

# Identification of New Antigenic Peptide Presented by HLA-Cw7 and Encoded by Several MAGE Genes Using Dendritic Cells Transduced with Lentiviruses<sup>1</sup>

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Antigens encoded by *MAGE* genes are of particular interest for cancer immunotherapy because they are tumor specific and shared by tumors of different histological types. Several clinical trials are in progress with MAGE peptides, proteins, recombinant poxviruses, and dendritic cells (DC) pulsed with peptides or proteins. The use of gene-modified DC would offer the major advantage of a long-lasting expression of the transgene and a large array of antigenic peptides that fit into the different HLA molecules of the patient. In this study, we tested the ability of gene-modified DC to prime rare Ag-specific T cells, and we identified a new antigenic peptide of clinical interest. CD8<sup>+</sup> T lymphocytes from an individual without cancer were stimulated with monocyte-derived DC, which were infected with a second-generation lentiviral vector encoding *MAGE-3*. A CTL clone was isolated that recognized peptide EGDCAPEEK presented by HLA-Cw7 molecules, which are expressed by >40% of Caucasians. Interestingly, this new tumor-specific antigenic peptide corresponds to position 212–220 of *MAGE-2*, -3, -6, and -12. HLA-Cw7 tumor cell lines expressing one of these *MAGE* genes were lysed by the CTL, indicating that the peptide is efficiently processed in tumor cells and can therefore be used as target for antitumoral vaccination. The risk of tumor escape due to appearance of Ag-loss variants should be reduced by the fact that the peptide is encoded by several MAGE genes. *The Journal of Immunology*, 2004, 172: 2232–2237.

Human tumors bear Ags that are recognized by autologous T lymphocytes and that are highly specific for tumors. Some of these Ags are shared by many tumors of various histological types (1, 2). The genes that code for these Ags are known. Clinical trials involving defined tumor-specific shared Ags have been performed in melanoma patients, and tumor regressions have been observed, even in patients with advanced disease (3–6). The vaccines consist of peptides, protein, recombinant viruses carrying the sequence coding for the Ag, and dendritic cells (DC)<sup>3</sup> loaded with defined antigenic peptides or with the entire protein (3–7). Loading DC with the entire protein has the advantage that peptides binding to a broad set of HLA molecules can be processed from the protein. However, the protein is delivered in limited amounts, resulting in a transient presentation of antigenic peptides, mostly presented on HLA class II molecules. An approach that avoids the drawbacks of this transient presentation would be the genetic modification of the DC *ex vivo*, using an entire Ag-coding sequence (8, 9). Gene-modified DC offer a major advantage by

providing a long-lasting expression of the entire array of peptides that fit into the patient's HLA molecules.

Recently, HIV-1-derived lentiviral vectors emerged as a powerful tool for gene delivery into DC (10–17). The performance and safety of the new vectors have been improved considerably compared with the first generation (12, 18–23). We have shown that the triple helix-containing, self-inactivating lentiviral vector is very efficient in transducing nonproliferating, monocyte-derived DC (16). DC transduced with lentiviruses stimulated Ag-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells *in vitro* and *in vivo*; in particular, they were able to prime CTL responses against melanoma differentiation Ags (12–17).

Gene *MAGE-3* is expressed in many tumors of various histological types, but is silent in normal cells, with the exception of male germline cells and placenta, which do not express MHC molecules and therefore cannot present antigenic peptides to T cells (24). Protein *MAGE-3* was shown to be located in the cytosol (25). We have fused the coding sequence of *MAGE-3* with the coding sequence of the first 80 aa of the human invariant chain (Ii), and have shown that some anti-*MAGE-3* CD4<sup>+</sup> T cells can recognize cells transduced with Ii-*MAGE-3* constructs, but not cells expressing *MAGE-3* (26). It has been reported that signals within the Ii could be used to target endogenously synthesized protein to the class II Ag-processing compartments (27). Interestingly, we have observed that cells expressing the Ii-*MAGE-3* constructs can also present very efficiently peptides on HLA class I molecules.

To prove that DC transduced with lentiviruses carrying a Ii-*MAGE-3* construct are able to prime rare Ag-specific CD8<sup>+</sup> T cells and with the purpose of identifying new antigenic peptides, we stimulated blood T cells from an individual without cancer with gene-modified autologous DC. An anti-*MAGE-3* CTL was isolated that lysed relevant tumor targets. The antigenic peptide is presented by HLA-Cw7, which is frequently expressed in Caucasians. Importantly,

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell;  $\beta_2m$ ,  $\beta_2$ -microglobulin; Ii, invariant chain; tNGFR, truncated nerve growth factor receptor.

the MAGE-3 antigenic peptide is also encoded by several other MAGE genes.

## Materials and Methods

### Cell lines, medium, and reagents

The PhoenixAmpho cell line, the K562 cell line, and the 293T cell line were cultured in DMEM (BioWhittaker, Walkersville, MD) containing 10% FCS (Harlan, Ad Horst, The Netherlands), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine (BioWhittaker).

The EBV B cell lines and the tumor cell lines (LB1017-HNSC, LB2077-MEL, CP50-MEL, LB30-MEL, LB37-NSCLC, MZ-MEL-7) were cultured in IMEM (BioWhittaker) supplemented with 10% FCS, 0.24 mM L-asparagine, 0.55 mM L-arginine, 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

Human recombinant IL-4, IL-1 $\beta$ , and IL-6 were produced in our laboratory. The cytokine GM-CSF was purchased from Novartis (Brussels, Belgium). IL-2, IL-7, IL-12, and TNF- $\alpha$  were purchased from PeproTech (Rocky Hill, NJ). PGE<sub>2</sub> and PHA were obtained from Sigma-Aldrich (St. Louis, MO).

### Generation of DC

PBMC were isolated from buffy coat preparations of an individual without cancer by Lymphoprep NYCOMED density centrifugation (Nycomed Pharma AS, Oslo, Norway). Subsequently, CD14<sup>+</sup> cells were separated by magnetic sorting using the VarioMACS technique (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), following the manufacturer's instructions. Monocytes were differentiated into DC in X-VIVO 15 medium (BioWhittaker) supplemented with 1% heat-inactivated human AB serum (PAA Laboratories, Linz, Austria) at a cell density of  $1 \times 10^6$  DC/ml, with GM-CSF (1000 U/ml) and IL-4 (100 U/ml). Cells were incubated in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. On day 3 of culture, DC were lentivirally transduced. On day 6 of culture, the DC were matured at a cell density of  $5 \times 10^5$  DC/ml X-VIVO 15 containing 1% human AB serum in a cytokine mix of IL-1 $\beta$  (100 U/ml), IL-6 (1000 U/ml), TNF- $\alpha$  (100 U/ml), and PGE<sub>2</sub> (1  $\mu$ g/ml) (28).

### Lentivirus production and characterization

The multiple attenuated packaging plasmid pCMV $\Delta$ R8.9 and the VSV.G-encoding plasmid pMD.G were a kind gift from D. Trono (University of Geneva, Geneva, Switzerland). The transfer vector pHR'ripCMVhul80 MAGE-3-Ires-tNGFR SIN (referred to as pHR'Ii-MAGE-3), encoding the first 80 aa of the human Ii fused to the entire MAGE-3 coding sequence, has been described (16).

The VSV.G-pseudotyped HIV-1-derived viral particles were generated by cotransfection of the transfer vector, the VSV.G-expressing construct pMD.G, and the packaging construct pCMV $\Delta$ R8.9 into 293T cells. The lentivirus-containing supernatant was collected 48 and 72 h after transfection, filtered through a 0.22- $\mu$ m-pore-size filter, and concentrated 200 $\times$  via ultracentrifugation in a Beckman SW28 rotor (Optima LE-80K ultracentrifuge; Beckman Coulter, Palo Alto, CA) for 140 min at 19,500 rpm. The virus stock was stored at  $-80^\circ\text{C}$  in X-VIVO 15 containing 1% human AB serum.

Viral titers were determined by infection of 293T cells with serial dilutions of the vector stock. Seventy-two hours after infection, the number of tNGFR-positive cells was scored to determine the titer. The titer was calculated as follows: (percentage of tNGFR-positive cells  $\times$  number of cells transduced)  $\times$  dilution factor/(100  $\times$  transduction volume).

### Transduction of human DC

The transduction of DC was performed, as recently described (16). Briefly,  $1 \times 10^6$  day 3 DC resuspended in 1 ml of X-VIVO 15 containing 1% human AB serum, protamin sulfate (10  $\mu$ g/ml; LeoPharma, Thornhill, Ontario, Canada), IL-4 (100 U/ml), and GM-CSF (1000 U/ml) were infected with recombinant lentiviruses at a multiplicity of infection of 15. The immature transduced DC were cultured at a cell density of  $1 \times 10^6$  cells/ml until day 6. Subsequently, day 6 DC were matured, as described above.

### Cryopreservation of DC

DC were frozen in cryotubes in 1 ml of 20% albumin solution with 10% DMSO (Sigma-Aldrich) at  $1-5 \times 10^6$  DC/vial. The DC were slowly frozen to  $-80^\circ\text{C}$  using a cryofreezing container (rate of cooling  $-1^\circ\text{C}/\text{min}$ ; Nalgene, Hereford, U.K.) and subsequently stored in liquid nitrogen. Thawing of the cryopreserved DC was performed in a  $37^\circ\text{C}$  waterbath until small ice crystals were visible. Cold HBSS (Invitrogen, Paisley, U.K.) was added dropwise. The DC were pelleted in a precooled centrifuge and resuspended

in 5 ml of prewarmed X-VIVO 15 medium containing 1% human AB serum. After a resting period of 15 min, the cells were counted using trypan blue.

### Flow cytometry

All stainings were performed for 30 min on ice in PBS containing 1% BSA and 0.02% sodium azide. FITC- or PE-conjugated mAbs specific for CD80, CD83, and CD86 were purchased from BD PharMingen (San Jose, CA). The anti-human tNGFR (clone HB8737) and anti-HLA-DR (clone L243) Abs were affinity purified and biotinylated in our lab. Biotinylated Abs were detected with streptavidin-PE (BD PharMingen). The stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest software. All stainings were compared with irrelevant isotype control Abs purchased from BD PharMingen.

### Semiquantitative RT-PCR

Total RNA was extracted from the DC using the SV Total RNA Isolation System (Promega, Madison, WI). Total RNA (1  $\mu$ g) was converted into first-strand cDNA using random hexamers and SuperScript II reverse transcriptase, according to the manufacturer's recommendations (Superscript First Strand Synthesis System for RT-PCR; Invitrogen). cDNA was amplified using BIOTAQ DNA polymerase (Biolone, London, U.K.), following the manufacturer's instructions in a PerkinElmer GeneAmp PCR system (PerkinElmer, Wellesley, MA). The RT-PCR was performed using the Access RT-PCR System (Promega). The MAGE-A3 cDNA was amplified using the MAGE-A3-sense, 5'-CCCAGATCTGGGGAGTGTCTCGTCCGA-3' and the MAGE-A3-antisense, 5'-CCCCTCGAGTCACTCTCCCCCTCTC TC-3' primers. To assess the RNA and cDNA quality, an RT-PCR for the housekeeping gene  $\beta$ -actin was performed.

### CD8<sup>+</sup> responder T cells and mixed lymphocyte-DC culture

After isolation of the CD14<sup>+</sup> monocytes from the PBMC of the donor, autologous, CD8<sup>+</sup> responder T cells were enriched by magnetic sorting using the VarioMACS technique (Miltenyi Biotec GmbH), following the manufacturer's instructions.

Mature, lentivirally transduced day 7 DC were harvested, washed in X-VIVO 15, and mixed with autologous CD8<sup>+</sup> responder T cells in a round-bottom 96-well plate at a T cell-DC ratio of 10:1 in 200  $\mu$ l of X-VIVO 15 supplemented with 1% human AB serum in the presence of IL-6 (1000 U/ml) and IL-12 (10 ng/ml). On days 7 and 14, autologous, lentivirally transduced DC were thawed and used to restimulate the CD8<sup>+</sup> lymphocytes in medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). The CD8<sup>+</sup> responder T cells were assessed on day 21 for their capacity to recognize autologous non- or retrovirally transduced EBV B cells in an IFN- $\gamma$  secretion assay.

### Retroviral transduction of EBV-transformed B cells and 293T cells

The retroviral pMFG vectors, encoding the MAGE-3 cDNA and the transduction procedure of EBV B cells, were previously described (26, 29). The human embryonal kidney cell line 293T was retrovirally transduced using the same procedure.

### IFN- $\gamma$ assay

In the IFN- $\gamma$  secretion assay,  $5 \times 10^3$  T cells were cocultured with  $2 \times 10^4$  stimulator cells (EBV B cells) in a round-bottom 96-well plate in 200  $\mu$ l of IMEM, containing 10% AB serum, L-asparagine, L-arginine, and L-glutamine (complete IMEM), and supplemented with 25 U/ml IL-2. After 24 h, the supernatant was collected, and its IFN- $\gamma$  content was determined in ELISA (Endogen, Woburn, MA), following the manufacturer's instructions.

### CD8<sup>+</sup> T cell clone

The CD8<sup>+</sup> T cell microcultures that specifically recognized autologous MAGE-3-expressing EBV B cells were cloned by limiting dilution in a round-bottom 96-well plate, using irradiated, allogeneic PBMC ( $3 \times 10^4$  PBMC) and LG2 EBV B cells ( $3 \times 10^4$  cells) as feeder cells. Irradiated, autologous, retrovirally transduced EBV B cells were added as stimulator cells ( $3 \times 10^4$  cells). The cytokines IL-2 (50 U/ml), IL-4 (5 U/ml), and IL-7 (5 ng/ml), as well as PHA (0.1  $\mu$ g/ml) were added. The established CD8<sup>+</sup> T cell clone was grown in complete IMEM supplemented with the same cytokines and PHA and passaged with feeder and stimulator cells ( $1 \times 10^6$  LG2 EBV B and  $2 \times 10^5$  autologous, retrovirally transduced EBV B cells/well) in a 24-well plate at 7- to 10-day intervals.

### Transfection of non- and retrovirally transduced 293T cells

**Plasmids.** To clone the MAGE-3 coding sequence into the expression vector pCI, a PCR was performed on the plasmid pTZ18R-MAGE-3 (sense, 5'-CCCCCATGG<sub>NcoI</sub>CTCTTGAGCAGAGGAGTC-3'; antisense, 5'-GGGAGATCT<sub>BglII</sub>TCACTCTCCCCTCTCAAAAC-3'). During this PCR, a restriction site is incorporated at both extremities of the gene: the 5' *NcoI* site contains the MAGE-3 start codon. The 3' *BglII* site is preceded by a stop codon. The resulting PCR product was cloned into the pCR2.1 vector. After sequence analysis, the MAGE-3 cDNA was excised as an *EcoRI-EcoRI* fragment and cloned into the *EcoRI*-digested pCI vector, resulting in pCI-MAGE-3.

**Transfection.** Non- or retrovirally transduced 293T cells ( $1 \times 10^6$  cells/6 well) were cotransfected via the calcium-phosphate method with 5  $\mu\text{g}$  of HLA-Cw\*0701- or HLA-A\*0101-encoding plasmid, 5  $\mu\text{g}$  of  $\beta_2$ -microglobulin ( $\beta_2\text{m}$ )-encoding plasmid, and 4  $\mu\text{g}$  of MAGE-2, -A3, -A6, -A12, or tyrosinase-encoding plasmid. Transfected cells were incubated for 24 h at 37°C and 5%  $\text{CO}_2$ . The transfectants were then used as targets in a standard  $^{51}\text{Cr}$  release assay.

### Standard $^{51}\text{Cr}$ release assay

Target cells (transfected 293T cells or tumor cells) were labeled with 100  $\mu\text{Ci}$  of  $\text{Na}^{51}\text{CrO}_4$  for 1 h and extensively washed. Five thousand target cells were then cocultured for 4 h with the CTL at E:T ratios varying from 10:1 to 1:1 in a total volume of 200  $\mu\text{l}$ /well in a 96-well round-bottom plate. Spontaneous  $^{51}\text{Cr}$  release (culture medium) and maximal release (2.5% SDS) were determined on each plate. To determine the amount of  $^{51}\text{Cr}$  released by lysed cells, 50  $\mu\text{l}$  of the coculture supernatant was added to 150  $\mu\text{l}$  of scintillation fluid and counted in a beta counter. The specific lysis was calculated as follows: percentage of lysis = (percentage of experimental  $^{51}\text{Cr}$  release - spontaneous  $^{51}\text{Cr}$  release)  $\times$  100/(maximal release  $^{51}\text{Cr}$  release - spontaneous  $^{51}\text{Cr}$  release).

### Peptide recognition assays

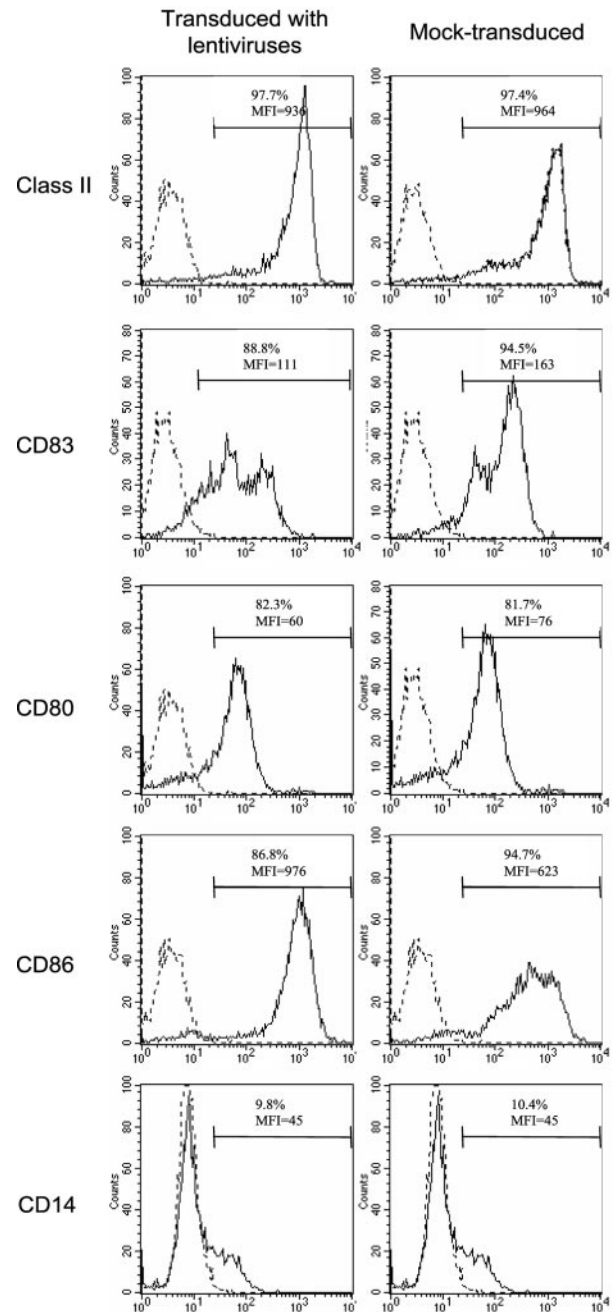
Peptides were synthesized on solid phase using F-moc for transient  $\text{NH}_2$ -terminal protection, and were characterized using mass spectrometry. Lyophilized peptides were dissolved at 2 mg/ml in DMSO/10 mM of acetic acid and stored at -20°C. Autologous EBV B cells were loaded with peptide at a density of  $2 \times 10^6$  cells/ml in serum-free IMEM with 10  $\mu\text{g}/\text{ml}$  peptide for 2 h at 37°C. Before use, the peptide-loaded EBV B cells were washed twice in IMEM. These cells were used as stimulators in an IFN- $\gamma$  assay. To test peptide recognition in a  $^{51}\text{Cr}$  release assay, peptides were distributed at 10  $\mu\text{M}$  in 50  $\mu\text{l}$  of serum-free medium and further diluted (dilution 3 $\times$ ) to 1 nM. Autologous,  $^{51}\text{Cr}$ -labeled EBV B cells, plated at  $1 \times 10^3$  targets in 100  $\mu\text{l}$  of serum-free medium, were incubated for 15 min with the peptides in the presence of 3  $\mu\text{g}/\text{ml}$   $\beta_2\text{m}$ . Subsequently,  $5 \times 10^3$  effector T cells were added in 50  $\mu\text{l}$  of medium containing 1% human AB serum. To determine the amount of  $^{51}\text{Cr}$  released by lysed cells, 50  $\mu\text{l}$  of the coculture supernatant was added to 150  $\mu\text{l}$  of scintillation fluid and counted in a beta counter. The specific lysis was calculated, as described above.

## Results

### *In vitro* priming of an anti-MAGE-3 CTL clone by DC transduced with lentiviruses

Monocyte-derived DC of blood donor MD0101, an individual without cancer, were transduced at an early stage of differentiation with HIV-1-derived, triple helix-containing, self-inactivating lentiviral particles. The viral vector contained the coding sequence of a fusion protein containing the first 80 aa of the human Ii and the MAGE-3 protein (pHR/Ii-MAGE-3). Maturation of the DC was induced on day 6 by the addition of a mixture of inflammatory cytokines. An average of 80% (range  $\pm$  9%,  $n = 3$ ) of the DC stained positive for tNGFR. Expression of tNGFR could still be detected 1 wk after transduction. To assess the duration of MAGE-3 expression, we have performed a qualitative RT-PCR on cDNA obtained from 7- and 10-day-old pHR/Ii-MAGE-3-transduced DC, showing that mRNA for Ii-MAGE-3 was still present 4 days and 1 wk after transduction (16).

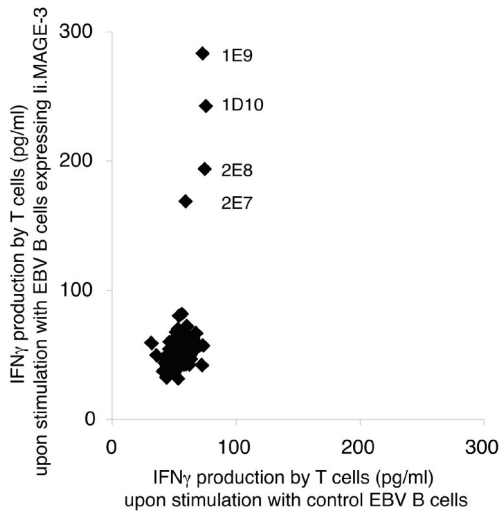
The lentivirally transduced DC showed a mature phenotype that was similar to their mock-transduced counterparts (Fig. 1).



**FIGURE 1.** Phenotype of DC transduced with lentiviruses. DC were differentiated from monocytes in the presence of IL-4 and GM-CSF. Immature DC were transduced on day 3 with a second-generation, triple helix-containing lentivirus containing the coding sequence of an Ii-MAGE-3 fusion protein. Maturation of the lentivirally transduced DC was induced on day 6 by the addition of a mixture of inflammatory cytokines: IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and PGE $_2$ .

The transduced DC were distributed in 96 microwells ( $1 \times 10^4$  cells) and were used to stimulate autologous CD8 $^+$  T lymphocytes ( $1 \times 10^5$  cells) in the presence of IL-6 and IL-12. Two restimulations were performed at weekly intervals with transduced DC in the presence of only IL-2 and IL-7. Aliquots of each microculture were tested on day 24 for their ability to secrete IFN- $\gamma$  specifically upon contact with cells expressing Ii-MAGE-3 (Fig. 2). Four positive microcultures were detected. The lymphocytes from microculture 1E9 were cloned by limiting dilution and stimulated with irradiated autologous MAGE-3-expressing EBV B cells. CTL



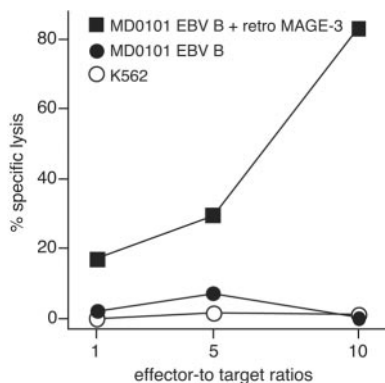


**FIGURE 2.** Presence of anti-MAGE-3 T cells in cultures primed with DC transduced with lentiviruses. CD8<sup>+</sup> T cells from an individual without cancer were stimulated with autologous DC infected with a lentiviral vector containing the coding sequence of an li-MAGE-3 fusion protein. After three stimulations at weekly intervals, 5,000 T cells from each microculture were cocultured with 20,000 autologous EBV B cells transduced or not with a retrovirus-encoding li-MAGE-3. The production of IFN- $\gamma$  was measured after overnight coculture by ELISA. The IFN- $\gamma$  production upon contact with li-MAGE-3-transduced EBV B cells (y-axis) is plotted against the IFN- $\gamma$  produced upon contact with control EBV B cells (x-axis). Microcultures containing anti-MAGE-3 T cells are indicated.

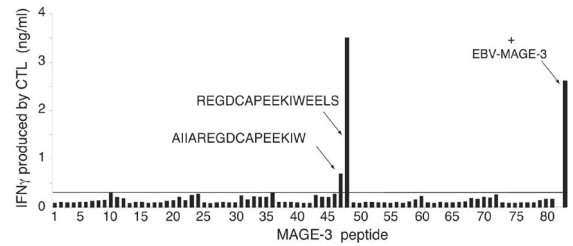
clone MD0101-1E9-4A7 was obtained that lysed specifically autologous EBV B cells expressing MAGE-3 (Fig. 3). This CTL clone will be further referred to as clone MD1.

*Identification of the MAGE-3 antigenic peptide*

CTL MD1 was stimulated with autologous EBV B cells incubated with each of a set of 81 peptides of 16 aa, overlapping by 12 residues and covering the complete MAGE-3 protein sequence. Two overlapping peptides were recognized by the CTL (Fig. 4). Shorter peptides were tested, and the shortest optimal peptide proved to be EGDCAPEEK (Fig. 5). Fifty percent of lysis of the target cells was obtained with only 2 nM of peptide. EGDCAPEEK corresponds to the position 212–220 of the MAGE-3 protein sequence. Recogni-



**FIGURE 3.** Lysis of cells expressing MAGE-3 by CTL clone MD1. Autologous EBV B cells, transduced or not with retrovirus containing the coding sequence of MAGE-3, and K562 cells were <sup>51</sup>Cr labeled for 1 h and incubated with the autologous CTL clone at the indicated E:T ratios. Cr release was measured after 4 h. The data shown represent the average of triplicate cultures.



**FIGURE 4.** Identification of two long overlapping peptides recognized by CTL clone MD1. Autologous EBV B cells were pulsed with 10  $\mu$ g/ml of each of a set of 16-aa-long MAGE-3 peptides. The peptide-pulsed EBV B cells ( $2 \times 10^4$ ) were cocultured with CTL clone MD1 ( $5 \times 10^3$  T cells). EBV B cells expressing MAGE-3<sup>+</sup> were used as positive control cells. The production of IFN- $\gamma$  by the CTL clone was measured by ELISA after overnight coculture.

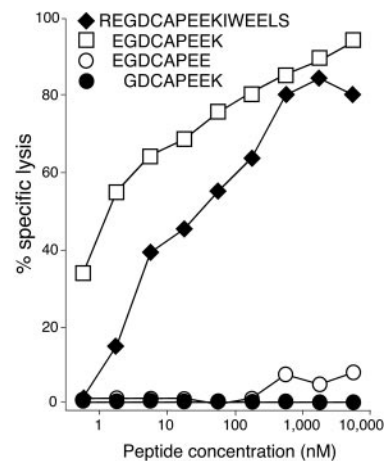
tion by the CTL was lost for peptides shorter either at the N terminus or the C terminus (Fig. 5).

*Identification of the presenting HLA molecule*

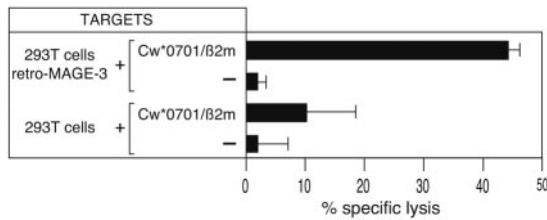
Blood donor MD0101 was serologically typed HLA-A2, A3, B7, and Cw7. To identify the HLA-presenting molecule, EBV B cells of different donors, loaded with peptide REGDCAPEEKIWEELS, were used to stimulate clone MD1 to produce IFN- $\gamma$ . Only the HLA-Cw7 EBV B cells were recognized by CTL MD1 (data not shown). To confirm these results, 293T cells, which were transiently transfected with an HLA-Cw\*0701 and a  $\beta_2$ m cDNA. Only 293T cells expressing MAGE-3 and transfected with HLA-Cw\*0701/ $\beta_2$ m were lysed by CTL MD1 (Fig. 6).

*MAGE-3 peptide EGDCAPEEK is encoded by several other MAGE genes*

Peptide EGDCAPEEK and several amino acids flanking the C terminus of the antigenic peptide are not only coded by MAGE-3, but also by other members of the MAGE gene family (Fig. 7A). CTL MD1 was able to lyse 293T cells transiently transfected with HLA-Cw\*0701 together with either MAGE-2, -3, -6, or -12, indicating that the antigenic peptide can be processed from several MAGE proteins (Fig. 7B).



**FIGURE 5.** Titration of different MAGE-3 peptides. Autologous EBV B cells were <sup>51</sup>Cr labeled and incubated with 3-fold dilutions of synthetic peptides. Autologous CTL MD1 was subsequently added at an E:T ratio of 5:1. Cr release was measured after 4 h. The concentrations indicated in the figure are the concentrations during the 4 h of incubation.



**FIGURE 6.** The MAGE-3 antigenic peptide is presented to CTL MD1 by HLA-Cw\*0701. The 293T cells were transduced or not with a retrovirus containing the coding sequence of MAGE-3. Each of these sublines was transiently transfected with an HLA-Cw\*0701 and a  $\beta_2m$  cDNA. One day after transfection, transfected cells were  $^{51}Cr$  labeled for 1 h and incubated with the autologous CTL clone at an E:T ratio of 1:1. Cr release was measured after 4 h. The data shown represent the average of triplicate cultures.

*Lysis of tumor cell lines by CTL clone MD1*

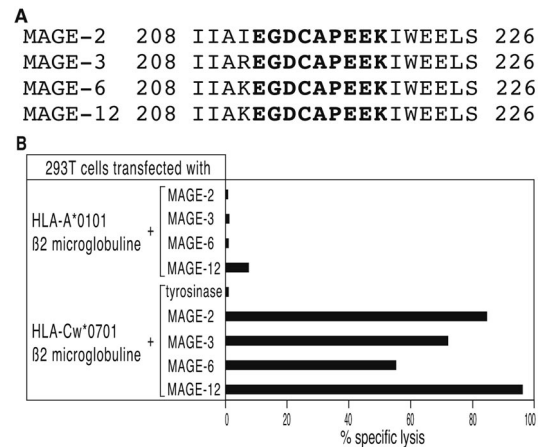
Because CTL clone MD1 was primed by DC, it was important to verify that tumor cells also process the MAGE Ag. We tested a number of HLA-Cw7 tumor cell lines from different histological types: melanomas, a head and neck squamous cell carcinoma, and a nonsmall cell lung carcinoma. The tumor cell lines expressed either MAGE-6 alone or several of the MAGE genes encoding the antigenic peptide (Fig. 8). They were efficiently lysed, whereas autologous EBV B cells or control K562 cells were not.

**Discussion**

Our results clearly show the ability of DC transduced with lentiviruses to prime rare Ag-specific CD8<sup>+</sup> T cells. In this method, we used autologous EBV-transformed B cells, which were retrovirally transduced to express MAGE-3, to screen for MAGE-3-specific T cells and to further subclone primed CTL. These cells were chosen because we needed a continuous source of autologous cells expressing MAGE-3. We could have used autologous DC modified to express MAGE-3, e.g., by electroporation or transduced with a vector system. However, such cells are often available in limited numbers.

Noteworthy, the Ii-MAGE-3 fusion protein, which is normally targeted into the class II-processing compartments, is also processed and presented efficiently by HLA class I molecules. The presentation by the two types of HLA-presenting molecules will be an advantage in a clinical setting using gene-modified DC.

The use of gene-modified DC for the identification of new antigenic peptides is an attractive alternative to the peptide consensus approach, in which a protein sequence is searched for the presence of peptides bearing consensus anchor residues. Because DC are transduced with a complete coding sequence, the Ag has to be



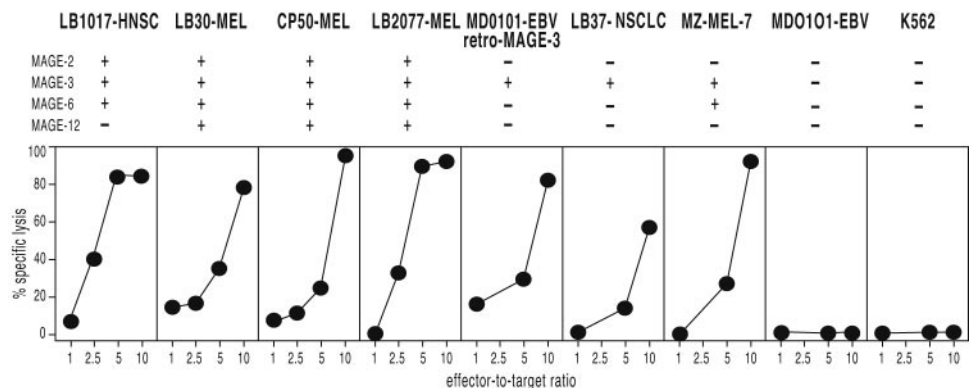
**FIGURE 7.** Peptide EGDCAPEEK is processed from several MAGE proteins. *A*, The amino acid sequence of homologous regions of proteins MAGE-2, -A3, -A6, and -A12 is shown. The peptide EGDCAPEEK is indicated in bold. *B*, 293T cells were transiently cotransfected with the cDNA containing the coding sequence of different MAGE proteins or tyrosinase, together with HLA-Cw\*0701, HLA-A\*0101, and  $\beta_2m$  cDNA. One day after transfection, the 293T cells were  $^{51}Cr$  labeled for 1 h and incubated with the CTL clone MD1 at an E:T ratio of 5:1. Cr release was measured after 4 h. The data shown represent the average of triplicate cultures.

processed by the DC, and we can surmise that the peptides would also be processed in the tumors expressing the relevant gene. A few antigenic peptides would have been missed by the peptide consensus approach, either because they result from posttranslational modifications, or because they do not contain the consensus anchor residues (29–33). The same is true for peptide EGDCAPEEK described in this study that is presented to CTL by HLA-Cw7 molecules and does not contain the proposed anchor residues for HLA-Cw7: a tyrosine, leucine, or phenylalanine at the C terminus (34–36).

HLA-Cw7 molecules seem to be expressed by ~41% of Caucasians, 40% of Blacks, and 28% of Orientals (36). Tumors expressing one of the genes coding for peptide EGDCAPEEK are very frequent: 81% of metastatic melanomas, 74% of esophageal carcinomas, 62% of infiltrating bladder carcinomas, 61% of squamous cell lung carcinomas, and 60% of head and neck tumors (F. Brasseur, unpublished observations). Therefore, the peptide EGDCAPEEK is a promising target for antitumoral vaccination in a substantial cohort of cancer patients.

Because peptide EGDCAPEEK is encoded by several MAGE genes, the risk of tumor escape by loss of expression of the gene coding the antigenic peptide should be reduced. The loss of expression of HLA-Cw7 could also result in Ag-loss tumor variants,

**FIGURE 8.** Lysis of HLA-Cw7 tumor cells expressing MAGE genes by CTL clone MD1. Melanoma cell lines (MEL), a head and neck squamous cell carcinoma cell line (HNSC), and a nonsmall cell lung carcinoma cell line (NSCLC) were used as targets. Cells were  $^{51}Cr$  labeled and incubated with CTL clone MD1 at the indicated E:T ratios. Cr release was measured after 4 h. The data shown represent the average of triplicate cultures.



but this can be counteracted by activation of NK cells that detect the loss of HLA-Cw7 molecules.

In future trials, it will be essential to have reliable monitoring of the CTL response against the immunizing Ag. One promising possibility, which nevertheless restricts the analysis to certain epitopes, is the use of a set of relevant peptides in combination with soluble HLA tetramers to label T cell receptors directly. Only such a detailed analysis of the anti-MAGE-CTL responses of patients will provide information on the immunogenicity of the various MAGE epitopes. Because HLA-Cw7 molecules are widely expressed, the identification of Cw7-restricted peptide EGDCAPEEEK is of importance.

## References

- Boon, T., J. C. Cerottini, B. Van den Eynde, P. van der Bruggen, and A. Van Pel. 1994. Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.* 12:337.
- Van der Bruggen, P., Y. Zhang, P. Chau, V. Stroobant, C. Panichelli, E. S. Shultz, J. Chapiro, B. J. Van den Eynde, F. Brasseur, and T. Boon. 2002. Tumor-specific shared antigenic peptides recognized by human T cells. *Immunol. Rev.* 188:51.
- Marchand, M., N. van Baren, P. Weynants, V. Brichard, B. Dreno, M. H. Tessier, E. Rankin, G. Parmiani, F. Arienti, Y. Humblet, et al. 1999. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int. J. Cancer* 80:219.
- Marchand, M., V. Brichard, N. van Baren, and P. G. Coulie. 2001. Biological and clinical developments in melanoma vaccines. *Exp. Opin. Biol. Ther.* 1:497.
- Thurner, B., I. Haendle, C. Roder, D. Dieckmann, P. Keikavoussi, H. Jonuleit, A. Bender, C. Maczek, D. Schreiner, P. von den Driesch, et al. 1999. Vaccination with MAGE-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J. Exp. Med.* 190:1669.
- Jager, D., E. Jager, and A. Knuth. 2001. Vaccination for malignant melanoma: recent developments. *Oncology* 60:1.
- Fong, L., and E. G. Engleman. 2000. Dendritic cells in cancer immunotherapy. *Annu. Rev. Immunol.* 18:245.
- Kirk, C. J., and J. J. Mule. 2000. Gene-modified dendritic cells for use in tumor vaccines. *Hum. Gene Ther.* 11:797.
- Jenne, L., G. Schuler, and A. Steinkasserer. 2001. Viral vectors for dendritic cell-based immunotherapy. *Trends Immunol.* 22:102.
- Schroers, R., I. Sinha, H. Segall, I. G. Schmidt-Wolf, C. M. Rooney, M. K. Brenner, R. E. Sutton, and S. Y. Chen. 2000. Transduction of human PBMC-derived dendritic cells and macrophages by an HIV-1-based lentiviral vector system. *Mol. Ther.* 1:171.
- Gruber, A., J. Kan-Mitchell, K. L. Kuhlen, T. Mukai, and F. Wong-Staal. 2000. Dendritic cells transduced by multiply deleted HIV-1 vectors exhibit normal phenotypes and functions and elicit an HIV-specific cytotoxic T-lymphocyte response in vitro. *Blood* 96:1327.
- Chinnasamy, N., D. Chinnasamy, J. F. Toso, R. Lapointe, F. Candotti, R. A. Morgan, and P. Hwu. 2000. Efficient gene transfer to human peripheral blood monocyte-derived dendritic cells using human immunodeficiency virus type 1-based lentiviral vectors. *Hum. Gene Ther.* 11:1901.
- Metharom, P., S. Takyar, H. Q. Xia, K. A. Ellem, G. E. Wilcox, and M. Q. Wei. 2001. Development of disabled, replication-defective gene transfer vectors from the Jembrana disease virus, a new infectious agent of cattle. *Vet. Microbiol.* 80:9.
- Esslinger, C., P. Romero, and H. R. MacDonald. 2002. Efficient transduction of dendritic cells and induction of a T-cell response by third-generation lentivectors. *Hum. Gene Ther.* 13:1091.
- Firat, H., V. Zennou, F. Garcia-Pons, F. Ginhoux, M. Cochet, O. Danos, F. A. Lemonnier, P. Langlade-Demoyen, and P. Charneau. 2002. Use of a lentiviral flap vector for induction of CTL immunity against melanoma: perspectives for immunotherapy. *J. Gene Med.* 4:38.
- Breckpot, K., M. Dullaers, A. Bonehill, S. van Meirvenne, C. Heirman, C. de Greef, P. van der Bruggen, and K. Thielemans. 2003. Lentivirally transduced dendritic cells as a tool for cancer immunotherapy. *J. Gene Med.* 5:654.
- Dyall, J., J. B. Latouche, S. Schnell, and M. Sadelain. 2001. Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes. *Blood* 97:114.
- Zufferey, R., D. Nagy, R. J. Mandel, L. Naldini, and D. Trono. 1997. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat. Biotechnol.* 15:871.
- Zufferey, R., T. Dull, R. J. Mandel, A. Bukovsky, D. Quiroz, L. Naldini, and D. Trono. 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* 72:9873.
- Zufferey, R., J. E. Donello, D. Trono, and T. J. Hope. 1999. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* 73:2886.
- Follenzi, A., L. E. Ailles, S. Bakovic, M. Geuna, and L. Naldini. 2000. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat. Genet.* 25:217.
- Miyoshi, H., U. Blomer, M. Takahashi, F. H. Gage, and I. M. Verma. 1998. Development of a self-inactivating lentivirus vector. *J. Virol.* 72:8150.
- Zennou, V., C. Petit, D. Guetard, U. Nerhbas, L. Montagnier, and P. Charneau. 2000. HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 101:173.
- Gaugler, B., B. Van den Eynde, P. van der Bruggen, P. Romero, J. J. Gaforio, E. De Plaen, B. Lethe, F. Brasseur, and T. Boon. 1994. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J. Exp. Med.* 179:921.
- Kocher, T., E. Schultz-Thater, F. Gudat, C. Schaefer, G. Casorati, A. Juretic, T. Willmann, F. Harder, M. Heberer, and G. C. Spagnoli. 1995. Identification and intracellular location of MAGE-3 gene product. *Cancer Res.* 55:2236.
- Schultz, E. S., B. Lethe, C. L. Cambiaso, J. Van Snick, P. Chau, J. Corthals, C. Heirman, K. Thielemans, T. Boon, and P. van der Bruggen. 2000. A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by CD4<sup>+</sup> cytolytic T lymphocytes. *Cancer Res.* 60:6272.
- Sanderson, S., K. Frauwrith, and N. Shastri. 1995. Expression of endogenous peptide-major histocompatibility complex class II complexes derived from invariant chain-antigen fusion proteins. *Proc. Natl. Acad. Sci. USA* 92:7217.
- Jonuleit, H., U. Kuhn, G. Muller, K. Steinbrink, L. Paragnik, E. Schmitt, J. Knop, and A. H. Enk. 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* 27:3135.
- Chaux, P., V. Vantomme, V. Stroobant, K. Thielemans, J. Corthals, R. Luiten, A. M. Eggermont, T. Boon, and P. van der Bruggen. 1999. Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4<sup>+</sup> T lymphocytes. *J. Exp. Med.* 189:767.
- Skipper, P. L. 1996. Influence of tertiary structure on nucleophilic substitution reactions of proteins. *Chem. Res. Toxicol.* 9:918.
- Meadows, L., W. Wang, J. M. den Haan, E. Blokland, C. Reinhardus, J. W. Drijfhout, J. Shabanowitz, R. Pierce, A. I. Agulnik, C. E. Bishop, et al. 1997. The HLA-A\*0201-restricted H-Y antigen contains a posttranslationally modified cysteine that significantly affects T cell recognition. *Immunity* 6:273.
- Schultz, E. S., Y. Zhang, R. Knowles, J. Tine, C. Traversari, T. Boon, and P. van der Bruggen. 2001. A MAGE-3 peptide recognized on HLA-B35 and HLA-A1 by cytolytic T lymphocytes. *Tissue Antigens* 57:103.
- Luiten, R. M., N. Demotte, J. Tine, and P. van der Bruggen. 2000. A MAGE-A1 peptide presented to cytolytic T lymphocytes by both HLA-B35 and HLA-A1 molecules. *Tissue Antigens* 56:77.
- Falk, K., O. Rotzschke, B. Grahovac, D. Schendel, S. Stevanovic, V. Gnau, G. Jung, J. L. Strominger, and H. G. Rammensee. 1993. Allele-specific peptide ligand motifs of HLA-C molecules. *Proc. Natl. Acad. Sci. USA* 90:12005.
- Chaux, P., R. Luiten, N. Demotte, V. Vantomme, V. Stroobant, C. Traversari, V. Russo, E. Schultz, G. R. Cornelis, T. Boon, and P. van der Bruggen. 1999. Identification of five MAGE-A1 epitopes recognized by cytolytic T lymphocytes obtained by in vitro stimulation with dendritic cells transduced with MAGE-A1. *J. Immunol.* 163:2928.
- Marsh, G. E. S., P. P. Parham, and D. L. Barber. 2000. Cw\*07-Cw7. In *The HLA Facts Book*. Academic Press, New York, p. 257.