

# Sequential Immune Escape and Shifting of T Cell Responses in a Long-Term Survivor of Melanoma<sup>1</sup>

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Immune-mediated control of tumors may occur, in part, through lysis of malignant cells by CD8<sup>+</sup> T cells that recognize specific Ag-HLA class I complexes. However, tumor cell populations may escape T cell responses by immune editing, by preventing formation of those Ag-HLA complexes. It remains unclear whether the human immune system can respond to immune editing and recognize newly arising escape variants. We report an example of shifting immune responses to escape variants in a patient with sequential metastases of melanoma and long-term survival after surgery alone. Tumor cells in the first metastasis escaped immune recognition via selective loss of an HLA haplotype (HLA-A11, -B44, and -Cw17), but maintained expression of HLA-A2. In the second metastasis, immune escape from an immunodominant MART-1-specific T cell response was mediated by HLA class I down-regulation, resulting in a failure to present this epitope, but persistent presentation of a tyrosinase-derived epitope. Consequent to this modification in tumor Ag presentation, the dominant CTL response shifted principally toward a tyrosinase-targeted response, even though tyrosinase-specific CTL had been undetectable during the initial metastatic event. Thus, in response to immune editing of tumor cells, a patient's spontaneous T cell response adapted, gaining the ability to recognize and to lyse "edited" tumor targets. The observation of both immune editing and immune adaptation in a patient with long-term survival after surgery alone demonstrates an example of immune system reactivity to counteract the escape mechanism(s) developed by tumor cells, which may contribute to the clinical outcome of malignant disease. *The Journal of Immunology*, 2005, 174: 6863–6871.

**T** lymphocytes are believed to be the principal effectors in an active immune surveillance network that protects immunocompetent individuals from expansion of neoplastic lesions (1). Accordingly, patients with advancing melanoma spontaneously develop cytotoxic T cell responses to multiple melanoma-associated Ags (MAA)<sup>3</sup> (2, 3), and these MAA-specific immune responses can be augmented by tumor vaccines (4, 5). However, these responses impart selective pressure on tumor cells, often leading to immune editing and selection of variant tumor

cells that escape immune recognition by any of several mechanisms, including tumor Ag down-regulation (6), MHC down-regulation or loss (7, 8), defective Ag processing (9–11), and secretion of immunosuppressive cytokines (12). The finding of immune editing in cancer patients prompts concern that immunotherapy may ultimately fail as tumors undergo repeated rounds of selection and immune evasion. Extended survival after such immune editing would require the human immune system to respond spontaneously and to refocus responses against newly arising tumor variants in an adaptive manner. However, this phenomenon has not been well studied.

As mechanisms of immune escape are more clearly delineated, it is equally important to understand mechanisms by which the human immune system may overcome tumor immune editing. In the present report, we document a case of immune adaptation in a long-term survivor of metastatic melanoma. After the tumor evaded immune recognition through a combination of defects, including the ablation of presentation of an immunodominant MART-1 peptide, the host's immune repertoire expanded to include recognition of a previously cryptic peptide from tyrosinase presented by the tumor cells in vivo. Significantly, the tyrosinase-derived epitope subsequently became the immunodominant Ag. This patient's long-term survival, without specific immunotherapy, suggests that adaptive evolution of the immune response may be a protective mechanism that can overcome immune escape by tumor cells. These findings underscore the potential for active immunization as a tool to increase the magnitude of nonimmunodominant T cell responses and to broaden the tumor Ag-specific repertoire.

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<sup>3</sup> Abbreviations used in this paper: MAA, melanoma-associated Ag; LMP, low-molecular-weight protein; LN, lymph node; TIN, tumor-infiltrated node;  $\beta_2$ m,  $\beta_2$ -microglobulin; MDP, melanocyte differentiation protein; APM, Ag-processing machinery.

## Materials and Methods

### Patient

The patient, designated VMM5, was diagnosed with primary melanoma of the upper back without evidence of metastatic disease and subsequently treated at the University of Virginia (Table I). The primary tumor was removed surgically with a wide margin. Approximately 5 years later, palpable regional metastases were detected in the right cervical and supraclavicular lymph nodes (LN). Neck dissection was performed, with removal of 24 LN, 12 of which contained tumor. The tumor bulk was substantial, but there was no other evidence of disease and no additional therapy. Approximately 6 years later, a 6-cm-diameter mass was discovered in the supraclavicular fossa, at the periphery of the prior neck dissection surgical site; this tumor was removed surgically and was found to be a LN essentially replaced by a tumor, and no other evidence of disease was found. The patient remained clinically free of tumor recurrence for an additional 6 years (>16 years after initial diagnosis) and died from unrelated disease, without evidence of melanoma. In all cases, PBL and tumor-infiltrated nodes (TIN) were obtained for research purposes by written informed consent, under University of Virginia Institutional Review Board protocol HIC-5202.

### Tumors and cell lines

Metastatic melanoma deposits were surgically removed from patient VMM5 at two time points. Cells of TIN resected from the first metastatic event (TIN-A) were cryopreserved and used to generate the tumor cell line VMM-5A; likewise, cells of TIN from the second metastatic event (TIN-B) were cryopreserved and used to generate the tumor cell line VMM-5B. VMM5B cells lack functional  $\beta_2$ -microglobulin ( $\beta_2m$ ) and, thus, do not express stable surface HLA class I Ags (C.-C. Chang, unpublished results). Cell surface HLA class I expression was restored by stably transfecting VMM5B cells with a wild-type  $\beta_2m$  cDNA (VMM5B- $\beta_2m$ ). A control cell line stably transfected with empty vector is designated VMM5B-neo. Surgically resected melanoma deposits were also obtained from three additional HLA-A2<sup>+</sup> patients: VMM87, VMM119, and VMM162. Other melanoma cell lines include DM6 and DM331, which are both HLA-A2<sup>+</sup> (a gift of Drs. H. Seigler and T. Darrow, Duke University, Durham, NC). DM6 cells express multiple melanocytic differentiation Ags (including gp100, MART-1, and tyrosinase), whereas DM331 cells do not express these Ags (6). T2 is a human T/B cell hybrid that lacks TAP but expresses HLA-A\*0201 Ags (13) (a gift of Dr. P. Cresswell, Yale University, New Haven, CT). HLA class I phenotype for all tissue isolates was determined either by PCR using allele-specific primers or by microcytotoxicity assay using autologous lymphocytes (One Lambda).

### Lymphocytes and T cell lines

Lymphocytes were obtained from peripheral blood and from LN specimens TIN-A and TIN-B of patient VMM5. To generate short-term cell lines, lymphocytes were cultured in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum (Sigma-Aldrich), 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (Pen-Strept; Invitrogen Life Technologies)).

A long-term CTL line was generated from TIN-A by culture of its lymphocytes and tumor cells in complete medium supplemented with 20 U/ml IL-2 (Chiron Corporation) in 24-well tissue culture plates, and restimulation in vitro first with irradiated autologous VMM5A melanoma cells and subsequently with irradiated allogeneic HLA-A\*0201<sup>+</sup> melanoma cells DM6, as described (14). This T cell line is referred to as CTL-A. Short-

term cultures of VMM5 TIN-B lymphocytes (CTL-B) were also performed in complete medium containing IL-2. A Tyr<sub>369-377</sub>-specific CTL line was derived, as described (5), from patient VMM119, who had been vaccinated with a mixture of four peptides including Tyr<sub>369-377D</sub> (YMDGTMSQV).

### Peptides

The HLA class I Ag-associated peptides KTWGQYWQV (gp100<sub>154-162</sub>) (15), YLEPGPVTA (gp100<sub>280-288</sub>) (16), AAGIGILTV (MART-1<sub>27-35</sub>) (17, 18), YMDGTMSQV (Tyr<sub>369-377D</sub>) (19), GILGFVFTL (influenza matrix protein M1 peptide), and YLKKIKNSL (malaria CSP<sub>334-342</sub>) (20) were synthesized with a free amide N terminus and free acid C terminus by standard Fmoc chemistry using a model AMS422 peptide synthesizer (Gilson), and then purified to >98% purity by reverse-phase HPLC on a C-8 column (Vydac) at the University of Virginia Biomolecular Core Facility. Purity and identity were confirmed using a triple quadrupole mass spectrometer (Finnigan).

### Monoclonal and polyclonal Abs

The anti-MART-1 mAb A103 and anti-tyrosinase mAb T311 were purchased from Novocastra and from Vector Laboratories, respectively. Additional murine mAbs, used for tumor cell characterization, are listed in Table II and were developed as described (21-29). The anti-idiotypic mAb MK2-23 (30) and mouse IgG2a (BD Biosciences) were used as isotype controls. R-PE-conjugated goat anti-mouse Fc $\gamma$  F(ab')<sub>2</sub> and goat anti-mouse IgG Abs were purchased from DakoCytomation and from Amersham Biosciences, respectively.

### Cytotoxicity assays

Cell-mediated lysis of target cells was determined using a standard 4-h <sup>51</sup>Cr release assay. Briefly, <sup>51</sup>Cr-labeled target cells were plated at 1-2 × 10<sup>3</sup> cells/well in triplicate on 96-well V-bottom plates (Costar) with the indicated ratio of effector cells in a final volume of 200  $\mu$ l. Wells containing either culture medium or 1 M HCl in place of the effector cells served as spontaneous and maximum <sup>51</sup>Cr release controls, respectively. The specific lysis of targets was calculated by the following formula: percentage of specific lysis = ((experimental lysis - spontaneous lysis)/(maximal lysis - spontaneous lysis)) × 100.

### ELISPOT assays

TIN-derived lymphocytes were cultured for 14 days with IL-2 (20 U/ml; Chiron) before evaluation by ELISPOT assay. For ELISPOT evaluation, lymphocytes were mixed with equal numbers of peptide-pulsed (40  $\mu$ g/ml) or unpulsed APC in Immulon 2 flat-bottom plates (Dynatech) coated with anti-IFN- $\gamma$  mAbs (M-700A; Endogen). Responder cell numbers ranged from 100,000 to 5,000 per well. Cells were incubated in plates for 18 h. After extensive washing with 0.025% Tween 20, plates were incubated with biotin-labeled secondary Ab to IFN- $\gamma$  (M-701B; Endogen), washed, and incubated with avidin conjugated with alkaline phosphatase (BD Pharmingen). After washing, plates were developed with the 5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma-Aldrich) in 1% low melting agarose. The numbers of blue spots, corresponding to the numbers of cells secreting IFN- $\gamma$ , were counted in each well. Each sample was tested in triplicate at each of several dilutions of lymphocytes. The frequency of T cells reactive to peptide Ag was calculated by the following formula: specific T cells = ((average number of spots produced by lymphocytes incubated with peptide-pulsed APC) - (number of spots produced by lymphocytes incubated with unpulsed APC))/number of cells loaded per well.

Table I. Clinical and immunological events in patient history

	Time Postdiagnosis (years)			
	0	4.7	10.6	16.3
Clinical course	2.1-mm melanoma of R posterior shoulder.	Detection and resection of 12 metastatic nodes (TIN-A).	Detection and resection of 1 large metastatic node (TIN-B).	No evidence of melanoma. Death from unrelated causes.
Immunodominant CTL epitope		MART-1 <sub>27-35</sub>	Tyrosinase (Tyr <sub>369-377D</sub> )	
CTL line generated		CTL-A	CTL-B	
Mechanisms of tumor escape		Loss of HLA-A11, B44, and CW17	Down-regulation of HLA class I surface expression, $\beta_2m$ point mutation.	
Tumor line generated		VMM5A	VMM5B	

Table II. *mAbs used in this study*

mAb	Specificity	References
W6/32	Anti- $\beta_2$ m-associated HLA-A, -B, -C, -E, and -G H chain	21, 22
LGIII-147.4.1	Anti- $\beta_2$ m-associated HLA-A H chain (excluding -A23, -A24, -A25, -A32)	23
B1.23.1	Anti- $\beta_2$ m-free and -associated HLA-B and -C H chain	24
HCA-2	Anti- $\beta_2$ m-free HLA-A (excluding -A24), -B7301, and -G H chain	25, 26
HC-10	Anti- $\beta_2$ m-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, -B (excluding -B5702, -B5804, and -B73) H chain	25–27
NOB-1	Anti-TAP1	S. Ferrone, unpublished results
NOB-2	Anti-TAP2	S. Ferrone, unpublished results
TO-3	Anti-tapasin	28
TO-5	Anti-calnexin	28
TO-2	Anti-ERp57	24
TO-11	Anti-calreticulin	28
SY-5	Anti-20S proteasome constitutive $\beta$ subunit $\delta$	S. Ferrone, unpublished results
SJJ-3	Anti-20S proteasome constitutive $\beta$ subunit MB1	S. Ferrone, unpublished results
NB-1	Anti-20S proteasome constitutive $\beta$ subunit Z	S. Ferrone, unpublished results
SY-1	Anti-20S proteasome inducible subunit LMP2	S. Ferrone, unpublished results
HB-2	Anti-20S proteasome inducible subunit LMP7	S. Ferrone, unpublished results
TO-7	Anti-20S proteasome inducible subunit LMP10	S. Ferrone, unpublished results
763.74	Anti-human high molecular weight-MAA (HMW-MAA)	29

### Flow cytometry

Cell surface staining was performed as follows. Following three washings with PBS containing 1% BSA (PBS/BSA), cells ( $5 \times 10^5$ ) were incubated for 30 min at 4°C with an excess of primary mAb in 100  $\mu$ l of PBS/BSA. Following three washings with PBS/BSA, cells were incubated with an optimal amount of R-PE-labeled goat F(ab')<sub>2</sub> Abs specific to mouse Ig (DakoCytomation) in 100  $\mu$ l of PBS/BSA in the dark for 30 min at 4°C. Cells were washed with PBS/BSA three times with 0.5% paraformaldehyde/PBS (Sigma-Aldrich), and analyzed with FACScan (BD Biosciences). Flow cytometry of intracellular Ag expression was performed as described (31). Briefly, cells were fixed with 2% paraformaldehyde (Sigma-Aldrich), heat-denatured with microwave, and permeabilized with 0.1% saponin before incubation with an optimal amount of mAb. The anti-idiotypic mAb MK2-23 was used as an isotype control. The binding of primary mAb was detected by incubation with an optimal amount of R-PE-goat anti-mouse IgG Ab (DakoCytomation) followed by analysis with FACScan (BD Biosciences).

### Western blot analysis

One million tumor cells were lysed in 100  $\mu$ l of lysis buffer composed of 10 mM Tris (pH 7.5), 5 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, and protease inhibitor mixture (Sigma-Aldrich). Proteins were separated on a 15% denaturing SDS-PAGE gel for detection of MART-1, and 10% gel for detection of tyrosinase. The separated proteins were transferred to nitrocellulose membrane (Roche) before blocking with 5% skim milk and adding primary Abs. Ags were detected by secondary Abs and evaluation with the ECL System (Amersham Biosciences).

### Immunohistochemistry

Tumor cells were pelleted and paraffin embedded. Sections of tumor cell blocks were prepared and stained with anti-MART-1 Ab A103 (DakoCytomation), anti-tyrosinase Ab T311 (Vector Laboratories), or anti-gp100 Ab HMB45 (BioGenex). Staining was processed using a Ventana Automated System (Clinical Laboratory, Pathology Department, University of Virginia).

### RT-PCR and DNA sequence methods

Total RNA was isolated from tumor cell lines and from TIN samples using an RNeasy Kit (Qiagen) according to the manufacturer's instructions. Full-length MART-1 cDNA was amplified with the Access RT-PCR System (Promega) using 5' and 3' primers with the sequences 5'-CCAAAG GAGAACATTAGATGTC and 5'-AGACAGAGGACTCTCATTAAAGG, respectively. The genetic sequence of each amplification product was determined with an Applied Biosystems 377 Prism DNA Sequencer using BigDye terminator chemistry and TaqDNA polymerase (University of Virginia Biomolecular Core Facility).

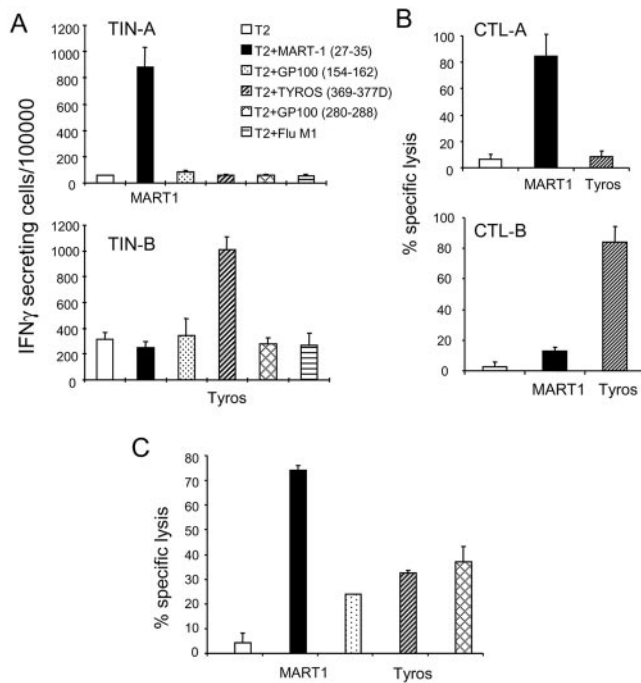
### Reconstitution of MART-1 and tyrosinase expression in VMM5B- $\beta_2$ m cell line by infection with vaccinia virus recombinants

Expression of MART-1 and tyrosinase proteins was reconstituted in VMM5B- $\beta_2$ m cells using recombinant vaccinia viruses expressing the respective full-length proteins. Cells infected in parallel with an identical vaccinia construct expressing influenza M1 protein were used as a negative control. Viruses were constructed as described (32), titrated, and tested for appropriate expression using HLA-A2-restricted CTL (data not shown). VMM5B- $\beta_2$ m cells were infected with 10 PFU/ml vaccinia virus for 30 min in HBSS supplemented with 0.1% BSA, 1.6 mM MgSO<sub>4</sub>, and 1.8 mM CaCl<sub>2</sub>, and then cultured for 6 h in RPMI 1640 medium supplemented with 10% FCS to allow transcription and expression of vaccinia-encoded proteins. Following vaccinia infection, targets were labeled in 100  $\mu$ Ci of Na<sup>51</sup>CrO<sub>4</sub> for 2 h, and then standard <sup>51</sup>Cr release assays were performed using MART-1 or tyrosinase-specific CTL to evaluate recognition of MART-1<sub>27–35</sub> or Tyr<sub>369–377D</sub> epitopes.

## Results

### The dominant immune response shifts from MART-1 to tyrosinase-derived epitopes in sequential metastatic recurrences

The patient VMM5 developed metastatic melanoma in LN ~5 years after initial resection of primary disease, and metastatic disease reoccurred in regional LN a second time 6 years later. Tumor-involved nodes were collected at each occurrence of disease (TIN-A and TIN-B, respectively). To determine the endogenous responses against melanocyte differentiation protein (MDP)-derived Ags, lymphocytes from TIN-A and TIN-B were cultured for 14 days in the presence of tumor cells from the corresponding TIN and IL-2, and analyzed for peptide reactivity in an ELISPOT assay. The dominant reactivity from TIN-A was to the MART-1<sub>27–35</sub> peptide, AAGIGILTV, whereas the dominant response from TIN-B was against the Tyr<sub>369–377D</sub> peptide YMDGTMSQV (Fig. 1A). For further analysis, long-term CTL lines were generated from TIN-A and TIN-B (CTL-A and CTL-B, respectively). CTL-A recognized at least six peptide epitopes associated with the HLA-A2 Ag, including MART-1<sub>27–35</sub> (14, 16, 33), but did not recognize the Tyr<sub>369–377D</sub> epitope (Fig. 1B). CTL generated from PBL collected at the time of TIN-A resection, and for several years thereafter, likewise were lytic against targets bearing MART-1<sub>27–35</sub> but not the Tyr<sub>369–377D</sub> epitope (data not shown). In contrast to CTL-A, the CTL-B lines, which were restimulated with the VMM5A melanoma cell line, demonstrated significant reactivity



**FIGURE 1.** CTL reactivity to tumor-associated Ags. **A**, TIN-A and TIN-B were cultured in medium containing IL-2 for 2 wk. These tumor-infiltrating lymphocytes were evaluated by IFN- $\gamma$  ELISPOT assay for reactivity to each of several HLA-A2-restricted melanoma peptides such as YLEPGPVTA (gp100<sub>280-288</sub>), KTWGQYWQV (gp100<sub>154-162</sub>), AAGIGILTV (MART-1<sub>27-35</sub>), YMDGTMSQV (Tyr<sub>369-377D</sub>), and GILGFVFTL (influenza matrix M1<sub>58-66</sub>). Data are means + SD for triplicate measurements within an assay. **B**, Peptide specificity of CTL-A line produced from TIN-A and CTL-B line produced from TIN-B. T2 cells were pulsed with AAGIGILTV, and YMDGTMSQV at 10  $\mu$ g/ml, and then washed and evaluated by standard  $^{51}$ Cr release assay for lysis by CTL-A and CTL-B lines. CTL-A were lytic for MART-1<sub>27-35</sub>-pulsed T2 target cells, CTL-B were lytic for Tyr<sub>369-377D</sub>-pulsed T2 targets. Data are shown for an E:T ratio of 10:1. The shading of the bars representing each peptide is consistent for all graphs in this figure. **C**, PBL collected 1 year before the second LN metastases were stimulated with VMM5 and DM6 tumor cells. CTL produced after several stimulations were lytic for T2 target cells pulsed with MART-1<sub>27-35</sub> as well for Tyr<sub>369-377D</sub>-pulsed T2 targets and gp100 peptide-pulsed targets.

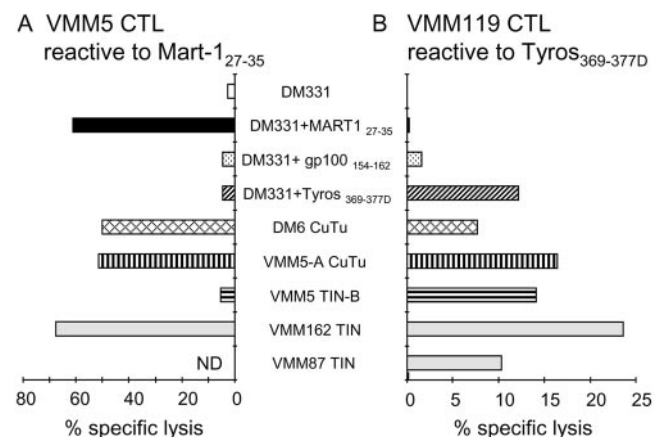
against the Tyr<sub>369-377D</sub> epitope but significantly decreased reactivity against MART-1<sub>27-35</sub> (Fig. 1B). PBL harvested 1 year before detection of the second LN metastasis, and stimulated in the same way, recognized the Tyr<sub>369-377D</sub> epitope (Fig. 1C). Thus, whereas Tyr<sub>369-377D</sub>-specific CTLs are virtually absent from the first metastatic outgrowth, Tyr<sub>369-377D</sub>-specific CTLs are abundant in the second metastatic outgrowth and became apparent in the peripheral blood in advance of the second tumor recurrence. MART-1<sub>27-35</sub>-specific CTLs, which were abundant in the initial metastatic lesion, are present in significantly smaller numbers in TIN-B and CTL-B. Collectively, these data demonstrate a significant change in immunodominance, suggesting that immune editing by the tumor may change the presentation of MDP-derived epitopes, leading the immune system to adapt dynamically by shifting the major antitumor CD8 response to a different Ag.

*The shift in immunodominant CTL response is associated with a loss of MART-1 epitope-MHC complexes on tumors from the second LN metastasis (TIN-B)*

The concurrent loss of MART-1 reactivity and development of tyrosinase reactivity within T cell populations infiltrating the

TIN-B metastasis suggested an immune-editing event that would immunologically distinguish this tumor from the original LN metastasis (TIN-A). To test this possibility, we performed cytotoxicity assays using CTL with defined specificity to determine whether HLA-A2-MART-1<sub>27-35</sub> peptide complexes and HLA-A2-Tyr<sub>369-377D</sub> peptide complexes are differentially expressed on tumor cells isolated from TIN-A and TIN-B. Long-term in vitro-restimulated CTL-A, which had lost reactivity against other MDP epitopes but maintained strong, restricted reactivity to the MART-1<sub>27-35</sub> epitope, effectively lysed cultured tumor cells from TIN-A (VMM5A), but not TIN-B tumor cells ex vivo (Fig. 2A). CTL-A also lysed specific-epitope-pulsed MART-1-negative targets, MART-1-expressing melanoma cell line DM6, and MART-1-expressing melanoma cells from TIN of patient VMM162. These CTL failed to lyse the MART-1-negative melanoma cell line DM331 or MART-1-negative tumor cells from TIN of patient VMM87. Lysis of nonautologous ex vivo and cultured tumor cells that express HLA-A2 and MART-1 Ags suggests that the failure of CTL-A to lyse TIN-B tumor ex vivo was independent of culture conditions of the target cells. Collectively, these data are consistent with the loss of MHC-associated MART-1<sub>27-35</sub> epitope on the surface of TIN-B tumor cells.

To determine whether the failure of TIN-A CTL to lyse TIN-B tumor was Ag specific or was due to a more global defect in Ag presentation by TIN-B-derived tumor cells, lytic activity by HLA-A2-restricted Tyr<sub>369-377D</sub>-reactive CTL (VMM119 CTL) was evaluated (Fig. 2B). In contrast to VMM5-derived CTL-A, the VMM119 CTL lysed VMM5A tumor cells and TIN-B tumor cells ex vivo as well as ex vivo tumor from TIN of VMM87 and VMM162, and the HLA-A2<sup>+</sup> Tyr<sub>369-377D</sub>-expressing melanoma cells DM6. These CTL failed to lyse the HLA-A2<sup>+</sup> tyrosinase-negative DM331 melanoma cells. Thus, both TIN-A-derived VMM5A cells and TIN-B melanoma cells express HLA-A2-Tyr<sub>369-377D</sub> complexes, suggesting that the failure of CTL-A to



**FIGURE 2.** TIN-A tumor cells, but not TIN-B tumor cells, are lysed by MART-1-specific CTL. T cell lines with restricted reactivity to MART-1<sub>27-35</sub> (VMM5-AG) (**A**) or to Tyr<sub>369-377D</sub> (VMM119 T cells) (**B**) were assayed for cytotoxicity against multiple fresh and cultured tumor specimens and peptide-pulsed targets, in a  $^{51}$ Cr release assay. The VMM5-AG CTL line (**A**) is a long-term-cultured T cell line from VMM5 TIN-A, with restricted reactivity to the MART-1<sub>27-35</sub>. The VMM119 CTL line (**B**) was generated from a LN draining a site of vaccination with the Tyr<sub>369-377D</sub> peptide, with resultant restricted reactivity to that tyrosinase peptide. DM331 is a tyrosinase-negative, MART-1-negative HLA-A2<sup>+</sup> melanoma cell line. The tumor ex vivo preparations VMM87 and VMM162 and the melanoma line DM6 are HLA-A2<sup>+</sup>, tyrosinase<sup>+</sup>, and MART-1<sup>+</sup>. VMM87 was not tested with the MART-1 CTL. Both CTL lines lysed DM331 minimally or not at all, but DM331 pulsed with MART-1 or tyrosinase peptides were lysed.

recognize TIN-B melanoma cells may be due to loss of MHC-associated MART-1<sub>27-35</sub>, and not simply loss of all HLA-A2 expression.

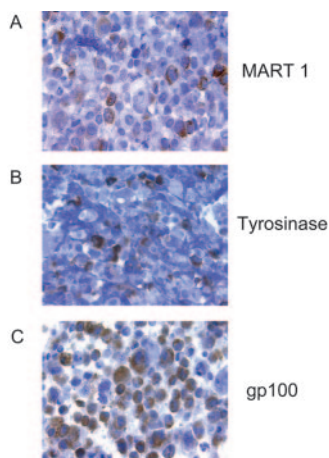
#### TIN-B melanoma cells express MDPs

The failure of MART-1<sub>27-35</sub>-specific CTL to recognize and to lyse TIN-B-derived tumor could arise from loss of MART-1 expression at the protein level. However, immunohistochemistry demonstrates TIN-B melanoma cell expression of MART-1 protein, as well as tyrosinase and gp100 proteins (Fig. 3). Western blot analysis confirmed that MART-1 and tyrosinase are expressed both in VMM5A and TIN-B cells (Fig. 4). An additional high molecular mass band was present in TIN-B sample stained for MART-1 expression, but the significance of this finding is not clear.

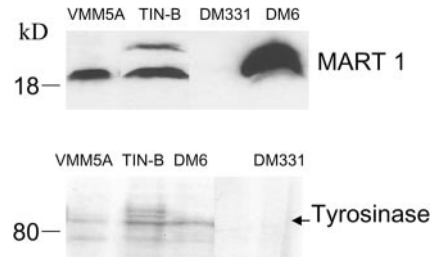
We next evaluated the *MART-1* gene in TIN-B-derived melanoma cells for mutations. Sequencing of RT-PCR-amplified products obtained from TIN-B ex vivo and from VMM5A cultured cells confirmed that the entire MART-1 coding sequence in TIN-B tumor matched the sequence obtained from VMM5A cells, as well as the published wild-type MART-1 sequence (GenBank accession no. NM 005511; data not shown). In summary, these data suggest the failure of MART-1<sub>27-35</sub>-specific CTL to lyse TIN-B melanoma is not resultant to abrogated or altered expression of MART-1 protein.

#### Expression profiles of the Ag-processing machinery (APM) components in VMM5A and VMM5B melanoma cell lines

We next hypothesized that the failure of MART-1<sub>27-35</sub>-specific CTL to lyse TIN-B melanoma may be the result of altered tumor cell presentation of the MART-1<sub>27-35</sub> epitope. Therefore, we evaluated the expression of APM components by TIN-A- and TIN-B-derived cell lines, VMM5A and VMM5B, respectively, to evaluate possible changes in the expression of these molecules. Intracellular flow cytometric analyses demonstrated that the major components of the APM are present in both cell lines, although the levels of low-molecular-weight protein (LMP)-7, LMP10, TAP1, tapasin, and HLA-A, -B, -C H chain expression was slightly lower in VMM5B cells than in VMM5A cells (Fig. 5). Additionally, there appears to be a subset of VMM5B cells with loss of TAP2. However, both lines express the necessary components to process Ag for presentation, and these data fail to explain the differential expression of MART-1<sub>27-35</sub> by VMM5B cells.



**FIGURE 3.** TIN-B tumors express melanoma differentiation proteins MART-1, tyrosinase, and gp100. Immunohistochemical analysis of tumor cells from TIN-B. The tumor cell suspension was made into a cell block and stained for MART-1 (A), tyrosinase (B), and gp100 (C) expression using A103, T311, and HMB45 Abs, respectively. The dark brown cytoplasmic staining represents expression of the respective tumor Ags.



**FIGURE 4.** Evaluation of MART-1 and tyrosinase expression in melanoma cell lines. VMM5A, DM6, DM331, and tumor cells from TIN-B ex vivo were tested for expression of MART-1 and tyrosinase by Western blot. MART-1 and tyrosinase expression was detected with A103 and T311 Abs, respectively. Goat anti-mouse IgG conjugated with peroxidase were used as secondary Ab. Lysate from equivalent number of tumor cells was loaded from each cell type.

#### TIN-B melanoma cells down-regulate surface HLA-A2 expression

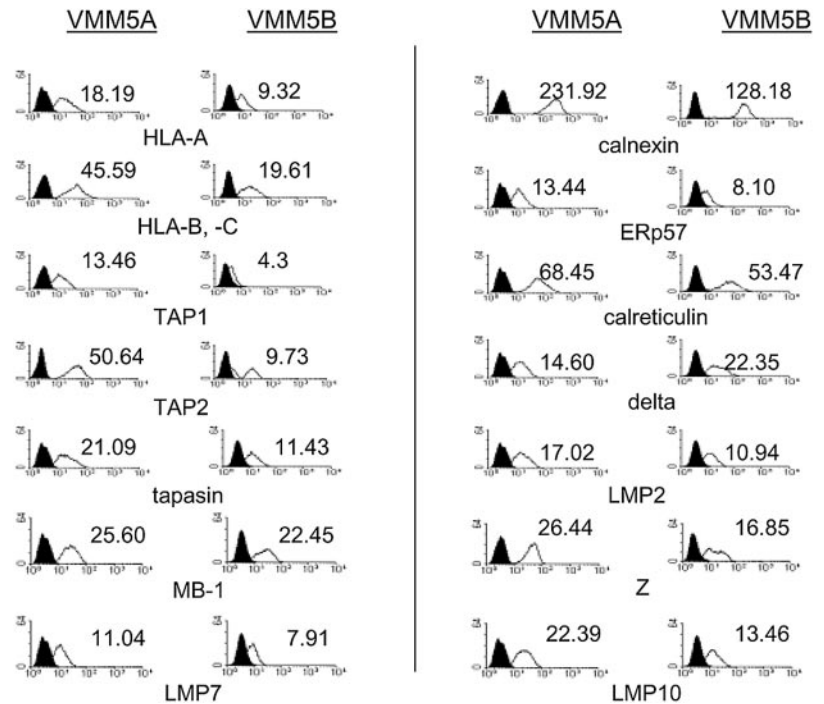
To evaluate whether loss of recognition of TIN-B melanoma cells by MART-1-specific T cells was associated with down-regulation of surface HLA class I Ag, we evaluated tumor cells by cell surface staining with HLA class I-specific Ab and also evaluated tumor cells by DNA typing to assess for allelic loss. Both the cultured melanoma cell lines VMM5A and VMM5B were negative for HLA-A11, B44, and Cw17 Ags, although PBL tested positive for these alleles, both by serologic and DNA typing (data not shown). Thus, HLA-A11, B44, and Cw17 allelic loss was an Ag escape mechanism that was acquired early in the course of the disease and may not be responsible for the described differential HLA-A2-peptide complex expression by tumor cells in the two lesions.

We next evaluated surface expression of HLA class I Ags by cell surface staining with HLA class I-specific mAb combined with cell surface staining for melanoma cells by their expression of the melanoma-specific HMW Ag (Ab 763.74) (29). Interestingly, two melanoma cell populations were identified in TIN-B (Fig. 6A). One included >80% of melanoma cells and was characterized by very low HLA class I Ag expression. This phenotype is likely to have been caused by loss of one  $\beta_2m$  gene and by a point mutation in the other  $\beta_2m$  gene copy (C.-C. Chang, unpublished results). The remaining ~12% of melanoma cells maintain HLA class I Ag expression (Fig. 6A). The melanoma cell line VMM5B was derived from TIN-B and, thus, represents a subset of TIN-B cells. By surface staining, cells in this melanoma cell line have almost completely lost cell surface HLA class I expression (Fig. 6B); however, some HLA-A, -B, -C H chain expression was detected by intracellular staining of VMM5B melanoma cell line (see Fig. 5). The functional significance of this mutation is demonstrated by the restoration of surface HLA class I Ag on VMM5B cells transfected with a plasmid containing DNA for wild-type  $\beta_2m$  under the control of a constitutive promoter (Fig. 6B). Furthermore, this mutation in  $\beta_2m$  was present in a subpopulation of tumor cells in TIN-B, suggesting that some cells in TIN-B had reduced expression of HLA-A2 by the same mechanism. Recognition and lysis of tumor cells from TIN-B by tyrosinase-reactive CTL ex vivo (see Fig. 2) confirms that at least a portion of the cells in the tumor deposit retained expression in vivo of sufficient HLA-A2 Ag for T-cell recognition.

#### Cell surface presentation of HLA-A2-associated MART-1 epitope is limited by competition from tyrosinase-derived peptides

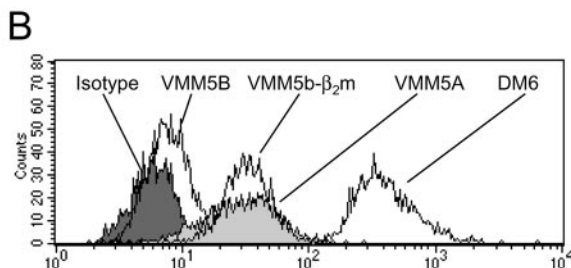
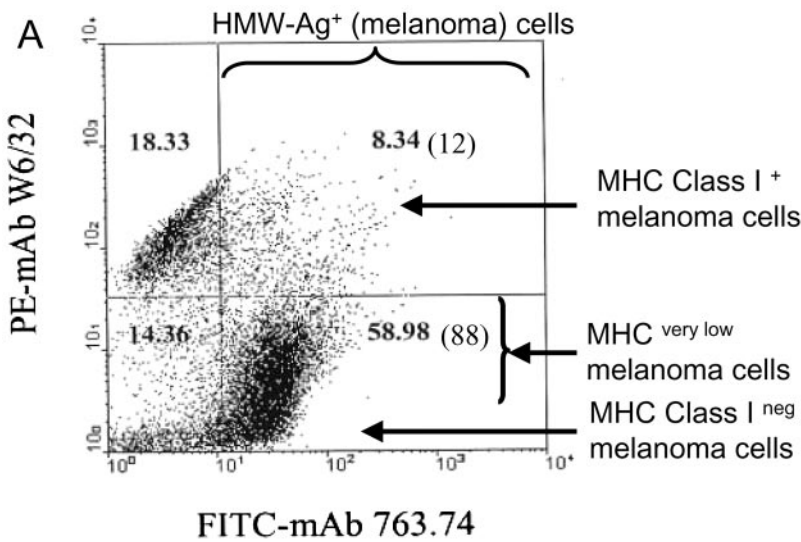
HLA class I Ag down-regulation provided many of the melanoma cells in the second metastasis of patient VMM5 with an escape

**FIGURE 5.** Both TIN-A and TIN-B tumor cells express components of the APM. Comparison of VMM5A and VMM5B tumor cell lines by intracellular staining with a panel of mAb against HLA class I H chains, TAP1, TAP2, tapasin, ER-chaperone proteins calnexin, calreticulin, ERp57, and proteasome components  $\delta$ , LMP2, MB1, LMP7, Z, and LMP10 under denaturing conditions. Numbers indicate the mean fluorescence intensity (MFI of the stained population (open histogram). Closed histogram shows isotype control.



mechanism from T cell recognition and destruction. However, a subset of melanoma cells retained low-level expression of HLA class I surface Ag. These latter cells appear to have lost their susceptibility to MART-1<sub>27-35</sub>-specific CTL, but were susceptible to recognition by Tyr<sub>369-377D</sub>-specific CTL.

These findings raise the possibility that HLA class I Ag down-regulation influences the presentation of distinct Melanoma Ag-derived peptides, likely because of their differential binding affinity to HLA-A2 Ag. Studies of Valmori et al. (34) suggested that MART-1<sub>27-35</sub> binds to HLA-A2 Ag with relatively low affinity.



**FIGURE 6.** Analysis of HLA class I and  $\beta_2m$  cell surface expression on surgically removed second metastatic tumor lesion. *A*, TIN-B cells were separated *ex vivo* by trypsin treatment and were analyzed by flow cytometry after staining with anti-HLA class I mAb W6/32 and anti-HMW-MAA mAb 763.74. The numbers represent the percentage of gated cells in each quadrant; numbers in parentheses represent the percentage of HMW-MAA<sup>+</sup> melanoma cells with or without mAb W6/32 staining. *B*, Tumor cell lines were analyzed by flow cytometry after staining with anti-HLA class I Ag mAb W6/32.

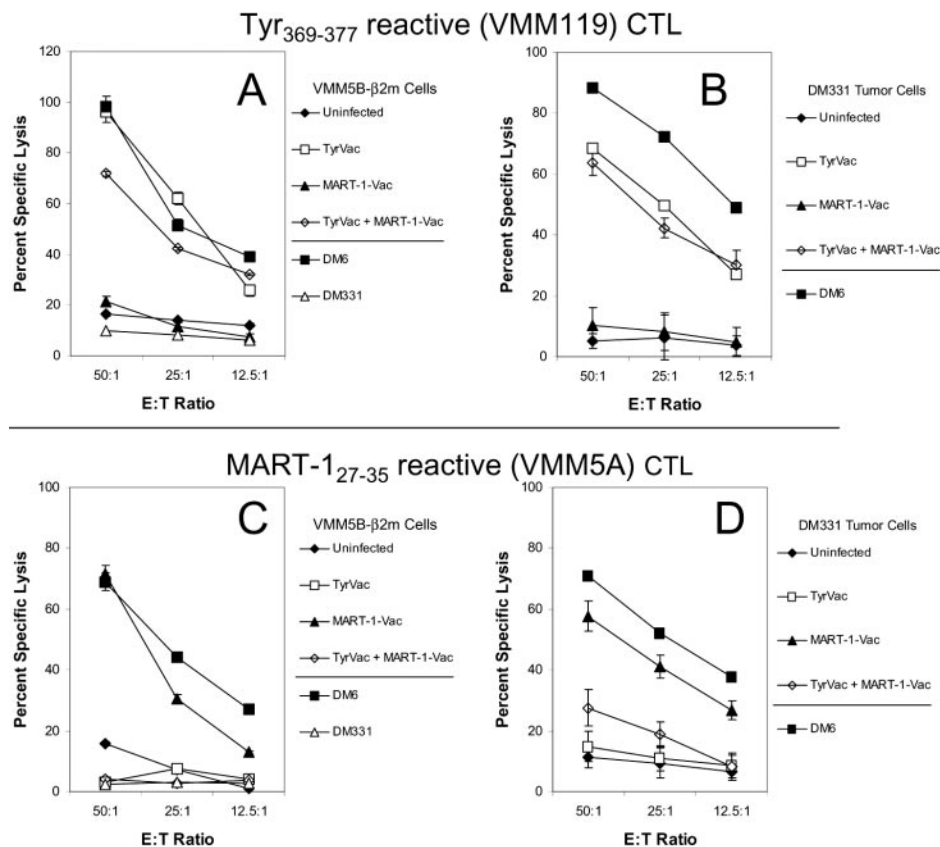
Furthermore, Engelhard et al. (35) demonstrated that HLA-A2 Ag has a significantly lower binding affinity for MART-1<sub>27-35</sub> than for Tyr<sub>369-377D</sub> (IC<sub>50</sub> of 950 and 74 nM, respectively), and mass spectrometry studies of peptides eluted from class I molecules of the human melanoma cell line DM6 indicated surface MART-1<sub>27-35</sub> to be present at 100-fold copies per cell less than Tyr<sub>369-377D</sub>.

We hypothesized that presentation of HLA-A2-MART-1<sub>27-35</sub> complexes may be functionally abrogated in cells with low HLA-A2 Ag expression, because other peptides with higher affinity for HLA-A2 compete for binding sites in a HLA class I molecule-limited environment. Limited availability for HLA-A2 Ag may arise either from decreased expression of HLA class I gene products, or from the failure to form stable peptide-HLA-A2 complexes in the absence of functional  $\beta_2m$  (C.-C. Chang, unpublished results). Therefore, we evaluated the possibility that Tyr<sub>369-377D</sub> peptides compete for free HLA-A2 molecules and prevent sufficient cell surface presentation of MART-1<sub>37-35</sub> peptides for T cell recognition. To test this hypothesis, recombinant vaccinia viruses expressing full-length human MART-1 (MART-Vac) or tyrosinase (Tyr-Vac) were used to drive overexpression of these proteins, singly or in combination, in TIN-B melanoma cells stably transfected to express wild-type  $\beta_2m$  (VMM5B  $\beta_2m$ ), which reconstituted functional HLA class I assembly and cell surface expression (see Fig. 6B). We then evaluated the lytic activity of Tyr<sub>369-377D</sub><sup>-</sup> and MART-1<sub>27-35</sub>-specific CTL (VMM119 and

VMM5A, respectively) against uninfected or vaccinia-infected tumor cells. Expression of tyrosinase and MART-1 in infected cells was confirmed by intracellular staining and Western blot analysis using specific Abs (data not shown). Tyr-Vac infection led to processing and presentation of Tyr<sub>369-377D</sub> epitope by TIN-B- $\beta_2m$  cells, as evidenced by specific lysis by VMM119 T cells (Fig. 7, A and B); likewise MART-1<sub>27-35</sub>-specific VMM5A CTL effectively lysed MART-1-Vac-infected tumor cells (C and D). However, whereas VMM119 T cells lysed either TyrVac-infected or Tyr-Vac/MART-Vac-coinfected melanoma cells with equal efficiency (Fig. 7A), VMM5A CTL failed to lyse coinfecting melanoma cells (C). Both CTL efficiently lysed HLA-A2<sup>+</sup> DM6 melanoma cells, which express both tyrosinase and MART-1, but neither CTL mediated lytic activity against HLA-A2<sup>+</sup> tyrosinase- and MART-1-deficient DM331 cells (Fig. 7, B and D). The absence of surface HLA-A2-MART-1<sub>27-35</sub> complexes consequent to the overexpression of tyrosinase is not unique to the TIN-B-derived tumor cell lines, because the phenomenon was also evident using vaccinia vectors to drive tyrosinase and MART-1 expression in DM331 cells (Fig. 7, B and D).

## Discussion

We report here the adaptive evolution of a melanoma Ag-specific cellular immune response in a long-term survivor of melanoma who had two recurrences in regional LN. The immune response to



**FIGURE 7.** MART-1<sub>27-35</sub> and Tyr<sub>369-377D</sub> epitopes compete for class I binding. VMM5B cells, derived from TIN-B, have down-regulated HLA class I surface expression due to a mutation in the  $\beta_2m$  gene (C.-C. Chang, unpublished results). Normal HLA class I expression was reconstituted by transfection with the  $\beta_2m$  cDNA and designated VMM5B- $\beta_2m$ . MART-1 ( $\blacktriangle$ ), tyrosinase ( $\square$ ), or both ( $\diamond$ ) were overexpressed by infection of VMM5B- $\beta_2m$  cells with specific recombinant vaccinia vectors. Lysis of uninfected target cells ( $\blacklozenge$ ), tyrosinase and MART-1-expressing melanoma DM6 ( $\blacksquare$ ) and tyrosinase- and MART-1-negative melanoma DM331 ( $\triangle$ ) were assessed as controls. A and B, Tyr<sub>369-377D</sub>-reactive (VMM119) CTL recognized and lysed tyrosinase-expressing targets, regardless of MART-1 expression. Tyr<sub>369-377D</sub>-expressing DM6 were lysed, and Tyr<sub>369-377D</sub>-negative DM331 cells were not lysed by these CTL, but CTL lysed TyrVac-infected DM331. C and D, MART-1<sub>27-35</sub> (VMM5A-G) CTL recognized and lysed MART-1-expressing targets, but coexpression of tyrosinase and MART-1 by TyrVac and MART-1-Vac was associated with significantly decreased lysis of both VMM5B- $\beta_2m$  (C) and DM331 (D). MART-1<sub>27-35</sub>-expressing DM6 were lysed, and MART-1<sub>27-35</sub>-negative DM331 cells were not lysed by these CTL. Data are means  $\pm$  SD for triplicate determinations within an assay. Representative data from one of four similar experiments are shown.

the original tumor metastasis (TIN-A) was polyvalent, with immunodominance manifested against the MART-1<sub>27-35</sub> epitope (AAGIGILTV), which was expressed by TIN-A tumor cells. Disease progression was heralded by a change in the immune repertoire of the patient's systemic CTL response to melanoma Ags. The CTL response in the second metastasis (TIN-B) was directed primarily against the Tyr<sub>369-377D</sub> epitope (YMDGTMSQV), to which there had been no detectable response in TIN-A. Response to the MART-1<sub>27-35</sub> peptide, which had dominated the TIN-A tumor recognition, was lost in TIN-B. This change in the repertoire of the CTL response to autologous melanoma cells in vivo corresponds temporally and spatially to a loss of MART-1<sub>27-35</sub> presentation in Ag expression by the melanoma cells in TIN-B.

Detailed evaluation of tumor cells from TIN-A and TIN-B revealed evidence for immune editing and selection of a variety of immune escape mutants during tumor progression. HLA-A11, B44, and Cw17 allelic loss was characteristic of this tumor at least since the time of the first metastasis. More recent phenomena were down-regulation of HLA class I expression in a subset of tumor cells, and loss of functional HLA class I Ag expression due to a point mutation in the  $\beta_2m$  gene (C.-C. Chang, unpublished results). Down-regulation and loss of MDP expression was yet another ongoing process in at least one of the tumor cell subsets. Loss of expression of HLA class I molecules and of MAA presumably provided survival advantage for the tumor cells in the setting of a host MAA-specific response.

The second metastatic recurrence in this patient differed from the first in the loss of presentation of the MART-1<sub>27-35</sub> epitope, even though MART-1 protein was expressed at comparable levels in both metastases. Because TIN-B tumor cells were lysed by tyrosinase-reactive CTL restricted by HLA-A2, and because TIN-B tumor cells expressed unmutated MART-1, absence of recognition by MART-1<sub>27-35</sub>-reactive CTL cannot be explained simply by loss of HLA-A2 expression or by lack of expression of the protein. Proteasome immunosubunits in dendritic cells can prevent presentation of the MART-1<sub>27-35</sub> epitope (36), but the VMM5B cell line derived from TIN-B does not appear to have a higher expression of proteasome immunosubunits (LMP2, LMP7, and LMP10), and most of the other components of the APM appear to be preserved in TIN-B cells. TAP2 down-regulation could potentially lead to changes in processing and presentation of selected peptides, and may well have been another immune escape mechanism for a subset of cells. However, transfection of cells with wild-type  $\beta_2m$  and MART-1 restored recognition by MART-1<sub>27-35</sub> reactive T cells. Thus, changes in Ag processing alone do not explain the loss of MART-1<sub>27-35</sub> complexes.

We have explored an alternate hypothesis to explain a failure of TIN-B tumor cells to present the MART-1 peptide. We hypothesized that competition of peptides for HLA class I Ag binding may be a contributing factor to the selective loss of presentation of epitopes with low affinity for HLA-A2 molecules. The MART-1<sub>27-35</sub> epitope has low-affinity binding to HLA-A2 Ag, with an IC<sub>50</sub> of 950 nM, whereas the Tyr<sub>369-377D</sub> peptide is a strong binder with IC<sub>50</sub> of 74 nM (35). Furthermore, the epitope frequency on the cell surface of melanoma cells is much lower for MART-1<sub>27-35</sub> peptide than for Tyr<sub>369-377D</sub> peptide (35). In the setting of HLA class I Ag down-regulation on the tumor cells, the low number of MHC molecules limits the number of peptides that can be presented, such that peptides with low affinity for the MHC molecule may be available at too low a copy number to permit recognition by epitope-specific T cells.

This hypothesis was supported by our experiments in which tyrosinase and MART-1 proteins were re-expressed in the VMM5B- $\beta_2m$  tumor cell line using vaccinia vectors (Fig. 7).

When MART-1 or tyrosinase proteins were expressed in VMM5B- $\beta_2m$  cells separately, each peptide was processed and presented in a context sufficient for recognition by peptide-specific CTL. However, recognition of HLA-A2/MART-1<sub>27-35</sub> by peptide-specific CTL was significantly diminished upon coexpression of MART-1 and tyrosinase proteins in the same cells. This result was not unique to the VMM5- $\beta_2m$  cell line, but was repeated with DM331 cells with a similar result. Collectively, these data suggest that the number of HLA class I molecules usually is not limiting. However, in tumor cells with dysregulated HLA class I expression or disrupted HLA class I stability, the MHC molecules become limiting, and surface presentation of the weakly binding MART-1<sub>27-35</sub> is significantly diminished as more strongly binding epitopes compete for limited HLA class I docking sites. We propose a model in which down-regulation of surface HLA class I Ag expression does not simply decrease the total number of HLA class I Ag-peptide complexes, but also alters the proportion of specific epitopes presented on the cell surface according to peptide affinity for MHC. As far as we know, this novel mechanism of tumor escape and epitope selection has not been previously described.

Despite multiple immune escape mechanisms, there remains persistent expression of the Tyr<sub>369-377D</sub> epitope on tumor cells in TIN-B. Therefore, the immune response that has arisen in that node (and systemically) against that epitope is an adaptive response, suggesting an appropriate change in immune repertoire. The findings are 1) that tumor cells have evolved toward an Ag-loss phenotype in the setting of a T cell response to tumor Ags, 2) that the Ag-loss phenotype included loss of the immunodominant epitope of the host T cells but retained expression of a cryptic epitope (Tyr<sub>369-377D</sub>), and 3) that the CTL repertoire has changed simultaneously, in a compensatory and adaptive manner, with dominant targeting of the previously cryptic epitope.

The fact that tumor did not appear for 5 years after the original recurrence, and the fact that the patient remained disease-free after surgical resection, both suggest that this immune response may have had clinical relevance. However, the tumor did recur despite this adaptive response, and this suggests that the adaptive response was inadequate to control this particular metastasis completely. Multiple immune escape mechanisms occurred over time. A large subset of cells in TIN-B down-regulated expression of class I HLA molecules due to a point mutation in the  $\beta_2m$  gene. This change would have permitted escape from recognition by HLA-restricted T cells. However, that change may also have made those cells more sensitive to NK-mediated lysis. The subsequent systemic control after surgery suggests that the tumor cells with the most complete immune escape phenotype either had not metastasized beyond this tumor deposit, or that other systemic mechanisms such as NK cells controlled micrometastases beyond this deposit. In any event, this presentation is a reminder that surgical intervention for isolated metastases can succeed in some cases.

Another example of what we consider an adaptive immune response has been described by Coulie and his associates (37) in a patient treated with tumor vaccines as well as surgery, where loss of MHC expression by a tumor was followed by development of a novel response by CTL bearing killer inhibitory receptors that targeted cells with loss of certain MHC molecules. By contrast, the present report shows evidence of an adaptive immune response in a patient treated only with surgery. Also, in this case, the adaptive response occurs by a different and more classic mechanism, responding to immune escape by retargeting to a CTL epitope still presented on the tumor after the selective loss of presentation of a previously immunodominant peptide. This observation also suggests the importance of developing immune therapy directed against multiple Ags simultaneously. As we learn more about

mechanisms by which tumors may evade immune recognition, it is encouraging that the human immune system, even in this elderly patient, has the plasticity to evolve in an adaptive manner in response to immune escape by the tumor.

It is tempting to speculate that development of such an adaptive immune response may predict a favorable clinical outcome. In this regard, unraveling the cellular and molecular events that govern such changes will be worthy of investigation. The resulting information will not only improve our understanding of the delicate interactions between tumor cells and the immune system, but may also suggest relevant therapeutic strategies to prevent tumor progression in the setting of immune escape.

## Disclosures

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