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High Frequency of Functionally Active Melan-A–Specific T Cells in a Patient with Progressive Immunoproteasome-Deficient Melanoma

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ABSTRACT

Tumor-reactive T cells play an important role in cancer immunosurveillance. Applying the multimer technology, we report here an unexpected high frequency of Melan-A–specific CTLs in a melanoma patient with progressive lymph node metastases, consisting of 18 and 12.8% of total peripheral blood and tumor-infiltrating $CD8^+$ T cells, respectively. Melan-A–specific CTLs revealed a high cytolytic activity against allogeneic Melan-A–expressing target cells but failed to kill the autologous tumor cells. Loading of the tumor cells with Melan-A peptide reversed the resistance to killing, suggesting impaired function of the MHC class I antigen processing and presentation pathway. Mutations of the coding region of the HLA-A2 binding Melan-$A_{26–35}$ peptide or down-regulation of the MHC class I heavy chain, the antigenic peptide TAP, and tapasin could be excluded. However, PCR and immunohistochemical analysis revealed a deficiency of the immunoproteasomes low molecular weight protein 2 and low molecular weight protein 7 in the primary tumor cells, which affects the quantity and quality of generated T-cell epitopes and might explain the resistance to killing. This is supported by our data, demonstrating that the resistance to killing can be partially reversed by pre-exposure of the tumor cells to IFN-γ, which is known to induce the immunoproteasomes. Overall, this is the first report of an extremely high frequency of tumor-specific CTLs that exhibit competent T-cell–effector functions but fail to lyse the autologous tumor cells. Immunotherapeutic approaches should not only focus on the induction of a robust antitumor immune response, but should also have to target tumor immune escape mechanisms.

INTRODUCTION

Although the prognosis for patients with advanced malignant melanoma remains poor, a number of promising immunotherapeutic approaches for the treatment of metastatic disease have been developed over the past decade. Complete and durable responses of some patients to cytokines such as interleukin (IL)-2 and IFN-α (1, 2) and more recently specific immunotherapeutic strategies such as vaccination with tumor-associated antigens (TAAs; refs. 3 and 4) or the adoptive transfer of TAA-specific T cells (5–7) provide the rationale for investigation of additional specific immunological approaches.

Malignant melanoma is exceptional among solid tumors because of its immunogenic properties. Existence of MHC-restricted CTLs with specificity for TAA has been demonstrated in melanoma patients. Tumor-specific CTLs have directly been implicated in spontaneous melanoma regression, occurring more frequently in melanoma than in other malignant diseases (8). Moreover, the presence of tumor-infiltrating lymphocytes (TILs) correlates with clinical outcome in vertical-growth–phase melanoma (9).

Numerous T-cell epitopes of melanoma cells have been identified, and several genes encoding the respective proteins have been cloned (10). Melan-A, belonging to the group of melanocyte differentiation antigen (Ag), is an ideal target epitope, because of its high immunogenicity and preferential expression on melanoma cells (11).

Taking advantage of the possibility to track Ag-specific T cells by appropriate multimer staining, low but consistent frequencies of T cells recognizing TAA could be detected in peripheral blood lymphocytes (PBLs) and/or TILs from the majority of cancer patients (12, 13). Why these responses may fail to effectively prevent tumor progression is still an unsolved question (12, 13). Functional analysis of TAA-specific T cells was either hampered by the need of prolonged *in vitro* culture, which can reverse impaired function (14), or revealed overt functional defects in the TAA-specific T cells. Lee *et al.* (15) reported that tyrosinase-specific T cells were selectively functionally inactive in a patient with melanoma, whereas others demonstrated that especially TILs of melanoma patients lack the effector cell phenotype necessary for cytotoxic activity (12). In some cases, tumor escape mechanisms such as failure of epitope expression, either due to lack of Ag or lack of presenting MHC complexes, also contributed to the progress of the disease (16).

In this report, we present a melanoma patient with an extremely high frequency of 18% Melan-A–specific T cells of total $CD8^+$ PBLs. Surprisingly, thorough analysis of fresh Melan-A–specific T cells either circulating in the peripheral blood or infiltrating metastatic lymph node showed T cells with effector phenotype and preserved cytolytic function. The isolated tumor cells revealed resistance to the attack by effector lymphocytes, but frequently described evasion mechanisms such as Ag mutation or loss could not be detected. However, analysis of the proteasome pathway revealed a decreased expression of the subunits of the low molecular weight protein (LMP) 2 and LMP7 as possible explanation for the tumor evasion.

This study, performed as a direct *ex vivo* analysis of PBLs, TILs, and primary tumor cells in a melanoma patient with a robust antitumor immune response, contributes to an explanation of the overall still disappointing clinical results of T-cell–based immunotherapies.

MATERIALS AND METHODS

Patient Characteristics. The patient under study (patient MeL162) was a 60-year-old man who had a superficial spreading malignant melanoma (Clark level II) that was excised from the back site in July 1999. In June 2001, he underwent resection of parailiac and left inguinal lymph node metastases. The patient received adjuvant chemo-immunotherapy with temozolomide and IFN-α for 2 months. In August 2001, he developed a relapse with an extensive retroperitoneal lymph node mass. In October 2001, he was included in a phase I clinical protocol designed to evaluate the feasibility and toxicity of adoptively transferred Melan-A–specific CTLs (7). He received four infusions of 0.25 to 11×10^8 Melan-A–specific CTLs i.v. at 2-week intervals together with low-dose IL-2. Computer tomography after four CTL infusions showed progressive disease of the lymph node metastases. The patient was then set on oral

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chemotherapy with trofosfamide but died in July 2002 due to progressive disease. The adoptive T-cell therapy protocol was designed and conducted in accordance with the Declaration of Helsinki, approved by the Institutional Ethics Committee, and registered with the regulatory state authority. The patient gave written informed consent before enrolling in the study.

Peptides. The following HLA-A2 binding peptides were used: natural (EAAGIGILTV) or modified (ELAGIGILTV) Melan-A_{26-35} decapeptides, $gp100_{280-288}$ (YLEPGPVTA) peptide, and influenza $matrix_{58-66}$ (GILGFVFTL) peptide. The modified Melan-A peptide used in the clinical study was prepared under good manufacturing practice conditions by Clinalfa AG (Laufelingen, Switzerland), and the other peptides were prepared by Bachem Biochemica GmbH (Heidelberg, Germany).

Media and Reagents. Lymphocytes and monocytes were cultured in supplemented RPMI 1640 (standard culture medium, M′; ref. 7). The following recombinant human cytokines and proteins were used at concentrations indicated in parentheses: IL-1β (10 ng/mL), IL-4 (500 units/mL), IL-6 (1000 units/mL), tumor necrosis factor-α (10 ng/mL), transforming growth factor-β (5 ng/mL; all from CellGenix, Freiburg, Germany), granulocyte-macrophage colony–stimulating factor (500 units/mL; Essex Pharma and Novartis Pharma, Basel, Switzerland), IFN-γ (100 units/mL; Promocell GmbH, Heidelberg, Germany), and prostaglandin-E_2 (1 μg/mL; Pharmacia & Upjohn, Erlangen, Germany).

Major Histocompatibility Complex/Peptide Multimers and Monoclonal Antibodies. Phycoerythrin (PE) or allophycocyanin-labeled HLA-A*0201 multimers that had been folded around ELAGIGILTV (Melan-A_{26-35}), YMDGTMSQV (Tyrosinase$_{369-377}$), IMDQVPFSV ($gp100_{209-217M}$), and SLYNTVATL ($HIVgag_{77-85}$) were synthesized by Beckman Coulter (Fullerton, CA). Monoclonal antibodies (mAbs) were from Becton Dickinson (San Jose, CA), except anti-CD4–allophycocyanin, anti-CD14–FITC (both from Coulter Immunology, Hialeah, FL), anti-CD16–FITC (PharMingen, San Diego, CA), anti-CD27–FITC, anti-CD45RA–allophycocyanin, anti-CD62L–FITC (all from Caltag, Burlingame, CA), anti-CD28–FITC (Immunotech, Marseille, France), anti-perforin–FITC (Ancell, Bayport, MN), and anti-granzyme B–FITC (Hoelzel Diagnostika, Cologne, Germany). Anti-CCR7 rat IgG mAb 3D12 was kindly provided by Dr. M. Lipp (Max Delbrück Institute, Berlin, Germany). Tumor cells were stained with mAbs against HLA class I (W6/32; Dako, Glostrup, Denmark), HLA-A2 (clone BB7-2; American Type Culture Collection, Manassas, VA), CD80 (PharMingen), anti-fibroblast mAb (AS02; Dianova, Hamburg, Germany), MEL-1 (clone R24), and MEL-2 (clone B5.2; both from Signet Laboratories Inc., Dedham, MA), identifying the GD3 ganglioside of human melanoma and the melanoma chondroitin sulfate protein, respectively. FITC-labeled annexin-V was purchased from PharMingen.

Isolation of Patient's Peripheral Blood Mononuclear Cells and *In vitro* Generation of Melan-A–Specific Cytotoxic T Lymphocyte. Peripheral blood mononuclear cells (PBMCs) were obtained from the melanoma patient MeL162 by leukapheresis on October 2001 (5 weeks before the first T-cell transfer and 2 months after adjuvant chemo-immunotherapy), isolated by ficoll separation, and cryopreserved. Melan-A–specific CTL lines were generated as described previously (7, 17). In brief, purified $CD8^+$ T cells were stimulated with dendritic cells pulsed with the appropriate peptide (30 μg/mL) and human β_2-microglobulin (10 μg/mL) in 96-well plates in M′ medium supplemented with 10% human AB serum and 1 to 2% T-cell growth factor (8). After four cycles of stimulation, phenotypic and functional analysis of T cells was performed.

Preparation of Primary Tumor Cells and Tumor-Infiltrating Lymphocytes and Culture of Tumor Cell Lines. Fresh tumor material (MeL 162) was obtained from a parailiac lymph node metastasis that was resected in June 2001 (before any systemic treatment). This material was used for the analysis of TILs and primary tumor cells. The isolation of tumor cells and TILs has been described previously (8, 18). The allogeneic melanoma cell lines Na-8-MEL (HLA-$A2^+$, Melan-A^-), Me 275, and Me 290 (HLA-$A2^+$, Melan-A^+) and T_2 cells (HLA-$A2^+$, $TAP^{-/-}$; ref. 19) were maintained in M′ medium plus 10% FCS.

Phenotype Analysis of T Cells. T cells were stained with multimers for 30 minutes at room temperature and incubated with appropriate mAbs for 20 minutes at 4°C. Cells were either immediately analyzed or sorted into defined populations on a FACSCalibur or a FACSVantage (Becton Dickinson), respectively, using the CellQuest software (Becton Dickinson). For intracellular staining, cells were stained with cell surface mAbs, fixed, permeabilized in PBS-1% paraformaldehyde-2% glucose-5 mmol/L sodium azide for 20 minutes at room temperature, and incubated with mAbs for intracellular Ag in PBS-0.1% saponin for 20 minutes at 4°C. For combination of multimer and annexin-V staining, cells were washed with annexin-V buffer (PharMingen) after incubation with surface mAbs. Cells were resuspended in 100 μL of annexin-V buffer, and 1 μL of annexin-V-FITC was added. After incubation for 20 minutes at room temperature in the dark, cells were fixed with 0.5% paraformaldehyde.

Phenotype Analysis and Purification of Primary Tumor Cells. Tumor cells were incubated with the appropriate mAbs for 20 minutes at 4°C, fixed with 0.5% paraformaldehyde, and analyzed on a FACSCalibur. To prevent possible artifacts in the functional analysis caused by contamination with nonmalignant cells, primary tumor cells from patient MeL162 were positively selected with magnetic beads (Dynabeads; Dynal, Oslo, Norway). Cells were stained with anti-MEL-1 mAb, which was found to be optimal to separate between contaminating nonmalignant fibroblasts and melanoma cells, incubated with CELLection pan mouse IgG (Dynal) with a ratio of 10 beads for 1 estimated positive cell, and then separated using a magnet.

Intracellular Cytokine Staining and Interferon-γ Secretion Assay. Measurement of intracellular IFN-γ production was combined with multimer labeling. Purified $CD8^+$ T cells were incubated for 4 hours with T_2 cells at a 1:1 ratio with irrelevant HIV-1 $Pol_{476-484}$ (ILKEPVHGV) peptide, 1 μg/mL cognate peptide, or 1 μg/mL PMA-0.25 μg/mL ionomycin, respectively. After 1 h, 10 μg/mL brefeldin-A (Sigma, St. Louis, MO) was added. After 3 h, cells were stained with multimers and mAbs, fixed, permeabilized, and incubated with anti-IFN-γ–FITC in PBS-0.1% saponin for 20 minutes at 4°C. Cells to be activated were stained with multimers before activation.

Chromium Release Assay. The cytotoxic activity of T cells was measured by a conventional 4-hour ^{51}Cr release assay as described previously (7). E:T ratios were calculated based on the number of Melan-A-$multimer^+$ T cells in the effector cell population to accurately compare different T-cell populations. Melanoma cells MeL162 were treated with IFN-γ (100 units/mL) for 24 hours, where indicated.

Immunohistochemical Staining. For the detection of Melan-A expression in melanoma cells and characterization of the T-cell infiltrate in formalin-fixed tumor tissue embedded in paraffin, the anti-Melan-A (A103) mAb (Novocastra, Newcastle, United Kingdom) and anti-CD4 and anti-CD8 mAbs (both from Dako, Hamburg, Germany) were used. mAbs against HLA class I heavy chain (HC10), human β_2-microglobulin (L368), TAP1 (NOB1), TAP2 (NOB2), tapasin (TO3), LMP2 (SY-1), and LMP7 (HB2) were kindly provided by S. Ferrone (Roswell Park Cancer Institute, Buffalo, NY) and have been described elsewhere (20, 21). Immunohistochemical staining of tissue sections with HLA class I Ag-processing machinery component-specific mAbs was performed as described elsewhere (21). Bound mAbs were detected using the diaminobenzidine method and counterstained with hematoxylin. Normal lymphocytes and vessel endothelia were used in each specimen as internal control. Negative controls were performed by omitting primary antibodies.

Complementarity-Determining Region 3 Size Analysis of T-Cell Receptor Vα and Vβ Transcripts. The complementarity-determining region (CDR) 3 of the PCR-amplified [T-cell receptor (TCR)] Vβ1–24 and Vα1–29 transcripts was analyzed using a run-off procedure (22). The run-off products were run on an automated sequencer in the presence of fluorescent size markers. The length of DNA fragments and the fluorescence intensity of the bands were analyzed with the Base ImagIR software (LI-COR Biotechnology Division, Bad Homburg, Germany).

Reverse Transcription-Polymerase Chain Reaction Analysis of the Ag-Processing Machinery in Melanoma Cells. Total cellular RNA from unpurified and MEL-1–purified primary melanoma cells of patient MeL162 and three allogeneic tumor cell lines (one renal cell carcinoma and two melanoma cell lines) was isolated using standard methods. The primers and the conditions used for conventional and one-step reverse transcription-PCR analysis have been described previously (18, 23).

Sequencing of the Coding Region of the Human Lymphocyte Antigen-A2 Binding Melan-A Peptide in Melanoma Cells. A 405-bp fragment covering the entire coding region of the human Melan-A gene was amplified from reverse transcribed RNA (Superscript II; Invitrogen, Groningen, the Netherlands) from MeL162 and Me 275 melanoma cells, using Melan-A sense (5′-CCTGTGCCCTGACCCTAC-3′) and Melan-A antisense (5′-AGCATGTCTCAGGTGTCTCG-3′) primers. PCR products were precipitated and

sequenced from both ends using the above primers. Sequencing was performed by Geneart GmbH (Regensburg, Germany).

RESULTS

Phenotypic and Functional Analysis of Highly Expanded Circulating Tumor-Associated Antigens-Specific T Cells in a Patient with Progressive Melanoma. Using fluorescent HLA-A2/peptide multimers, we quantified Melan-A–specific $CD8^+$ T cells in PBMC from a HLA-$A2^+$ melanoma patient (patient MeL162) with rapidly progressive disease. As shown in Fig. 1*A*, we found an unexpected high frequency of circulating Melan-A–specific CTLs of 18.1% of total $CD8^+$ cells. T cells recognizing other HLA-A2–binding TAAs such as tyrosinase, but not gp100, were also detectable in patient's PBLs, although to a much lesser extent (see Fig. 1*A*).

Because selective anergy or functional defects of TAA-specific T cells have been proposed as possible immune escape mechanisms in tumor patients, we thoroughly characterized the native phenotypic state of the Melan-A–specific $CD8^+$ T cells in this patient (see Fig. 1*B*). Melan-A–specific T cells consisted of Ag-primed CTLs, showing either $CD45RA^+/CCR7^-$ terminally differentiated effector cells or $CD45RA^-/CCR7^-$ effector–memory cells (24). This finding is supported by the high expression of CD57 on the specific CTLs, recently defined as a marker for Ag-primed T cells (ref. 25; data not shown). The vast majority of Melan-A–specific CTLs were already in the intermediate ($CD27^+/CD28^-$) or late maturation stage ($CD27^-/CD28^-$); only 7% of the Melan-A–specific T cells simultaneously expressed CD27 and CD28, which is typical for the early stage of differentiation (refs. 26 and 27; see Fig. 1*B*). Melan-A–specific T cells expressed activation markers such as HLA-DR, CD69, CD95, but were negative for CD25 (see Fig. 1*B* for expression of HLA-DR and CD69). Annexin-V staining of Melan-A–specific CTLs revealed no difference in the frequency of apoptotic T cells in the Melan-A–specific T-cell population (0.03%) compared with Melan-A^- $CD8^+$ T cells (0.16%). We also performed an *ex vivo* analysis of the intracellular content of perforin and granzyme B, two major effector molecules in cytolytic activity (28). A high proportion of the Melan-A–specific T cells in this patient expressed both effector molecules (see Fig. 1*B*), thus suggesting a high cytotoxic potential of these cells. Furthermore, NKG2D, another molecule associated with cytolytic capacity (29), was expressed by all Melan-A–specific T cells (data not shown).

To assess the functional properties of the highly expanded Melan-A–specific T cells in this patient, cytokine production and cytotoxic activity of the specific T-cell population were determined. On short-term antigenic challenge with the Melan-A peptide, a high proportion of Melan-A–specific T cells produced IFN-γ (see Fig. 2*A*), which was comparable with that observed on nonspecific PMA/ionomycin stimulation.

Another important feature of $CD8^+$ effector T cells is the ability to lyse target cells expressing the cognate peptide. As shown in Fig. 2*B* the freshly isolated Melan-A–specific T cells revealed a high cytolytic activity against Melan-A–pulsed T_2 cells and HLA-$A2^+$ Melan-A–expressing allogeneic melanoma cells (see Fig. 2*B*).

To assess whether the Melan-A–specific cell population consists of clonally amplified T cells, analysis of CDR3 distribution of the TCRVα and Vβ families was performed in sorted Melan-A multimer–positive and –negative cells. As shown in Fig. 2*C*, Melan-A–specific T cells revealed an oligoclonal pattern with predominance of a particular CDR3 length for TCR Vβ3, Vβ7, and Vβ16, whereas other Vβ subfamilies such as Vβ1 were negative or showed a polyclonal pattern. CDR3 distribution of the TCR Vα families revealed a similar pattern (data not shown).

***In vitro* Generation and Adoptive Transfer of Melan-A–Specific T Cells in Patient MeL162.** Patient MeL162 was included in a phase I clinical protocol designed to evaluate the feasibility and toxicity of adoptively transferred Melan-A–specific CTLs (7). He received four i.v. infusions of $0.25–11 \times 10^8$ Melan-A–specific CTLs. The mean frequency of Melan-A multimer$^+$ T cells of total $CD8^+$ cells in the patient's T-cell product was of 51.7%, comparable with the frequencies observed in the other 10 melanoma patients (mean of 36.1%).

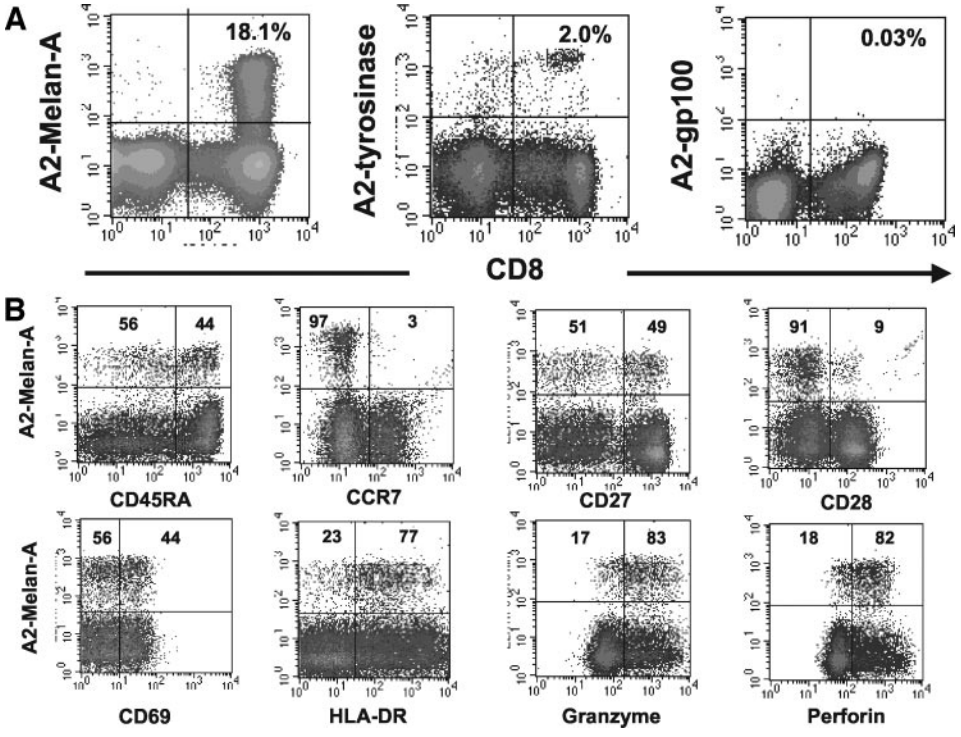

Fig. 1. Identification and phenotypic analyses of TAA-specific $CD8^+$ T cells by multimer staining from PBLs of melanoma patient MeL162. *A.* PBLs were stained with PE-conjugated Melan-A, tyrosinase, gp100 multimers, and allophycocyanin-conjugated anti-CD8 mAb. Dot plots are shown on gated $CD3^+$, $CD14^-$ small lymphocytes (by forward and side scatter). *Numbers* in the *top right quadrants* represent percentages of multimer$^+$/$CD8^+$ cells within total $CD8^+$ PBLs. *B.* Cells were stained with the Melan-A multimer and combinations of mAbs against surface antigens (*CD45RA*, *CCR7*, *CD27*, *CD28*, *CD69*, and *HLA-DR*) and intracellular proteins (*granzyme B* and *perforin*). *Numbers* in the *top quadrants* are the percentages of Melan-A multimer$^+$ cells with the corresponding phenotype.

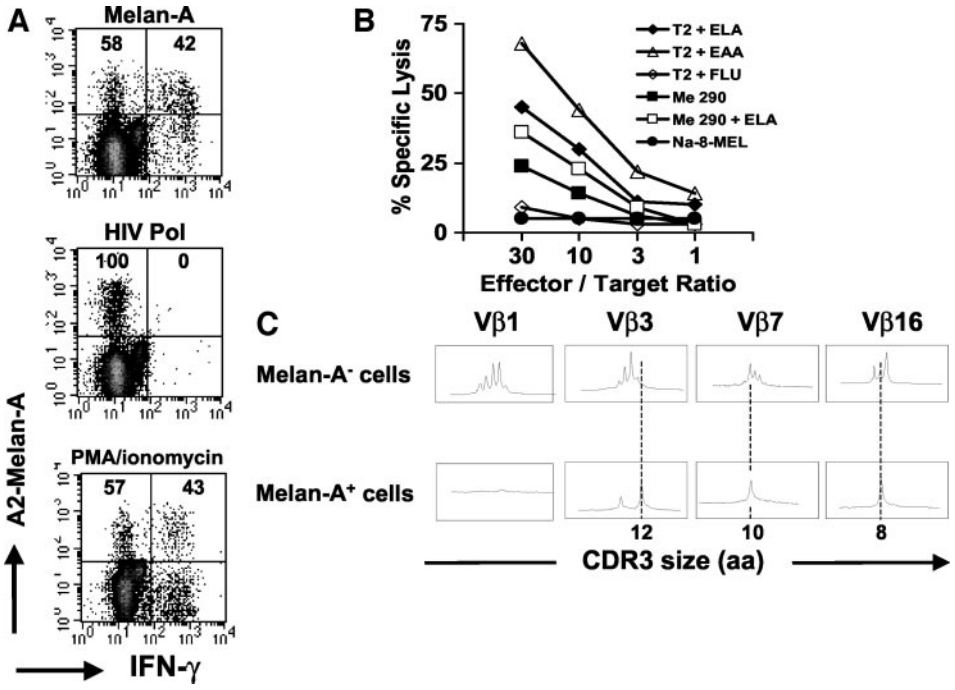

Fig. 2. Competent effector function and diverse oligoclonal TCR repertoire of circulating Melan-A–specific T cells. *A*, IFN-γ production of Melan-A–specific T cells in gated $CD8^+$ T cells obtained from patient MeL162 PBMC. $CD8^+$ T cells were stimulated either with Melan-A peptide (*top*), irrelevant HIV peptide (*middle*), or PMA/ionomycin (*bottom*). *Numbers* in the *top quadrants* are the percentages of Melan-A multimer$^+$ cells with the corresponding phenotype. *B*, cytolytic activity of Melan-A multimer$^+$ T cells, sorted from patient MeL162 PBMC, against T_2 target cells in the presence of the natural [EAAGIGILTV (*EAA*)] or modified Melan-A [ELAGIGILTV (*ELA*)] decapeptide or the control influenza matrix [GILGFVFTL (*FLU*)] peptide, or against melanoma cell lines Me290 ($A2^+$/*Melan-A*$^+$) ± ELA peptide or Na8 ($A2^+$/*Melan-A*$^-$). *C*, A2/Melan-A multimer-positive and -negative PBMCs were isolated by multimer-guided cell sorting. Total RNA was extracted, reverse transcribed, and amplified by reverse transcription-PCR using Vβ primers. Amplified cDNA was copied with a fluorescent Cβ primer in a run-off reaction and subjected to electrophoresis on an automated sequencer. The patterns obtained show the size in amino acids of the CDR3 region (*X axis*) and the relative fluorescence intensity (*Y axis*) of in-frame Vβ-Cβ amplification products.

Analysis of IFN-γ secretion of *in vitro*-generated Melan-A–specific CTLs revealed a frequency of 37.8% IFN-γ–secreting cells of total Melan-A$^+$ T cells on Melan-A restimulation compared with 2.6% responding to the control peptide (gp100).

To analyze whether infused Melan-A–specific CTLs localize at tumor sites, indium-111 (^{111}In) oxine labeling of Melan-A–specific CTLs was performed (7). ^{111}In-labeled Melan-A–specific CTLs from patient MeL162 demonstrated localization of transferred CTLs to the parailiac lymph node mass as early as 48 hours post infusion (data not shown).

Native Phenotype and Functional Activity of Tumor-Infiltrating Melan-A–Specific T Cells. To assess whether functionally active Melan-A–specific CTLs are also enriched at the tumor site, one part of the parailiac lymph node metastasis was mechanically dispersed into single-cell suspension; the other part used for immunohistochemical analysis. Staining of the parailiac lymph node metastasis with lymphocyte markers revealed that the tumor was infiltrated by numerous $CD8^+$ T cells, whereas only very few $CD4^+$ T cells could be detected (data not shown).

Analysis of primary TILs revealed a nearly identical frequency of TAA-specific T cells at the tumor site compared with patient's PBLs, *i.e.*, 12.9% Melan-A, 2.1% tyrosinase, and 0.03% gp100 multimer$^+$ T cells within total $CD8^+$ cells, respectively (see Fig. 3*A*). The Melan-A–specific T-cell population consisted almost exclusively of $CD45RA^-/CCR7^-$ memory effector T cells with a uniform expression of CD27. Furthermore, specific T cells were highly activated as

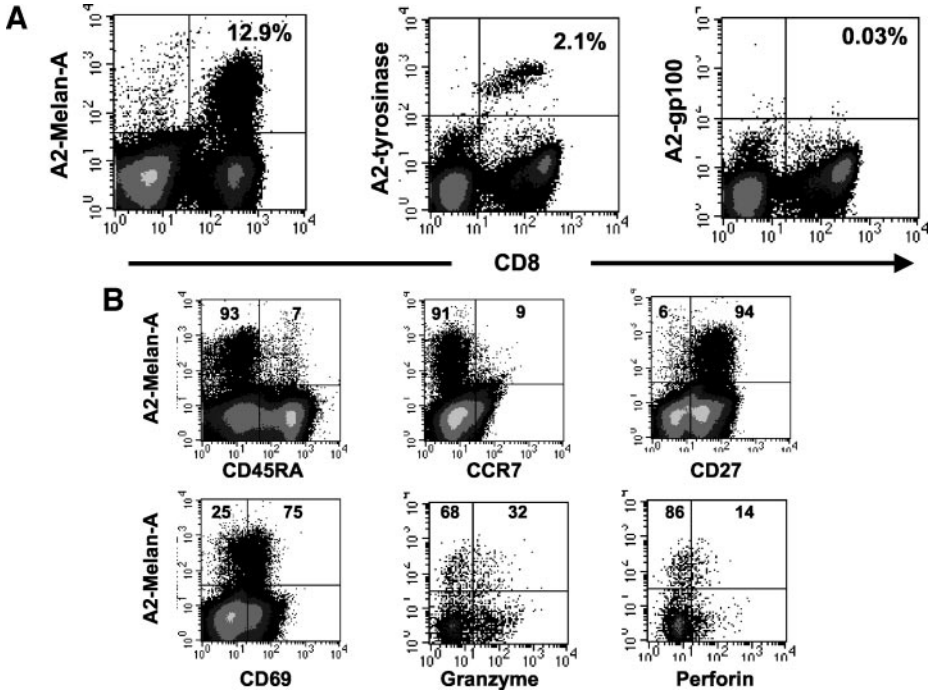

Fig. 3. Identification and phenotypic analyses of TAA-specific $CD8^+$ T cells by multimer staining isolated from lymphocytes infiltrating metastatic lymph node lesion of patient MeL162. *A*. TILs were stained with multimers and analyzed as described in Fig. 1*A*. *B*. Cells were stained with the Melan-A multimer and combinations of mAbs against surface Ag (*CD45RA*, *CCR7*, *CD27*, and *CD69*) and intracellular proteins (*granzyme B* and *perforin*). For additional details, see legend of Fig. 1*B*.

demonstrated by the expression of CD69 (Fig. 3*B*) and CD95 (data not shown). Part of the Melan-A–specific T cells were also positive for perforin and granzyme B, however, the intracellular content of perforin was clearly reduced in the specific TIL population compared with Melan-A^+ T cells in peripheral blood (see Figs. 1*B* and 3*B*).

Functional analysis of freshly isolated TILs revealed a high cytolytic activity against Melan-A–pulsed T_2 cells and HLA-$A2^+$ Melan-A–expressing allogeneic melanoma cells (Fig. 4*A*), indicating a well-preserved functional activity of tumor-infiltrating Melan-A–specific T cells.

Melanoma Cells from Patient MeL162 Are Resistant to Lysis by Melan-A–Specific T Cells. We were next interested whether either the highly expanded population of Melan-A–specific T cells freshly isolated from the lymph node metastasis (Fig. 4*A*) and peripheral blood (Fig. 4*B*) or *in vitro*-generated (four rounds of stimulation with Melan-A–pulsed dendritic cells) autologous (Fig. 4*C*) or allogeneic (Fig. 4*D*) HLA-$A2^+$ Melan-A–specific CTL lines were capable of killing the patient's melanoma cells. Despite being functionally active, none of the Melan-A–specific effector cells were able to efficiently lyse the melanoma cells from patient MeL162. In contrast, Melan-A–pulsed T_2 cells and allogeneic HLA-$A2^+$ Melan-A–expressing melanoma cells were lysed by all different effector cells. *In vitro* stimulation of patient's Melan-A–specific CTLs did not lead to substantial enhancement of lysis compared with freshly isolated T cells (Fig. 4*B* and *C*). Of interest, the resistance to lysis could be partially reversed by exogenous loading of the tumor cells with the Melan-A peptide (see Fig. 4*A–D*). These observations prompted us to investigate how the patient's melanoma cells may escape from functional Ag-specific immune recognition.

Preserved Expression of Human Lymphocyte Antigen Class I and Melan-A in the Patient's Tumor Cells. Phenotypic analysis of melanoma cells was performed on patient MeL162 tumor cells and two allogeneic HLA-$A2^+$ melanoma cell lines that were lysed by HLA-$A2^+$ Melan-A–specific CTLs. The patient's tumor cells and the allogeneic melanoma cells were positive for CD54, CD95, and bcl-2, whereas they were negative or only showed low levels of expression of CD80 and CD86 (data not shown). HLA class I and particularly HLA-A2 were highly expressed in the patient's tumor cells (data not shown). Furthermore, the coding region of the HLA-A2 binding Melan-A_{26-35} peptide of the patient's melanoma cells and of Me 275 cells demonstrated neither mutations nor polymorphisms when compared with the published sequence (GenBank accession no. NM_005511; data not shown). Moreover, immunohistochemical staining and reverse transcription-PCR analysis of the tumor cells of patient MeL162 revealed a strong expression of Melan-A (see Fig. 5*A* for staining with a cytoplasmic Melan-A mAb) as well as gp100, tyrosinase, and NY-ESO-1 (data not shown).

Down-regulation of LMP2 and LMP7 Expression in the Patient's Melanoma Cells Correlates with Escape from Immune Recognition by Melan-A–Specific Cytotoxic T Lymphocyte. Because the tumor cells did not exhibit alterations in the HLA class I surface expression, the expression of several components of the Ag-processing and presentation pathway was investigated. As shown in Fig. 6*A*, reverse transcription-PCR analysis revealed a substantial down-regulation of LMP2 and LMP7 expression in the patient's purified melanoma cells compared with autologous nonmalignant cells and three allogeneic tumor cell lines. In contrast, the expression of molecules involved in the translocation of the peptides from the cytosol to the endoplasmic reticulum such as TAP1 and TAP2 and the chaperone tapasin was not altered compared with control cells (Fig. 6*A*). The reverse transcription-PCR data could be partially confirmed by immunohistochemical staining of the patient's parailiac lymph node metastasis using intracytoplasmic mAbs against components of the HLA class I Ag-processing machinery. As demonstrated in Fig. 5*E*, a complete lack of LMP7 staining could be observed in MeL162. LMP2 was expressed on MeL162 cells (Fig. 5*C*), but revealed a predominant nuclear staining and only moderate cytoplasmic expression. Two lymph node metastases from other melanoma patients were used as a control and revealed a strong cytoplasmic staining with LMP2 and LMP7 (see Fig. 5*D* and *F* for patient MeR214).

To determine whether the resistance to lysis in MeL162 melanoma cells by Melan-A–specific T cells can be reversed by pre-exposure to

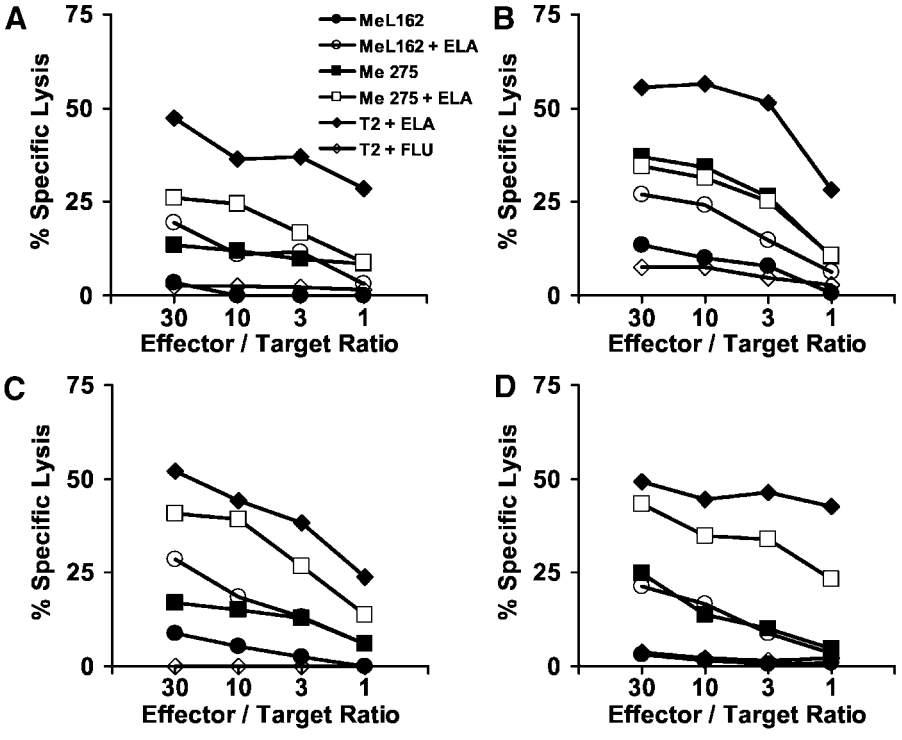

Fig. 4. Melanoma cells from patient MeL162 are resistant to lysis by autologous and allogeneic Melan-A–specific CTLs. Four different T-cell populations were used as effectors against melanoma cells from patient MeL162: autologous Melan-A–specific T cells freshly isolated from the lymph node metastasis (*A*) or the peripheral blood (*B*), and *in vitro*-generated autologous (*C*) or allogeneic (*D*) HLA-A2–matched Melan-A–specific CTL lines. ^{51}Cr-labeled target cells were either T_2 target cells in the presence of the modified Melan-A [ELAGIGILTV (*ELA*)] decapeptide or the control influenza matrix [GILGFVFTL (*FLU*)] peptide and autologous (*MeL162*) or allogeneic (*Me 275*; $A2^+$/Melan-A^+) melanoma cells ± ELA peptide, respectively.

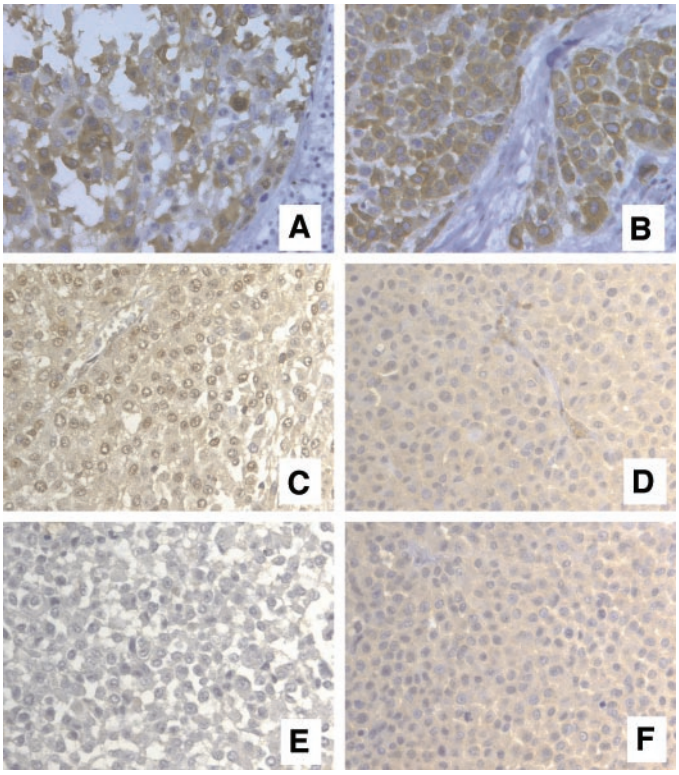

Fig. 5. Immunohistochemical analysis of Melan-A, LMP2, and LMP7 expression in lymph node metastases from melanoma patients. Staining of formalin-embedded tumor specimens from patient MeL162 (*A*, *C*, and *E*) and patient MeR214 (*B*, *D*, and *F*) was performed with anti-Melan-A (*A* and *B*, ×400), anti-LMP2 (*C* and *D*, ×400), and anti-LMP7 (*E* and *F*, ×400) mAbs. Bound antibody was detected using the diaminobenzidine method. Representative fields of the tissue sections examined are shown.

IFN-γ, which is known to induce the immunoproteasomes, patient's tumor cells were treated or not with IFN-γ (100 units/mL) for 24 hours and used as targets against an autologous Melan-A–specific CTL. As shown in Fig. 6*B* only melanoma cells exposed to IFN-γ were efficiently lysed by Melan-A–specific T cells.

In summary, the immunoproteasome deficiency of the patient's melanoma cells might lead to an altered quantitative and qualitative Ag presentation and subsequently to decreased lysis by Ag-specific T cells.

DISCUSSION

TAA-specific T cells have been shown to play an important role in cancer immunosurveillance. Here, we report an unexpected high frequency of circulating Melan-A–specific CTLs of approximately one-fifth of the total $CD8^+$ T-cell pool which is, to our knowledge, the highest reported frequency of circulating $CD8^+$ T cells recognizing a single TAA. In general, the frequency of TAA-specific T cells being triggered by the tumor itself or by different vaccination strategies was reported between 0.0001 and 3% (30). One possible explanation for the frequent failure of spontaneously induced as well as therapeutically induced TAA-specific T cells to control the disease may be their insufficient number. In viral infections such as EBV, which are eliminated or at least controlled by the immune system, the frequency of T cells specific for a single EBV epitope is very high, comprising nearly one-half of the $CD8^+$ T-cell population in some cases (31). In line with this hypothesis, Dudley *et al.* (5) demonstrated impressive clinical responses in melanoma patients by autologous T-cell therapy after nonmyeloablative chemotherapy. TAA-specific T cells made up to 90% of the $CD8^+$ T-cell pool, suggesting that the number of T cells might play an important role for a successful immune response. However, in our case, the patient had rapidly progressing lymph node metastases despite the presence of this high number of TAA-specific T cells.

The phenomenon of paradoxical coexistence between tumor and tumor-specific T cells has been of interest for many years (12). Several mechanisms of tumor-induced immune defects have been proposed, including alterations in signal transduction (32), functional unresponsiveness (15), and immunosuppressive environment (33), leading to defective maturation and functional tolerance of tumor-specific T cells at the tumor site (34). Alternatively, active immune regulatory mechanisms such as $CD4^+CD25^+$ T cells might impede any endogenous immune response to cancer cells (35).

According to the widely accepted models to determine the maturation phenotype (24, 36), circulating Melan-A–specific T cells in our patient consisted of Ag-experienced T cells that were "ready to kill." In contrast to a previous report describing anergic tyrosinase-specific CTLs (15), Melan-A–specific CTLs freshly isolated from our patient revealed an unimpaired functional activity. Furthermore, apoptosis of activated T cells, suggested as one mechanism of tolerance (37), could also be excluded.

Functional TAA-specific T cells circulating in the periphery do not automatically indicate competent TILs (34, 38). Analysis of the T cells infiltrating the lymph node revealed a similar pattern of a highly expanded population of Melan-A–specific $CD8^+$ showing a memory/effector phenotype, but decreased perforin and granzyme content, suggesting impairment of the T cells induced by the tumor. Despite the substantially decreased content of perforin in Melan-A–specific TILs, which has been already reported in larger series of patients with melanoma (34, 38), these T cells revealed a strong cytolytic activity against Melan-A–expressing target cells without any prior stimula-

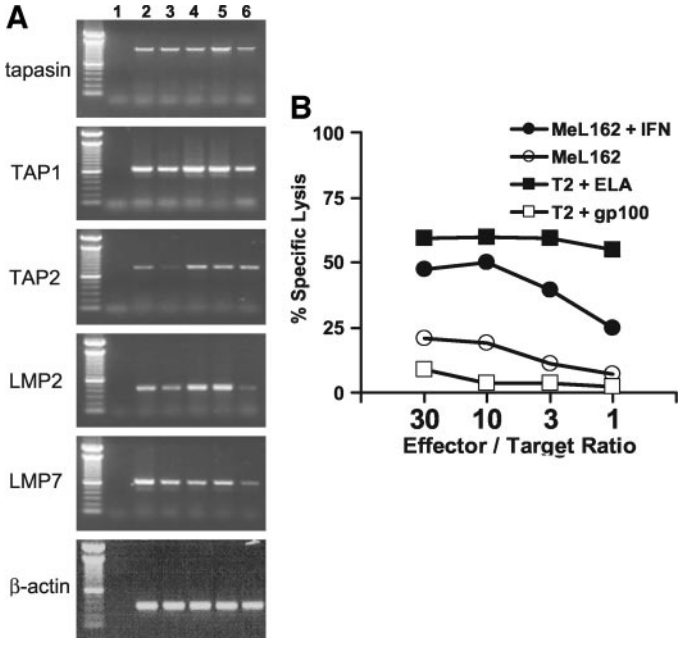

Fig. 6. LMP2 and LMP7 deficiency in primary melanoma cells resistant to killing against autologous Melan-A–specific CTLs can be partially reversed by pre-exposure of tumor cells to IFN-γ. *A*. RNA from different tumor cells was extracted, reverse transcribed into cDNA, and analyzed for the expression of tapasin, TAP1, TAP2, LMP2, LMP7, and β-actin by reverse transcription-PCR. *Lane 1*, H_2O; *Lane 2*, renal cell carcinoma line; *Lanes 3* and *4*, allogeneic melanoma lines; and *Lanes 5* and *6*, nonpurified and purified melanoma cells from patient MeL162. *B*. Autologous tumor cells (MeL162) were treated or not with IFN-γ (100 units/mL) for 24 h, labeled with ^{51}Cr, and used as targets. T_2 target cells pulsed either with the modified Melan-A (*ELA*) or with the $gp100_{280-288}$ peptide were used as positive and negative controls, respectively. *In vitro*-generated Melan-A–specific CTLs from patient MeL162 were used as effector cells.

tion. Several explanations for this paradox are possible: (*a*) a low proportion of perforin-containing TILs being capable to exert the granule exocytosis pathway is sufficient for lysis, as suggested in animal models (39); and (*b*) the CTLs mediate cell death by cross-linking of death receptors such as Fas on the target cells (40).

An essential question is how these functionally active Melan-A–specific T cells could expand *in vivo* up to one in five circulating $CD8^+$ T cells? One possible explanation for the existence of an expanded population of Melan-A–specific T cells is the transfer of MHC class I–restricted Ag from tumor cells to allophycocyanin *in vivo*, thereby eliciting TAA-specific T cells through cross-priming (41). In line with this hypothesis, we found a superior stimulatory capacity of the dendritic cells from our particular patient when compared with those of other melanoma patients (data not shown).

Although the patient's tumor revealed a brisk infiltrate of $CD8^+$ T cells, no clear signs of tumor cell apoptosis or necrosis could be observed. This observation was confirmed *in vitro*, demonstrating that the patient's tumor cells were largely resistant to killing by autologous and allogeneic effector cells. Reversal of the resistance of the patient's tumor cells to killing after exogenous loading with the Melan-A peptide, however, clearly argues against a defect in the apoptotic machinery of the tumor cells. It rather strongly suggests a defect either in the HLA class I Ag-processing machinery or in the presentation of the epitope. Loss or decreased expression of TAA, which is associated with disease progression in melanoma (42) and has been observed spontaneously in the presence of specific T cells (12), or point mutation within the HLA class I–presented T-cell epitope (43) could be excluded.

Furthermore, we could not detect any loss, down-regulation, or functional impairment of the HLA class I molecule or defects in the HLA class I Ag-processing machinery such as TAP or tapasin. Whereas the TAP molecule is required for peptide loading of the HLA class I molecules, the proteasome subunits LMP2 and LMP7 are important for the production of Ag capable of binding to the class I molecules, which plays an important role in the generation of peptides to be presented to T cells (44). Interestingly, we observed a down-regulation of the constitutive expression of LMP2 and LMP7 in our patient's tumor cells compared with other melanoma cells used as controls. In contrast to our data, Morel *et al.* (45) have demonstrated that the Melan-A peptide is not processed efficiently by the immunoproteasome. In our case, we have a situation *in vivo* where the LMP2 and LMP7 subunits should have been induced by the IFN-γ released locally by specifically activated TAA-reactive T cells, yet we not only fail to see such an increase, but the expression levels of the IFN-γ–inducible subunits are relatively reduced. Perhaps, subtle changes in immunoproteasome composition may lead to different outcomes in efficiency of Ag presentation in tumor cells.

Of interest, Zippelius *et al.* (34) recently demonstrated that Melan-A–specific cells failed to produce IFN-γ in metastatic lymph nodes and nonlymphoid tissue metastases. These findings also allow reconciliation of the observation of rather robust effector functions in the periphery with a state of local antigen-specific tolerance in metastatic lymph nodes.

Altogether, these data suggest a decreased presentation of the Melan-A peptide on HLA-A2 molecules due to LMP deficiency as a potential mechanism for insufficient lysis of patient's tumor cells. Moreover, a tumor-induced state of local functional tolerance of specific CD8 T cells may aggravate their failure to effectively counter tumor growth.

These findings have profound implications for cancer immunotherapy and may provide one explanation of why malignant tumors do not respond to specific immunotherapeutic regimens despite the induction of a strong TAA-specific T-cell response.

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