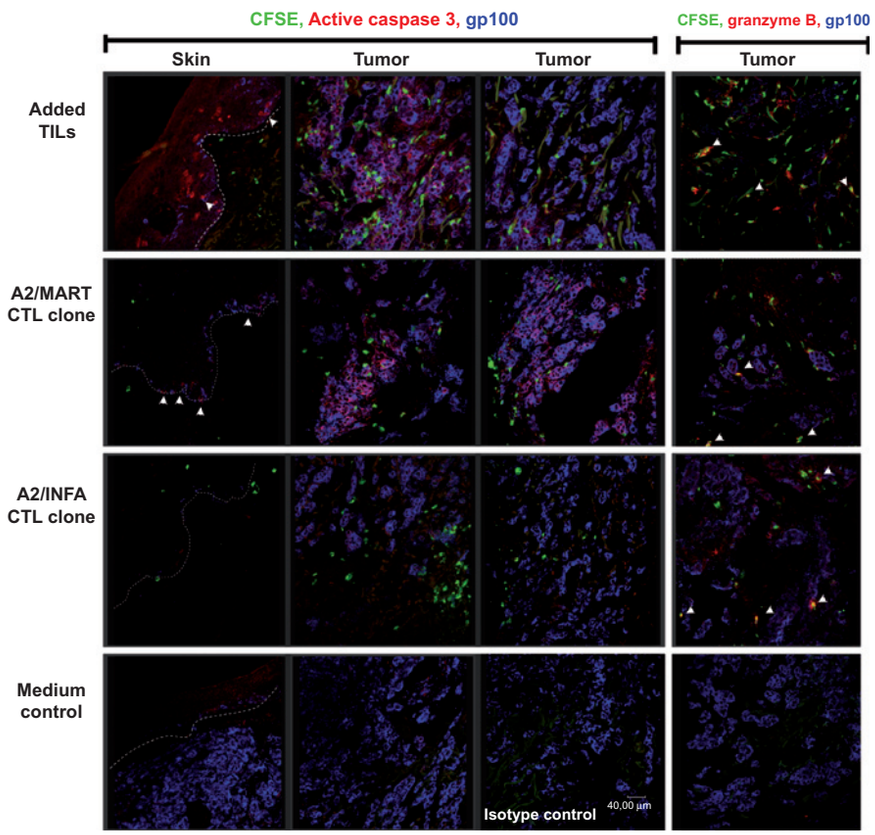

Figure 1. T-cell migration and T-cell–mediated apoptosis of melanoma cells/melanocytes in the tumor/skin tissue. CLSM analysis of tumor/skin tissue (patient 5) after coculture with CFSE-labeled autologous TIL (top), A2/MART-1–specific CTL clone (second panel), A2/influenza-specific CTL clone (third panel), or without cells (medium control, bottom panel). Infiltration of added T cells in the skin (left column) or tumor tissue (middle column) was visualized by CFSE (green); apoptosis of melanoma cells/melanocytes was detected by active caspase-3 (red) in combination with gp100 (blue) staining (left + middle columns, see arrows). Middle columns show 2 representative pictures, illustrating the observed heterogeneity in T-cell infiltration among different tumor areas. Activation of T cells was visualized by CFSE together with polarized granzyme B (red) expression (right column, see arrows). Dashed line indicates basal membrane of the skin. White scale bar for CLSM panels = 40 μm.

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clone was able to kill melanoma cells efficiently (infiltration:apoptosis index = 0.3:1), whereas the control HLA-A2/influenza virus–specific CTL did not induce apoptosis of melanoma cells (infiltration:apoptosis index = 24:1; Table 5), indicating that the melanoma cells are susceptible to T-cell–mediated killing. Considering the mixed antigen specificities present in TIL, as compared with the MART-1–specific T cells, TIL seemed quite efficient in inducing apoptosis in the melanoma cells.

In contrast to patient explants described above, infiltration of added autologous PBL from patient 18 did not result in apoptosis of melanoma or melanocytes, whereas the HLA-A2/MART-1 CTL clone induced melanocyte/melanoma cell apoptosis. However, the enriched $CD8^+$ PBL population slightly improved the killing of the tumor cells (data not shown), suggesting that numbers of specific T cells were too low in the PBL to induce detectable levels of apoptosis. The other explants ($n = 4/6$) were not evaluable for T-cell killing activity because of high levels of necrotic or endogenous apoptotic melanoma cells already present in the tumor tissue, and not induced by experimental conditions, as evidenced by the apoptosis found in freshly frozen, noncultured biopsies (data not shown). Despite the limited number of evaluable tumor/skin explants, our results show that this *ex vivo* equivalent of cellular interactions in the tumor environment can be used to test the potential efficacy of T cells to kill tumor cells, in the presence of the potential negative influence of the tumor microenvironment.

Discussion

This study integrates for the first time the immunophenotypical and functional features of intratumoral T cells with antigen expression and escape mechanisms of melanomas. Antigen specificity and functional activation of T cells isolated from tumor, adjacent normal skin, and peripheral blood of melanoma patients were studied as well as the expression of HLA class-I, HLA-A2, melanocyte differentiation antigens, and T-cell inhibitory factors in the tumor tissue.

We detected melanocyte antigen-specific $CD8^+$ T cells in tumor, adjacent normal skin, and peripheral blood of patients with advanced stage melanoma. Higher frequencies of melanocyte differentiation antigen-specific $CD8^+$ T cells were found in the melanoma tissue than in peripheral blood, indicating that these T cells predominantly accumulate in the tumor. The antigen-specific $CD8^+$ T cells analyzed here did not always produce cytokines or become activated upon antigenic stimulation. In addition, in 5 of 17 patients (= 29%) TIL, but not autologous PBL, produced cytokines after specific stimulation. The nonresponsiveness of tetramer-reactive T cells in PBL in our assays cannot be explained by a naive phenotype of the T cells, not able to respond in short-term activation assays, because the PBL in our analyses were cultured prior to functional testing, thereby changing the activation phenotype of the T cells.

These results clearly show that T-cell responses could be missed when only PBL and not TIL are used to evaluate the presence of functional T-cell responses in patients. Whereas most immunotherapeutic studies focus on T-cell effector function (i.e., cytokine production) in the peripheral blood, our findings however show the importance of TIL analysis and have major implications for immunomonitoring. We show here that it is clinically feasible to analyze infiltrating T cells in tumor tissue from melanoma patients, by taking a biopsy of a metastatic lesion and isolate the T cells by culture. Moreover, T-cell activation during vaccination regimens can be tested in skin biopsies of the vaccination site, as we have previously published for the analyses of skin immunity in vitiligo patients (17) and in ongoing studies on melanoma biopsies from patients treated with DNA vaccination (FV, JH, unpublished). The importance of analyzing TIL is supported by the finding that the level of melanoma-specific TIL, but not circulating melanoma-specific T cells, may predict survival in resected advanced stage melanoma patients (20). In our study, it is possible that T cells from the patients respond to other (melanoma) antigens in addition to the investigated melanocyte differentiation antigens (21) or recognize antigens in another HLA allele context than HLA-A2.

Interestingly, specific stimulation of the T cells revealed 2 groups of patients: (i) patients with ($n = 10/17$) and (ii) patients without cytokine-producing $CD8^+$ T cells ($n = 7/17$). Moreover, considerable differences were found between the 2 patient groups in the expression of HLA class-I and HLA-A2 molecules. $HLA\text{-}A2^+$ patients with no cytokine-producing $CD8^+$ T cells displayed significant loss of HLA-A2 compared with $HLA\text{-}A2^+$ patients with cytokine-producing $CD8^+$ T cells (Table 4). It is possible that the high HLA-A2 expression found in the tumor of patients with cytokine-producing T cells was the result of upregulation by local IFN-γ production (22). Recently, it has been described that favorable clinical outcome for stage II melanoma patients is strongly associated with preserved HLA class-I antigen expression and the presence of granzyme B^+ TILs (4). Loss of HLA class-I or HLA-A2 antigens abrogates tumor recognition by antigen-specific T cells. Our findings further suggest that alterations in HLA class-I expression have great impact on the $CD8^+$ T-cell effector function (e.g., cytokine production), leading to poor antitumor responses. In fact, significant lower transcriptional levels of HLA class-I components have been found in progressing melanoma metastases than in regressing ones (23). Remarkably, we were able to isolate and enrich $HLA\text{-}A2^+$ tetramer–positive $CD8^+$ T cells from melanoma tissue that lost their HLA-A2 expression. Probably, infiltration of these cells preceded the downregulation of HLA-A2 in these tumors and remaining low HLA-A2 expression or possibly antigen cross-presentation provided by $HLA\text{-}A2^+$ stromal cells was sufficient for these cells to survive. The melanoma tissues in this study showing (partial) loss of HLA expression may contain malignant melanoma initiating cells (MMIC), which have been described to evade immune responses by T-cell modulation (24, 25). These stem

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cell-like cells express the transporter ABCB5, conferring resistance to chemotherapy, as well as the nerve growth factor receptor CD271, and are characterized by enhanced capacity for self renewal and differentiation. MMIC show decreased expression of melanoma differentiation antigens and HLA molecules, allowing immune evasion. Moreover, MMIC can actively downmodulate T-cell activation by expressing the negative costimulatory molecules B7.2 and PD-1. However, whereas PD-1 was found on a minor proportion of T cells infiltrating in the tumor tissue, the melanoma tissues analyzed in this study did not show clear indications of PD-1 expression on melanoma cells (unpublished).

Comparable infiltration of $CD8^+$ and $CD4^+$ T cells was detected in the tumor tissue, suggesting that $CD8^+$ T-cell infiltration was not the limiting factor (Table 1). In contrast to HLA-A2, the $CD8^+$ T-cell effector function (i.e., cytokine production) in these 2 patient groups did not correlate to tumor-escape mechanisms nor to the survival of patients. With regard to T-cell inhibitory factors, the PD-1/PD-L1 pathway and FoxP3 Treg cells did not play an important role in determining T-cell function in this patient population.

Other mechanisms responsible for nonfunctional T cells might involve self-tolerance induction. Self-reactive T cells with high avidity for melanocyte differentiation antigens are depleted during early T-cell development. However, the immunologic tolerance to (self) melanocyte differentiation antigens is reversible (26, 27) or not complete as shown by our results of cytokine-producing $CD8^+$ T cells following melanocyte antigenic peptide stimulation (Table 3). Because cytokine production by T cells after nonspecific PMA/ionomycin stimulation was observed with T cells from all patients (Supplementary Fig. S1 and data not shown), the inability to produce cytokines after specific stimulation in some patient T cells may not be due to general defects in the cytokine production/secretion machinery. Yet, the considerable cytokine production by $CD8^+$ T cells after specific stimulation, found in 10 of 17 patients, did not correlate with the upregulation of CD107a (Table 3). This suggests that upregulation of CD107a required more stringent stimulation conditions than cytokine production. Also, it is possible that these T cells are not fully effective in their antitumor response and this may explain the advanced/progressive disease status of these patients.

In this study, we introduced the tumor/skin explant assay that enables us to test the effector function of T cells in the tumor microenvironment, within the intact tissue architecture. We showed that within the tumor tissue, the melanoma cells were susceptible to T-cell–mediated lysis. Autologous TIL can be effective in inducing apoptosis of melanoma cells within the tumor upon reactivation by culturing outside the tumor environment. The cultured TIL of patient 5 showed considerable cytokine production upon specific stimulation in the functional analysis. This suggests impaired effector function of the endogenous TIL *in vivo* rather than involvement of immune-escape mechanisms in this patient. Generation of tumor-reactive TIL by removing these cells from their suppressive tumor environment to generate and expand potent antitumor effector T cells, is already an established method and is applied for adoptive cell therapy (ACT) in melanoma patients (28). With this method, the ACT has shown objective clinical response rates between 49% to 72% in patients with metastatic melanoma (29). The tumor/skin explant assay was based on the well-characterized skin explant model used for transplantation and vitiligo research (17–19). By incorporating the influence of the tissue environment on T-cell effector function, the tumor/skin explant assay provides essential information on the efficacy of T-cell responses to attack tumor cells within the melanoma tissue. The tumor/skin explant assays thereby complement the currently used T-cell monitoring techniques, such as tetramer analyses, cytokine production, or cytotoxicity assays, and represents a valuable immunomonitoring tool to predict the potential antitumor effect of an immunotherapeutic strategy in a patient-specific setting.

To conclude, our study proposes that the effector function (e.g., cytokine production) of TIL and the characteristics of melanoma tissue, in particular, melanocyte antigens and HLA class-I expression, are important parameters that are informative for the potential outcome of immunotherapy in melanoma patients. Discriminating patients with functional antimelanoma effector T cells from those with nonfunctional T cells may help to select those patients who may benefit from immunotherapy.

Disclosure of Potential Conflicts of Interest

C.J.M. Melief is employed for 0.75 FTE as CSO of ISA Pharmaceuticals and has stock appreciation rights in ISA. The other authors declare no conflict of interest.

Authors' Contribution

E.P.M. Tjin: designed, performed research, and wrote the article; D. Konijnenberg: performed experiments; G. Krebbers: assisted with immunohistochemistry; H. Mallo: recruited patients for this study; J.W. Drijfhout, and K.L.M.C. Franken: synthesized tetramers; C.M.A.M. v.d. Horst: provided nevi materials; J.D. Bos: supervised the project; O.E. Nieweg and B.B.R. Kroon: provided melanoma patient materials; J.B.A.G. Haanen: supervised the clinical aspects of this study; C.J.M. Melief: designed and supervised the project; F.A. Vyth-Dreese: designed and supervised the project; R.M. Luiten: designed and supervised the project.

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