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A Tumor-Infiltrating Lymphocyte from a Melanoma Metastasis with Decreased Expression of Melanoma Differentiation Antigens Recognizes MAGE-12

Monica C. Panelli,* Maria P. Bettinotti,† Kate Lally,† Galen A. Ohnmacht,* Yong Li,* Paul Robbins,* Adam Riker,* Steven A. Rosenberg,* and Francesco M. Marincola†*†

Twenty separate tumor infiltrating lymphocyte (TIL) bulk cultures and a tumor cell line were originated simultaneously from a fine needle aspiration biopsy of a metastasis in a patient with melanoma (F001) previously immunized with the HLA-A*0201-associated gp100:209–217(210 M) peptide. None of the TIL recognized gp100. However, 12 recognized autologous (F001-MEL) and allogeneic melanoma cells expressing the HLA haplotype A*0201, B*0702, Cw*0702. Further characterization of F001-MEL demonstrated loss of gp100/Pmel17, severely decreased expression of other melanoma differentiation Ags and retained expression of tumor-specific Ags. Transfection of HLA class I alleles into B*0702/Cw*0702-negative melanoma cell lines identified HLA-Cw*0702 as the restriction element for F001-TIL. A cDNA library from F001-MEL was used to transfect IFN-γ-stimulated 293 human embryonal kidney (293-HEK) cells expressing HLA-Cw*0702. A 100-gene pool was identified that induced recognition of 293-HEK cells by F001-TIL. Subsequent cloning of the pool identified a cDNA sequence homologous, except for one amino acid (aa 187 D → A), to MAGE-12. Among 25 peptide sequences from MAGE-12 with the HLA-Cw*0702 binding motif, MAGE-12: 170–178 (VRIGHTYIL) induced IFN-γ release by F001-TIL when pulsed on F001-EBV-B cells at concentrations as low as 10 pg/ml. Peptide sequences from MAGE-1, 2, 3, 4a, and 6 aligned to MAGE-12: 170–178 were not recognized by F001-TIL. In summary a TIL recognizing a MAGE protein was developed from an HLA-A*0201 expressing tumor with strongly reduced expression of melanoma differentiation Ags. Persisting tumor-specific Ag expression maintained tumor immune competence suggesting that tumor-specific Ags/melanoma differentiation Ags may complement each other in the context of melanoma Ag-specific vaccination. *The Journal of Immunology, 2000, 164: 4382–4392.

During the last decade, several genes that encode melanoma Ags (MA)2 recognized by tumor-infiltrating lymphocytes (TIL) in association with HLA class I molecules have been identified (1). The two genes encoding the MA predominantly recognized by TIL from patients bearing the HLA-A*0201 phenotype have been cloned, sequenced, and have been called MART-1/Melan-A and gp100/Pmel17 (2–5). With the exception of melanocytes and retina, no normal tissues express these MA and no expression of these gene products has been observed in cancers other than melanoma. Therefore, these Ags appear to represent molecules associated with the melanocyte lineage and are thus called melanoma differentiation Ags (MDA). MDA are believed to be tumor rejection Ags because TIL used for their identification were associated with in vivo tumor regression when adoptively transferred, in combination with human recombinant IL-2 (hrIL-2), to patients with metastatic melanoma (6). In particular, a correlation was noted between therapeutic efficacy of TIL and gp100/Pmel17 reactivity (7). Furthermore, the development of vitiligo, a disease characterized by immune destruction of melanocytes, has been associated with clinical response to immunotherapy (8). Thus, it was deduced that clinically relevant anti-cancer immune responses are directed against Ags that are, like MDA, expressed also by melanocytes.

Because MDA are not related to the oncogenic process and represent a remnant of the melanocytic origin, the maintenance of their expression confers no survival advantage to tumor cells. Thus, their expression is often lost or severely decreased, particularly in melanoma metastases (9). We and others have also noted that loss of MDA expression occurs more frequently in patients undergoing Ag-specific vaccination (10–13). Analysis of 532 melanoma metastases from 204 patients undergoing gp100/Pmel17-specific vaccination revealed that the frequency of gp100/Pmel17-negative metastases was significantly increased after vaccination (26% vs 14%) (13).

A second group of MA comprises tumor-specific Ags (TSA) (14, 15). These MA are expressed by tumors of different histology and not by normal cells with the exception of spermatocytes and spermatogonia (16). These latter cells do not express HLA class I molecules, and, therefore, the host immune system is not exposed to these MA in nontumor-bearing individuals (17). Among them, those that appear to exert a predominant role are those coded by the MAGE-A family of genes (18). TSA are variably expressed in tumors in correlation with a genome-wide demethylation process associated with tumor progression (19, 20). Thus, it is possible
that, as tumor cells dedifferentiate during the neoplastic process, MDA expression decreases while TSA expression remains stable or even increases (15). TSA have been predominantly isolated by repeated in vitro stimulation with tumor cells of T cells from patients with prolonged survival after immune treatment (15, 21). Furthermore, minimal epitopic sequences from TSA used as vaccines have been reported to induce tumor regression when administered in the absence of other concomitant therapy to patients with advanced melanoma (22, 23). Interestingly, MAGE-specific CTL have been only seldom and indirectly identified among TIL populations. In these rare cases, either epitope-driven in vitro stimulation (24) or expansion of subdominant clonal populations appeared necessary for the identification of these T cells (25, 26). The difficulty in identifying MAGE-recognizing TIL has caused observers to question the frequency and intensity with which T cell responses against MAGE proteins may naturally develop in vivo and has been attributed to the “cryptic” nature of these MA compared with MDA (27).

The different pattern of tissue expression and diversity in the observed immune responses has produced uncertainty about the biological significance of these two categories of MA. In this study, we identified in a bulk TIL population a predominant CD8+ T cell specific for a protein encoded by a MAGE gene (MAGE-12). This finding introduces a conceptual bridge between the biological significance of MDA and TSA. It has been demonstrated that the same molecule may effectively or ineffectively induce immune responses depending upon the strategy adopted for Ag delivery and the environment in which the Ag is presented to the host immune system (28–31). Thus, qualitative and/or quantitative differences in the way the antigenic molecule is presented to the host immune system might more significantly shape the observed immune response than its pattern of gene expression and tissue distribution. By identifying a TIL that could recognize a TSA (MAGE-12), we demonstrate that there is no absolute difference in immunogenic potential of TSA and MSA within the tumor microenvironment. However, the less frequent observation of TSA-recognizing TIL suggests a relative difference among these molecules, perhaps related to quantitative aspects of the efficiency of processing and presentation.

Materials and Methods

Cell lines

The bulk melanoma cell line 624-MEL (HLA-A*0201/0301, B1+42/0702, CW*0702/0802) was generated by limiting dilution from a metastasis. In this study, two clones from 624-MEL were used that are characterized by a similar pattern of MA and HLA allele expression with the exception of HLA-A*0201. One clone (624.38-MEL) maintains expression of this allele, while the other (624.28-MEL) does not because of aberrant pre-mRNA splicing of the HLA-A*0201 transcript (32). A375-MEL and SK23-MEL were purchased from the American Type Culture Collection (Manassas, VA). 293-HEK cells were maintained in complete medium (CM) consisting of RPMI 1640 (Biofluids) supplemented as described for CM except 7.5% FBS. Normal human epithelial melanocytes (NHME) generated from human foreskin (Wistar Institute, Philadelphia, PA) and expanded as described (37) in melanocyte growth medium (Clonetics, San Diego, CA).

Peptides

Peptides were produced by solid-phase synthesis techniques and solubilized in sterile water or DMSO (Sigma, St. Louis, MO) according to their biochemical characteristics. Peptide identity was confirmed by mass spectral analysis. Relevant peptide sequences are shown throughout the text.

Expansion and characterization of F001-TIL from FNA

Using a 23-gauge needle, cells were aspirated from an axillary metastasis of patient F001. The material obtained from the FNA was immediately suspended in Iscove’s Medium (IM) (Biofluids) supplemented as described for CM except with the exception of 10% heat inactivated human AB serum (Gemini Bioproducts, Calabasas, CA). Bulk TIL cultures were initiated in a Costar 24-well plate (Corning, Corning, NY) at a concentration of 5 × 10^5 cells/well in 2 ml of IM supplemented with 6000 IU/ml hrIL-2 (Chiron, Emeryville, CA). Twenty separate cultures were attempted. Two melanoma cell lines were expanded in different wells by plating in 24-well plates material from the same FNA in CM without hrIL-2. Preliminary characterization of two distinct cell lines demonstrated that they were practically identical in MA and HLA molecule expression. Therefore, for the remainder of the manuscript they will be referred to as F001-MEL. MA recognition by F001-TIL was assessed by IFN-γ release assay: 1 × 10^5 effector cells were plated with 5 × 10^4 stimulator cells in 96-well round-bottom plates in 200 μl CM. After 24 h incubation at 37°C, the plates were centrifuged and the supernatant harvested for analysis by ELISA (Endogen, Cambridge, MA). IFN-γ is reported as pg/ml secreted by 5 × 10^5 effector cells in 24 h, and values double the background and >100 pg/ml were considered positive.

FACS staining of F001-MEL and F001-TIL

Cell-surface expression of HLA and MA by FACS staining has been previously described (38). In particular the following mAbs were used for evaluation of expression of HLA-class I alleles: KS-1 for HLA-A*0201 (33), GAP-A3 for HLA-A*0301 (39), and SFR8-B6 (40) recognizing the public epitope HLA-Bw6 common to HLA-B*0801 and -B*0702 present in F001 genotype. Abs used to characterize F001-TIL included: anti-human CD8-FITC, CD4-PE, TCR-FITC, CD45RA-FITC, CD45RO-PE, CD95L, CD11b (MAC-1)-PE, CD44(gg)-1-FITC, CD152(CTLA-4)-PE, (PharMingen, San Diego, CA), CD56-PE, CD28-FITC, CD62L-PE, CD154-PE, (Becton Dickinson, San Jose, CA), and FITC-anti-human CD49a, CD49b, CD49d, CD49e, (Serotec, Raleigh, NC). Primary staining with the purified Ab CD95L was followed by secondary staining with FITC-goat anti-mouse IgG.

Cloning of HLA-B*0702 and -Cw*0702

The HLA-B*0702 and Cw*0702 alleles were isolated from total RNA from F001-EBV-B cells using the RNaseky kit (Qiagen, Chatsworth, CA). RNA (4 μg) was transcribed to first-strand cDNA using the SuperScript Preamplification System (Life Technologies, Grand Island, NY). The following F001-MEL locus-specific primers were designed for amplification of HLA-B*0702 cDNA: 5′UT-B-1, 5′-CTCCTCCAGACCAGCGAGATGC-3′ and 3′B-3 utl, 5′-GGTCCTCTAAAGACACGTCGTCAGAGGC-3′.

The F001-Locus specific primers were designed for amplification of HLA-Cw*0702 cDNA: 5′UT-C-2, 5′-TCTCCCCAAGGCGCAGATGC-3′ and 3′B-3 utl, 5′-GTGCTTCAACAGAGCGCAGAGAGCGG-3′. The following reaction mix contained 3 μl cDNA, 4 μl 1 μF Turbo DNA polymerase (Stratagene) 10 μl of 15 mmol/ml of the 5′ primer, and 10 μl of the 3′ primer, 0.8 μl of 10 μM dNTPs, 4 μl 10X buffer. PCR analysis were conducted in a Perkin-Elmer Thermal Cycler 9600 (Branchburg, NJ) using the following parameters: 1 cycle of 94°C for 1 min, 30 cycles of 94°C for 45 s, 62°C for 45 s, 72°C for 45 s, 1 cycle of 72°C. The HLA-B*0702 cDNA was then cloned into the pCR12 TOPO vector (Invitrogen, Carlsbad, CA) using the TA Cloning Kit (Invitrogen), and the construct was confirmed to contain the HLA-B*0702 gene by sequencing. HLA-B*0702 was then inserted into the eukaryotic retroviral expression vector pCRV3 (kindly provided by Dr. R. F. Wang, Bethesda, MD), and the correct insertion was confirmed by sequencing after digestion of pCRV3-B*0702 with BgIII plus HindIII (Life Technologies). The transfected of relevant tumor cell lines (553-MEL and 1495-MEL) was performed by plating 2.5 × 10^5 cell/well in six-well plates in 3 ml of CM. After 24 h, the CM was aspirated and 1 ml of retroviral supernatant diluted 1:2 or 1:3 in CM was added to each well and incubated
overnight. On the following day, the HLA-B*0702-transduced tumor cells were harvested and resuspended at 1 × 10^6/ml for FACS analysis and cytokine release assay.

HLA-Cw*0702 cDNA was cloned in vector pT7blue (Novagen, Madison, WI) using the Original TA Cloning Kit (Invitrogen). The pT7blue was confirmed to contain the HLA-Cw*0702 gene by sequencing. The HLA-Cw*0702 gene was subcloned into the episomal vector pC-NEO (Promega, Madison, WI) by digestion with Xbal/Smal (Promega). The Xbal/Smal/Cw*0702 insert, in pC-NEO, was electrophoresed on a 1% gel, and gel was extracted using the QIAquick gel extraction kit (Qiagen). The Xbal/Smal/Cw*0702 insert was ligated to pC-NEO using T4 ready-to-go ligation mix from Pharmacia (Piscataway, NJ). Proper orientation of the insert into the two constructs was confirmed by sequencing. An unexpected ATG codon in the episomal vector pC-NEO-Cw*0702 preceding the natural initiation codon of the Cw*0702 gene was corrected by site-directed mutagenesis (ATG→CTG).

The vector was then used to transfect Cw*0702-negative tumor cell lines (537-MEL and 1495-MEL). Transfection was conducted as follows. First, 100 μl of lipofectamine (2 mg/ml stock; Life Technologies) mix consisting of 40 μg of lipofectamine per ml of DMEM supplemented with 5 ml/L glutamine was added to each well of a 96-well flat-bottom Costar plate. Next, 1 μl of 200 ng/ml plasmid DNA (pC-NEO+Cw*0702) was added to each well, and the plate incubated for 20 min at room temperature. After incubation, 1 μl of 1 × 10^6 DH10B cells were added to the well, mixed with lipofectamine solution, and incubated overnight. On the following day, the supernatant was removed, and effector cells were added to a concentration of 5 × 10^5/200 μl CM and incubated for 24 h. The supernatant was tested for the release of INF-γ.

Construction of F001-MEL cDNA library

A cDNA library was constructed from F001-MEL. Total RNA was isolated using RNeasy (Qiagen) with a total recovery of 2.5 mg RNA from 6 × 10^6 tumor cells. A total of 16 μg mRNA were then isolated using the polyATtract mRNA isolation system (Promega). cDNA synthesis with directional random priming was performed using Orient Express cDNA System (Novagen). After EcoRI/HindIII linker ligation and enzyme digestion, the cDNA was size fractionated by column chromatography using the Life Technologies pGem3Zf+ cDNA size fractionation column. Two groups of nine fractions each were selected on the basis of the fraction volume and the total volume of the eluate and were pooled into fraction A (larger fragments) and fraction B (smaller fragments). Fraction A and fraction B cDNA fragments with EcoRI/HindIII linkers were subsequently cloned into the cloning vector V-mut (Vical, San Diego, CA) digested with EcoRI and HindIII. Then, 100 ng of vector V-mut was ligated to 1 or 2 μl of insert (fraction A or B) fragments using T4 ligase incubation at 16°C for 16 h. The ligation reactions were then diluted 1:5 and used to transform Escherichia coli cells. Next, 20 μl E. coli DH10B-cell competent cells (Invitrogen) were transferred to a 1-mm sterile cuvette (model 610; BTX Genetronix, San Diego, CA) on ice, and 1 μl of the diluted ligation reaction mixture was added to the cells. Immediately after addition of the ligation mixture, the cells were electroporated with 1.5 kV pulsing. After electroporation, E. coli cells were transferred to a Falcon tube (model # 2059) containing 1 ml of SOC medium (Biofluids, Rockville, MD) and incubated on a shaker at 37°C for 1 h. After incubation, 10, 100, and 200 μl of the transformation reactions were plated on Luria-Bertani Agar plates supplemented with 30 μg/ml kanamycin and incubated overnight. Growth of colonies was used to establish the titer of transformed E. coli and to assess the average size of cDNA inserts in fraction A and B. Accurate titers were obtained in plates inoculated with 10 μl of transformation reaction. The average size of cDNA fragments from 20 randomly chosen colonies demonstrated that fraction B cDNA inserts ranged in size from 0.5 to 1.5 kilobases. Because the size of the known MA is in this range, fraction B ligated to V-mut was chosen to screen F001-TIL reactivity. Subsequently, E. coli DH10B cells transformed with fraction B were diluted to 100 cells/1.2 ml of Super Broth (Advanced Biotechnologies, Columbia, MD) supplemented with 30 μg/ml kanamycin and inoculated into 96-deep well blocks (Edge Biosystems, Gaithersburg, MD) at 1.2 ml/well. Pools of 100 bacteria per well were incubated at 37°C shaking at 300 rpm for 48 h. After incubation, plasmid DNA was isolated from each pool using the Qiaprep 96 Turbo Miniprep kit (Qiagen). Plasmid DNA of each pool was eluted in 150 μl buffer EB. On average, 25–30 μg of plasmid DNA were isolated from each pool.

Screening of F001-MEL cDNA library by transfection of 293-HEK

Each cDNA pool was used to transfect 293-HEK cells (HLA-A*0301, B*0702, Cw*0702). To enhance the expression of Cw*0702, 293-HEK cells were incubated for 48 h with 1000 IU/ml IFN-α before transfection. Then, 100 μl of serum- and antibiotic-free DMEM (s/a-DMEM) supplemented with glutamine and containing 40 μg/ml lipofectamine (Life Technologies) were added to each well of a 96-well flat-bottom plate. Next, 1 μl of each cDNA library-pool (corresponding to 200–300 ng plasmid DNA) was then added. The DNA-lipofectamine mix was incubated for 20 min at room temperature. After incubation, 1 × 10^6 293-HEK cells were added to each well in 100 μl s/a-DMEM. Plates were then centrifuged at 500 rpm for 5 min and incubated for 24 h at 37°C. The following day, plates were centrifuged again at 500 rpm for 5 min. The supernatant was aspirated and discarded. A total of 4 × 10^5 TIL in 200 μl of IM were added in each well, and the plates were incubated for another 24 h at 37°C. After incubation, plates were centrifuged at 1000 rpm for 5 min, and the supernatant were harvested for detection of INF-γ.

Subcloning of cDNA pools and identification of target gene

A consistently reactive cDNA pool containing an estimated 100 gene transcripts was subcloned to identify the gene coding the MA recognized by F001-TIL. This pool was plated on Luria-Bertani Agar plate supplemented with 30 μg/ml kanamycin, and 400 single colonies were picked and tested by transfection of 293-HEK cells as described for the screening of the cDNA pools. Positive clones were subcloned at higher dilution to exclude possible contamination of plasmid DNA. Two subclones were identified that stimulated F001-TIL upon transfection of 293-HEK. These pools were restriction enzyme digested with HindIII and EcoRI and sequenced by automated fluorescent method using an ABI 277 DNA analyzer (Applied Biosystems, Foster City, CA). The DNA sequence obtained was then compared with reported sequences using the BlastSearch program of GenBank (National Library of Medicine, National Institutes of Health, Bethesda, MD).

Quantitative assessment of MAGE 1–12 mRNA expression in cell lines

Quantitative assessment of MA mRNA expression was evaluated with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Norwalk, CT) using uniplex quantitative real-time PCR (qRT-PCR) (41). Each tube contained a Taqman probe that targeted a single gene of interest. Each probe consisted of an oligonucleotide with a 5′ reporter and a downstream 3′ quencher dye. Reporter/quencher dyes were analyzed by dual analysis based upon the different emission wavelength maxima. Uniplex qRT-PCR was performed using different reaction tubes for the target gene of interest and for the endogenous reference housekeeping transcript. A parallel tube contained the probe specific for the endogenous reference housekeeping gene β-actin. Primers were labeled with a reporter dye, 6-carboxyfluorescein (6-FAM, λem = 518 nm) quenched by 6-carboxytetramethylrhodamine (TAMRA). Cycling of cDNA involved denaturation at 95°C for 15 s, annealing/extension at 60°C for 1 min for a total of 40 cycles. Absolute measurement of mRNA copy number was performed with a standard curve for each gene of interest and for β-actin mRNA, dividing the test gene amount by the housekeeping gene amount. The final value represented the absolute number of mRNA copies per 10^3 copies of β-actin mRNA. The sets of primers and labeled probes used for qRT-PCR are presented elsewhere (42) with the exception of MAGE-12, for which the following primers and probes were designed: MAGE-12, 6FAM-TGGTCCGCATCGG and 5′-GAGGCACCATCGCG and 5′-CTCAGACGCTCG TGACAAGG.

Routine and high-resolution HLA typing

Patients’ and cell lines’ HLA type was screened using genomic DNA amplification by sequence-specific primer PCR as previously described (43). To confirm the identity of relevant alleles, cDNA from cell lines was sequenced after locus-specific cDNA amplification. Evaluation of surface expression of HLA alleles was achieved by FACS analysis when mAb specific for a given allele were available. Surface expression of HLA-Cw alleles was demonstrated by complement-mediated cytotoxicity using pre-fabricated trays (One Lambda, Canoga Park, CA).

Results

Development and characterization of a TIL (F001-TIL)/tumor (F001-MEL) pair from a melanoma metastasis

A patient with metastatic melanoma (F001) had undergone vaccination with gp100:209–217(210M) peptide in IFA in combination with systemic IL-12. A pretreatment FNA had demonstrated high expression of MAGE 1–12. Following transient reduction in...
size, the metastasis grew rapidly and failed to respond to further treatment including systemic high-dose hrIL-2. At the time of rapid growth of the metastasis, the tumor cells had totally lost expression of gp100/Pmel17 according to immunocytochemical staining of material from a second FNA obtained at that point (38). In vitro expansion of material from the FNA in the presence of hrIL-2 yielded 20 separate bulk TIL cultures. None of the cultures recognized gp100:209–217 (target of the vaccine), perhaps explained by the loss of gp100/Pmel17 by the tumor. However, 12 TIL cultures could recognize the autologous tumor F001-MEL. TCR Vβ-based molecular typing by PCR demonstrated that the 12 tumor-specific TIL cultures used predominantly Vβ7s2 (38). Other Vβ7s2 rearrangements were excluded by directed heteroduplex analysis (38). Subcloning of the Vβ7s2 amplification product and sequencing identified a unique Vβ7s2-TTY-Jβ2.1 rearrangement for all cultures. Thus, one of the cultures (culture 9), chosen as representative and used for identification of the epitopic determinant, will be referred to as F001-TIL. F001-TIL consisted of CD8+, CD45 R0/high/RAtot+ T cells. This culture was also CD44+, CD56-, CD49b-, and CD94-positive. F001-MEL has been partially characterized previously (38). The neoplastic origin of this line was demonstrated by electron microscopy and karyotyping. F001-MEL did not express HLA class II but expressed high levels of HLA class I molecules. In particular, HLA-A*0201 and -A*0301, HLA-B*0702 and -B*0801 were highly expressed without pretreatment with IFN-γ (data not shown). Also, detectable levels of HLA-Cw*0701 and/or -Cw*0702 could be demonstrated by complement-mediated cytotoxicity without pretreatment with IFN-γ. Sequencing of F001-MEL cDNA confirmed expression and excluded mutations in all six HLA class I alleles. gp100/Pmel17 expression was lost in F001-MEL, while other MDA such as MART-1/MelanA and tyrosinase were expressed at low levels both at the protein and mRNA level (data not shown). Quantitative RT-PCR confirmed no or low levels of expression of MDA (gp100/Pmel17, MART-1/MelanA, tyrosinase, and TRP-1) and relatively high expression of TSA (MAGE-1, MAGE-3, MAGE-12, and NY-ESO-1) in comparison with other cell lines (Table I).

Identification of HLA-Cw*0702 as the restriction element associated with the F001-TIL epitope

Functional characterization demonstrated that F001-TIL did not recognize gp100:209–217 targeted by the vaccination or other HLA-A*0201-associated epitopes. However, F001-TIL could recognize the autologous tumor (F001-MEL) and other melanoma cell lines matched for HLA-B*0702 and -Cw*0702 but not -Cw*0701 (differing from HLA-Cw*0702 by two amino acids) (Fig. 1). No cell lines could be identified that expressed singularly either HLA-B*0702 or -Cw*0702, presumably because these alleles are strongly linked in the ancestral haplotype HLA-B7, -Cw7 (44). Because F001-TIL could not recognize autologous or HLA-matched EBV lines, it was concluded that a new MA epitope had been identified in association with either HLA-B*0702 or -Cw*0702.

To test whether the epitope recognized by F001-TIL was presented in association with HLA-B*0702, melanoma cell lines mismatched for this allele and expressing HLA alleles that did not contain the HLA-Bw6 public specificity were transduced with pCRV3-B*0702. FACS analysis was used to assure the presence of the HLA-B*0702 allele on tumor cells after transduction using SRF8-B6 mAb specific for HLA-Bw6. 553-MEL cells were used for transfection of this allele because this cell line HLA phenotype is -A2, 23; -B44 (Bw4/Bw6), and -Cw3, 4, HLA-Bw6-expressing, pCRV3-B*0702-transduced 553-MEL cells were recognized by an archival HLA-B*0702-restricted CTL clone recognizing a HLA-B*0702-associated gp100/Pmel17 epitope but were not recognized by F001-TIL (Fig. 2). Surface expression of the HLA-Cw*0702 on transfected tumor cells was confirmed by complement-mediated cytotoxicity. Transfection of 537-MEL, a HLA-Cw*0702-negative, MAGE-12-expressing melanoma cell lines (537-MEL and 1495-MEL) with pCI-neo-Cw*0702, consistently restored recognition by F001-TIL (Table II). Similar results were obtained using another melanoma cell line (1495-MEL, data not shown).

### Table I. MA mRNA expression in melanoma cell lines relative to NEHM

<table>
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<th>MDA</th>
<th>NEHM</th>
<th>F001-MEL</th>
<th>624.38-MEL</th>
<th>1102-MEL</th>
<th>SK-23-MEL</th>
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* Tumor Ag mRNA expression was assessed by qRT-PCR (see Materials and Methods). mRNA copy number for each Ag was standardized over the amount of β-actin mRNA within the same sample. Values obtained with NEHM were used as comparison for values obtained from the various cell lines. For MDA, NEHM mRNA levels were arbitrarily considered + + +. Therefore, + + + = mRNA levels ranging within the same logarithmic unit of NEHM mRNA, + + = 1 logarithmic unit below NEHM mRNA, and − = one or more than two logarithmic units lower than NEHM. For TSA, NEHM mRNA levels were arbitrarily considered negative. Negative (−) = mRNA levels ranging within the same logarithmic unit of NEHM mRNA. +, + +, and + + + = mRNA levels one, two, and three logarithmic units above NEHM mRNA levels, respectively.
However, expression of HLA-Cw*0702 could be restored by culturing 293-HEK with IFN-\(\gamma\) or IFN-\(\alpha\) for 48 h. Thus, IFN-\(\alpha\) (not cross-reacting with IFN-\(\gamma\) in ELISA)-treated 293-HEK cells were used as target for transfection of F001-MEL cDNA library. Efficiency of transfection was based on the expression of green

![FIGURE 1. IFN-\(\gamma\) release by F001-TIL cocultured with different melanoma cell lines plus the autologous EBV-B cell line (F001-EBV) and a breast cancer cell line (MCF7-BRE). The legend on the right panel details the HLA class I phenotype of the cell lines. Underlined alleles for HLA-B and -Cw loci point at matches with patient F001. All HLA-B*07** and HLA-Cw*07** typings were confirmed by sequencing of cDNA obtained from each cell line expressing such specificities.](http://www.jimmunol.org/)

![FIGURE 2. IFN-\(\gamma\) release by F001-TIL and by the gp100-specific HLA-B*0702-restricted M5-CTL cocultured with 553-MEL cell lines transfected with pCRV3-EGFP or pCRV3-B*0702. Expression of pCRV3-EGFP was confirmed by fluorescence microscopy, while expression of HLA-B*0702 was confirmed by FACS analysis using SRF8-B6 mAb.](http://www.jimmunol.org/)
fluorescent protein (GFP) by 293-HEK cells transfected with V-mut-GFP (data not shown). Twelve hundred pools containing an estimated 100 genes each were generated. A consistently positive F001-TIL-reactive pool was in this fashion identified. Subcloning of this pool identified two pure subclones that induced IFN-γ release by F001-TIL upon transfection of 293-HEK cells. Restriction enzyme digestion with \( \text{Hin} \) \( \text{D-III} \) and \( \text{Eco} \) \( \text{R-I} \) identified an 800-bp band. Minipreps from both subclones were sequenced in both directions, and in both cases an insert of 780 bp was identified homologous to the published sequence of MAGE-12 except for one amino acid (aa 187 D→A) (Fig. 3). Sequencing of genomic DNA from F001-EBV-B with MAGE-12-specific primers (18) confirmed the aa 187 D→A difference from the previously published sequence, suggesting a novel MAGE-12 allele.

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Nucleotide and deduced amino acid sequences of MAGE-12 derived from analysis of pool-20 cDNA from F001-MEL. This sequence is aligned with the MAGE-12 sequence (L18877) accessible through GenBank. Numbering of nucleotides and amino acids is arbitrarily presented from the first codon and amino acid. The epitope recognized by F001-TIL is underlined (aa 170–178). The variant residue and related nucleotide are also underlined in position 187. The variant was confirmed by sequencing of genomic DNA obtained from F001-EBV-B cells.
MAGE-12:170–178 is the epitope recognized by F001-TIL

Mapping of the F001-TIL epitope was performed by synthesis of 25 peptides from the MAGE-12 sequence based on the HLA-Cw*0702 binding motif (45). This library was screened by culturing F001-EBV-B cells with 10- and 1-µM concentrations of each peptide (Table III). In this fashion, four candidate peptides could be identified that could stimulate IFN-γ release by F001-TIL at 1-µM concentration. Titration of these candidate epitopes identified one (MAGE-12:170–178; VRIGHLYIL) that could stimulate IFN-γ release by F001-TIL at 10- to 100-pM concentration (Fig. 4 and 5). Alignment of this peptide with the peptide sequences of other MAGE proteins commonly expressed by tumor cells revealed limited homology (18). The closest sequences were those from MAGE-2 and MAGE-3 (two and three amino acid differences respectively). We then tested whether F001-TIL specificity was limited to MAGE-12:170–178 or included other MAGE proteins also expressed by F001-MEL at levels probably compatible with CTL recognition (46). Peptides deduced from aligned MAGE sequences homologous to MAGE-12:170–178 were synthesized; however, exogenous pulsing of F001-EBV with limiting dilutions of these peptides demonstrated that F001-TIL recognized exclusively MAGE-12:170–178 (Fig. 5).

Expression of MAGE-12 by melanoma cell lines

The frequency of expression of MAGE-1 through 12 transcripts was tested in 17 melanoma cell lines (data not shown). Only one cell line (1123-MEL) did not express MAGE-12. This finding explains why all HLA-Cw*0702-expressing melanoma cell lines cocultured with F001-TIL induced IFN-γ release. This analysis confirms previous reports suggesting that MAGE-12 is among the most frequently expressed MAGE proteins in cell lines derived from melanoma metastases (18). Quantitative RT-PCR demonstrated that the level of MAGE-12 mRNA expression was comparably close among most cell lines (Table IV), suggesting that the expansion of F001-TIL was not due to unusually high expression of this gene.

Discussion

We have previously monitored immune responses to MA-specific vaccination by immunochemical (47, 48) or molecular (49) analysis of material obtained from serial FNA of the same metastasis. This strategy allows comparative evaluation of the tumor targeted by the immune response at different time points relevant to treatment. Furthermore, FNA, by leaving the lesion in situ, allows the prospective documentation of the natural progression of the disease or its response to therapy (9). Recently, we have been able to expand tumor/TIL pairs from FNA material (50). This has allowed detailed functional and molecular testing of findings suggested by direct immunochemical or molecular testing of FNA (38, 50). Using this approach, we identified, in a patient with melanoma (F001), a metastasis whose loss of gp100/PMel17 expression could be best explained by immune selection following gp100/PMel17-based vaccination (38). Coincident to the loss of gp100/PMel17 expression was the rapid growth of the metastasis that had initially responded to immunization. It was also noted that, at the time of rapid growth, expression of other MDA was strongly reduced. Further characterization of the F001-MEL, FNA-derived autologous

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### Table III. Peptide sequences from MAGE-12 retrieved according to predicted binding to HLA-Cw*0702 a or Eluted HLA-Cw*0702 ligands (45)

<table>
<thead>
<tr>
<th>Start Position</th>
<th>No. of Amino Acids</th>
<th>Sequence</th>
<th>Half-Life of Dissociation b (minutes)</th>
<th>Recognition by F001-TIL of F001-EBV Pulsed with Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>69</td>
<td>9</td>
<td>STLPITINY</td>
<td>5,600</td>
<td>+</td>
</tr>
<tr>
<td>97</td>
<td>9</td>
<td>TFPDLLETSF</td>
<td>5,600</td>
<td>+</td>
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<td>116</td>
<td>9</td>
<td>LVHFILLFYK</td>
<td>19,200</td>
<td>+</td>
</tr>
<tr>
<td>123</td>
<td>9</td>
<td>LKVRAREPF</td>
<td>&lt;1,000</td>
<td>+</td>
</tr>
<tr>
<td>135</td>
<td>9</td>
<td>MLGSVRNF</td>
<td>8,000</td>
<td>+</td>
</tr>
<tr>
<td>139</td>
<td>9</td>
<td>VIRNFQDFFF</td>
<td>2,400</td>
<td>+</td>
</tr>
<tr>
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<td>VIFSKASEY</td>
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<td>+</td>
</tr>
<tr>
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<td>ASEYQLQVF</td>
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<td>+</td>
</tr>
<tr>
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<td>ELYQLVFGI</td>
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</tr>
<tr>
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<td>EVRIGHLY</td>
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<td>+</td>
</tr>
<tr>
<td>169</td>
<td>9</td>
<td>VIRGHLY</td>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>178</td>
<td>9</td>
<td>LVTCLGLSY</td>
<td>8,000</td>
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</tr>
<tr>
<td>181</td>
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</tr>
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<td>10</td>
<td>SRKMAELVHF</td>
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</tr>
<tr>
<td>111</td>
<td>10</td>
<td>RKMAELVHFL</td>
<td>NA</td>
<td>+</td>
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<td>175</td>
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</tr>
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<td>185</td>
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<td>SYDGLLGDQI</td>
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<td>+</td>
</tr>
<tr>
<td>196</td>
<td>11</td>
<td>VPTGILLIVL</td>
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</tr>
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<td>175</td>
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<td>+</td>
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</tr>
<tr>
<td>64</td>
<td>14</td>
<td>SPQGASTLPTTTINY</td>
<td>NA</td>
<td>+</td>
</tr>
</tbody>
</table>

a http://bimas.dcmi.nih.gov/cgi-bin/molbio/ken_parker_comboform
b Estimated half-life of dissociation of the HLA-Cw*0702/β2-microglobulin complex.

c The half-life of dissociation for these peptides was not analyzed with Parker’s program because these peptides were selected on the basis of elution analysis data (45).
melanoma cell line confirmed loss of gp100/PMel17 expression and severely decreased expression of other MDA such as MART-1/MelanA, tyrosinase, and TRP-1. Interestingly, however, this cell line had retained expression of most TSA, in particular those encoded by the MAGE-A family of genes.

Surprisingly, TIL cultures (F001-TIL) expanded with hrIL-2 from the same FNA could still recognize F001-MEL- and HLA-matched melanoma cells. Thus, we wondered about the nature of the MA recognized by F001-TIL in view of the loss of expression of common MDA such as MART-1/MelanA and gp100/PMel17 by the HLA-A*0201-expressing F001-MEL cell line. We have previously reported that TIL generated from metastases in patients carrying the HLA-A*0201 phenotype most frequently recognize MART-1/MelanA (~90% of the times) and/or gp100/PMel17 (~40% of the times) (7), only exceptionally other MDA (51).

Characterization of the antigenic determinant recognized by F001-TIL identified MAGE-12. Evidence of MAGE protein-specific T cells infiltrating tumors has been so far scant and indirect (24, 25).
More recently, Zorn and Hercend (26) have identified a subdominant T cell clone that recognized MAGE-6 in association with HLA-A*3402. The identification of that T cell provided evidence that T cell responses against MAGE proteins may naturally develop within the tumor microenvironment. Although the TIL clone, named 5G, was expanded from a regressing primary melanoma, recurrent metastases in the same patients contained T cells using a TCR rearrangement identical with 5G. This finding suggested that the presence of a MAGE-recognizing T cell at the tumor site was not in itself an absolute predictor of response. In this study, we confirm, with the documentation of 12 short-term bulk TIL cultures, that MAGE-specific T cells contribute to the anti-melanoma repertoire naturally populating the tumor microenvironment. Furthermore, the presence of a MAGE-12-specific TIL in rapidly progressing metastasis suggests a biologic role for this TSA-recognizing T cell similar to that of other MA-specific TIL previously identified. Interestingly, although molecular evidence of vaccine-reactive T cells could be gathered in the FNA from which F001-TIL was originated, none of the 20 TIL cultures attempted to demonstrate vaccine-specific (gp100/PMel17) reactivity (38). This failure to expand gp100/PMel17-reactive T cells could be best explained by the gradual loss of expression by the tumor of the Ag targeted by the vaccination and underlines the concept that functional dissociation might be expected between the tumor microenvironment.

The identification in a HLA-A*0201 patient of a MAGE-12-specific TIL within a MDA-depleted metastasis suggests a “cryptic” role of this MA. MAGE-12 and other MAGE genes are expressed by many tumors at levels compatible with CTL recognition (15, 46). However, in HLA-A*0201-expressing melanoma patients, it is possible that their level of expression is not sufficient to override the stimulus provided by MDA. It is not clear why in the context of HLA-A*0201 MART-1/MelanA and gp100/PMel17 exert an immunodominant role. Given the predominant expression of HLA-A over HLA-Cw alleles (52), it is possible that a MA associated with the latter might most commonly remain cryptic. Furthermore, immune dominance might depend on previous individual exposure to endogenous or environmental Ags mimicking MDA epitopes (53). An unusual abundance of MDA-derived, HLA-A*0201-associated epitopes due to preferential Ag processing and transport cannot be excluded, although such phenomenon has never been described. It is unlikely that high affinity of MDA-derived epitopes for the HLA-A*0201 molecule plays a significant role because most of these epitopes are characterized by intermediate affinity for this allele (7). Finally, it is unlikely that comparatively high level of protein expression may explain MDA immune dominance considering the stringent association for instance in the case of MART-1/MelanA and gp100/PMel17 exert an immunodominant role. Given the predominant expression of HLA-A over HLA-Cw alleles (52), it is possible that a MA associated with the latter might most commonly remain cryptic. Furthermore, immune dominance might depend on previous individual exposure to endogenous or environmental Ags mimicking MDA epitopes (53). An unusual abundance of MDA-derived, HLA-A*0201-associated epitopes due to preferential Ag processing and transport cannot be excluded, although such phenomenon has never been described. It is unlikely that high affinity of MDA-derived epitopes for the HLA-A*0201 molecule plays a significant role because most of these epitopes are characterized by intermediate affinity for this allele (7). Finally, it is unlikely that comparatively high level of protein expression may explain MDA immune dominance considering the stringent association for instance in the case of MART-1/MelanA and gp100/PMel17 exert an immunodominant role.

In this particular case, many concurrent events may have lead to the identification of F001-TIL. First, the expression of immunodominant MDA (MART-1/MelanA and gp100/PMel17) was lost. Second, F001-MEL did not demonstrate down-regulation of the HLA-B and -Cw alleles often noted in melanoma cell lines.
(37, 55), implying that in vivo the tumor could sustain the MAGE-12/HLA-Cw*0702-restricted reactivity of F001-TIL. Interestingly, however, all cell lines derived from patients carrying the HLA-Cw*0702 phenotype were recognized by F001-TIL. Among them, 624-MEL has been previously shown to lack detectable surface levels of Cw*0702 by complement-mediated cytotoxicity (55). Thus, it is possible that minimal HLA-Cw*0702 expression is sufficient for recognition by F001-TIL. Finally, F001-MEL demonstrated a quantitatively robust expression of most MAGE genes. Quantitative assessment of MAGE-12 mRNA levels excluded that F001-MEL expressed significantly more MAGE-12 transcript than most melanoma cell lines tested. Because a MAGE-12-specific mAb was not available, estimation of MAGE-12 expression was based on mRNA rather than protein measurements. However, a good correlation has been reported between the amount of mRNA expression and recognition by CTL specific for MAGE proteins (46, 56). Thus, it appears that exceptional over-expression of the target Ag was not the reason for the identification of F001-TIL. Thus, with this study we cannot conclusively explain the successful identification of a MAGE-12-recognizing TIL because neither levels of HLA nor MAGE-12 expression obviously altered F001-TIL interactions with relevant targets. Our best explanation remains the loss of expression of common MDA that might sustain a dominant T cell population in most, but not this, circumstances.

We and others have previously shown that melanomas can progressively lose expression of MDA in relation to neoplastic dedifferentiation or in response to immune selection (10–13). However, progressive loss of TSA has not been documented. It is possible that expression of TSA may be more stable during cancer progression because their expression is dependent upon an incremental genome-wide demethylation process associated with tumorigenesis (19, 20). In fact, expression of MAGE proteins is more frequent in metastatic than in primary melanoma (15), while the opposite seems to occur for MDA (57). Thus, despite the cryptic nature of TSA it can be hypothesized that T cell responses induced against MAGE proteins by exogenous stimulation (for instance with epitope-specific vaccines) may have more durable in vivo effectiveness. These immune responses could exert their action toward MA whose expression is characterized by higher stability in comparison to MDA. This may explain why CTL responses elicited by MAGE-based vaccines lead in the absence of concomitant treatment to tumor regression more often that MDA-based vaccines (22, 23, 58, 59) and for prolonged periods of time (21).

It has been postulated that development of TSA-specific T cells may follow rules different from that of MDA-specific T cells due to the different pattern of expression of the two categories of Ags (27). As a corollary, although identification of cryptic TSA-specific CTL is uncommonly observed, their effectiveness could be less limited by self-tolerance because, contrary to MDA, TSA are not present in HLA class I-expressing normal cells. The expansion of a TIL recognizing a MAGE protein from a rapidly progressing metastatic lesion suggests that the coexistence of TSA-specific TIL with TSA-expressing tumor cells is possible in the tumor microenvironment. This is, in essence, not biologically different from the coexistence noted between MDA-specific TIL and MDA-expressing melanoma cells. Thus, this finding supports the immunological view that it is not the distribution or the genetic origin of a particular molecule that determines the outcome of an immune response but, rather, the quality of the stimulus applied (28–31).

As previously noted by others (18), MAGE-12 was expressed in the majority of melanoma cell lines analyzed, and its expression was sufficient to induce recognition by F001-TIL. High avidity of the epitope/F001-TCR interaction was suggested by the picomolar range in which MAGE-12:170–178 could induce recognition of F001-EBV-B cells. This might explain why the reported low surface expression of HLA-Cw alleles (52) was not a factor limiting the recognition of melanoma cells by F001-TIL. Thus, the identification of MAGE-12:170–178 adds a new epitope in the context of HLA class I alleles different from HLA-A that could be used for Ag-specific vaccination. This epitope can be added to a growing list of other MA epitopes recently described in association with HLA-Cw such as MAGE-3 and BAGE epitopes associated with HLA-C*06:02 (60) and GAGE-1 and -2 epitopes associated with HLA-C*06:02 (61). The relatively high frequency of HLA-Cw*0702 in the melanoma population (~20%, data not shown) suggests that MAGE-12:170–178 could be used in one of five patients with melanoma and other cancers expressing this TSA (15). The inability of F001-TIL to cross-react with HLA-Cw*0701 (among other HLA-Cw*07 alleles closest to HLA-Cw*0702) suggests, based on our experience with other HLA/epitope combinations, that it is unlikely (54, 62, 63) that MAGE-12:170–178 can function as an immunogen in the context of other HLA-Cw*07 alleles.

The identification of a TIL culture recognizing a protein encoded by the MAGE-A family of genes breaks the dichotomy between the suggested biological significance of MDA and TSA within the tumor microenvironment. Furthermore, these experiments identify an immunogenic role for MAGE-12, frequently expressed by melanoma and tumors of other histology (15, 18). Finally TSA may have a role in the treatment of undifferentiated cancers that have lost expression of MDA.

References


