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Antigen Spreading Contributes to MAGE Vaccination-Induced Regression of Melanoma Metastases

Véronique Corbière[1], Jacques Chapiro[1,2], Vincent Stroobant[1,2], Wenbin Ma[1,2], Christophe Lurquin[1,2], Bernard Lethé[1,2], Nicolas van Baren[1,2], Benoît J. Van den Eynde[1,2], Thierry Boon[1,2], and Pierre G. Coulie[1]

Abstract

A core challenge in cancer immunotherapy is to understand the basis for efficacious vaccine responses in human patients. In previous work we identified a melanoma patient who displayed a low-level antivaccine cytolytic T-cell (CTL) response in blood with tumor regression after vaccination with melanoma antigens (MAGE). Using a genetic approach including T-cell receptor β (TCRβ) cDNA libraries, we found very few antivaccine CTLs in regressing metastases. However, a far greater number of TCRβ sequences were found with several of these corresponding to CTL clones specific for nonvaccine tumor antigens, suggesting that antigen spreading was occurring in regressing metastases. In this study, we found another TCR belonging to tumor-specific CTL enriched in regressing metastases and detectable in blood only after vaccination. We used the TCRβ sequence to detect and clone the desired T cells from tumor-infiltrating lymphocytes isolated from the patient. This CD8 clone specifically lysed autologous melanoma cells and displayed HLA-A2 restriction. Its target antigen was identified as the mitochondrial enzyme caseinolytic protease. The target antigen gene was mutated in the tumor, resulting in production of a neoantigen. Melanoma cell lysis by the CTL was increased by IFN-γ treatment due to preferential processing of the antigenic peptide by the immunoproteasome. These results argue that tumor rejection effectors in the patient were indeed CTL responding to nonvaccine tumor-specific antigens, further supporting our hypothesis. Among such antigens, the mutated antigen we found is the only antigen against which no T cells could be detected before vaccination. We propose that antigen spreading of an antitumor T-cell response to truly tumor-specific antigens contributes decisively to tumor regression. *Cancer Res; 71(4); 1253–62. ©2011 AACR.*

Introduction

Melanoma patients with measurable metastatic disease have been vaccinated with defined tumor-specific antigens, with clear but limited clinical benefit. Tumor regressions that were sometimes complete and durable have been observed in only about 10% of vaccinated patients. The present work is part of our effort to understand the mechanisms of these regressions.

Focusing on patients vaccinated with an antigenic peptide encoded by gene *MAGE-A3* and presented by HLA-A1, antivaccine cytolytic T-cell (CTL) responses were monitored with a sensitive method (1). Antivaccine CTL responses were detected in 9 of 16 evaluated patients who showed tumor regression, and in 1 of 29 who did not (2–4). Six of the 10 detectable responses provided a blood frequency of antivaccine CTL precursors (CTLp) below 10^{-5} of the CD8 T cells, and T-cell receptor (TCR) analyses indicated that 7 of 10 responses were monoclonal. Even though the detection of antivaccine CTL responses correlated with the observation of tumor regressions, the low frequencies of these CTLs appeared insufficient to provide all the effectors of the tumor rejections.

We therefore used blood lymphocytes stimulated with autologous tumor cells to broaden our analysis to CTLs recognizing other tumor antigens. In all 7 patients tested, antitumor CTLps were found at high frequencies, from 10^{-4} to 3×10^{-2} of blood CD8 cells (5). Surprisingly, they were already present at high frequency prior to vaccination.

To evaluate the contributions to tumor rejection of the antivaccine and of these other "antitumor" CTLs, their specificities and distribution into tumors were analyzed in melanoma patient EB81. This patient showed complete regression of cutaneous metastases following vaccination with a recombinant canarypox virus containing a minigene encoding antigenic peptides MAGE-A3$_{168-176}$ and MAGE-A1$_{161-169}$, and booster injections of the peptides (6). The frequency of antivaccine CTL was 3×10^{-6} of the blood CD8 T cells (2), whereas those of antitumor CTL were 3×10^{-4} and 3×10^{-3} before and after vaccination, respectively (5). Antitumor CTL clones were established that recognized antigens encoded by genes *MAGE-C2* and *gp100* (5). T lymphocytes infiltrating metastases were analyzed using both TCR-specific PCR amplifications and

Authors' Affiliations: [1]de Duve Institute, Université Catholique de Louvain and [2]Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium

Corresponding Author: Pierre G. Coulie, de Duve Institute, Université catholique de Louvain, Avenue Hippocrate 74, UCL 7459, B-1200 Brussels, Belgium. Phone: 32-2-7647581; Fax: 32-2-7647590.
E-mail: pierre.coulie@uclouvain.be

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TCRβ cDNA libraries prepared with RNA extracted from the tumor samples (7). Analysis of TCR sequence libraries indicated the presence of several highly repeated clonotypes, most of which corresponded to the antitumor CTL clones derived from blood lymphocytes. Another clonotype, which we called 101, caught our attention because it was present at high frequencies in metastases collected after vaccination but had not been detected, either in blood or in a metastasis, before vaccination.

In the present study we show that this TCR belongs also to a tumor-specific CTL, recognizing a mutated antigen. We will discuss how this observation, together with our previous results, leads us to propose that both ''clonal'' spreading and ''antigen'' spreading contribute to tumor regressions triggered by vaccination.

Materials and Methods

Patient and cell lines

Melanoma patient EB81 received 4 cutaneous vaccinations with a recombinant canarypox virus (ALVAC) carrying a minigene encoding antigenic peptides MAGE-3.A1 and MAGE-1.A1, followed by vaccinations with the peptides without adjuvant (6). Cell lines EB81-MEL.2, EB81-EBV-B, LG2-EBV-B, and K562 were cultured in Iscove's medium (Life Technologies) supplemented with 10% fetal calf serum (FCS; MP Biomedicals), L-arginine, L-asparagine, and L-glutamine (AAG). Mixed lymphocyte peptide cultures (MLPC) and culture of CTL clones were carried out in Iscove's medium with AAG and human serum, referred to hereafter as complete medium. 293-EBNA cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS.

Derivation of CTL clone 101 and functional assays

Cells from the lymph node metastasis were labeled with anti-CD8 coupled to phycoerythrin and anti-Vβ5 coupled to fluorescein (BD Biosciences Pharmingen), sorted at 1 cell per well on a FACSVantage, and stimulated with 125 ng/mL of Phytohemagglutinin (PHA HA16, Murex Biotech Ltd.) or 5,000 irradiated (100 Gy) EB81-MEL.2 cells pretreated with IFN-γ (100 U/mL for 48 hours), and 50,000 irradiated allogeneic LG2-EBV-B cells in complete medium with 100 μmol/L methyltryptophan, 50 U/mL of recombinant human interleukin 2 (rhIL-2) 0.5 ng/mL of rhIL-4, and 10 ng/mL of rhIL-7. Lymphocytes were restimulated weekly with cytokines and irradiated tumor and feeder cells. Around day 14, aliquots of the microcultures were screened for TCRβ 101 with a clonotypic PCR (7). Lytic activity was tested in standard chromium release assays. For peptide recognition assays, ^{51}Cr-labeled EB81-EBV-B cells were incubated with peptides for 30 minutes at room temperature and washed before CTL addition at an effector-to-target (E/T) ratio of 10.

cDNA library screening and analysis of the *CLPP* transcript

The cDNA library of EB81-MEL.2 was prepared as described (8). Microcultures of 50,000 293-EBNA cells were cotransfected using Lipofectamine (Invitrogen) with 50 ng of vector pcDNA1/Amp containing an HLA-A*0201 sequence and 50 ng of plasmid DNA extracted from 768 pools of ± 100 bacterial colonies. CTLs (5,000) were added after 24 hours, and after an overnight coculture, TNF produced in the supernatant was measured with the WEHI-164c13 bioassay (9, 10).

To analyze the nucleotide variation of cDNA clone 57/2, cDNA from EB81-MEL.2 tumor cells and blood mononuclear cells was used in PCR amplifications with OPC1583 (5′-CATCCAGGCAGAGGAGATCAT) and OPC1584 (5′-GCCCAGCTCTCAGGTGCT; Fig. 4). PCR products were purified and sequenced. The effect of IFN-γ on *CLPP* expression was evaluated by quantitative reverse transcriptase PCR (RT-PCR) with OPC1583, OPC1584, and FAM-5′AGCCTGCAGGTGATCGAGTCCGC-TAMRA.

Western blotting

Western blots were performed with either mouse monoclonal IgG2b anti–caseinolytic protease P (anti-CLPP) antibody (Sigma-Aldrich), and peroxydase-coupled goat antimouse anti-IgG2b (Jackson Immunosearch), or mouse anti-β-actin antibodies (Sigma) and peroxydase-coupled goat antimouse IgG (R&D Systems).

Anti-CLPP T-cell frequencies in blood

Peripheral blood mononuclear cells (PBMC) were thawed in medium with 10 μg/mL of DNase I (Roche) and resuspended in PBS containing DNase I, HLA-A2 tetramers folded with CLPP peptide ILDKVLVHL and coupled to phycoerythrin, and control A2 tetramers containing peptide MAGE-C2$_{336–344}$ ALKDVEERV and coupled to phycoerythrin-Cy5. After 10 minutes, anti-CD8 antibodies coupled to allophycocyanin were added for 15 minutes more of incubation. Cells were then washed and fixed. New anti-CLPP CTL clones were derived using our MLPC/tetramer/cloning method as described (1).

Antigen processing experiments

Processing was analyzed in cell lines 293-SP and 293-IP, which were described previously and contain exclusively standard proteasomes or immunoproteasomes, respectively (11). 293-SP and 293-IP cells were plated at 80,000 cells per microwell 48 hours before cotransfection, using Lipofectamine, with 50 ng of pcDNA1/Amp containing the HLA-A2 construct and various amounts of pCEP4 containing *CLPP* or *Melan-A/MART-1* cDNA clones. After 24 hours, 1,500 cells of CTL clone 101 or 5,500 cells of anti-Melan-A.A2 CTL clone LB373-CTL-246/15 were added, in medium with 25 U/mL of IL-2. Cells transfected with the HLA-A2 construct alone were incubated with 0.3 μmol/L of the CLPP peptide or 3 μmol/L of Melan-A peptide EAAGIGILTV for 1 hour at 37°C before addition of CTL 101 and LB373-CTL-246/15, respectively. After 24 hours, supernatants were collected and their IFN-γ content measured by ELISA. For the *in vitro* digestion experiments, 20S standard or immunoproteasomes were purified from human erythrocytes and human LCL-721 cells treated over 7 days with 100 U/mL of IFN-γ, respectively (11). Digestions were performed by incubating 5 μg of CLPP precursor peptide EAQEFGILDKVLVHLPQDGE, which was 90% pure, with 7.5 μg of purified standard or immunoproteasomes in 50 μL of

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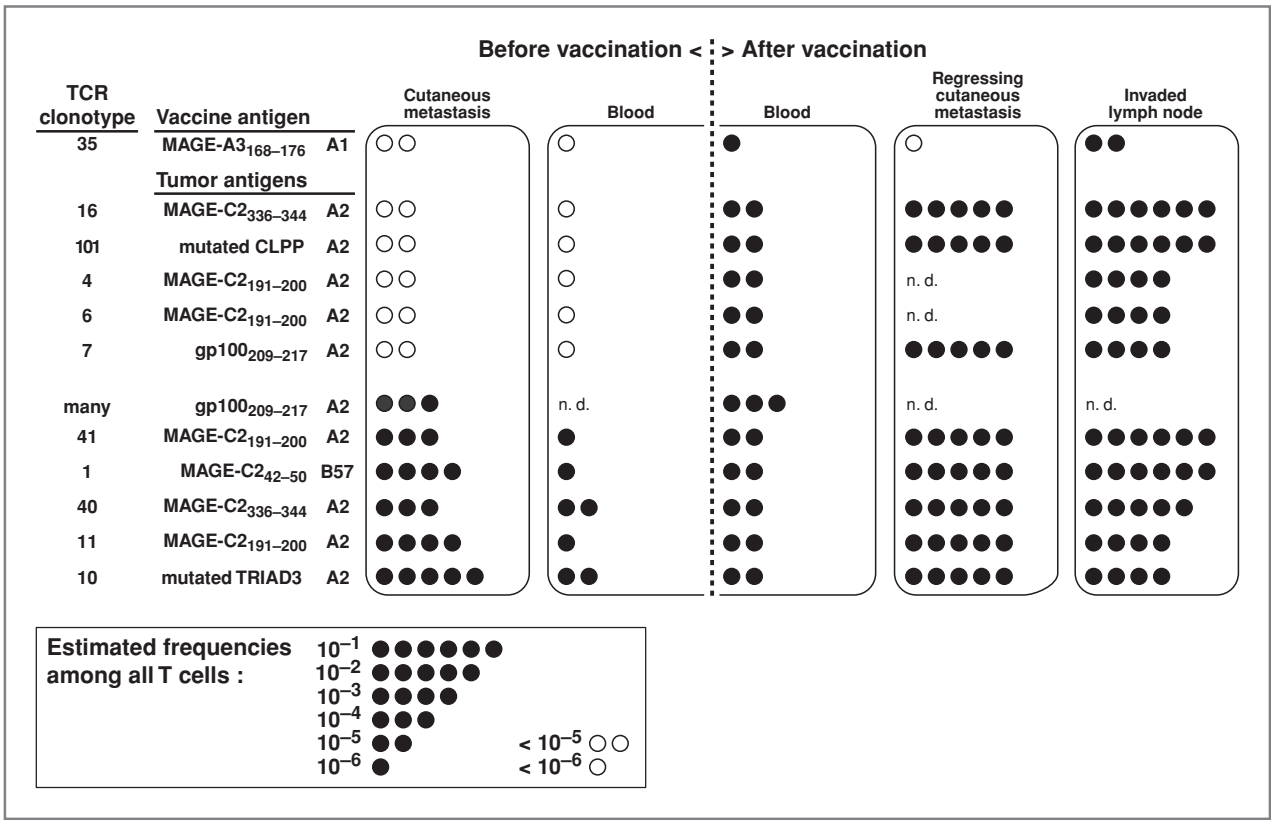

Figure 1. Overview of the distribution of antitumor CTL clonotypes in the blood and tumors of patient EB81. Frequencies among T lymphocytes are represented for the antivaccine CTL clone and for antitumor CTL clones grouped according to their undetectability or detectability before vaccination. Target antigens are indicated on the left: MAGE-C2 peptides presented by HLA-A2 (8) or HLA-B57 (45), peptide 209–217 from Pmel17/gp100 (5), mutated peptides from CLPP (this report), and TRIAD3 (Corbière et al., in preparation)

10 mmol/L Tris-HCl, pH 7.5, at 37°C. Aliquots of 10 μL were collected at each time point, and digestion stopped with 1 μL of 10% trifluoroacetic acid. The amount of residual precursor peptide was estimated by high-performance liquid chromatography/mass spectrometry (HPLC/MS) analysis. The kinetics of digestion differed significantly between the 2 types of proteasomes: about 85% of the precursor was degraded by standard proteasomes after 90 minutes and by immunoproteasomes after 36 hours. We observed similar differences with precursors of other antigenic peptides. For the T-cell recognition assay, the digests were lyophilized and resuspended in water. One thirtieth was added on 30,000 T2 cells for 1 hour at 37°C, before addition of IL-2 and 500 CTL. After 24 hours, the supernatants were collected for IFN-γ measurement.

Results

A summary of our observations in patient EB81 is presented in Fig. 1. The antivaccine T-cell response consisted of a single CTL clone recognizing peptide MAGE-A3$_{168–176}$ on HLA-A1 (2). This clone was not detected in a regressing metastasis, and was modestly enriched in a lymph node metastasis as compared with blood (7). Antitumor CTLs, which recognized tumor antigens distinct from the vaccine antigens, showed enormous enrichment in tumors, being about 10,000 times more frequent than the antivaccine T cells (7). They belonged to 2 groups according to their presence or absence before vaccination.

Obtaining a T-cell clone expressing TCR 101

Clonotype 101 was identified in TCRβ cDNA libraries prepared with RNA extracted from tumor tissues and did not correspond to any of the CTL clones derived from the blood (5, 7). The enrichment of TCR 101 in tumors relative to the blood suggested that it belonged to tumor-specific T cells. To verify this hypothesis, we first had to isolate a T-cell clone bearing this receptor.

TCR 101 corresponded to a Vβ5.5-Jβ2.7 rearrangement. A clonotypic PCR amplification was applied to cDNA extracted from CD4 or CD8 T cells purified from a lymph node metastasis resected from patient EB81 and in which we knew that TCR 101 was expressed (7). The results indicated that TCR 101 was expressed by CD8 T cells. To isolate a T-cell clone, cells of this metastasis were labeled with anti-CD8 and anti-Vβ5 antibodies. The double-stained cells, representing 4% of the CD8 lymphocytes, were cloned and restimulated with either phytohemagglutinin-A or autologous melanoma cells (Fig. 2). The best growing clones were screened by PCR for TCR 101 expression. Four CD8 T cell clones proved positive and CTL clone 734A/1 was kept for further experiments. We will refer to this clone as CTL 101.

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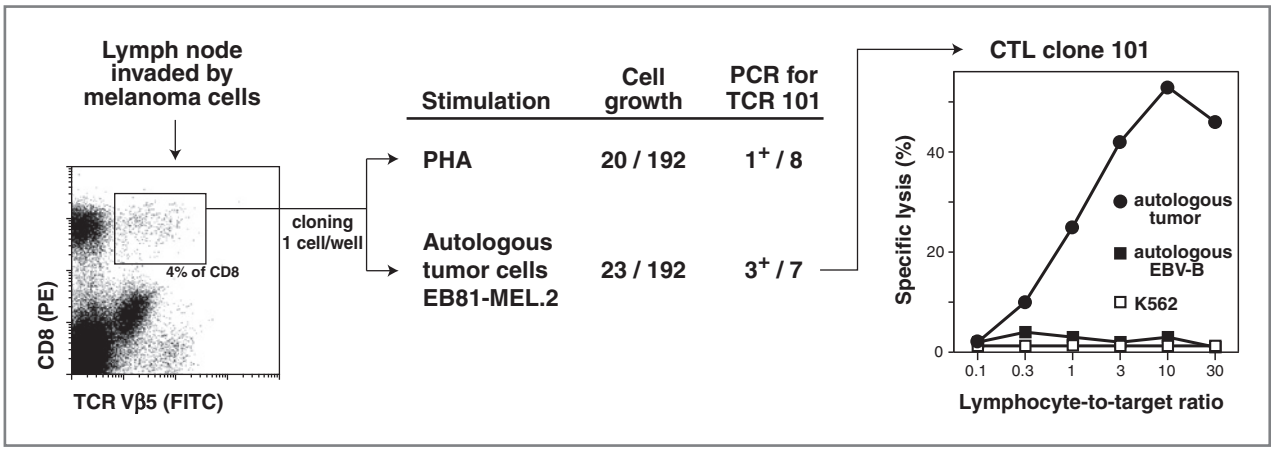

Figure 2. Derivation and lytic activity of CTL clone 101. Cells of a metastatic lymph node from patient EB81 were labeled with anti-CD8α and anti-TCR-Vβ5 antibodies. The $CD8^+$ $Vβ5^+$ T cells were sorted, cloned, and stimulated with either phytohemagglutinin (PHA) or irradiated autologous tumor cells pretreated with IFN-γ. All microcultures contained IL-2, IL-4, IL-7, and feeder cells. Cloning efficiencies were similar in the 2 sets of microcultures. The clonotypic PCR for TCR 101 was used on cDNA extracted from those clones that showed the best proliferation rates. The lytic activity of one clone expressing TCR 101 is shown. ^{51}Cr-labeled targets included EB81-MEL.2 cells, a clonal line derived from the EB81-MEL cells, treated over 2 days with IFN-γ, autologous EBV-B cells, and the NK target cells K562. Chromium release was measured after 4 hours.

CTL clone 101 lysed autologous tumor cells treated with IFN-γ, but did not lyse either autologous Epstein-Barr virus (EBV)-transformed B cells or natural killer (NK) target cells K562 (Fig. 2). It produced TNF and IFN-γ, but no IL-2, IL-4, IL-5, or IL-10 after stimulation with tumor cells, with a reduction in the presence of anti-HLA-A2 monoclonal antibody BB7.2 (data not shown). CTL 101 did not recognize any of 10 allogeneic HLA-A2 melanoma lines, nor did it recognize any antigenic peptide of a set corresponding to shared melanoma antigens presented by HLA-A2. We concluded that TCR 101 belonged to an HLA-A2-restricted CTL that appeared to recognize an individual antigen on the melanoma cells of patient EB81.

Identification of the gene coding for the antigenic peptide

A cDNA library prepared with mRNA of EB81-MEL.2 cells was cloned into expression vector pCEP4 (8). Plasmid DNA extracted from 768 pools of 100 recombinant bacteria was cotransfected into 293-EBNA cells with an HLA-A*0201 construct, and the transfectants were screened for recognition by CTL 101. Two pools were found positive and one was subcloned. cDNA clone 57/2 was obtained, which transferred antigen expression (Fig. 3). It was 876 bp long, contained a polyadenylation signal and a poly(A) tail, and corresponded to the gene coding for CLPP, a mitochondrial protein of 277 amino acids.

Identification of the antigenic peptide

The sequence of cDNA 57/2 differed in one nucleotide from the *CLPP* sequences present in databanks (BC002956 and NM006012). A cytidine-to-thymidine substitution changes a proline into leucine in the putative protein (Fig. 4A). Leucine at position 2 or at the carboxyterminus of a peptide can anchor it to HLA-A2 molecules (12). Here, the leucine residue resulting from the mutation corresponded to the carboxyterminus of a 9-amino acid peptide, ILDKVLVHL, containing another leucine at position 2. This peptide could sensitize $HLA\text{-}A2^+$ EBV-transformed B cells to lysis by CTL 101, with half-maximal effect at 1 nmol/L (Fig. 4B). ILDKVLVHL was the optimal antigenic peptide, as longer or shorter peptides were recognized much less efficiently. The wild-type peptide, with proline at its carboxyterminus, was poorly recognized, with half-maximal lysis requiring 10 μmol/L of peptide (Fig. 4B).

To confirm that the C-to-T transition was a mutation, cDNA prepared from the EB81-MEL.2 tumor cell line and from autologous blood mononuclear cells was used in PCR amplifications with primers shown in Fig. 4A. At the location of the substitution, the products amplified from blood cDNA contained only cytidine, whereas those amplified from tumor cDNA contained a mixture of cytidine and thymidine, confirming the presence of a mutation. The mutation was also present in cDNA prepared from a cutaneous metastasis resected from patient EB81, indicating that it had occurred *in vivo*.

We did not investigate the oncogenic potential of the CLPP mutation. CTL 101 did not recognize any of 10 HLA-A2

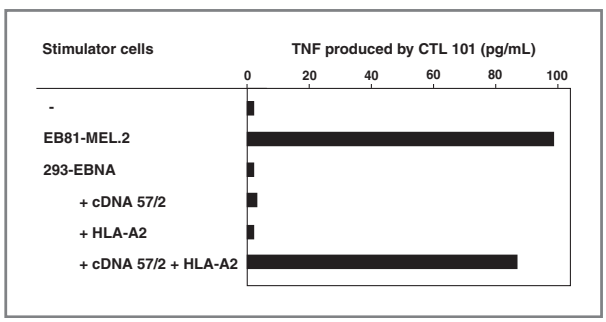

Figure 3. Identification of a cDNA clone encoding the target antigen of CTL clone 101. CTL 101 was stimulated by EB81-MEL.2 cells treated with IFN-γ, or by 293-EBNA cells cotransfected with vectors pCEP4 containing cDNA clone 57/2 and pcDNA3 containing an HLA-A*0201 sequence. TNF released in the medium was measured with a bioassay.

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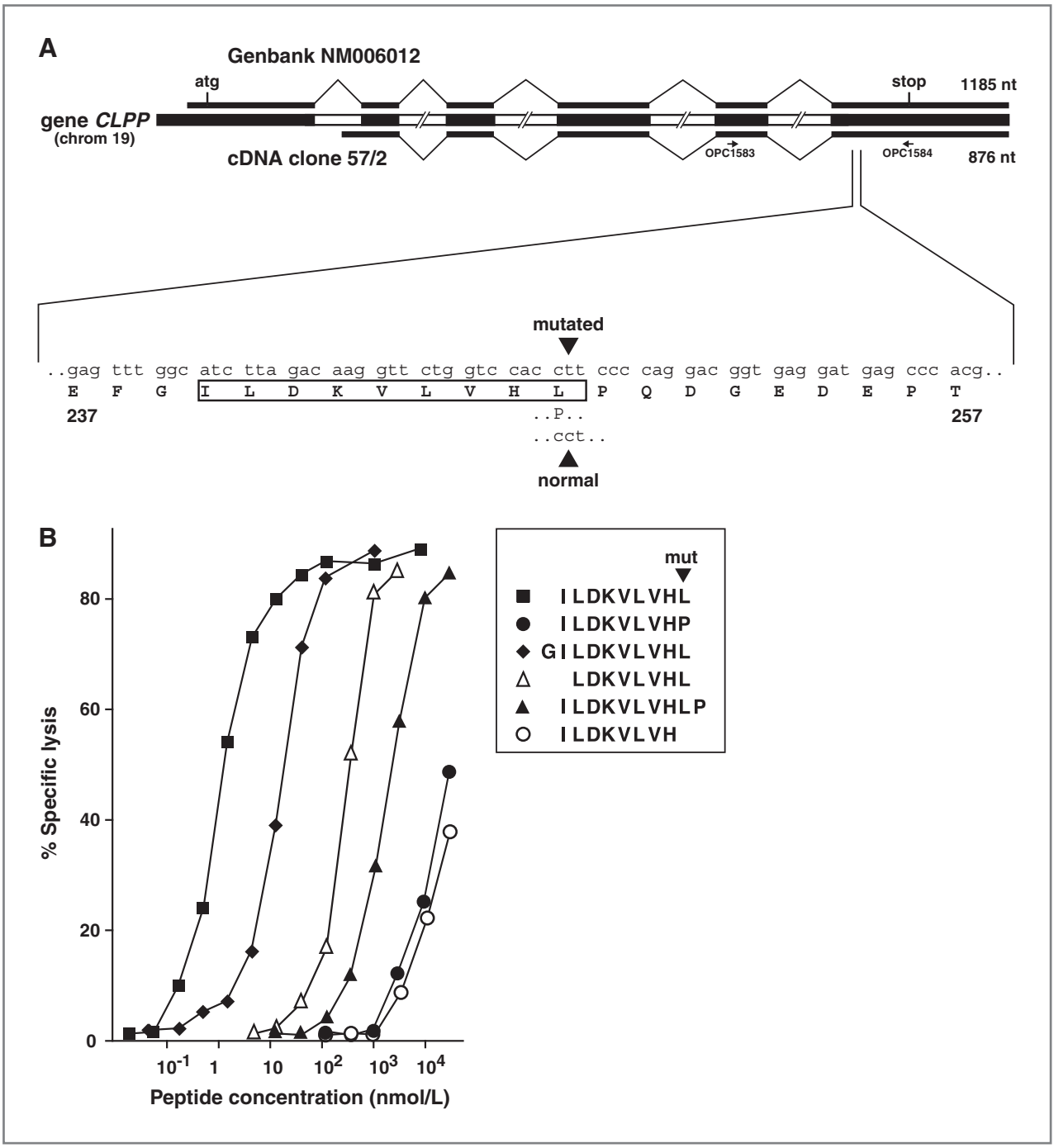

Figure 4. Identification of the antigenic peptide. A, gene *CLPP* is represented with introns and exons as open and filled boxes, respectively. The structure of cDNA clone 57/2 is compared with that of a complete cDNA sequence present in databanks (NM006012). The antigenic peptide is boxed. PCR primers are shown as arrows. B, titration of the antigenic peptide. ^{51}Cr-labeled autologous EBV-B cells were incubated for 30 minutes at room temperature with the indicated concentrations of peptides. CTL clone 101 was added at an E/T cell ratio of 10, and chromium release was measured after 4 hours.

melanoma lines, suggesting that the mutation is not frequent in such tumors. It is probably individual to the tumor of patient EB81, being one of the hundreds of passenger nonsynonymous mutations that can be present in a melanoma (13).

The immunologic consequence of the mutation, which changes proline into leucine at the peptide carboxyterminus, is probably to allow peptide processing, as most proteases do not cleave after proline. The mutation also creates an aggretope, as carboxyterminal leucine, but not proline, anchors antigenic peptides into HLA-A2 molecules (14).

cDNA 57/2 corresponds to an incompletely spliced *CLPP* transcript that contains part of intron 1 and lacks exon 1,

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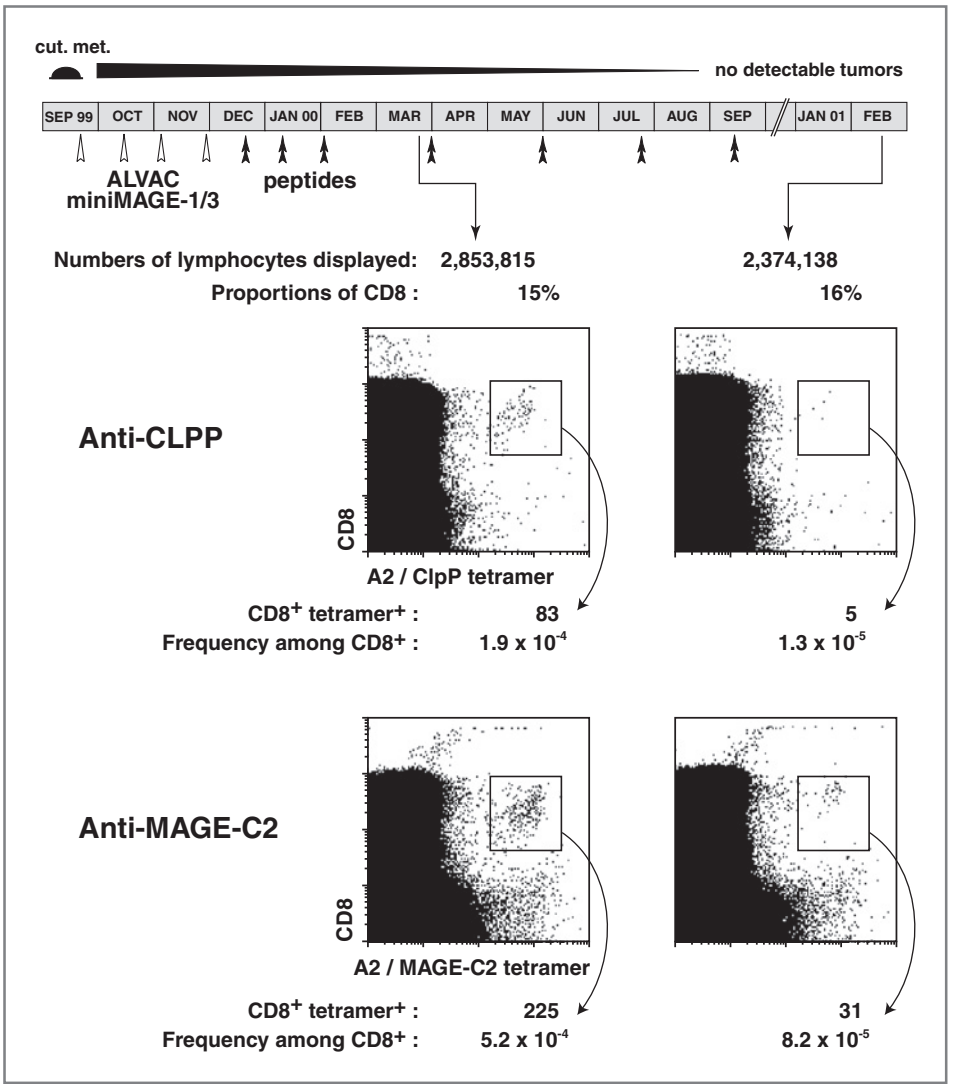

Figure 5. *Ex vivo* detection of anti-CLPP.A2 and anti-MAGE-C2.A2 T cells with tetramers. PBMCs collected from patient EB81 6 and 17 months after the onset of vaccination were labeled with HLA-A2 tetramers folded with the mutated CLPP peptide and coupled to phycoerythrin, HLA-A2 tetramers folded with peptide MAGE-$C2_{336–344}$ and coupled to phycoerythrin-Cy5, and anti-CD8 antibodies coupled to allophycocyanin. About 4 million cells were analyzed for each time point, and the plots include only lymphocytes gated on forward- and wide-angle light scatter. Clusters of $CD8^+$ $tetramer^+$ cells are boxed.

which codes for a mitochondrial targeting sequence of 56 amino acids (Fig. 4A). In cDNA 57/2 the first canonical initiation codon is in exon 2, in the CLPP reading frame. Thus its translated product is expected to be cytosolic instead of mitochondrial. Considering that it could be a misfolded defective ribosomal product (DRiP), targeted to proteasomes much more efficiently than the normal CLPP protein (15, 16), we verified that CTL 101 recognized cells transfected with a complete mutated *CLPP* transcript. Such a cDNA clone was found in the cDNA library, which transferred antigen expression (data not shown).

The anti-CLPP CTL response of patient EB81

A clonotypic PCR was used previously to estimate the frequency of CTL 101 at less than 7×10^{-7} and 10^{-4} of blood CD8 cells collected before and 6 months after the onset of vaccination, respectively (7). Labeling the same batch of post-vaccination PBMCs with an HLA-A2/CLPP tetramer indicated a frequency of 1.9×10^{-4} among CD8 cells (Fig. 5), suggesting that 50% of the anti-CLPP T cells had another TCR than CTL 101. We derived 4 independent anti-CLPP CTL clones from postvaccination blood lymphocytes: 2 clones expressed TCR 101 whereas the other 2 carried different TCRs, confirming polyclonality (data not shown).

In PBMCs collected 17 months after the onset of vaccination, when the patient had become clinically disease-free, anti-CLPP T cells could still be detected *ex vivo* with tetramers (Fig. 5). Their frequency had dropped more than 10-fold. A similar decrease was observed for CD8 T cells recognizing peptide MAGE-$C2_{336–344}$ (Fig. 5). In PBMCs collected 24, 36, and 48 months after the first vaccine injection, frequencies of anti-CLPP and of anti-MAGE-C2 T cells remained stable around 10^{-5} and 10^{-4} of the CD8 T cells, respectively (data not shown).

The role of IFN-γ in the presentation of the mutated CLPP antigen

We observed that efficient lysis of EB81-MEL.2 by CTL 101 required pretreatment with IFN-γ. Typically, lysis of untreated tumor cells did not exceed 20%, reaching 60% with IFN-γ

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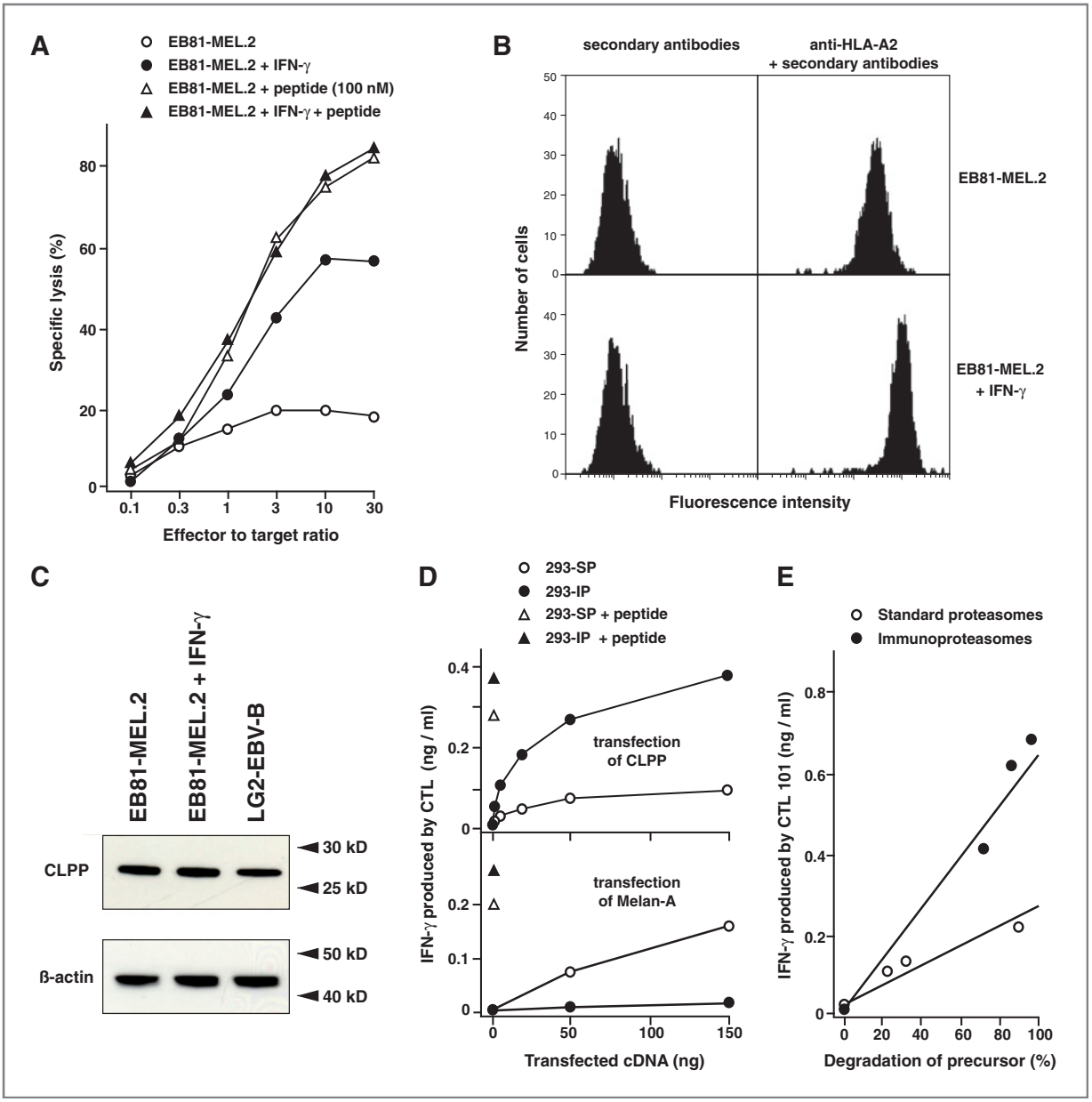

Figure 6. Effect of IFN-γ on the processing of the CLPP peptide. A, effect of IFN-γ on the sensitivity of melanoma cells EB81-MEL.2 to lysis by CTL clone 101. Tumor cells treated with and without 100 U/mL of IFN-γ for 48 hours were used as target cells in a standard lysis assay. They were pulsed or not with the CLPP peptide for 30 minutes and washed before CTL addition. B, effect of IFN-γ on HLA-A2 surface expression. Tumor cells treated with and without 100 U/mL of IFN-γ for 48 hours were labeled with anti-HLA-A2 monoclonal antibody BB7.2 followed by goat antimouse Ig antibodies coupled to FITC. C, no effect of IFN-γ on CLPP protein levels. Cell lysates from EB81-MEL.2 cells treated with and without 100 U/mL of IFN-γ for 48 hours, and from an EBV-transformed B-cell line as a control, were analyzed by Western blot with anti-CLPP and anti-β-actin antibodies. D, optimal antigen processing depends on immunoproteasomes. The indicated limiting amounts of *CLPP* or *Melan-A* cDNA constructs were cotransfected with 50 ng of an *HLA-A2* construct into 293-SP or into 293-IP cells. CTL 101 was added 24 hours later, and the amount of IFN-γ produced after 24 hours was measured by ELISA. To control HLA-A2 expression, 293-SP and 293-IP cells transfected with the HLA-A2 plasmid alone were peptide-pulsed (0.3 μmol/L for CLPP and 3 μmol/L for Melan-A) for 1 hour before addition of the CTL clones. E, better processing of the CLPP peptide by immunoproteasomes. Precursor peptide EAQEFGILDKVLVHLPQDGE was digested with purified 20S standard or immunoproteasomes. At different time points, the digests were incubated with T2 cells and tested for CTL recognition, and the amount of residual precursor peptide was estimated by HPLC/MS analysis.

(Fig. 6A). However, IFN-γ did not increase CLPP mRNA (data not shown) or protein (Fig. 6C) expression in EB81-MEL.2 cells. In addition, untreated EB81-MEL.2 cells already carried a high amount of surface HLA-A2 molecules, only 3-fold lower than that observed on autologous EBV-B cells, and IFN-γ increased this level only slightly (Fig. 6B). Finally, incubating untreated EB81-MEL.2 cells with the CLPP antigenic peptide increased lysis to ±80% at CTL/target ratios of 10 or more (Fig. 6A), indicating that HLA expression was not a limiting factor. These results suggested that IFN-γ increased tumor

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recognition by CTL through another mechanism than CLPP or HLA upregulation.

We surmised that IFN-γ increased the processing of the antigenic peptide. Preliminary experiments using proteasome inhibitors suggested that the CLPP peptide was processed by the proteasome. In cells exposed to IFN-γ, the 3 catalytic subunits of the proteasome are replaced by their inducible counterparts to constitute the immunoproteasome, and several antigens are processed differently by the 2 proteasome types (11, 17–19). To examine whether immunoproteasomes processed the CLPP antigenic peptide better than standard proteasomes, we used 2 complementary approaches. In the first, *CLPP* cDNA was cotransfected with an HLA-A2 construct into 293 cells, which contain standard proteasomes (293-SP), and into 293 cells that were made to stably express the 3 immunosubunits of the proteasomes and therefore contain only immunoproteasomes (293-IP; ref. 11). After 24 hours, transfected 293-IP cells stimulated the CTL better than transfected 293-SP (Fig. 6D). In the same experiment, the opposite result was observed for the Melan-A/MART-1 antigenic peptide EAAGIGILTV, which is known to be better produced by standard proteasomes (ref. 19; Fig. 6D).

To confirm better processing of the mutated CLPP peptide by immunoproteasomes, we conducted digestions of a synthetic precursor peptide with purified standard proteasomes or immunoproteasomes and compared the capacity of the digests to sensitize HLA-A2 cells to recognition by CTL clone 101. Immunoproteasomes produced more of the antigenic peptide than standard proteasomes, leading to a 2-fold increase in IFN-γ production by the CTL (Fig. 6E). This is in line with the results obtained with the cellular approach (Fig. 6D). Altogether, these results suggested that IFN-γ increased presentation of the CLPP peptide because of its better processing by immunoproteasomes.

Discussion

The main result of this work is that vaccination of patient EB81 with MAGE-A antigens was followed by the appearance in blood and in regressing metastases of CTL recognizing a tumor-specific mutated antigen against which no CTLs were found prior to vaccination. We already knew that after vaccination, a new wave of tumor-specific CTL clones became detectable in the blood of the patient (clonotypes 16, 4, 6, and 7 in Fig. 1; ref. 5). Three clones recognized MAGE-C2 peptides. However, these peptides were also recognized by other CTL clones, which were already present in blood and metastasis collected prior to vaccination (clonotypes 41, 40, and 11 in Fig. 1). Thus this new wave of anti-MAGE-C2 clones is a ''clonal spreading'' of the antitumor response, that is, new CTL clones against previously targeted antigens. On the contrary, the CLPP antigen was not targeted by the spontaneous prevaccination antitumor CTL response. Thus anti-CLPP CTL 101 is part of *antigen spreading*, that is, new CTL clones against previously ignored antigens.

Clonal and antigen spreading are not infrequent following cancer immunotherapy. Reactivity to MAGE-C2 was documented after vaccination with MAGE-A peptides (20), to MAGE-A12 after vaccination with gp100 (21), to MAGE-A3 and carcinoembryonic antigen after MUC1 vaccination (22), to gp100 and tyrosinase after Melan-A/MART-1 vaccination (23), to HER-2/neu epitopes different from those present in a vaccine (24–26), and to various prostate antigens after poxviral vaccination with prostate-specific antigen (27). Infusion of anti-Melan-A CTL clones was followed by increased frequencies of additional anti-Melan-A clones (28, 29). Transfer of an anti-NY-ESO-1 CD4 T-cell clone led to responses against Melan-A and MAGE-A3 (30). Interestingly, most of these reports concern patients who showed a tumor response following immunotherapy.

Our analyses of 2 vaccinated patients (5, 7, 20) lead us to believe that clonal and antigen spreading are much more frequent than reported and that they are required to obtain a complete and durable clinical response. In patient EB81, antigen spreading towards an antigen encoded by a mutated gene may have played a decisive role in the tumor regression. For example, levels of MAGE-C2 proteins could be heterogenous, as shown in hepatocellular carcinomas (31), whereas there could be a more homogenous expression of the CLPP antigen. We can of course not ascertain that antigen spreading to the mutated CLPP antigen played a crucial role in the tumor regression of patient EB81. A relapse with a CLPP antigen loss would be a very strong argument, but the patient fortunately remains clinically disease-free. In the context of antigen spreading it is worth pointing out the paradoxical status of the mutated tumor-specific antigens: Impossible to use in generic vaccines because differing from one patient to another, they might nevertheless cause or contribute to tumor regressions following vaccination with other tumor antigens. In the tumor microenvironment, the activation through antigen spreading of CTL clones that are potent for tumor rejection could be rare, explaining that in some patients a tumor regression started only after several months of persistent vaccination (ref. 32; and unpublished observations).

CLPP is, together with CLPX, a component of ClpXP, an ATP-dependent serine protease that catalyzes unfolding and degradation of misfolded mitochondrial proteins (33). CLPP is highly conserved between bacteria and eukaryota. In bacteria, ClpXP is a barrel-shaped oligomer composed of a double heptameric ring of ClpP bordered by hexameric rings of ClpX or ClpA, with a proteasome-like overall architecture (34–36). ClpX and ClpA present the substrates in an unfolded conformation to the ClpP protease (33). Human CLPP, identified through its homology to the *Escherichia coli* ClpP (37), contains an N-terminal mitochondria targeting sequence (37). The protein was located by antibodies in the mitochondrial matrix, apparently in association with the inner mitochondrial membrane (38). CLPP processively degrades unknown substrates and generates peptides of about 7 to 8 residues which might exit at the equatorial regions of the CLPP barrel (36).

A few observations pointed to the recognition by T cells of antigenic peptides naturally processed from mitochondrial proteins. The murine class Ib molecules H2-M3 present to alloreactive CTL formylated peptides from the N-terminus of proteins encoded by the mitochondrial genome (39). A peptide

whose sequence matched that of an enzyme encoded by mitochondrial DNA was eluted from the H-2K^b molecules of thymocytes (40). Human CTL could be primed *in vitro* to a peptide of pyruvate dehydrogenase, a mitochondrial autoantigen in primary biliary cirrhosis, but there was no proof that this peptide was naturally processed (41). The mutated CLPP peptide described here is the first antigen derived from a mitochondrial protein and presented to human CD8 T cells by classical HLA class I molecules.

An antigenic peptide derived from a mitochondrial protein encoded within the nuclear genome could be processed through several pathways. The complete protein, or DRiP, that is, prematurely terminated or misfolded polypeptides, could be processed by proteasomes in the cytosol and transported to the estrogen receptor through tandem affinity purification. In this classical pathway, the antigenic peptide derives from cytosolic CLPP polypeptides before addressing to mitochondria. Another possibility is that CLPP is degraded by intramitochondrial proteolytic systems such as Pim1, other proteases of the AAA family (42, 43), or the CLPP system itself. The resulting peptides are actively transported across the inner mitochondrial membrane into the intermembrane space, then cross the outer membrane by passive diffusion through porins or through the general import pore of the outer membrane (44). Our results of antigen presentation by 293 cells expressing immunoproteasome subunits indicate a classical proteasome-dependent processing of the mutated CLPP peptide recognized by CTL 101.

Better processing by immunoproteasomes was also observed for another EB81-MEL antigenic peptide, MAGE-C2$_{336–344}$ (8, 11). There the reason for differential processing was a destructive peptide cleavage by standard but not by immunoproteasomes. Peptide MAGE-C2$_{336–344}$ was recognized by CTL 16. CTL 16 and CTL 101 were the most represented T-cell clones in the invaded lymph node of patient EB81, representing 9% and 7%, respectively, of all T cells present (Fig. 1; ref. 7). They were also the most represented clones in a regressing cutaneous metastasis, with 3% for each clone (7). Local production of IFN-γ in the regressing metastases may have shaped the antigenic repertoire of the tumor cells, favoring the amplification of CTL recognizing antigens better processed by immunoproteasomes. The source of IFN-γ is likely to be the activated CTL themselves, resulting in positive feedback. In the prevaccination metastases there were probably fewer activated T cells, and less IFN-γ, produced. It decreased the probability of activating anti-CLPP T cells and favored resistance to anti-MAGE-C2$_{336–344}$ CTL (Fig. 1). Gene expression profiling of pre- and postvaccination metastases of patient EB81 indicated a barely detectable level of IFN-γ expression in the prevaccination sample, and a 5-fold higher expression in the regressing cutaneous metastasis (unpublished observations), which is compatible with this scenario.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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Antigen Spreading Contributes to MAGE Vaccination-Induced Regression of Melanoma Metastases

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