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Abstract:

The basis of intra-tumoral and systemic T-cell reactivity toward cancer remains unclear. In particular the role that peripheral stimuli play in shaping the acquired immune response toward cancer is still poorly understood. In this study we document the surfacing of systemic immunity toward a nine-residue cryptic epitope from a member of the Melanoma Antigen family (MAGE-12:170-178), following temporary regression of a single melanoma metastasis, in response to vaccination against another molecule (gp100/PMel17). This emergence was unlikely to be related to unusually high expression of MAGE-12 by the tumor, or by the influence of analog epitopes to MAGE-12:170-178. Since MAGE-12 was unlikely to be expressed at sites other than the tumor, the demonstration of MAGE-12:170-178 reactivity in post- but not pre-vaccination circulating lymphocytes suggests that the systemically observed immune response was influenced by events induced by the vaccine at the tumor site or in draining lymph nodal areas. Possibly, as suggested by pre-clinical models, immunologic ignorance is the default response toward cancer unless unusual stimulatory conditions occur. Surfacing of MAGE-12 specificity occurred in association with loss of gp100/PMel 17 targeted by the vaccine. This finding suggests that vaccinations might have effects beyond their intrinsic specificity and may trigger broader immune responses through epitope spreading by inducing changes within the tumor microenvironment. This may have important practical implication for the development of immunization strategies.

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Introduction

The identification of tumor antigens and the principles of immunotherapy have provided an excellent tool for dissecting the molecular immunology of tumor/host interactions (1). There has been considerable interest in the concepts of immunotherapy as well as multiple attempts to immunologically enhance the anti-tumor response. The principals of immunotherapy allow for an elegant solution to diseases for which many conventional treatments are non-specific and frequently inefficient. As more has become known about the immune system and its interactions with both self and foreign peptides, investigators have become more successful in shaping modalities with potential to harness the immune system into an effective anti-tumor response.

Multiple vaccine-based approaches to anti-tumor therapy are currently being undertaken. In particular, the molecular identification of the genes encoding tumor antigens recognized by T cells has sparked interest in their utilization as vaccines against cancer. Contrary to most vaccinations, which are aimed at priming the immune system against future pathogens, immunizations against cancer are given as a surrogate for the apparent weak immunogenicity of tumors themselves (2, 3). Specific epitopes can be determined, frequently by isolating tumor infiltrating lymphocytes (TIL) and identifying the peptide/HLA complex they recognize. The specific peptide can then be synthesized, and injected into the patient, in the presence or absence of cytokines. These vaccines are simple to administer in an out-patient setting and have been shown to incur systemic immunity to the peptide of interest (4,5,6). Unfortunately systemic immunity does not always equal clinical regression of the tumor.

Currently used peptide vaccines are based upon two types of tumor antigens (TA); tumor specific antigens (TSA) and melanoma differentiation antigens (MDA). TSA are specific to the tumor and are generally not present in normal cells. They are frequently fetal antigens and are not expressed in adult cells until far along in tumorogenesis when the de-differentiation of the cell leads to demethylation of the genome and to expression of molecules which are not normally expressed (7). An example of TSA are the melanoma antigen family of genes (MAGE), a family of 12 genes and gene products which have been shown to be expressed in many melanomas. The other type of TA, MDA, are found in normal melanocytes and may be critical to the normal function of the cell. These molecules are not critical to the tumor and are frequently down-regulated as the tumor becomes increasingly anaplastic (11). Examples of MDA include gp100/PMel17, and MART-1/MelanA.

Extensive trials have been undertaken using certain MDA, specifically gp100:209-217(210M), a modified form of a peptide derived from the gp100/PMel17 molecule, presented in the context of HLA*A201. This peptide was analyzed on its ability to induce a measurable immune response, and its ability to induce tumor regression. Data from this study demonstrated that on the basis of *in vitro* immunologic assays, 91% of patients studied could be successfully immunized with this synthetic peptide, and 13 of 31 patients (42%) receiving the peptide vaccine plus IL-2 had objective cancer responses (1). Many patients who were HLA*A201 positive and who expressed the gp100/PMel17 molecule have undergone vaccination with this peptide.

Although gp100:209-217(210M) vaccine has demonstrated clinical success, there have been many questions as to why this molecule should induce such a strong immune

response, and why this molecule should appear to be immunodominant over all of the potential antigens a cell can express. Of the millions of possible epitopes a cancer cell expresses, only certain ones induce specific immune responses in the host, and these responses can be demonstrated over and over again in a multitude of hosts (8). Questions about this phenomenon of immunodominance remain unanswered. Furthermore, questions about other antigens exist, and their potential immunogenicity if they could be expressed in the absence of other, more dominant antigens.

The MAGE family of gene products became of interest to our group with the discovery of a TIL which recognized MAGE-12 in a melanoma metastasis of a patient (F001) who had been undergoing vaccinations with the gp100:209-217(210M) peptide vaccine (9). This patient's tumor was noted to have expression of gp100/PMel17 prior to vaccination, and was noted to have a good initial response to the gp100:209-217(210M) vaccine, experiencing significant shrinkage of his tumor. However, after a period of time, his tumor was noted to re-grow. Analysis of peripheral blood mononuclear cells (PBMC) demonstrated that he retained immunity to gp100/PMel17, however, a fine needle aspiration (FNA) of the tumor demonstrated that it had lost expression of gp100/PMel17 (10). Interestingly, lymphocytes were noted to be infiltrating the patient's tumor. Studies of this TIL identified MAGE-12 as the restriction element within the context of HLA Cw*0702. (9).

The identification of a TSA-recognizing TIL was an unusual finding and provided a conceptual bridge between the MDA-directed immune reactivity commonly observed at tumor site and the TSA reactivity more frequently observed among circulating T cells. Differences in the levels of immune reactivity toward the two categories of tumor antigens

might be attributed to their tissue expression pattern. Some investigators, however, suggested that qualitative and/or quantitative differences in the way antigenic molecules are presented to the host might shape the immune response more significantly (2, 12, 3).

Statement of Purpose and Hypothesis.

Several hypotheses attempt to explain the insurgence of reactivity toward the cryptic MAGE-12 epitope in this patient. First, the tumor might have been characterized by unusually high expression of MAGE-12 and/or HLA-Cw*0702 capable of overriding the commonly dominant stimulus provided by MDA (8). Second, the loss of expression of MDA after vaccination (10) might have allowed the unmasking of less prominent T-cell populations. Third, the patient's reactivity toward MAGE-12 might have been unusually high due to priming by exposure to analog epitopes of MAGE-12:170-178 as suggested for other TA (13). Fourth, peptide analogs of MAGE-12:170-178 from other MAGE family genes (14) might have exerted super-agonist or antagonist action and uniquely shaped the immunogenicity of MAGE-12 (15). Finally, MAGE-12-specific cytotoxic T-cell (CTL) could have risen in response to vaccine-induced modifications of the tumor environment (16). These hypotheses were tested by this study.

Materials and Methods

All experiments were performed by Ms. Lally with the exception of the experiments detailed in the sections labeled "Detection of Cw*0702 surface expression by complement dependent cytotoxicity" and "HLA phenotyping of patients and cell lines".

Cell lines.

Two clones from the melanoma cell line 624-MEL (HLA-A*0201/0301, B*1402/0702, Cw*0702/0802) characterized by identical pattern of MA and HLA allelic expression with the exception of HLA-A*0201 were used. One clone (624.38-MEL) maintains expression of this allele, while the other (624.28-MEL) does not due to aberrant splicing of the HLA-A*0201 transcript (17). A375 MEL, SK23-MEL and 293-HEK primary embryonic kidney cells were purchased from the American Type Culture Collection, Rockville, MD. 397-MEL, 537-MEL, 836-MEL, 888-MEL, 938-MEL, 1102-MEL, 1123-MEL, 1280-MEL, 1359-MEL, 1495-MEL are archival cell lines derived from surgically removed melanoma metastases (18). F001-MEL, F002-MEL and F010-MEL consist of early passage (<5 passages) cell lines derived from FNA of melanoma metastases. The B-lymphoblastoid cells, F001-EBV, were transformed from patient F001 PBMC. All cell lines were maintained in complete medium (CM) consisting of RPMI 1640 (Biofluids, Rockville, MD) with 10 mM hepes buffer, 100 U/ml penicillin-streptomycin (Biofluids), 10 μg/ml Ciprofloxacin (Bayer West Haven, CT), 0.03% L-glutamine (Biofluids), 0.5 mg/ml amphotericin B (Biofluids) and 10% heat-inactivated fetal bovine serum (Biofluids). F001-MEL cells were maintained in Iscoves (Biofluids) supplemented as described for CM. 293-HEK cells were maintained in DMEM (Biofluids) supplemented as described for CM except 7.5% FBS. Normal human epithelial

melanocytes (NHEM) generated from human foreskin were donated by Dr. Mernhard Herlyn (Wistar Institute, Philadelphia, PA) and expanded in melanocyte growth medium (MGM, Clonetics, San Diego, CA). The F001-TIL culture recognizing MAGE-12:170-178 was generated from patient F001 (10, 9) and expanded in CM supplemented with 10% human AB serum (Biofluids) and 6,000 IU/ml IL-2.

Peptides.

Peptides were produced by solid phase synthesis techniques and solubilized in sterile water or dimethylsulfoxide (DMSO, Sigma, St Louis, MO) according to their biochemical characteristics. Peptide identity was confirmed by mass spectral analysis. The following peptides were used: MAGE-12:170-178 (VRIGHLYIL) and analog peptides from other MAGE family genes (MAGE-1: DPTGHSYVL; MAGE-2: VPISHLYIL; MAGE-3: DPIGHLYIF; MAGE-4a: DPASNTYTL; MAGE-6: DPIGHVYIF), MART-1:27-35 (AAGIGILTV, abbreviated as MART-1), gp100:209-217 (ITDQVPFSV, abbreviated as g209), the modified gp100:209-217(210M) (19) (IMDQVPFSV, abbreviated as g209-2M), FluM1:58-66 (GILGFVFTL, abbreviated as FluM1) and Histone H3.3:40-48 (RYRPGTVAL).

TCR V β PCR analysis.

A previously described set of 35 primers was selected to amplify 45 functional Vβ (10). Each primer mix was composed of 10 x PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 1.25 U of AmpliTaq Gold, 0.5 μl of cDNA, 0.5 μM Vβ primer, 0.5 μM TC-1 constant region primer (AYACCAGTGTGGCCTTTT), and water up to 20 μl final

reaction volume. PCR was run using the following protocol: initial activation of the enzyme at 94°C for 9 min; 10 high-stringency cycles of 94°C for 30 sec for denaturation, 65°C for 1 min for annealing and 72°C for 1 min for elongation; 20 low-stringency cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min; final extension at 72°C for 10 min. After PCR, 6 µl of the product and 6 µl of BPB-loading buffer were mixed and run on a 1% agarose gel for 45 min. at 150 V. The gel was stained with Vistra Green (Amersham Life Science Inc., Arlington Heights, IL) 1:10000 dilution in 1x TBE for 50 min and analyzed on a FluorImager 595 (Molecular Dynamics, Inc. Mountain View, CA).

Analysis of MAGE 1-12 mRNA expression in cell lines.

Expression of MAGE 1-12 gene products was evaluated by standard RT-PCR using published primer sequences (14). Two µl cDNA from each cell line were PCR amplified at a final volume of 50 µl and overlaid with mineral oil. The final reaction mix contained 2.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ, USA) and 10 pM of each primer in a 50 nM KCL, 1.5 mM $MgCl_2$, 25 mM Tris HCL pH 8.9 and 200 µM of each dNTP solution. The reactions were carried out in a Perkin-Elmer Thermocycler Model 9600 using the following parameters: one cycle at 96°C for 15 minutes, 30 cycles at 96°C for 30 seconds, 62°C for 30 seconds, 72°C for one minute. Eight µl of each PCR product were electrophoresed through a 2% Agarose gel containing ethidium bromide and a 100 bp ladder (Gibco, BRL Gaithersburg, USA). The fluorescent DNA bands were observed on a 302-nm UV transilluminator.

Quantitative assessment of MAGE-12 mRNA expression was evaluated with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer) utilizing uniplex real time

quantitative RT-PCR (qRT-PCR). 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 utilizing different reaction tubes for the target gene of interest and for the endogenous reference (β-Actin). Probes were labeled with a reporter dye, 6-carboxyfluorescein (6-FAM, λ_{max}=518 nm) quenched by 6-carboxytetramethylrhodamine (TAMRA). Cycling of cDNA involved denaturation at 95^0 C for 15 sec., and annealing/extension at 60^0 C for 1 min. for a total of forty 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 10^5 copies of β-Actin mRNA. The sets of primers and labeled probes used for qRT-PCR have been previously published (20) with the exception of MAGE-12 for which we designed the forward primer (5'-TGGCATCGAGGTGGTGG-3'), the reverse primer (5'-CCCAGGCAGGTGACAAGG-3') and the probe (6FAM-TGGTCCGCATCGGCCACTTGTAC-TAMRA).

Screening of normal human tissues for MAGE-12 expression.

A 96 well plate containing 1X, 10X, 100X and 1000X concentration of various normal tissues was analyzed (1X is approximately equal to 1 pg). The following tissues were studied: brain, heart, kidney, spleen, liver, colon, lung, small intestine, muscle, stomach, testis, placenta, salivary gland, thyroid gland, adrenal gland, pancreas, ovary, uterus, prostate, skin, PBL, bone marrow, fetal brain, fetal liver. The dried cDNAs were

dissolved in the following reaction mix: 10 pmol of each primer, 50 nM KCl, 1.5 mM $MgCl_2$, 25 mM Tris HCl pH 8.9, and 200 μM of each dNTP solution and PCR reactions were carried as for the tumor cell lines.

Generation of CTL by in vitro stimulation of PBMC

PBMC were separated from the peripheral blood of HLA-A*0201/Cw0702 expressing patients with melanoma by centrifugation of Ficoll-Hypaque gradients and used as cyropreserved samples. PBMC were thawed and their systemic reactivity against various epitopes was assessed by repeated stimulation with 1 μM peptide administered to the cultures after an overnight rest (21). Twenty-four hours after cognate stimulation 600 IU/ml of interleukin-2 were added to the cultures. Cultures were replenished of IL-2 every other day. Stimulation with peptide was repeated at weekly intervals by exogenous loading of irradiated autologous PBMC (30 gy) as antigen presenting cells with 1 μM peptide. Irradiated, exogenously pulsed peptides were then co-cultured with responding cells at a 1:1 ratio.

Tumor-reactive CTL were also generated using HLA matched cell lines as previously described (22), PBMC were cultured in CM supplemented with 10% heat-inactivated human AB serum. Either irradiated (5,500 rads) tumor cells (1:10 stimulator/responder ratio) or peptide (1 μM) were added to 4 x 10^6 PBMC in 24 well Costar plates. The following day and every other day thereafter 300 IU/ml IL-2 (Chiron Co., Emeryville, CA) were added. CTL cultures were re-stimulated weekly either with irradiated (3,000 rads) autologous PBMC (2 x 10^6 cells/ml) pulsed with 1μM peptide or irradiated tumor cells at responder/stimulator ratios ranging between 1:3 and 1:10.

Assessment of antigen recognition by CTL

Epitope specificity of CTL was determined by IFN-γ mRNA production in response to 2-hour stimulation with relevant or irrelevant epitopes. cDNA from each culture was assayed for IFN-γ production by quantitative real-time PCR (23). Absolute measurement of mRNA copy number was performed with a standard curve for each gene of interest. In each sample IFN-γ mRNA levels were normalized per 10^5 copies of CD8 mRNA.

Inhibition of MAGE-12 by other MAGE peptides

Exposure to potential agonists or antagonists was provided by 3 x 10^6 293-HEK cells treated with IFN-α for 24 hours to enhance HLA-Cw*0702 surface density (24) and pulsed for 2 hours at 37 C with 1 μM peptide. Cells were washed of excess peptide and exposed to 3 x 10^6 F001-TIL. Aliquots containing 2 x 10^5 TIL were removed at time zero and every two hours up to 6 hours and tested for IFN-γ release by qRT-PCR. After 24 hours each culture was split into two cultures. 293-HEK cells (10^6) pulsed with MAGE-12:170-178 or the irrelevant HLA-Cw*0702 binding histone H3.3:40-48 peptide were added to each culture. Aliquots containing 2 x 10^5 TIL were removed at time zero and every two hours up to 6 hours from each culture and tested for IFN-γ transcript level by quantitative real-time PCR.

*Detection of Cw*0702 surface expression by complement dependent cytotoxicity.*

HLA*Cw0702 levels of expression were judged by Complement Dependent Cytotoxicity (CDC) of cell lines previously cultured with or without IFN-α(500 mg/ml) for 48 hours.

HLA phenotyping of patients and cell lines.

Molecular and serological analysis of patient and cell line HLA phenotypes was performed by CDC, sequence-specific primer PCR or automated sequencing as appropriate (9).

Results

Identification of MAGE-12 reactivity in TIL and circulating lymphocytes from patient F001 who experienced temporary regression of cancer in response to MDA-specific vaccination.

F001-TIL and F001-MEL have been previously characterized (9). These lines were expanded simultaneously from a FNA of a melanoma metastasis that had originally regressed in response to vaccination with the HLA-A*0201-associated gp100:209-217(210M) peptide administered subcutaneously in incomplete Freund's adjuvant. F001-MEL lacked expression of gp100/PMel17 and other MDA and retained expression of TSA of the MAGE family. Twelve identical clones from that FNA (combined here as F001-TIL) recognized the HLA-Cw*0702 associated MAGE-12:170-178 epitope. In this study we tested whether the immune reactivity against MAGE-12 observed within the tumor was associated with detectable systemic reactivity toward the same epitope. PBMC obtained from the patient at the time of the FNA were stimulated with MAGE-12:170-178, MART-1, gp100/PMel17 (abbreviated as g209 in the figures and text) or gp100:209-217(210M) (abbreviated as g209-2M in the figures and text). The immune reactivity elicited toward the various TA was compared by documenting the kinetics of specific IFN-γ transcription by various cultures in response to relevant stimulation (23) (Figure 1A). No TA-specific reactivity could be detected directly in PBMC. One week after the first stimulation, CTL cultures elicited by stimulation with g209 or g209-2M demonstrated specific expression of IFN-γ mRNA. Specific reactivity toward either MAGE-12 or MART-1/MelanA appeared only after two weeks and one re-stimulation. This is not surprising since the patient had recently received g202-2M-based vaccine. Interestingly, the reactivity toward MAGE-12

paralleled reactivity toward MART-1/MelanA. Phenotypic characterization of the CTL induced by stimulation of PBMC with MAGE-12:170-178 demonstrated selective expansion of a Vβ7s2 identical to the Vβ expressed by F001-TIL (10). This suggested that systemic and local responses were mediated by expansion of an identical T-cell clone.

Expression of TA on HLA class I alleles by F001-MEL.

F001-MEL expressed most MDA including gp100/Mel17 and MART-1/Melan A below the threshold of recognition by high-avidity TA-specific CTL (20). However, this tumor cell line retained expression of most TSA (9, 10) and could be still recognized by F001-TIL. Here we further characterized the level of expression of MAGE family genes in this and other tumor cell lines (Table IA). Notably, F001-MEL expressed most of the MAGE family genes. The only MAGE genes not expressed by F001-MEL (MAGE-5, 7 and 8) were not expressed by any other melanoma cell line suggesting that these genes are uncommonly expressed in the context of metastatic melanoma. Since the amount of expression of a given TA might influence its potential immunogenicity, we used quantitative real-time PCR to test whether F001 expressed unusual amounts of MAGE-12 compared with other melanoma cell lines (Table IB). F001-MEL did not appear to express unusually high amounts of MAGE-12 transcript compared with other melanoma cell lines, suggesting that levels of MAGE-12 expression were not, by themselves, a factor responsible for the development of MAGE-12 reactive TIL in this patient.

Since the level of HLA class I expression modulates T-cell recognition of tumor cells (25), it is possible that F001-MEL expressed unusually high levels of HLA-Cw*0702 associated with the MAGE-12:170-178 recognition. CDC suggested that HLA-Cw*0702

surface expression was relatively higher in F001-MEL than most melanoma cell lines since it could be detected without preconditioning with IFN-α or IFN-γ (24). However, this did not appear to play a critical role in eliciting F001-TIL reactivity. Other melanoma cell lines, characterized by undetectable levels of HLA-Cw*0702 expression by CDC could elicit similar IFN-γ release. Of six cell lines tested, three (624.28, 624.38 and SK-23) had HLA-Cw*0702 surface density below the threshold of detection by CDC without pre-treatment with IFN-α and became susceptible to CDC after such a treatment. The other three (938-MEL 1280-MEL and F001-MEL) were susceptible to CDC independent of IFN-α pre-treatment. However, all cell lines triggered similar levels of IFN-γ expression when co-cultured with F001-TIL. Thus, the most significant functional difference between this tumor cell line and other archival cell lines was the reduced expression of MDA.

The expression of MAGE-12 in a panel of normal tissues revealed only trace levels of transcript in lungs and high levels in the testis at the highest concentration of cDNA (approximately 1 ng). At 0.1 ng MAGE-12 expression was barely identified in lung tissue but was still clearly identifiable in the testis. MAGE-12 expression was not observed in any other normal tissue including brain, heart, kidney, spleen, liver, colon, small intestine, muscle, stomach, placenta, salivary gland, thyroid gland, adrenal gland, pancreas, ovary, uterus, prostate, skin, PBL, bone marrow, fetal brain and fetal liver (data not shown).

Effect of antigen presentation by tumor cell lines on MAGE-12 recognition.

To evaluate whether loss of expression of MDA by F001-MEL might have facilitated the detection of MAGE-12:170-178 reactivity, we repeatedly stimulated *in vitro* post-vaccination PBMC from patient F001 with allogeneic tumor cells as previously

described (22). PBMC received repeated stimulation *in vitro* with a melanoma cell line expressing MAGE-12, MART-1/MelanA and gp100/PMel17 and HLA-A*0201/Cw*0702 alleles (624.38-MEL). Parallel PBMC cultures were also performed with 624.28-MEL, a clone from the same cell line with identical pattern of expression of TA and HLA-Cw*0702 but that had lost expression of HLA-A*0201, the molecule associated with the presentation of the MDA-derived immunodominant epitopes. The purpose of this experiment was to test whether lack of stimulation with supposedly "immunodominant" epitopes of gp100 or MART-1 could facilitate the identification of other cryptic epitopes. Since MART-1 and gp100 immune dominance has been principally associated with peptides presented in the context of HLA-A*0201, we selected two clones, 624.28 and 624.38, that express identical amounts of MART-1 and gp100. Both were derived from 624-MEL, however 624.28 has lost expression of HLA-A*0201 and cannot present peptides in the context of this molecule(17). In our opinion this model has the advantage of subtracting the expression only of the gp100-HLA-A*0201 and the MART-1/HLA-A*0201 associated epitopes maintaining the immunogenic potential of these two antigens in association with other HLA alleles shared with the patient's PBMC (HLA-B*0702 and Cw*0702). In three separate experiments, stimulation of PBMC with 624.28 allowed detection of MAGE-12-specific, but not MART-1/MelanA-specific, CTL that could recognize the parental 624-MEL cell line (Figure 2, gray bars). In parallel experiments PBMC stimulated with 624.38 failed to demonstrate MAGE-12 specificity, developed MART-1/MelanA reactivity and could also recognize 624-MEL (Figure 2, white bars). The recognition of 624-MEL by the 624.28-MEL induced, but not the 624.38-induced, CTL could also be enhanced by pulsing with MAGE-12:170-178. Thus, the lack of

induction of HLA-A*0201-associated activity in 624.28-elicited cultures might have facilitated the detection of reactivity toward MAGE-12:170-178, most often masked by the coexistence of HLA-Cw*0702 and HLA-A*0201 within the same ancestral haplotype (26).

*Immune reactivity toward MAGE-12 in HLA-Cw*0702 bearing individuals with metastatic melanoma.*

MAGE-12:170-178 immune reactivity in patient F001 was then compared to that of three other HLA-A*0201/-Cw*0702-expressing patients with advanced melanoma who, unlike patient F001, were vaccine-naïve. PBMC from F001 and the other patients were repeatedly stimulated with MAGE-12:170-178 and the HLA-A*0201 associated Flu M1:58-66 epitope, an irrelevant peptide (Figure 3). Although it was possible to elicit brisk FluM1-specific responses in all patients, MAGE-12:170-178-specific responses could be detected only in patient F001.

Epitope spreading following successful vaccination with a MDA-specific epitope induces MAGE-12:170-178 reactivity.

To evaluate whether the unusual detection of a MAGE reactive TIL was related to the recent vaccination, we compared MAGE-12 recognition by PBMC obtained from patient F001 before and after the treatment had induced temporary tumor regression. This was done by comparative *in vitro* stimulation of pre- and post-vaccination PBMC with the MAGE-12:170-178 peptide. In three consecutive experiments we could not induce MAGE-12 reactivity in PBMC obtained before vaccination. However, we consistently identified MAGE-12:170-178-specific reactivity in PBMC obtained after two vaccinations

with g209-2M that had caused a dramatic, although temporary, shrinkage of the tumor (Figure 1B). This data suggests that during the period lasting from when the pre-immunization sample and the post-immunization samples were obtained, a stimulus secondary to the vaccination induced an increase in circulating precursor T-cells capable of responing to *in vitro* stimulation with MAGE-12:170-178. Since only the tumor tissues expressed MAGE-12, it is reasonable to suggest that the enhancement of MAGE-12 reactivity in circulating T-cells was related to events occurring within the tumor environment in response to the vaccine.

MAGE-12:170-178 analog peptides from other MAGE-1 genes do not interfere with MAGE-12:170-178 immunogenicity.

We then tested whether various MAGE family genes encompassing peptide sequences analogous to MAGE-12:170-178 could interfere in MAGE-12 immunogenicity. All of the members of the MAGE family have sequences which are homologous to MAGE-12:170-178. In particular, MAGE-2 and MAGE-3 have sequences which differ in only two to three residues from MAGE-12:170-178 and these differences are compatible with the HLA-Cw*0702 binding motif (9). Assuming similar binding affinity of these homologous sequences to HLA-Cw*0702, it is possible that these natural analogs might exert agonist or antagonist effects on MAGE-12:170-178 stimulatory properties, as has been shown for other artificial analog peptides in the context of MART-1/MelanA (13, 15). As a consequence, different patterns of MAGE gene expression by various tumors might contribute to global differences in immune reactivity toward determinants sharing sufficient homology. To test the possibility of interference of these epitopes with F001-TIL

reactivity, we co-cultured this TIL with 293-HEK cell line (pretreated with IFN-α to induce HLA-Cw*0702 expression) which was pulsed with 1 μM concentrations of analogs from MAGE-1, MAGE-2, MAGE-3, MAGE-4a and MAGE-6. Lack of interference in recognition of MAGE-12:170-178 would exclude competitive or synergistic effects of the peptides whether due to lack of binding to HLA or to direct agonistic/antagonistic effects. None of the analogs could stimulate F001-TIL. To test for MAGE-12:170-178 antagonism, F001-TIL was exposed to various analogs pulsed onto IFN-α treated 293-HEK cells for 24 hours. TIL were then re-exposed to MAGE-12:170-178 or an HLA-Cw*0702-associated control epitope (histone H3.3:40-48). No modulation of IFN-γ transcription in response to stimulation with the cognate MAGE-12:170-178 was produced by any of the analogs (data not shown). Thus, it is unlikely that competing analogs from other MAGE family gene products play a significant role in modulating MAGE-12 responses in this or other patients.

Discussion.

The frequent identification of MART-1/MelanA and gp100/PMel17 reactive CTL from patients with metastatic melanoma expressing HLA-A*0201 suggests that these TA may play an "immune dominant" role in the context of this HLA class I restriction element. In particular, MART-1/MelanA immune dominance is restricted to a unique epitope (MART-1:27-35) and the molecular basis for this phenomenon remains unclear (8). Conflicting theories have been evoked to explain the predominant role that this TA seems to play. We noted that MART-1/MelanA reactivity is more readily elicited in patients with melanoma compared with normal individuals (27). This finding suggested that tumors can prime the host immune reactivity and, since MART-1/MelanA is also expressed by normal melanocytes, that the tumor microenvironment might provide a quantitatively and/or qualitatively stronger stimulus for the host immune system than the epidermis. Others confirmed this finding and suggested that differences in immune reactivity between normal and melanoma bearing individuals correlate with a respective predominance of naïve and memory CTL in the two populations (28, 29).

Loftus et al. (13) suggested that MART-1:27-35 immune dominance might be explained by biochemical commonalties shared by MART-1:27-35 and analog epitopes to which individuals might become repeatedly exposed during their life time. Others have suggested a central explanation for MART-1/MelanA immune dominance. MART-1:27-35 is a peptide sequence characterized by low affinity for HLA-A*0201 and, therefore, negative TCR selection toward this epitope might be less strictly enforced during embryogenesis (21).

It is now clear that immune reactivity toward cancer can be influenced by peripheral stimuli artificially provided in the form of wild type or modified epitope determinants (25, 30). This is well illustrated by the acquisition of gp100/PMel17 reactivity by patient F001 in response to vaccination. However, evidence that human tumors can naturally shape the host immune reactivity has remained indirect (8, 28, 29). Pre-clinical models suggest that epitope spreading occurs in association with tumor rejection following vaccination with a single immunodominant CTL epitope (16). Thus, under immune-mediated perturbations of the tumor microenvironment, an additional signal is induced that complements the weak signal ordinarily provided by TA-bearing cancer cells (2). Antigen spreading, however, has never been demonstrated to occur in response to anti-cancer treatments in humans. This study documents the positive conversion of a patient's immune reactivity toward a TSA (MAGE-12) following successful vaccination utilizing a single epitope from another TA (**see Figure 1 b**). Since MAGE-12 is not expressed in normal tissues (31), this finding can best be explained by priming of the immune system by vaccine-induced inflammatory reactions within the tumor. Alternatively, tumor antigens shed by dying tumor cells could have been transported to regional lymph nodes where this induction could have occurred. Since it is not known whether MAGE-12 was expressed by the tumor before vaccination, it cannot definitively be concluded that the only change occurring during treatment was related to co-stimulatory factors. However, MAGE-12 is expressed in approximately 80% of melanoma metastases and the short-term kinetics of its expression in response to immune manipulation appears to be extremely stable (32). The expression of the associated HLA-Cw*0702 allele might have played a role in the emergence of this unusual reactivity. However, increases in the

constitutive expression of HLA alleles are unusual in cancer unless an altered cytokine microenvironment (i.e. increased IFN-γ levels) could have been induced by immune cells accrued by the vaccine.

The most significant implication of this study is that tumor microenvironment or the draining lymph nodes can influence, under appropriate conditions, the host immune reactivity and that systemically documented immune reactivity is a vestige of a past immunogenic eruption. CTL elicited from PBMCs shared TCR utilization with F001-TIL suggesting a common origin for the tumor-reactivity seen in this patient (10). It remains unclear how the tumor cells, whose immunogenicity resounded systemically, could escape the immune effects they created. To the best of clinical testing, this patient was the bearer of only this tumor mass suggesting that the immune reactions caused by the vaccine converged in the lesion studied. It is possible that upon temporary disappearance of the lesion the priming conditions created by the vaccine extinguished and failed to sustain a clinically effective immune reactivity. Exogenous sustenance of such reactivity by vaccines might provide the continuity of stimulation that the tumor microenvironment seems to lack. The demonstration of epitope spreading may have also practical implications because it suggests that alternatives are available when loss of TA targeted by vaccines occurs. In particular, the identification of MAGE-12:170-178 might be of broader significance than previously suspected as this epitope has recently been shown to play a role as antigen in cancers other than melanoma (33).

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Figure legends

*Figure 1. **A.*** Detection of epitope-specific IFN-γ transcript by quantitative real-time PCR in PBMC (time 0) and CTL cultures (at various time points) obtained from patient F001 after two subcutaneous administrations of g209-2M peptide in incomplete Freund's adjuvant. CTL were sensitized *in vitro* by repeated weekly exposure to 1 μM MAGE-12:170-178, MART-1:27-35, g209 or g209-2M peptides and interleukin-2 (300 IU/ml). Epitope-specificity was tested by exposing the T-cell cultures to relevant and irrelevant (Flu M1:58-66) 1 μM peptide for two hours before preparation of cDNA for assessment of IFN-γ transcript by quantitative real-time PCR. The labels describe the peptide used for PBMC induction / peptide used for specificity testing. Data are presented as average of four experiments ± SEM. Student t-test p-value for data after a second and third in vitro sensitization are all < 0.001 when relevant (solid symbols) stimulation is compared to the respective irrelevant (open symbols) stimulation with Flu. ***B.*** Detection of epitope-specific IFN-γ transcript by quantitative real-time PCR in PBMC obtained from patient F001 before and after vaccination with g209-2M peptide in incomplete Freund's adjuvant as in panel ***A***. PBMC were sensitized *in vitro* by repeated weekly exposure to MAGE-12:170-178, g209 and g209-2M peptides and interleukin-2 (300 IU/ml). Epitope-specificity was tested by exposing the T-cell cultures to 1 μM peptide for two hours before preparation of cDNA for assessment of IFN-γ transcript. In both panels, results are normalized as IFN-γ mRNA copies per 10^6 CD8 mRNA copies (23). Data are presented as average of four experiments ± SEM. Student's t-test p-value for pre- vs. post-vaccination culture results < 0.001 for all specificities presented.

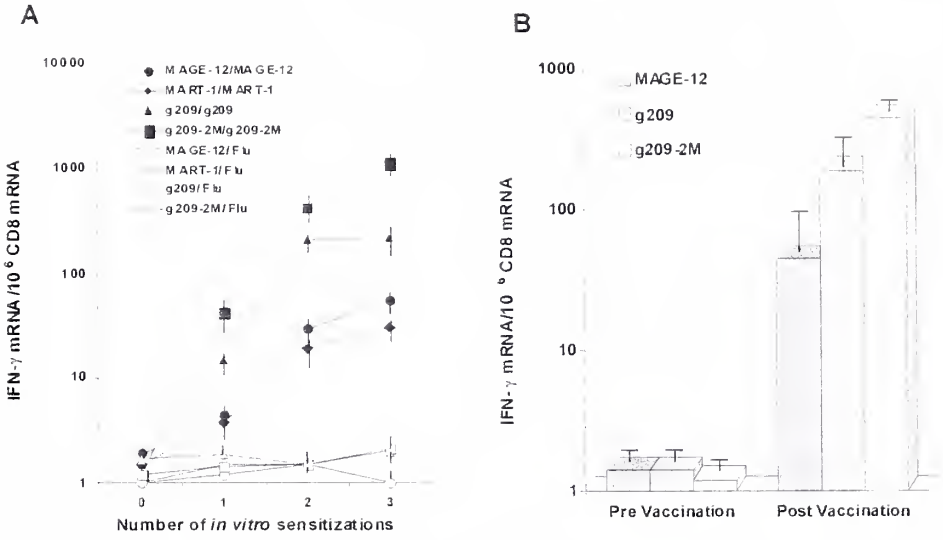
A
10000
1000
100
10
1
IFN-γ mRNA/10^6 CD8 mRNA
MAGE-12/MAGE-12
MART-1/MART-1
g209/g209
g209-2M/g209-2M
MAGE-12/Flu
MART-1/Flu
g209/Flu
g209-2M/Flu
0
1
2
3
Number of in vitro sensitizations
B
1000
100
10
1
IFN-γ mRNA/10^6 CD8 mRNA
MAGE-12
g209
g209-2M
Pre Vaccination
Post Vaccination

Figure 2. Modulation of epitope-specific T-cell induction by stimulation with melanoma cells expressing different HLA class I alleles. Patient F001 PBMC were stimulated weekly in presence of IL-2 (300 IU/ml) with the HLA-A*0201 and Cw*0702 expressing cell line clone 624.38 MEL (white bars) or a clone (624.28 MEL) characterized by HLA-A*0201 allelic loss (17) but retaining expression of Cw*0702 (gray bars). These clones have been previously characterized and express similar amounts of MART-1/MelanA and MAGE-12. After three stimulation the cultures were tested by two-hour exposure to 1 μM MART-1:27-35, 1μM MAGE-12:170-178 or to 624-MEL pretreated with IFN-α (500 μg/ml x 48 hours) to enhance HLA class I molecule expression. In addition, 624-MEL was pulsed with 1μM MAGE-12:170-178 (624-MEL + MAGE). Results are presented as ratio of IFN-γ mRNA copies per 10^6 CD8 mRNA copies in tested sample over the same parameter in cultures not exposed to peptide. Data are presented as average of three experiments ± SEM. Student t-test p-value comparing cultures induced with 624.28 with those induced with 624.38 are as follows: <0.05 for MART-1, <0.001 for MAGE-12, non-significant for 624-MEL and <0.01 for 624-MEL exogenously loaded with 1 μM MAGE-12:170-178 peptide

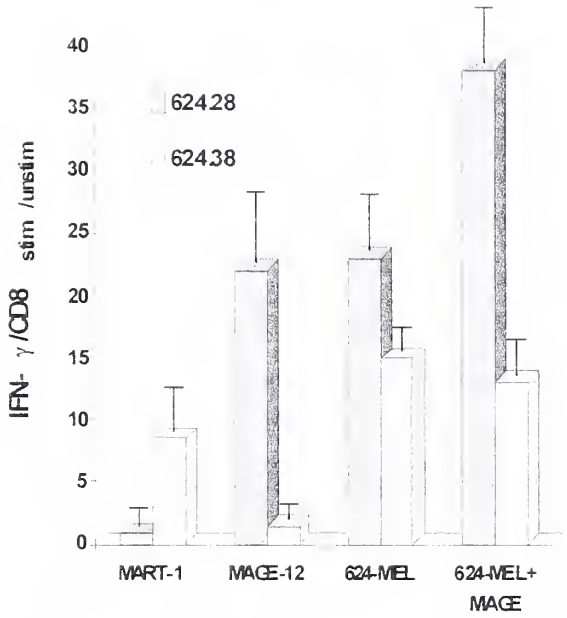
IFN- γ/CD8 stim /unstim
40
35
30
25
20
15
10
5
0
6242.8
6243.8
MART-1
MAGE-12
624-MEL
624-MEL+ MAGE

Figure 3. Induction of MAGE-12:170-178 reactivity in PBMC from four HLA-Cw*0702 expressing patients with metastatic melanoma. PBMC from four patients with metastatic melanoma expressing HLA-Cw*0702 (as well as HLA-A*0201) were stimulated weekly with 1μM MAGE-12:170-178 peptide or 1μM Flu M1:58-66 peptide and interleukin-2 (300 IU/ml). Patient # 1 is F001. Epitope-specificity was tested by exposing the T-cell cultures to 1μM MAGE-12:170-178 or Flu M1:58-66 peptide for two hours before preparation of cDNA for assessment of IFN-γ transcript by quantitative real-time PCR. Results are presented as IFN–γ mRNA copies per CD8 mRNA copies in tested sample over the same parameter in cultures not exposed to peptide. Data are presented as average of three experiments ± SEM.

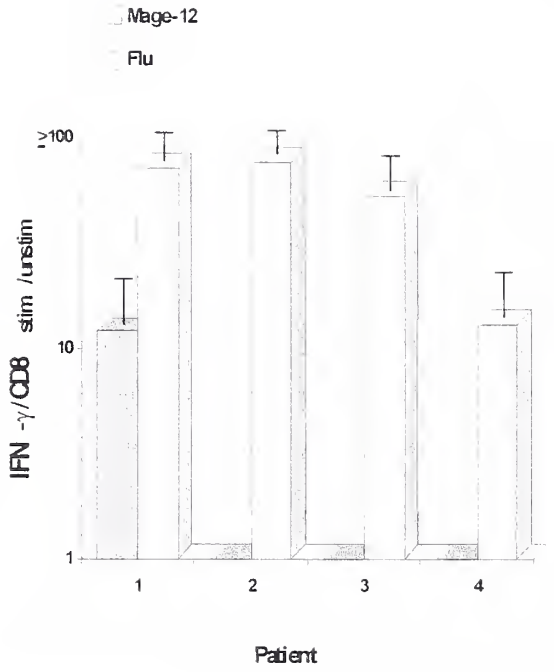
Mage-12
Flu
>100
10
1
IFN -γ/CD8 stim /unstim
1
2
3
4
Patient

Table I

Quantitative and qualitative assessment of MAGE gene expression in melanoma cell lines

	A												B
Cell Line	MAGE protein												MAGE 12
	1	2	3	4a	5	6	7	8	9	10	11	12	
NHEM	-	-	-	-	-	-	-	-	-	-	-	-	1
F001-MEL	+	+	+	+	-	+	-	-	+	+	+	+	590
F001-EBV	-	-	-	-	-	-	-	-	-	-	-	-	4
F002-MEL	+	+	+	+	-	+	-	-	-	+	+	+	4700
F010-MEL	-	+	+	-	-	+	n.d.	n.d	-	-	-	+	1200
397-MEL	+	+	+	-	-	+	-	-	+	+	+	+	3800
537-MEL	+	+	+	+	-	+	-	-	-	+	-	+	1500
624.28-	+	+	+	+	-	+	-	-	+	+	+	+	n.d.
624.38-	+	+	+	+	-	+	-	-	+	+	+	+	1500
836-MEL	+	+	+	+	-	+	-	-	-	+	+	+	1100
888-MEL	+	+	+	-	-	+	-	-	+	+	-	+	n.d.
938-MEL	+	+	+	+	-	+	-	-	+	+	+	+	1000
1102-MEL	-	+	+	+	-	+	-	-	-	-	-	+	550
1123-MEL	-	-	+	-	-	-	-	-	-	-	-	-	15
1280-MEL	-	+	+	-	-	+	-	-	-	-	-	+	15
1359-MEL	-	+	+	-	-	+	-	-	-	-	-	+	51
1495-MEL	+	+	+	+	-	+	-	-	+	+	+	+	1850
A375-MEL	+	+	+	+	-	+	-	-	-	+	+	+	250
SK-23-	-	+	+	-	-	+	-	-	-	-	-	+	1200

A. The identity of amplified products was confirmed by sequencing using PCR products from F001-MEL. (n.d. = not done)

B. Expression of MAGE-12 mRNA copies was measured quantitatively by real-time PCR as MAGE-12 mRNA copy number over β–Actin mRNA copy number. Data were further normalized by providing a ratio of MAGE-12/β-Actin transcript levels in cell lines over that of normal human epithelial melanocytes (NHEM). Each sample was analyzed in duplicate and average values are presented.

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