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# A MAGE-A1 HLA-A*0201 Epitope Identified by Mass Spectrometry[1]

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## ABSTRACT

**Peptides presented by HLA-A*0201 molecules on the surface of the human breast carcinoma cell line KS24.22 after IFN-γ induction were analyzed by the "Predict-Calibrate-Detect" approach, which combines epitope prediction and high-performance liquid chromatography mass spectrometry. One of the predicted epitopes, MAGE-A1$_{278–286}$ (KVLEYVIKV), was found to be presented by HLA-A*0201, with an estimated copy number of 18 molecules/cell. HLA-A*0201 transgenic mice (HHD mice) were used to generate CTL lines that stained positive with an HLA-A*0201 tetramer folded around the KVLEYVIKV peptide and killed peptide-loaded mouse target cells expressing HLA-A*0201. IFN-γ-treated or -nontreated HLA-A*0201 expressing HeLa cells transiently transfected with a plasmid expressing the *MAGE-A1* gene stimulated *in vitro* cytokine production by the CTL lines. Moreover, IFN-γ-treated KS24.22 cells, but not IFN-γ-treated HLA-A*0201$^+$ MAGE-A1$^-$ cells or IFN-γ-treated HLA-A*0201$^-$ MAGE-A1$^+$ cells, were killed by these CTLs. Thus, the combination of HLA epitope prediction, peptide analysis, and immunological methods is a powerful approach for the identification of tumor-associated epitopes.**

## INTRODUCTION

Tumor-specific shared antigens, such as those of the *MAGE* gene family, are expressed by tumors of different histological types. Most of these are silent in normal cells but are expressed in male germ-line cells (1, 2). Their immunogenicity, both for humoral and cellular immunity, raised the possibility of developing anticancer immunotherapies or vaccinations. CTLs, shown to be efficient antitumor effector cells, recognize antigens in the form of 8–12-residue peptides bound to allelic products of the MHC class I (3). The precise characterization of the immunogenic sequences from tumor antigens involved in the cytotoxic immune response, *i.e.*, the class I epitopes, is critical for the development of peptide-based therapies using peptide-loaded DCs[5] (4) or peptide vaccines (5) as well as for the *in vitro* monitoring of the natural or therapeutically induced antitumor immune response in cancer patients using the tetramer technology (6), ELISPOT assays, or cytotoxicity assays (7). Most tumor-specific class I epitopes recognized by CTLs have been identified using *in vitro*-stimulated human tumor-infiltrating lymphocytes or peripheral blood lymphocytes (1, 8, 9). This approach requires the difficult and time-consuming cell culture and characterization of human tumor-specific or peptide-specific CTLs. Moreover, many peptide-specific CTLs do not recognize HLA-matched tumor cells expressing the protein endogenously (10). To overcome these problems, we recently developed a MS-based approach, the PCD method, to identify tumor epitopes that associate with HLA class I molecules (11). Epitopes are predicted from the protein sequences of known tumor antigens, and the mixture of corresponding synthetic peptides is used for the calibration of a nanocapillary HPLC MS system. Extracted natural MHC ligands are analyzed the same way, and predicted tumor-associated peptides are identified by coelution and HPLC tandem MS. This method allows the rapid detection of new epitopes. Some of these, the subdominant or cryptic epitopes, might be ignored by the immune system of healthy people or cancer patients. Using this method, we identified previously two tumor-associated HLA-A*0201 ligands [one from CEA and one from p53 (11)]. Here, we report the MS-based identification of a new HLA-A*0201 tumor epitope derived from the MAGE-A1 protein, a tumor antigen expressed in ~40% of melanoma and in some other tumors (12) subsequent to a genome-wide demethylation process that occurs in many cancers (13). To confirm the presentation of this epitope at the surface of tumor cells and its relevance for antitumor immunity, we generated peptide-specific CTLs from HHD mice (14, 15). These mouse CTL lines are stimulated *in vitro* by HLA-A*0201$^+$ cells transfected with the *MAGE-A1* gene and kill specifically HLA-A*0201- and MAGE-A1-positive human tumor cells.

## MATERIALS AND METHODS

**Tumor Cell Lines.** HeLa cells were transfected with a plasmid coding for a chimeric HLA-A*0201 molecule containing the α3, transmembrane, and cytoplasmic domains of H-2D$^b$ (14) to give HeLa/A2A2D$^b$ transfectants. EL4S3-Rob/HHD cells are mouse β2m-deficient EL4 cells expressing the HHD molecule (14). The human breast carcinoma KS24 cell line (16, 17) was transfected with a plasmid coding for Her2/neu generating the KS24.22 cell line. The human breast carcinomas GAKL and K1HE were also derived from malignant pleural effusions of breast carcinoma patients. For these breast carcinoma cells, the expression of HLA-A*0201 was tested by immunofluorescence using the mAb BB7.2 (American Type Culture Collection, Manassas, VA) and by genomic PCR. The expression of the *MAGE-A1* gene was tested by RT-PCR using the pair of primers (5′-CGGCCGAAGGAACCTGACCCAG-3′ + 5′-GCTGGAACCCTCACTGGGTTGCC-3′) that amplifies a 421-bp DNA fragment from the MAGE-A1 sequence (18). The human lymphoblastoid cell line 721 was described previously (19). All cells were kept in RPMI 1640 enriched with 10% FCS, 2 mM L-glutamine and 1% penicillin/streptomycin (Life Technologies, Inc.) supplemented at 1 mg/ml G418 (Life Technologies, Inc.) for all transfectants (KS24.22, HeLa/A2A2D$^b$, and EL4S3Rob/HHD).

**Epitope Prediction.** Prediction of potential HLA-A*0201 ligands was carried out as described (20). Briefly, proteins were screened against a matrix pattern, which evaluates every amino acid within nonamer or decamer peptides fitting the HLA-A*0201 motif. Anchor residues are given values of 10; other residues, 0–10, reflect amino acid preferences for certain positions within the peptide. The theoretical maximum score for a candidate peptide is 36; scores

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[5] The abbreviations used are: DC, dendritic cell; PCD, Predict-Calibrate-Detect; MS, mass spectrometry; HPLC, high-performance liquid chromatography; HHD, HLA-A*0201 transgenic H2 class I-deficient; CEA, carcinoembryonic antigen; mAb, monoclonal antibody; TOF, time of flight; ESI, electrospray ionization; RT-PCR, reverse transcription-PCR; FCS, fetal calf serum; PE, phycoerythrin; FACS, fluorescence-activated cell sorter; IL-2, interleukin-2.

for abundant natural ligands are typically between 32 and 34. Such motif predictions are available using the database SYFPEITHI.[6]

**Peptides.** Peptides were synthesized in an automated peptide synthesizer 432A (Applied Biosystems, Foster City, CA) following the 9-fluorenylmethyloxycarbonyl/*tert*-butyl (Fmoc/tBu) strategy. After removal from the resin by treatment with trifluoroacetic acid:phenol:ethanedithiol:thioanisole:water (90:3.75:1.25:2.5:2.5 by volume) for 1 or 3 h (arginine-containing peptides), peptides were precipitated from methyl-*tert*-butyl ether, washed once with methyl-*tert*-butyl ether and twice with diethyl ether, and resuspended in water before lyophilization. Synthesis products were analyzed by HPLC (System Gold; Beckman Instruments, Fullerton, CA) and matrix-assisted laser desorption/ionization TOF MS (G2025A; Hewlett-Packard, Palo Alto, CA). Peptides of <80% purity were purified by preparative HPLC.

**Peptide Binding Assay.** T2 cells were used for binding studies. A 1 mM peptide stock solution in PBS 10% DMSO was made, and cells were incubated with the peptide at a final concentration of 50 $\mu$M in RPMI 1640 overnight at 37°C. HLA surface expression was monitored after staining with the primary antibody BB7.2 and a FITC-coupled goat antimouse IgG (Dianova, Hamburg, Germany) on a FACSCalibur cytometer (Becton-Dickinson, San Jose, CA). Results in Fig. 1 show the ratio of the mean fluorescence of the stained cells incubated with the peptide:the mean fluorescence of the stained cells incubated without peptide.

**Isolation of HLA-A2-bound Peptides.** HLA-A2-bound peptides were isolated according to standard protocols (21, 22) using the HLA-A2-specific antibody BB7.2, acid treatment, ultrafiltration, and fractionation by HPLC. Peptide-containing HPLC fractions were pooled, and aliquots corresponding to peptide extracts from $\sim 10^{10}$ cells were analyzed by nanocapillary HPLC ESI MS.

**HPLC-coupled MS.** Synthetic and natural peptide mixtures were analyzed as described (11) by a reversed-phase HPLC system (ABI 140D; Applied Biosystems) coupled to a hybrid quadrupole orthogonal acceleration TOF tandem mass spectrometer (Q-TOF; Micromass, Manchester, United Kingdom) equipped with an ESI source. Solvent A was 4 mM ammonium acetate adjusted to pH 3.0 with formic acid. Solvent B was 2 mM ammonium acetate in 70% acetonitrile/water adjusted to pH 3.0 with formic acid. As a modification of the described set-up, loading of typical sample volumes of 100 $\mu$l was achieved by preconcentration on a 300-$\mu$m * 5-mm C18 $\mu$-Precolumn (LC Packings, San Francisco, CA). A syringe pump (PHD 2000; Harvard Apparatus, Inc., Holliston, MA), equipped with a gastight 100-$\mu$l syringe (1710 RNR; Hamilton, Bonaduz, Switzerland), was used to deliver solvent and sample at a flow rate of 2 $\mu$l/min. For peptide separation, the preconcentration column was switched in line with a 75-$\mu$m * 250-mm, C18 column (LC Packings). A binary gradient of 25–60% B within 70 min was performed, applying a flow rate of 10 $\mu$l/min reduced to ~300 nl/min with a precolumn split using a TEE-piece (ZT1C; Valco, Schenkon, Switzerland) and a 300-$\mu$m * 150-mm C18 column as a backpressure device. A gold-coated glass capillary (PicoTip; New Objective, Cambridge, MA) was used as the needle in the ESI source. The integration time for the TOF analyzer was 3 s with an interscan delay of 0.1 s. A blank run was performed before any subsequent HPLC MS run to ensure that the system was free of any residual peptide.

For on-line nanocapillary HPLC MS/MS experiments, fragmentation of the parent ion was achieved at the given retention time by collision with argon atoms. Q1 was set to the mass of interest ±0.5 Da and an optimized collision energy applied. Fragmentation was completed after 60 s.

**Nanoflow ESI MS.** Gold-coated glass capillary nanoflow needles were obtained from Protana (normal type; Odense, Denmark). The needle was filled with 3 $\mu$l of the sample diluted in 50% methanol:water:1% formic acid and subsequently opened by breaking the tapered end of the tip under a microscope. A stable spray was observed applying a needle voltage of 800–1200 V, a backpressure of 2 psi, and a source temperature of 40°C. The estimated flow rate was 20–50 nl/min. For nanoflow ESI MS/MS experiments, fragmentation was achieved by collision with argon atoms. Q1 was set to the mass of interest ± 0.5 Da, and an optimized collision energy was applied. The integration time for the TOF analyzer was 1 s with an interscan delay of 0.1 s.

**Generation of Mouse CTLs and Cytotoxicity Assays.** HHD mice (14) were injected s.c. in the neck with 25 $\mu$g of synthetic KVLEYVIKV peptide mixed with 25 $\mu$g of the HBV core$_{128-140}$ helper epitope (23) and 50 $\mu$l of Titermax (Sigma). Ten days later, spleens were removed, and splenocytes were put in culture in 10 ml of Iscove's modified Dulbecco's medium supplemented with 10% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin (Life Technologies, Inc.), 50 $\mu$g/ml Gentamycin (Life Technologies, Inc.), $5 \times 10^{-5}$ M $\beta$-mercaptoethanol, and 1 mM sodium pyruvate (Sigma). Peptide was added at a final concentration of 1 $\mu$M. Three days later, 25 units of recombinant IL-2 (Proleukin; CHIRON) were added. At day 6, the cytotoxicity of the cultures was tested on 5000 unloaded or peptide-loaded EL4S3Rob/HHD cells in a 6-h $^{51}$Cr release assay. Specific lysis was calculated as follows: (experimental release − spontaneous release)/(total release − spontaneous release) × 100. CTL cultures exhibiting detectable peptide-specific cytotoxicity were restimulated weekly using $2 \times 10^7$ million peptide-loaded (1 $\mu$M peptide for 1 h), fresh irradiated (200 Gy) HHD splenocytes. Long-term cultures were used for cytolytic assays on 5000 human tumor cells previously treated or not treated for 48 h with 250 units/ml of human IFN-$\gamma$ (Boehringer Mannheim).

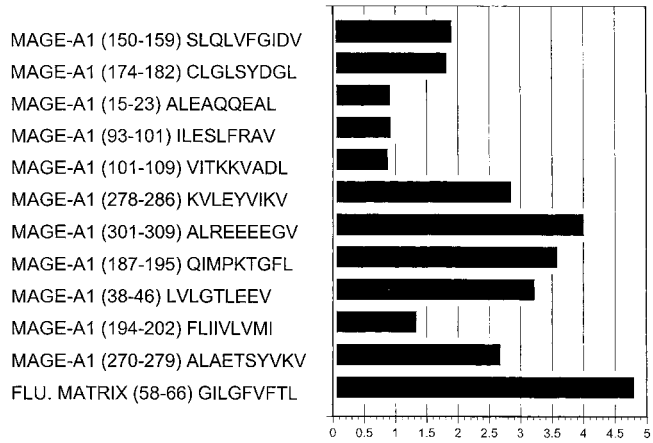


Fig. 1. Eleven MAGE-A1-derived potential HLA-A2-binding peptides were compared for their ability to stabilize HLA-A*0201 at the surface of T2 cells. *Brackets*, position of these sequences. X axis, the ratio between the mean fluorescence of T2 cells incubated overnight with 50 mM peptide and the mean fluorescence of T2 cells incubated overnight without synthetic peptide. The influenza matrix$_{58-66}$ epitope is used as a positive control.

**Cell Staining Using HLA-peptide Tetrameric Complexes.** HLA-peptide tetrameric complexes were produced as described previously (6). In brief, the heavy chain was modified by deletion of the transmembrane domain and COOH-terminal addition of a sequence containing the BirA enzymatic biotinylation site. The HLA-A2 heavy chain and $\beta$2-microglobulin were produced using a prokaryotic expression system (pET/HLA-A2 plasmid and bacteria kindly provided by Dr. Vincenzo Cerundolo), purified, and refolded *in vitro* by limiting dilution with the HLA-A*0201-binding peptides. The HLA-A*0201-binding peptides used were MAGE-A1$_{278-286}$ KVLEYVIKV and CEA$_{694-702}$ GVLVGVALI. The refolded complexes were purified by gel filtration (Superdex 75, Pharmacia) using fast protein liquid chromatography, biotinylated by BirA (Avidity, Denver, CO) in the presence of biotin (Sigma Chemical), ATP (Sigma Chemical), and $Mg^{2+}$ (Sigma Chemical). The biotinylated product was separated from free biotin by gel filtration and ion exchange (monoQ Pharmacia) using fast protein liquid chromatography. Tetramers were made by mixing biotinylated protein complexes with streptavidin-PE (Molecular Probes) at a molecular ratio of 4:0.8. Mouse CTLs ($2 \times 10^5$) were incubated with 10 $\mu$g/ml of tetrameric complexes on ice; after 15 min incubation, anti-CD8 antibody (Pharmingen) was added, and the samples were incubated on ice for an additional 15 min. After extensive washing with PBS containing 1% FCS, the samples were analyzed using a FACScalibur (Becton Dickinson).

**Plasmids, Transient Transfections, and Intracellular Cytokine Staining.** The MAGE-A1 cDNA (kindly provided by Dr. Pierre van der Bruggen) and the CEA cDNA (kindly provided by Dr. Cécile Gouttefangeas) were cloned in a pIRES-CD4t vector (kindly provided by Dr. Peter Gaines; Ref. 24). These plasmids were introduced into tumor cells using electroporation mixing $2 \times 10^7$ cells with 25 $\mu$g of DNA and pulsed at 225 V, 1050 $\mu$F. Forty-eight h after transfection, untreated or IFN-$\gamma$-treated cells were harvested and incubated with antihuman CD4-coated magnetic beads (Miltenyi Biotec). CD4-positive cells were sorted as recommended by the manufacturer (Miltenyi

[6] Available on our web page at http://www.uni-tuebingen.de/uni/kxi.

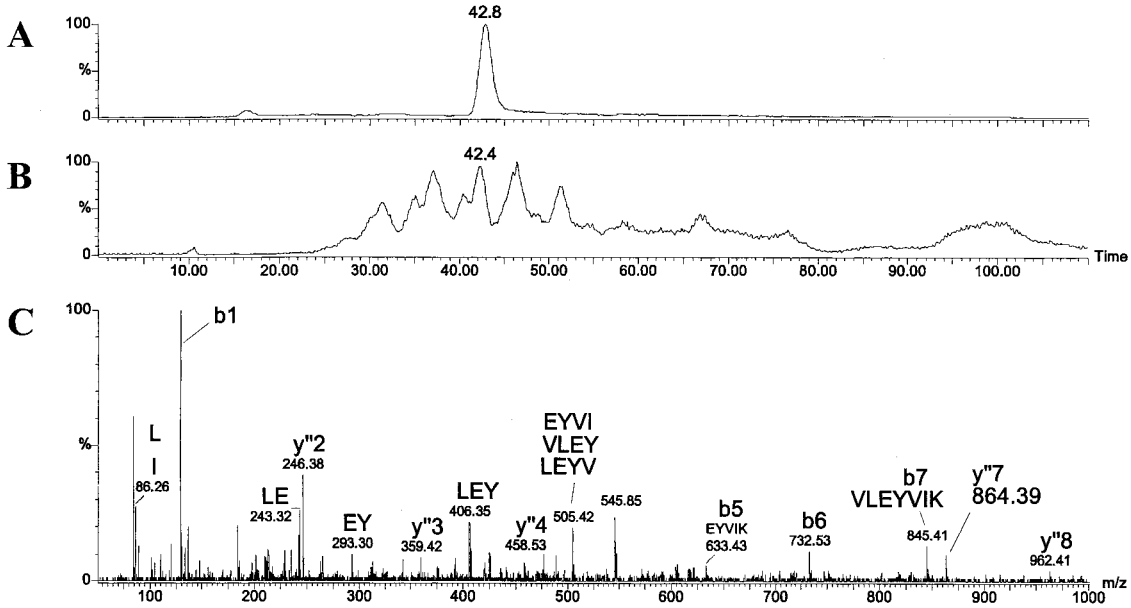


Fig. 2. Identification of the predicted tumor-associated peptide MAGE-A1$_{278-286}$ in a peptide mixture eluted from KS24.22 cells. *A*, the mass chromatogram of *m/z* 545.9, corresponding to $(M + 2H)^{2+}$ ions of MAGE-A1$_{278-286}$, for the synthetic peptide mixture. 720 fmol of synthetic MAGE-A1$_{278-286}$ were injected; the isobaric peptide MAGE-A1$_{150-159}$ elutes at 83.2 min exclusively as $(M + H)^{+}$ ions (data not shown). *B*, the mass chromatogram of *m/z* 545.9 for a mixture of HLA-A2-bound peptides extracted from KS24.22 cells, showing, among others, a peak at the same retention time as the synthetic MAGE-A1$_{278-286}$. Analysis of the spectra at this time confirmed that this peak was attributable to $(M + 2H)^{2+}$ ions. *C*, the collisionally induced dissociation mass spectrum recorded on $(M + 2H)^{2+}$ ions at *m/z* 545.9 during a time of 43.4 and 43.9 min. Minor variations in retention times are probably attributable to slight changes in the split ratio of the precolumn split.

Biotec). $CD4^{+}$ cells (50,000) were incubated with 500,000 anti-MAGE-A1$_{278-286}$ CTLs overnight in a medium supplemented with GolgiStop (Pharmingen). Then, cells were permeabilized and stained using the Cytofix/Cytoperm Plus kit and anti-CD4-FITC, anti-IFN-γ PE, and anti-CD8 cytochrome antibodies, according to the manufacturer's recommendations (Pharmingen). FACS analysis was performed using a FACSCalibur cytometer (Becton-Dickinson). Data in Fig. 4 show the IFN-γ and CD8 expression in gated CD4-negative lymphocytes.

## RESULTS

**Epitope Prediction.** Potential HLA-A*0201-binding peptides were predicted from protein sequences of reported tumor antigens using our prediction algorithm[6] (20). In addition to the reported list of predicted peptides from p53, CEA, Her2/neu, RAS, MDM2, and SSX2 (11), we also included peptides from MAGE-A1, MAGE-A3, MUC1, and additional peptides from Her2/neu and SSX2. A total of 80 peptides predicted to be the best binders were synthesized and tested for HLA-A*0201 binding by cell surface MHC class I stabilization assay. Fig. 1 shows the results obtained for peptides predicted from MAGE-A1 and for the immunodominant Influenza Matrix$_{58-66}$ HLA-A*0201 epitope as positive control. Although three of the MAGE-A1 peptides show no experimental binding, all of them were used to calibrate the HPLC MS system, because, to our knowledge, the stabilization assay is not sensitive enough to detect the binding of all natural ligands.

**Detection of a Predicted MAGE-A1 Epitope by an HPLC MS System.** The cell line KS24.22 is a Her2/neu-transfected variant of the KS24 human breast carcinoma cell line described previously (16). It expresses HLA-A*0201 as checked by mAb staining using BB7.2 and RT-PCR and the MAGE-A1 gene as checked by RT-PCR.[7] IFN-γ-treated (250 units/ml; 48 h treatment) KS24.22 cells ($3 \times 10^{10}$) were used for HLA-A*0201 purification and peptide extraction as described (21). Analysis of the peptide mixture eluted from HLA-A*0201 showed coelution of peptides with molecular masses corresponding to the self-ligands p68$_{168-176}$ (YLLPAIVHI) and PP2A$_{402-410}$ (SLLPAIVEL) selected as internal controls, as well as the predicted tumor-associated peptide MAGE-A1$_{278-286}$ (KVLEYVIKV; Fig. 2). The amino acid sequences of these three peptides were confirmed in a second experiment by on-line nanocapillary HPLC MS/MS, shown in Fig. 2 for MAGE-A1$_{278-286}$. Nanoflow ESI MS/MS of synthetic MAGE-A1$_{278-286}$ under identical conditions as in the nanocapillary HPLC MS/MS experiment confirmed further the identity of the peptide (data not shown). The self-peptide p68$_{168-176}$ was present at ~2.5 pmol/$10^{10}$ cells, as estimated by comparison of signal intensities of eluted ($3.81 \times 10^3$ counts/scan) and 3 pmol of synthetic peptide ($4.61 \times 10^3$ counts/scan). This corresponds to ~600 copies/cell assuming an overall yield of 25% of natural peptides after peptide extraction and HPLC. Signal intensities for MAGE-A1$_{278-286}$ were $4.19 \times 10^3$ counts/scan for 720 fmol of synthetic peptide and 450 counts/scan for the eluted peptide. MAGE-A1$_{278-286}$ was therefore present at ~300 fmol/$10^{10}$ cells, corresponding to ~18 copies/cell.

**Generation of Anti-MAGE-A1$_{278-286}$ HLA-A*0201-restricted Mouse CTLs.** HHD mice express at low level a chimeric monochain HLA-A*0201 molecule able to educate a diverse repertoire of $CD8^{+}$ cells (14). These mice received subcutaneous injections of a synthetic peptide corresponding to the MAGE-A1$_{278-286}$ epitope emulsified in Titermax adjuvant (Sigma) together with an H2-A$^{b}$ helper peptide from HBV core protein (25). Ten days after injection, splenocytes of immunized mice were stimulated *in vitro* with MAGE-A1$_{278-286}$ plus recombinant IL-2. After 6 days of culture, specific cytotoxicity of the CTL line was detected on mouse target cells (mutant EL4 cells expressing the HHD molecule) preincubated with MAGE-A1$_{278-286}$ (Fig. 3*A*). After few restimulations *in vitro* using irradiated, peptide-loaded fresh splenocytes from HHD mice, CTL lines consisting essentially of $CD8^{+}$ cells were obtained. These lines can be stained using a tetrameric fluorescent HLA-A*0201 molecule folded around the MAGE-A1$_{278-286}$ epitope (Fig. 3*B*). A control HHD mouse-

[7] B. Gückel, manuscript in preparation.

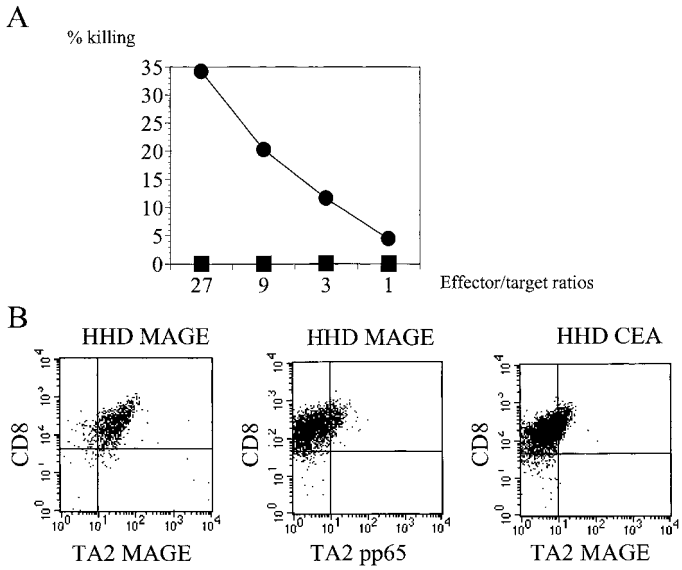


Fig. 3. Characterisation of the mouse CTL line restricted by HLA-A*0201 and specific for the MAGE-A1$_{278-286}$ epitope. *A*, cytotoxic response of the CTL line using unloaded (■) or peptide loaded (●) EL4S3-Rob/HHD as target cells. *B*, FACS analysis of the mouse HLA-A*0201-restricted CTL lines specific for the MAGE-A1$_{278-286}$ epitope (*HHD MAGE*) or for the CEA$_{694-702}$ epitope (*HHD CEA*) using CD8 antibodies and HLA-A*0201 tetramers folded around either the MAGE-A1$_{278-286}$ epitope (*TA2 MAGE*) or a CMVpp65-derived epitope (TA2 pp65).

derived CTL line generated against a CEA HLA-A*0201 epitope described previously (11) is not stained by this tetramer, and an irrelevant HLA-A*0201 tetramer folded around a cytomegalovirus epitope does not stain the anti-MAGE-A1$_{278-286}$ CTL lines. Altogether, these results show that we obtained homogeneous anti-MAGE-A1$_{278-286}$ cytotoxic lines from HHD mice.

**MAGE-A1-transfected Cells Stimulate Anti-MAGE-A1$_{278-286}$ HLA-A*0201-restricted Mouse CTLs.** To verify the presentation of MAGE-A1$_{278-286}$ at the surface of MAGE-A1$^+$ tumor cells, we used transiently transfected HeLa cells (HeLa/A2A2D$^b$) expressing a chimeric HLA-A*0201 molecule that, similarly to the HHD monochain molecule, is composed of the $\alpha$1 and $\alpha$2 domains of HLA-A*0201 and the $\alpha$3, transmembrane, and cytoplasmic domains of H2-D$^b$ to stimulate *in vitro* the anti-MAGE-A1$_{278-286}$ CTL lines. These HeLa cells were transfected with pIRES plasmids coding for both a truncated version of the human CD4 molecule and either the CEA or the MAGE-A1 antigens. Thirty-six h after transfection, CD4-positive cells were sorted using magnetic beads coated with antihuman CD4 antibodies. Sorted cells were then coincubated with anti-MAGE-A1$_{278-286}$ HHD-derived CTL lines for 12 h before the cells were permeabilized and stained for intracellular IFN-$\gamma$. Fig. 4 shows that IFN-$\gamma$-treated (or -nontreated; data not shown) HeLa cells expressing a chimeric HLA-A*0201 molecule and the MAGE-A1 protein can stimulate interferon-$\gamma$ production by CD8$^+$ anti-MAGE-A1$_{278-286}$ CTL lines. Peptide-loaded HeLa/A2A2D$^b$ cells induce a stronger stimulation of the CTL line, and, on the contrary, HeLa/A2A2D$^b$ cells transiently expressing the human CD4 molecule and the CEA antigen induce a background expression of IFN-$\gamma$ in some cells. This assay shows that MAGE-A1$_{278-286}$ is exposed at the cell surface after the processing of the cytoplasmic MAGE-A1 protein and loading on HLA-A*0201 molecules.

**Anti-MAGE-A1$_{278-286}$ HLA-A*0201-restricted Mouse CTLs Kill Specifically HLA-A*0201 and MAGE-A1-positive Human Tumor Cells.** We tested the recognition of human tumor cells by the anti-MAGE-A1$_{278-286}$ mouse CTL lines using cytotoxicity assays. Results of a representative experiment are shown in Fig. 5. All peptide-loaded HLA-A*0201$^+$ tumor cells are efficiently killed by the mouse anti-MAGE-A1$_{278-286}$ CTL lines, but only the IFN-$\gamma$-treated MAGE-A1$^+$ and HLA-A*0201$^+$ KS24.22 tumor cells are killed without prior incubation with the peptide. Neither IFN-$\gamma$-treated or -untreated HLA-A*0201$^+$ MAGE-A1$^-$ (GAKI; 721) nor IFN-$\gamma$-treated or -untreated HLA-A*0201$^-$ MAGE-A1$^+$ (KLHE) tumor cells are lysed by the HLA-A*0201-restricted anti-MAGE-A1$_{278-286}$ mouse CTL line. These results demonstrate that the MAGE A1$_{278-286}$ epitope is presented at the cell surface of human tumor cells expressing HLA-A*0201 and the MAGE-A1 tumor antigen.

## DISCUSSION

Using a recently described (11) combination of epitope prediction and HPLC mass spectrometry, the PCD method, we identified an HLA-A*0201-restricted MHC class I ligand derived from the tumor antigen MAGE-A1. *In vitro* cytokine secretion of peptide-specific CTL lines from HLA-A*0201 transgenic mice, as well as cytotoxicity assays, proved that the identified epitope is indeed presented at the cell surface of human HLA-A*0201 and MAGE-A1-positive cells. Consequently, this epitope is a new possible target for the development and monitoring of antitumor immunotherapies or vaccinations (4, 5). The presentation of the MAGE-A1$_{278-286}$ epitope is increased by IFN-$\gamma$ treatment, putting this peptide into the family of epitopes produced both by constitutive and immuno-proteasomes (26). Strikingly, the MAGE-A1$_{278-286}$ epitope is not the best HLA-A*0201 binder compared with other predicted MAGE-A1 epitopes (Fig. 1). Limiting steps in the antigen processing (proteasome-mediated degradation or generation of the correct epitope COOH terminus, TAP transport, loading on neosynthesized class I molecules) might favor the presentation of this MAGE-A1 epitope. Accordingly, our recently described cleavage prediction algorithm (27) indicates that proteasomes would perform a cleavage of the MAGE-A1 protein after the valine 286, which is the COOH-terminal anchor amino acid of the MAGE-A1 HLA-A*0201 epitope (data not shown).

Besides reporting a clinically important new tumor epitope, the first

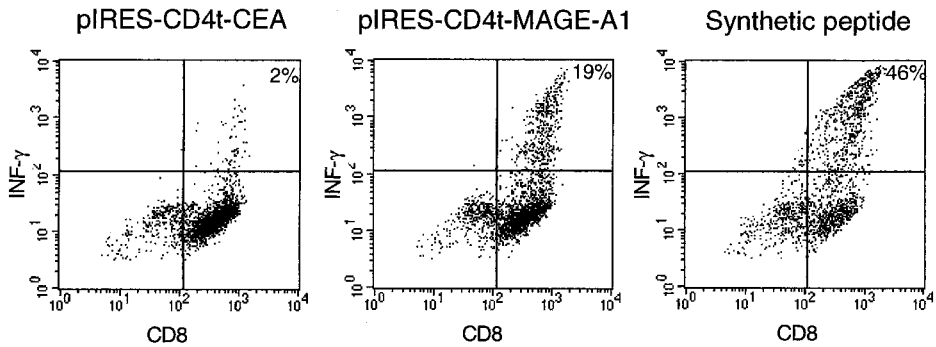


Fig. 4. FACS analysis of intracellular IFN-$\gamma$-stained MAGE-A1$_{278-286}$ CTL line incubated overnight with HeLa/A2A2D$^b$ cells transfected with either a pIRES-CD4t-CEA plasmid or a pIRES-CD4t-MAGE-A1 plasmid or pulsed with the MAGE-A1$_{278-286}$ synthetic peptide. *Numbers*, the percentage of IFN-$\gamma$-positive cells in the total CD8-positive cell population.

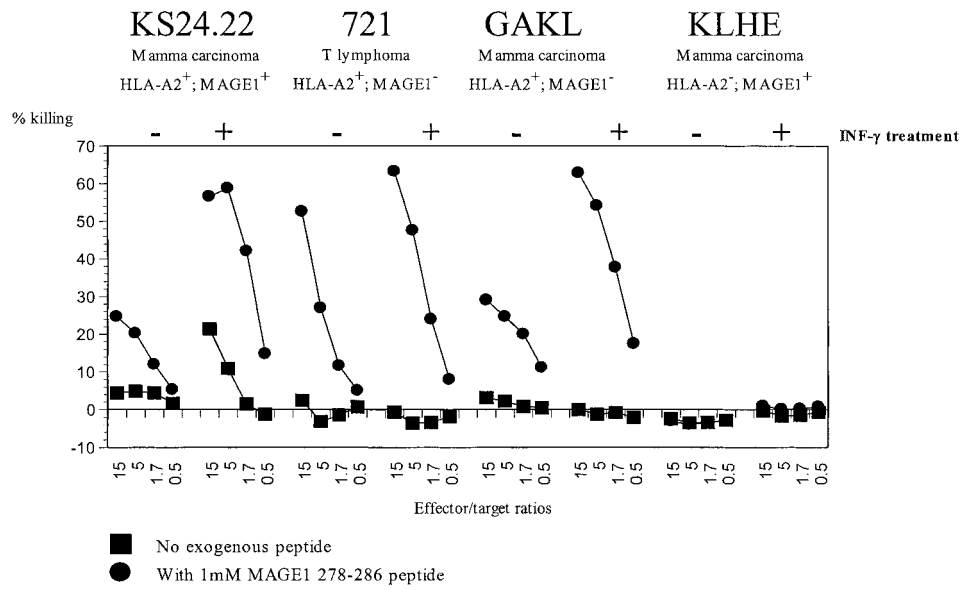


Fig. 5. Cytotoxic response of the MAGE-A1$_{278-286}$-specific CTL line using unloaded (■) or peptide-loaded (●) human tumor cells treated or not treated with INF-γ as target cells.

described HLA-A*0201-restricted epitope from the MAGE-A1 protein, our results demonstrate the potential of our PCD approach for high through-put identification of class I epitopes. Moreover, the CTL-independent characterization of HLA-class I restricted epitopes might allow the description of subdominant or cryptic CTL epitopes (28). These epitopes are often ignored by the immune system. As opposed to epitopes recognized during the development of the tumor, cryptic epitopes might be used in therapy to trigger the activation of antitumor cytotoxic T cells derived from the pool of naive CD8-positive cells. These effector cells might have a higher antitumor potential than tolerized or previously stimulated and possibly anergized CTLs (29, 30). Thus, such epitopes appear to be interesting targets for immunotherapy. In order to classify the MAGE-A1 epitope as a dominant or cryptic epitope, we stained PBLs with fluorescent HLA-A*0201 tetramers. We could not unambiguously detect CTLs specific for the MAGE-A1$_{278-286}$ epitope in the blood of healthy people or tumor patients. Functional assays indicating the presence of effector or memory anti-MAGE-A1$_{278-286}$ CTLs in blood or tumors are still required. Nevertheless, we found that the repertoire of human T cells contains precursors that can recognize the MAGE-A1$_{278-286}$ epitope in the context of HLA-A*0201. Indeed, it was possible to generate *in vitro* human anti-MAGE-A1$_{278-286}$ CTLs using antigen-presenting cells (mature DCs or activated B cells) loaded with 1 μM synthetic peptide. Unfortunately, these CTLs killed only peptide-loaded T2 cells but not MAGE-A1$^+$ HLA-A2$^+$ tumor cells treated or not with IFN-γ.[8] We do not know yet whether this negative result is due to a too-low affinity of the CTLs for their epitope or to the inhibition of killing through either KIRs expressed by the CTLs or specific anti-CTL-mediated killing mechanisms developed by the tumor target cells, *e.g.*, heat shock protein expression (31), Fas ligand (32), or serpin proteases (33). The recognition of the MAGE-A1$_{278-286}$ epitope by the human immune system and its clinical relevance will be evaluated in breast carcinoma patients vaccinated using DCs loaded with a cocktail of tumor epitopes which include the MAGE-A1 HLA-A*0201 peptide.[9]

We demonstrated the presentation of the MAGE-A1$_{278-286}$ epitope at the cell surface through the utilization of HLA-A*0201-restricted CTLs derived from HLA-class I transgenic mice (14). Injection of synthetic KVLEYVIKV in HHD mice allowed the fast generation of CTL lines that can be stained using an HLA-A*0201 tetramer folded around the MAGE-A1$_{278-286}$ peptide. This indicates that these mouse HLA class I-restricted CTLs have a high avidity for the peptide (34). Such CTL lines are able to kill peptide-pulsed, HLA-A2-positive human cells as well as IFN-γ-treated HLA-A*0201$^+$ and MAGE-A1$^+$ human tumor cells. Previously, many groups used HLA class I-restricted CTLs from transgenic mice to identify or to study human class I epitopes (15, 25, 34–36). Usually, one drawback of these HLA-A*0201-restricted mouse CTL lines is that they do not kill human cells expressing a wild-type class I molecule (37). This phenomenon might be attributable to the lack of interaction between mouse CD8 molecule and human class I molecules, resulting in weak interaction between effector and target cells. In contrast, we observed that CTL lines derived from HHD mice kill HLA-A*0201-positive human cells in an epitope-specific and CD8-independent way. This characteristic of HHD-derived CTLs might be attributable to the low expression level of the HHD molecule in the transgenic mice, which results in the development of T cells expressing high avidity T-cell receptors. These CTLs killed human peptide-loaded target cells with high efficiency; moreover, antigen-expressing KS24.22 cells were killed after IFN-γ treatment.

Although they are sensitive to the cytotoxic effector functions of the MAGE-A1$_{278-286}$-specific mouse CTL lines, human cells could not efficiently stimulate IFN-γ production in these killer cells (data not shown). Indeed, cytotoxicity and activation of effector CTLs are separate functions, cytotoxicity being most readily observed (38). In our case, full activation of the mouse effector cells required either very high epitope density (reached using peptide-loaded human cells; data not shown) or coactivation by CD8. This latter requirement is fulfilled using transfected human cells expressing the chimeric HLA-A*0201 molecule and which contain a mouse class I α3 domain. Thus, only HeLa cells expressing such a chimeric HLA-A*0201 molecule and the MAGE-A1 tumor antigen can fully activate the mouse peptide-specific CTL lines to produce IFN-γ. Altogether, these studies allowed us to describe further the advantages and the limitations of mouse CTL lines for the identification of HLA-class I-restricted epitopes and for the recognition of human tumor cells. Because of the low HLA-A*0201 expression, HHD mice-derived CTLs might have a higher affinity for their antigen than other HLA-

[8] B. Gückel, unpublished results.
[9] B. Gückel, ongoing studies.

A*0201 transgenic mice-derived CTLs. Direct comparison of the affinity of CTL lines derived from both transgenic mice is in progress. Nevertheless, by combining intracellular cytokine staining after CD8-dependent stimulation of the effectors with CD8 independent cytotoxicity assays using HHD-derived CTL lines, we unambiguously confirmed the presentation of the PCD-identified MAGE-$A1_{278-286}$ epitope at the surface of human cells. This combination of MS-based analysis and class I transgenic mice utilization is now used extensively for the identification of bacterial, viral, and tumoral epitopes.

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## REFERENCES

1. Boon, T. and van der Bruggen, P. Human tumor antigens recognized by T lymphocytes. J. Exp. Med., *183:* 725–729, 1996.
2. Lucas, S., De Plaen, E., and Boon, T. MAGE-B5, MAGE-B6, MAGE-C2, and MAGE-C3: four new members of the MAGE family with tumor-specific expression. Int. J. Cancer, *87:* 55–60, 2000.
3. Rammensee, H., Bachmann, J., and Stevanovic, S. MHC Ligands and Peptide Motifs. Molecular Biology Intelligence Unit. Landes Bioscience, 2000.
4. Brugger, W., Brossart, P., Scheding, S., Stuhler, G., Heinrich, K., Reichardt, V., Grunebach, F., Buhring, H. J., and Kanz, L. Approaches to dendritic cell-based immunotherapy after peripheral blood stem cell transplantation. Ann. NY Acad. Sci., *872:* 363–371, 1999.
5. Jager, E., Jager, D., and Knuth, A. CTL-defined cancer vaccines: perspectives for active immunotherapeutic interventions in minimal residual disease. Cancer Metastasis Rev., *18:* 143–150, 1999.
6. Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J., and Davis, M. M. Phenotypic analysis of antigen-specific T lymphocytes [published erratum appears in Science *280:* 1821, 1998]. Science, *274:* 94–96, 1996.
7. Jager, E., Nagata, Y., Gnjatic, S., Wada, H., Stockert, E., Karbach, J., Dunbar, P. R., Lee, S. Y., Jungbluth, A., Jager, D., Arand, M., Ritter, G., Cerundolo, V., Dupont, B., Chen, Y. T., Old, L. J., and Knuth, A. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. Proc. Natl. Acad. Sci. USA, *97:* 4760–4765, 2000.
8. Kawashima, I., Hudson, S. J., Tsai, V., Southwood, S., Takesako, K., Appella, E., Sette, A., and Celis, E. The multi-epitope approach for immunotherapy for cancer: identification of several CTL epitopes from various tumor-associated antigens expressed on solid epithelial tumors. Hum. Immunol., *59:* 1–14, 1998.
9. Duffour, M. T., Chaux, P., Lurquin, C., Cornelis, G., Boon, T., and van der Bruggen, P. A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes. Eur. J. Immunol., *29:* 3329–3337, 1999.
10. Van den Eynde, B. J. and van der Bruggen, P. T cell defined tumor antigens. Curr. Opin. Immunol., *9:* 684–693, 1997.
11. Schirle, M., Keilholz, W., Weber, B., Gouttefangeas, C., Dumrese, T., Becker, H. D., Stevanovic, S., and Rammensee, H. Identification of tumor-associated MHC class I ligands by a novel T cell independent approach. Eur. J. Immunol., *30:* 2216–2225, 2000.
12. Pellat-Deceunynck, C., Mellerin, M. P., Labarriere, N., Jego, G., Moreau-Aubry, A., Harousseau, J. L., Jotereau, F., and Bataille, R. The cancer germ-line genes *MAGE-1, MAGE-3* and PRAME are commonly expressed by human myeloma cells. Eur. J. Immunol., *30:* 803–809, 2000.
13. De Smet, C., De Backer, O., Faraoni, I., Lurquin, C., Brasseur, F., and Boon, T. The activation of human gene *MAGE-1* in tumor cells is correlated with genome-wide demethylation. Proc. Natl. Acad. Sci. USA, *93:* 7149–7153, 1996.
14. Pascolo, S., Bervas, N., Ure, J. M., Smith, A. G., Lemonnier, F. A., and Perarnau, B. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from $\beta$2 microglobulin ($\beta$2m) HLA-A2.1 monochain transgenic H-2Db $\beta$2m double knockout mice. J. Exp. Med., *185:* 2043–2051, 1997.
15. Faulkner, L., Borysiewicz, L. K., and Man, S. The use of human leucocyte antigen class I transgenic mice to investigate human immune function. J. Immunol. Methods, *221:* 1–16, 1998.
16. Guckel, B., Meyer, G. C., Rudy, W., Batrla, R., Meuer, S. C., Bastert, G., Wallwiener, D., and Moebius, U. Interleukin-12 requires initial CD80-mediated T-cell activation to support immune responses toward human breast and ovarian carcinoma. Cancer Gene Ther., *6:* 228–237, 1999.
17. Meyer, G. C., Batrla, R., Meuer, S. C., Wallwiener, D., Guckel, B., and Moebius, U. The potential of CD80-transfected human breast carcinoma cells to induce peptide-specific T lymphocytes in an allogenic HLA-A2.1+-matched situation. Cancer Gene Ther., *6:* 282–288, 1999.
18. Russo, V., Traversari, C., Verrecchia, A., Mottolese, M., Natali, P. G., and Bordignon, C. Expression of the *MAGE* gene family in primary and metastatic human breast cancer: implications for tumor antigen-specific immunotherapy. Int. J. Cancer, *64:* 216–221, 1995.
19. Kavathas, P., Bach, F. H., and DeMars, R. Gamma ray-induced loss of expression of HLA and glyoxalase I alleles in lymphoblastoid cells. Proc. Natl. Acad. Sci. USA, *77:* 4251–4255, 1980.
20. Rammensee, H., Bachmann, J., Emmerich, N. P., Bachor, O. A., and Stevanovic, S. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics, *50:* 213–219, 1999.
21. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H. G. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature, *351:* 290–296, 1991.
22. Seeger, F. H., Schirle, M., Gatfield, J., Arnold, D., Keilholz, W., Nickolaus, P., Rammensee, H. G., and Stevanovic, S. The HLA-A*6601 peptide motif: prediction by pocket structure and verification by peptide analysis. Immunogenetics, *49:* 571–576, 1999.
23. Milich, D. R., Hughes, J. L., McLachlan, A., Thornton, G. B., and Moriarty, A. Hepatitis B synthetic immunogen comprised of nucleocapsid T-cell sites and an envelope B-cell epitope. Proc. Natl. Acad. Sci. USA, *85:* 1610–1614, 1988.
24. Gaines, P. and Wojchowski, D. M. pIRES-CD4t, a dicistronic expression vector for MACS- or FACS-based selection of transfected cells. Biotechniques, *26:* 683–688, 1999.
25. Firat, H., Garcia-Pons, F., Tourdot, S., Pascolo, S., Scardino, A., Garcia, Z., Michel, M. L., Jack, R. W., Jung, G., Kosmatopoulos, K., Mateo, L., Suhrbier, A., Lemonnier, F. A., and Langlade-Demoyen, P. H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies. Eur. J. Immunol., *29:* 3112–3121, 1999.
26. Morel, S., Levy, F., Burlet-Schiltz, O., Brasseur, F., Probst-Kepper, M., Peitrequin, A. L., Monsarrat, B., Van Velthoven, R., Cerottini, J. C., Boon, T., Gairin, J. E., and Van den Eynde, B. J. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. Immunity, *12:* 107–117, 2000.
27. Kuttler, C., Nussbaum, A. K., Dick, T. P., Rammensee, H., Schild, H., and Hadeler, K. P. An algorithm for the prediction of proteasomal cleavages. J. Mol. Biol., *298:* 417–429, 2000.
28. Spencer, J. V. and Braciale, T. J. Incomplete CD8(+) T lymphocyte differentiation as a mechanism for subdominant cytotoxic T lymphocyte responses to a viral antigen. J. Exp. Med., *191:* 1687–1698, 2000.
29. Chen, Y. M., Yang, W. K., Whang-Peng, J., Tsai, W. Y., Hung, Y. M., Yang, D. M., Lin, W. C., Perng, R. P., and Ting, C. C. Restoration of the immunocompetence by IL-2 activation and TCR-CD3 engagement of the *in vivo* anergized tumor-specific CTL from lung cancer patients. J. Immunother., *20:* 354–364, 1997.
30. Lee, P. P., Yee, C., Savage, P. A., Fong, L., Brockstedt, D., Weber, J. S., Johnson, D., Swetter, S., Thompson, J., Greenberg, P. D., Roederer, M., and Davis, M. M. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat. Med., *5:* 677–685, 1999.
31. Dressel, R., Elsner, L., Quentin, T., Walter, L., and Gunther, E. Heat shock protein 70 is able to prevent heat shock-induced resistance of target cells to CTL. J. Immunol., *164:* 2362–2371, 2000
32. Walker, P. R., Saas, P., and Dietrich, P. Y. Role of Fas ligand (CD95L) in immune escape: the tumor cell strikes back. J. Immunol., *158:* 4521–4524, 1997.
33. Medema, J. P., de Jong, J., Bres, S., Schumacher, T., Tourdot, S., Toebes, M., Melief, K., and Offringa, R. Immune escape by tumors through inhibition of apoptotic pathways. ENII Conf., 2000.
34. Hernandez, J., Lee, P. P., Davis, M. M., and Sherman, L. A. The use of HLA A2.1/p53 peptide tetramers to visualize the impact of self tolerance on the TCR repertoire. J. Immunol., *164:* 596–602, 2000.
35. Escobar, P., Yu, Z., Terskikh, A., Holmes, N., Corradin, G., Mach, J. P., and Healy, F. Induction in transgenic mice of HLA-A2.1-restricted cytotoxic T cells specific for a peptide sequence from a mutated p21ras protein. Clin. Exp. Immunol., *116:* 214–219, 1999.
36. Colella, T. A., Bullock, T. N., Russell, L. B., Mullins, D. W., Overwijk, W. W., Luckey, C. J., Pierce, R. A., Restifo, N. P., and Engelhard, V. H. Self-tolerance to the murine homologue of a tyrosinase-derived melanoma antigen: implications for tumor immunotherapy. J. Exp. Med., *191:* 1221–1232, 2000.
37. Bullock, T. N., Colella, T. A., and Engelhard, V. H. The density of peptides displayed by dendritic cells affects immune responses to human tyrosinase and gp100 in HLA-A2 transgenic mice. J. Immunol., *164:* 2354–2361, 2000.
38. Valitutti, S., Muller, S., Dessing, M., and Lanzavecchia, A. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. J. Exp. Med., *183:* 1917–1921, 1996.

# A MAGE-A1 HLA-A*0201 Epitope Identified by Mass Spectrometry

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