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# Modified Vaccinia Virus Ankara for Delivery of Human Tyrosinase as Melanoma-associated Antigen: Induction of Tyrosinase- and Melanoma-specific Human Leukocyte Antigen A*0201-restricted Cytotoxic T Cells *in Vitro* and *in Vivo*[1]

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## ABSTRACT

**Vaccination with tumor-associated antigens is a promising approach for cancer immunotherapy. Because the majority of these antigens are normal self antigens, they may require suitable delivery systems to promote their immunogenicity. A recombinant vector based on the modified vaccinia virus Ankara (MVA) was used for expression of human tyrosinase, a melanoma-specific differentiation antigen, and evaluated for its efficacy as an antitumor vaccine. Stable recombinant viruses (MVA-hTyr) were constructed that have deleted the selection marker *lacZ* and efficiently expressed human tyrosinase in primary human cells and cell lines. Tyrosinase-specific human CTLs were activated *in vitro* by MVA-hTyr-infected, HLA-A*0201-positive human dendritic cells. Importantly, an efficient tyrosinase- and melanoma-specific CTL response was induced *in vitro* using MVA-hTyr-infected autologous dendritic cells as activators for peripheral blood mononuclear cells derived from HLA-A*0201-positive melanoma patients despite prior vaccination against smallpox. Immunization of HLA-A*0201/K$^b$ transgenic mice with MVA-hTyr induced A*0201-restricted CTLs specific for the human tyrosinase-derived peptide epitope 369–377. These *in vivo* primed CTLs were of sufficiently high avidity to recognize and lyse human melanoma cells, which present the endogenously processed tyrosinase peptide in the context of A*0201. Tyrosinase-specific CTL responses were significantly augmented by repeated vaccination with MVA-hTyr. These findings demonstrate that HLA-restricted CTLs specific for human tumor-associated antigens can be efficiently generated by immunization with recombinant MVA vaccines. The results are an essential basis for MVA-based vaccination trials in cancer patients.**

## INTRODUCTION

With the identification of an increasing number of TAAs[4] and their molecular cloning (1), the need for appropriate vector systems for vaccination and efficient presentation of these TAAs to the immune system is growing. Live attenuated viruses have been shown over decades to be most efficient in eliciting protective immunity. Therefore, the development of live recombinant vectors suitable as vaccines for cancer immunotherapy has become a goal of utmost importance.

A major subset of melanoma-specific TAAs are nonmutated differentiation antigens (1) that are specifically expressed by cells of neuroectodermal origin, *i.e.*, melanocytes and melanoma cells. Tyrosinase, the key enzyme in melanin synthesis, encodes a remarkable number of peptide epitopes presented by melanoma cells in the context of MHC class I and II molecules (2–6) for recognition by CD8+ and CD4+ T lymphocytes, respectively. Tyrosinase has been observed to be expressed in most melanomas, including amelanotic variants (7–9), and is therefore likely to be an attractive target for CTL-based immunotherapy of melanoma patients.

Despite the presence of melanoma-reactive CTL precursors in the immune repertoire (10) and the evidence that these T cells are being activated *in vivo* (11), their efficacy on and correlation with tumor regression *in vivo* is still uncertain. Because tyrosinase represents a normal self protein produced not only by melanoma cells but also by nonmutated tissues of the skin, eye, and central nervous system (12), the frequency of such CTLs and the affinity of their receptors could be limited due to self-tolerance (13). Strategies to activate and expand melanoma-reactive CTLs *in vitro* or *in vivo* may therefore be critical to obtain therapeutic T-cell responses.

Several methods have been applied to induce cellular antitumor immunity *in vivo*, but in practice, autologous tumor cell lysates (14) may not often be available, and peptide-based approaches (15) may lead to epitope-escape variants (16), select for low-avidity CTLs (17), or even induce tolerance (18). The use of recombinant viral vectors for cancer immunotherapy such as adeno- (19), fowlpox (20), or vaccinia viruses (21) is presently under investigation. In particular, recombinant vaccinia viruses extensively used as eukaryotic expression vectors (reviewed in Ref. 22) have been evaluated as anticancer vaccines encoding TAAs in murine models (23, 24) and have already entered clinical trials (25).

The application of live replicating vector-based vaccines in the clinic is hampered by their residual virulence. For safe treatment, including immunocompromised individuals, attenuated and avirulent viral vectors may be used preferentially. Several strains of vaccinia virus were developed to change its virulence (26). MVA proved to be extremely attenuated and replication deficient in mammalian cells, most likely as a result of deletions and mutations in the viral genome acquired through serial passages (27–29). A MVA-based vaccine for primary vaccination against smallpox was successfully tested in extensive field studies involving over 120,000 recipients, including many patients considered at risk for conventional vaccination (27). The inability of MVA to produce viral progeny in established or primary human cells (30) and its inherent avirulence even in immunosuppressed organisms (31) have been demonstrated. Because viral replication is blocked at a late stage of infection in nonpermissive cells, recombinant MVA allows a high level expression of heterologous genes (32). MVA is, therefore, an efficient viral vector system with an adequate safety profile for versatile use as a prophylactic and therapeutic vaccine in humans. Apart from viral diseases (33–35), MVA-based vaccines have already demonstrated their protective capacity in murine malaria (36) and artificial tumor models (37).

To further investigate the use of MVA as a potential anticancer

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[4] The abbreviations used are: TAA, tumor-associated antigen; MVA, modified vaccinia virus Ankara; MVA-hTyr, recombinant MVA expressing human tyrosinase; MVA-wt, nonrecombinant MVA; CEF, chicken embryo fibroblast; DC, dendritic cell; Tg, transgenic; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; MOI, multiplicity of infection; APC, antigen presenting cell; TNF, tumor necrosis factor.

vaccine, recombinant viruses that allowed the stable and efficient expression of the human *tyrosinase* gene were constructed and characterized. The recombinant tyrosinase protein was produced upon infection of nonpermissive primary human cells with MVA-hTyr. Targeting of human DCs resulted in efficient MHC class I-restricted presentation of the tyrosinase-derived peptide epitope 369–377 to allogeneic as well as autologous CD8+ CTLs. MVA-hTyr-infected autologous DCs induced a tyrosinase- and tumor-specific CTL response in two of four melanoma patients *in vitro*, although all individuals were preimmunized with vaccinia virus against smallpox during their childhood. Vaccination of A*0201/$K^b$-Tg mice with MVA-hTyr induced A*0201-restricted and melanoma-reactive CTLs *in vivo* that were specific for the human tyrosinase peptide 369–377.

## MATERIALS AND METHODS

**Mice.** The derivation of homozygous A*0201/$K^b$-Tg mice expressing a chimeric transgene that consists of the $\alpha$1 and $\alpha$2 domain of HLA-A*0201 and the $\alpha$3 domain of H2-$K^b$ has been described (38, 39). Mice were propagated and maintained in the animal facility at the Johannes Gutenberg-University of Mainz.

**Peptides.** The converted internal tyrosinase 369–377 peptide YMDGTMSQV (40) and the A/PR/8/34 influenza virus matrix protein M1 58–66 peptide GILGFVFTL (41), both of which bind to HLA-A*0201, were synthesized by Neosystem, SNPE group, (Frankfurt, Germany). Purity of peptides was ascertained by mass spectrometry, and high-performance liquid chromatography and proved to be >90%.

**Antibodies.** A polyclonal rabbit antipeptide serum directed against the COOH terminus of murine tyrosinase ($\alpha$-pep7) that cross-reacts with human tyrosinase (42) was kindly provided by V. J. Hearing (Laboratory of Cell Biology, NIH, Bethesda, MD). T311, a mouse monoclonal antibody reactive with human tyrosinase (7), was kindly provided by E. Stockert and L. J. Old (Ludwig Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, NY). MA2.1 is a mouse monoclonal antibody with blocking activity against HLA-A*0201 (ATCC HB-54).

**Virus.** MVA-wt, the vaccinia virus MVA cloned isolate F6, was routinely propagated and titered by end point dilution in CEFs to obtain a 50% tissue culture infectious dose ($TCID_{50}$). For *in vitro* and *in vivo* assays, MVA-wt was purified by ultracentrifugation through a 36% sucrose cushion.

**Cell Lines and Transfectants.** The monkey kidney cell line MA104 (Pasteur Mérieux, Paris, France) was used as a semipermissive animal cell line (28, 30). HLA-A*0201-positive human cell lines used were: the B-lymphoblastoid cell line SY9287 (a gift from R. Drillien, Strasbourg, France); the mutant cell line T2 (Ref. 43; a gift of P. Cresswell, Howard Hughes Medical Institute, New Haven, CT), which lacks expression of transporter associated with antigen processing, resulting in expression of predominantly empty A*0201 molecules; and the tyrosinase positive melanoma cell lines SK29-Mel-1 (44) and Malme-3 (ATCC HTB-64). The tyrosinase-positive, HLA-A*0201-negative human melanoma cell line MZ7-Mel served as a control. NA8-Mel +Tyr (a gift of A. Van Pel, Ludwig Institute for Cancer Research, Brussels, Belgium) and NA8-Mel +CDK4 were derived by transfection of the tyrosinase-negative, HLA-A*0201-positive melanoma cell line NA8-Mel (a gift of F. Jotereau, Inserm U211, Nantes, France) with the full-length *tyrosinase* gene (45) and a mutated *CDK4* gene (46), respectively. The plasmid pcDNAI/Amp (Invitrogen BV, NV Leek, the Netherlands) served as a transfer vector for the transfected genes. The murine lymphoma cell lines EL4 (ATCC TIB 39) and EL4-A*0201/Kb, stably transfected with the *A*0201/Kb* gene (38), were used as control cells. All cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS (Seromed, Biochrome KG, Berlin, Germany) at 37°C in a humidified 5% $CO_2$ atmosphere.

**DCs.** Monocytes were obtained from PBMCs by magnetic cell sorting (MACS; Milteny Biotech, Bergisch Gladbach, Germany) with anti-CD14 magnetic beads. DCs were generated as described (47). In brief, monocytes from HLA-A*0201-positive donors were cultured with interleukin 4 (400 IU/ml) and granulocyte-macrophage colony-stimulating factor (800 IU/ml) for 7 days. Differentiation of immature DCs (48) was ascertained by fluorescence-activated cell sorter analysis. Primary human cell cultures were grown in RPMI 1640 supplemented with 10% pooled human AB serum (Seromed) in a humidified 5% $CO_2$ atmosphere at 37°C.

**Induction of Human CTLs.** PBMCs were prepared from blood of four HLA-A*0201-positive melanoma patients by Ficoll Hypaque density centrifugation. All patients were vaccinated against smallpox during childhood. They were patients with stage I (W. E.), stage II (U. H.), or stage III melanoma (L. E. and M. A.). Autologous monocytes were prepared and maintained as described above to obtain immature DCs. Autologous monocyte-derived DCs were infected at an MOI of 10 for 3 h. After washing twice, $5 \times 10^5$ MVA-hTyr-infected DCs were cocultured with $2 \times 10^6$ autologous monocyte-depleted PBMCs for 7 days. Cultures were restimulated weekly using freshly prepared MVA-hTyr-infected autologous DCs at a responder to stimulator ratio of 2:1 and supplemented with 25 IU/ml IL-2. After four cycles of restimulation, bulk cultures were tested for tyrosinase-reactive CTLs.

**CTL Lines.** The human HLA-A*0201-restricted CD8+ CTL line IVSB, specific for the internal human tyrosinase peptide 369–377, was maintained as described (49). An A*0201-restricted murine CTL line specific for a peptide representing residues 58–66 of the A/PR/8/34 influenza virus matrix protein M1 was derived by peptide immunization of [huCD8 $\times$ A*0201/$K^b$]$_{F1}$ double-Tg mice and maintained by weekly restimulation as described (50, 51). This CTL line served as a negative control.

**Plasmid Construction.** The MVA vector plasmid pUCII LZ P7.5 was generated by insertion of a 290-bp DNA fragment containing the complete sequence of the vaccinia virus early/late promoter P7.5 into the MVA transfer plasmid pUCII LZ (52). A DNA sequence homologous to the 3′-end of the *Escherichia coli lacZ* gene was amplified by PCR using the primers 5′-CAG CAG CTG CAG CCC GAC CGC CTT ACT GCC GCC- 3′ and 5′-GGG GGG CGT CAG ATG GTA GCG ACC GGC GCT CAG- 3′ (sites for restriction enzyme *Pst*I are underlined). The resulting 330-bp *lacZ*-DNA fragment was cloned into a unique *Pst*I restriction site of pUCII LZ P7.5 between the *lacZ* gene expression cassette and flanking MVA-DNA sequences (flank 2) to generate the MVA vector plasmid pUCII LZdel P7.5. Subsequently, a 1.9-kb DNA fragment containing the entire coding sequence of the human *tyrosinase* gene was excised with *Eco*RI from plasmid pcDNAI/Amp-Tyr (53), modified by Klenow enzyme, and cloned into a unique *Sma*I restriction site of pUCII LZdel P7.5 to generate the vector plasmid pII LZdel P7.5-Tyr.

**Generation of Recombinant Viruses.** CEFs infected with MVA-wt at a multiplicity of 0.01 $TCID_{50}$ per cell were transfected with plasmid DNA, harvested, and processed as described previously (33). MVA expressing the human *tyrosinase* gene and transiently coexpressing $\beta$-galactosidase coding sequences (MVA-hTyr/LZ) was isolated by consecutive rounds of plaque purification in CEFs stained with 300 mg/ml 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside (Boehringer Mannheim, Mannheim, Germany; Ref. 32). MVA expressing only the human *tyrosinase* gene (MVA-hTyr) was isolated by three additional rounds of plaque purification screening for nonstained viral foci in the presence of 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside (300 mg/ml). The recombinant viruses were subsequently amplified in CEF monolayers, and viral DNA genomes were analyzed by Southern blot hybridization and PCR. High titer stocks of purified MVA-hTyr were prepared by centrifugation through a 36% sucrose cushion.

**Southern Blot Analysis of Viral DNA.** Cytoplasmic DNA isolated from virus-infected CEF cells was digested with *Hin*dIII, electrophoresed on a 0.6% agarose gel, transferred to a Hybond N+ membrane (Amersham Buchler, Life Science, Braunschweig, Germany), and hybridized to a DNA probe consisting of either the plasmid transfer vector pII LZdel P7.5 Tyr or a PCR-amplified, tyrosinase-specific fragment labeled with [$\alpha$-$^{32}$P]CTP. Prehybridization and hybridization was performed according to the QuikHyb protocol (Stratagene GmbH, Heidelberg, Germany). Membranes were washed twice with 2× sodium chloride/sodium citrate pH 7.0 solution (SSC)/0.1% SDS at room temperature for 15 min and twice with 0.1× SSC/0.1% SDS at 65°C for 30 min. The blots were exposed to a phosphorimaging plate (Type BAS-IIIs; FUJI Photo Film Co. Ltd., Tokyo, Japan) and visualized on a phosphorimaging analyzer (Fujix BAS 1000; FUJI).

**Analysis of Recombinant Gene Products.** Expression of heterologous tyrosinase was confirmed by immunoperoxidase staining of infected cells and by Western blot analysis of cell lysates. For the latter, proteins were resolved by electrophoresis on a SDS-8% polyacrylamide gel and electroblotted onto nitrocellulose for 2 h in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.6). The blots were blocked overnight at 4°C in a PBS

blocking buffer containing 1% BSA and 0.1% NP40 and then incubated for 1 h at room temperature with mAb T311 diluted 100-fold in blocking buffer. After being washed with 0.1% NP40 in PBS, the blots were incubated for 1 h at room temperature with $^{125}$I-labeled sheep anti-mouse IgG (Amersham) diluted 1000-fold in blocking buffer, washed again, and exposed to a phosphorimaging plate (Type BAS-IIIs; Fuji) for evaluation with a phosphorimaging analyzer (Fujix BAS 1000).

**TNF-$\alpha$-ELISA Assay.** CTLs generated from melanoma patients after *in vitro* stimulation with MVA-hTyr-infected autologous DCs were assayed for their ability to secrete TNF-$\alpha$ in coculture with HLA-A*0201-positive melanoma cells SK29-Mel-1 expressing tyrosinase or B-lymphoblastoid cells (LCL) SY9287 exogenously pulsed with tyrosinase-derived 369–377 peptide. Stimulator cells were infected for 4 h with MVA at an MOI of 10, extensively washed, and plated in 96-well plates at $5 \times 10^4$ cells/well. After an additional 11 h of incubation at 37°C, effector T cells ($2 \times 10^4$ cells/well) were added. T cells cocultured with unpulsed LCLs as well as melanoma cells and LCLs alone were used as negative controls. In addition, stimulator cells were incubated with the anti-HLA-A*0201 mAb MA2.1 to block CTL reactivity. Supernatants were harvested after 40 h, and the TNF-$\alpha$ content was determined by ELISA (R&D Systems, Wiesbaden, Germany). All assays were performed in triplicate.

**Chromium Release Assays.** The lytic activity of either *in vitro*-stimulated autologous CTLs derived from melanoma patients or allogeneic tyrosinase-specific CTL line IVSB was tested against MVA-hTyr-, MVA-wt-, or mock-infected primary human target cells in a 4-h standard $^{51}$Cr release assay. Briefly, HLA-A*0201-positive Malme-3 cells and HLA-A*0201-negative MZ7-Mel cells, as well as allogeneic or autologous HLA-A*0201-positive DCs, were infected for 3 h with MVA at an MOI of 10, washed once, labeled for 1 h at 37°C with 100 $\mu$Ci $Na^{51}CrO_4$, and then washed four times. Labeled target cells were plated in U-bottomed 96-well plates at $1 \times 10^4$ cells/well and incubated for an additional 8 h at 37°C. Fifteen h after infection, effector cells were incubated with the target cells at various E:T ratios. After 4 h, 100 $\mu$l of supernatant per well were collected, and the specific $^{51}$Cr release was determined. Assays were performed in triplicate.

**Vaccination of A*0201/K$^b$-Tg Mice and Induction of Tyrosinase-specific A*0201-restricted CTLs.** A*0201/K$^b$-Tg mice were inoculated i.p. with $2 \times 10^8$ infectious units (IU) of either MVA-hTyr or MVA-wt. Mice were either not boosted or boosted twice at days 28 and 49. Three weeks after the last vaccination, spleen cells of primed mice were cocultured with irradiated (3000 rads) and lipopolysaccharide-activated syngeneic spleen cell stimulators that had been pulsed with tyrosinase 369–377 peptide at 5 $\mu$g/ml and human $\beta_2$-microglobulin (Sigma Chemical Co., St. Louis, MO) at 10 $\mu$g/ml (38, 39). After 6 days of culture, the lytic activity of resultant CTL effector cells was determined at various E:T ratios in a 4-h standard $^{51}$Cr release assay (50). Targets were T2 cells that had been pulsed at 1 $\mu$M with either the tyrosinase 369–377 peptide, the unrelated A*0201-binding influenza M1 58–66 peptide, or no peptide. Recognition of endogenously presented tyrosinase 369–377 peptide was tested in a 5-h $^{51}$Cr release assay by using recombinant IFN-$\gamma$ (R&D Systems; 20 ng/ml for 15 h), pretreated or nonpretreated human melanoma cell lines, as well as SY9287 targets that had been infected with either MVA-hTyr, MVA-wt, or mock.

## RESULTS

**Construction and Isolation of MVA Recombinants Expressing the Human Tyrosinase Gene.** To allow the formation and convenient isolation of recombinant MVA expressing the human *tyrosinase* gene, we constructed the MVA transfer plasmid pUCII LZdel P7.5. This vector contains the well-characterized vaccinia virus early/late promoter P7.5 for expression of foreign genes, MVA-DNA sequences that target the insertion of foreign genes precisely to the site of a naturally occurring 2500-bp deletion within the *Hin*dIII N fragment of the MVA genome (28), and an expression cassette of the *Escherichia coli LacZ* marker gene designed to be inactivated by deletion after homologous intragenomic recombination. The *tyrosinase* gene was inserted into pUCII LZdel P7.5 to obtain pII LZdel P7.5-Tyr (Fig. 1*A*).

MVA-hTyr was formed in CEFs that were infected with MVA-wt and transfected with pII LZdel P7.5 Tyr. The recombinant virus was plaque purified. We screened first for virus isolates that transiently produced the reporter enzyme $\beta$-galactosidase (MVA-hTyr/LZ) and isolated viruses with a $\beta$-galactosidase-negative phenotype (MVA-hTyr) in subsequent rounds of plaque purification (Fig. 1*B*). Southern blot analysis of viral DNA confirmed the precise intragenomic re-

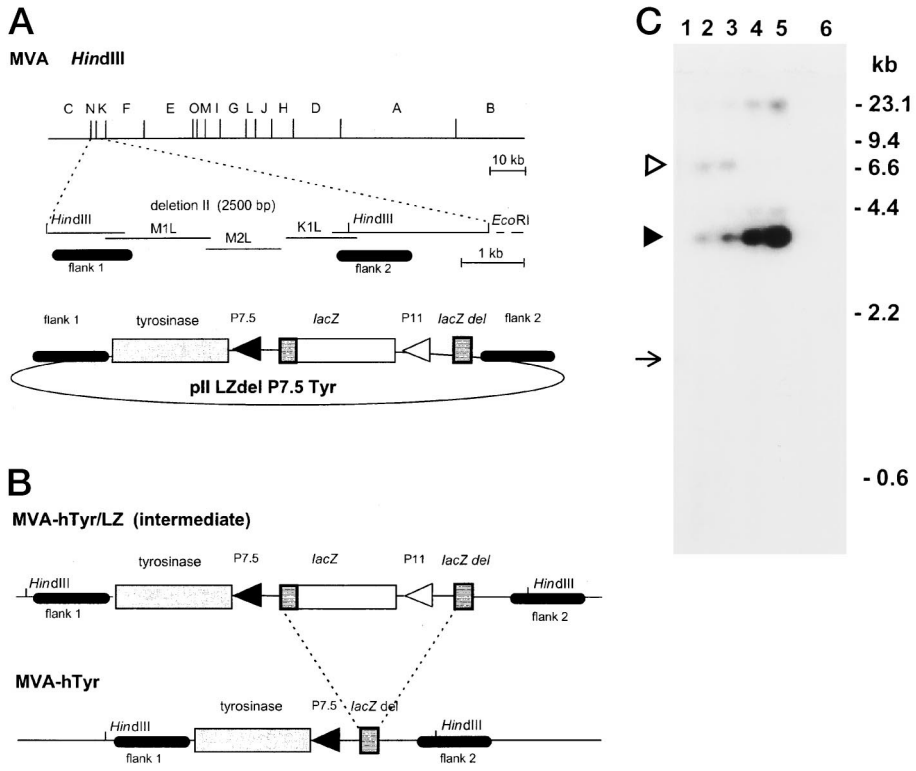


Fig. 1. Construction and characterization of MVA-hTyr. Schematics of the MVA genome and plasmid pII LZdel-P7.5-Tyr designed for transfer of the human *tyrosinase* gene (*A*) and the deletion of the *E. coli lacZ* marker gene during formation of MVA-hTyr via the unstable intermediate MVA-hTyr/LZ (*B*). *Top*, *Hin*dIII restriction endonuclease sites within the genome of MVA. MVA-DNA sequences (*flank 1* and *flank 2*) adjacent to deletion II within the *Hin*dIII N fragment were cloned to direct homologous recombination into the MVA genome. Expression cassettes for the tyrosinase and *E. coli lacZ* genes as well as a 330 bp-*lacZ* gene fragment (*lacZ*del) enabling the subsequent deletion of the *lacZ* reporter gene sequences were inserted between the MVA-DNA sequences. P11 and P7.5 refer to well-characterized late and early/late vaccinia virus-specific promoters, respectively. Southern blot analysis of viral DNA extracted from virus-infected CEF cells is shown. *C*, DNA from CEFs infected with MVA-wt (*Lane 1*), MVA-hTyr/LZ (*Lanes 2* and *3*), and MVA-hTyr (*Lanes 4* and *5*) was digested with *Hin*dIII and hybridized with $^{32}$P-labeled plasmid DNA (pII LZdel P7.5Tyr). Genomic DNA fragments containing the recombinant *tyrosinase* gene before (*open arrowhead*) or after (*filled arrowhead*) deletion of the *lacZ* gene sequences are indicated. The 900 bp *Hin*dIII N-fragment of MVA-wt is marked by an *arrow*. Double-stranded DNA was used as marker for molecular weight in kb pairs (*Lane 6*).

combination of homologous *LacZ* gene sequences, which resulted in the loss of marker gene sequences together with the transcriptional regulator, the vaccinia virus promoter P11 (Fig. 1*C*). In viral DNA obtained from CEFs infected with an intermediate plaque isolate of recombinant virus designated MVA-hTyr/LZ, we identified DNA fragments corresponding in size to viral genomes that contained either the complete (6600 bp) or the deleted (3200 bp) *E. coli lacZ* gene sequences. Because almost equal amounts of both viral genomes were observed after a single round of virus amplification, the deletion of the reporter gene appeared to be highly efficient, avoiding the possible disadvantage to coproduce an additional foreign antigen. Consistent with this finding, only the smaller 3200-bp DNA fragment was detected in viral DNA isolated from CEFs infected with the plaque-purified MVA-hTyr. In contrast, we obtained no hybridization signal from a DNA fragment corresponding in size to the original 900-bp *Hin*dIII N-fragment of nonrecombinant MVA-DNA, thereby confirming the absence of MVA-wt in recombinant virus isolates after plaque purification. After multiple passages in CEFs, identical hybridization patterns were found by Southern blotting in all isolates, proving the genetic stability of MVA-hTyr (data not shown).

**Expression of Human Tyrosinase.** To monitor the synthesis of recombinant tyrosinase, total cell extracts from infected human cell cultures were analyzed by Western blotting using the anti-tyrosinase mouse mAb T311 (Fig. 2 *A* and *B*). After MVA-hTyr infection of human monocytes (Fig. 2 *A*) and the human B lymphoblastoid cell line SY9287 (Fig. 2 *B*), a broad protein band within the range of $M_r$ 60,000–75,000 was detected. This band corresponds precisely to the expected size of human tyrosinase and most likely represents various glycosylated species of this protein (7, 8, 54). The heterologous protein was made as early as 6 h after infection with a peak production at 24 h. It was detectable in large amounts for at least 3 days. This observation indicated long lasting production of tyrosinase, despite the inability of MVA to replicate in human cells.

**Immunostimulatory Potential of Human APCs Infected with MVA-hTyr.** Because professional APCs are required for the primary induction of naive T cells, A*0201-restricted presentation of the tyrosinase 369–377 peptide epitope by MVA-hTyr was studied after infection of monocyte-derived DCs. Based on kinetics of tyrosinase expression (Fig. 2), CTL recognition was determined at 12–15 h after infection in a standard $^{51}$Cr-release assay (Fig. 3 *A-C*). We took advantage of a previously characterized CD8+ CTL line (IVSB) that recognizes a distinct tyrosinase peptide (amino acids 369–377) presented by HLA-A*0201. HLA-A*0201-positive DCs infected with MVA-hTyr were efficiently recognized by CTL line IVSB (Fig. 3*A*), demonstrating that the tyrosinase peptide 369–377 has been endogenously processed upon infection *in vitro*. Lysis of MVA-hTyr-infected DCs as opposed to Malme-3 melanoma target cells (Fig. 3*B*) was more efficient at lower E:T ratios. This finding could be due to a higher level of expression of class I MHC-peptide complexes and adhesion molecules by MVA-hTyr-infected professional APCs. Infection of Malme-3 cells with MVA-wt had no negative impact on the presentation of the inherent tyrosinase peptide 369–377, whereas infection with MVA-hTyr did scarcely enhance specific lysis of these targets (Fig. 3*B*). A*0201-negative MZ7-Mel cells were not recognized as expected (Fig. 3*C*).

We wished to assess the capability of MVA-hTyr to generate tyrosinase- and melanoma-specific effector CTLs from patient-derived precursor cells. Therefore, we stimulated PBMCs from four melanoma patients expressing the *HLA-A*0201* allele *ex vivo* by adding autologous monocyte-derived DCs infected with MVA-hTyr. As shown in Fig. 4*A*, coculture of the *in vitro* generated T cells from two of four patients (W. E. and L. E.) with MVA-hTyr-infected autologous DCs used as targets resulted in tyrosinase-specific lysis. The

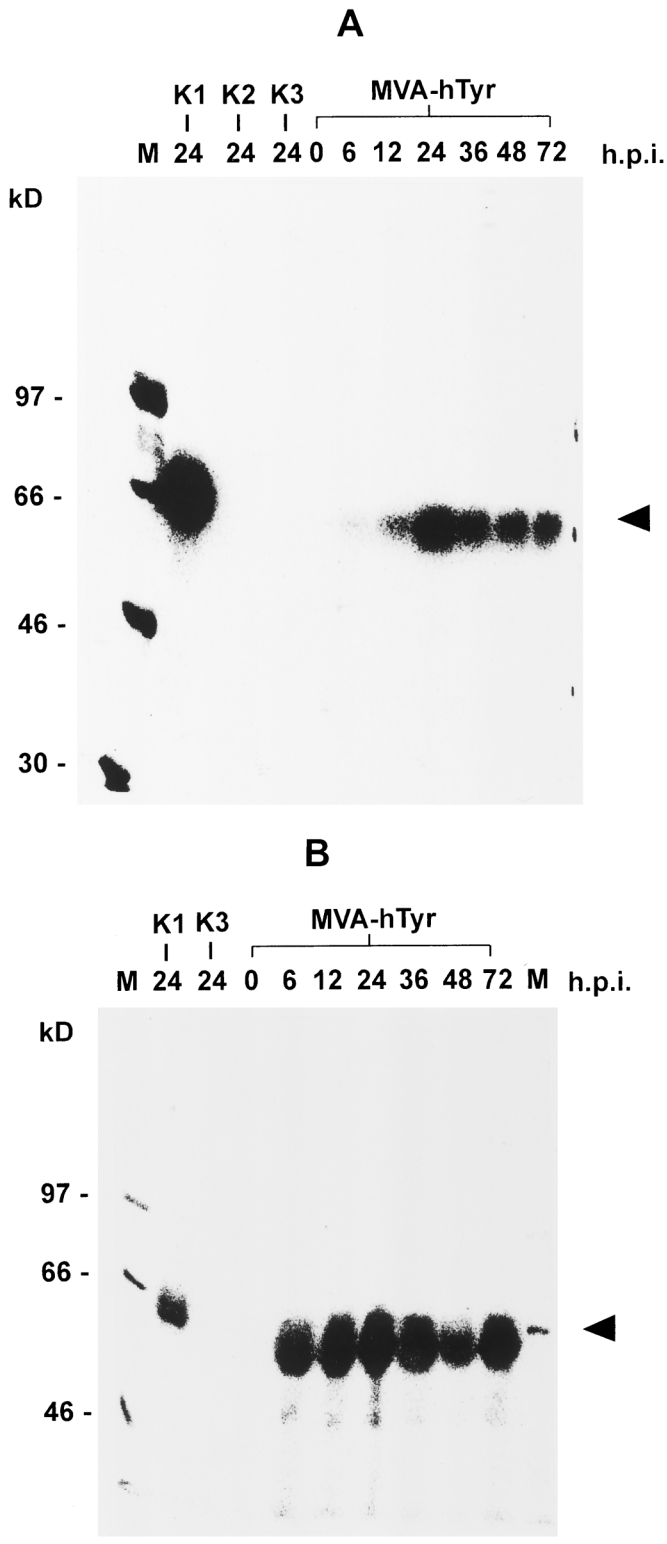


Fig. 2. Expression of the human *tyrosinase* gene by recombinant MVA-hTyr in human primary cells and cell lines. Radioimmunoblot analysis of monocytes (*A*) or SY9287 cells (*B*) infected with MVA-hTyr or MVA-wt (*Lane K3*). Cells were harvested at the indicated hours postinfection (*h.p.i.*). Cell lysates were separated by 8% SDS-PAGE. The blot from this gel was probed with anti-tyrosinase mAb T311 and $^{125}$I-labeled sheep anti-mouse IgG secondary antibody and visualized on a phosphorimager. MA104 cells, a semipermissive cell line for MVA, infected with MVA-hTyr (*Lane K1*) and MVA-wt (*Lane K2*) served as positive and negative controls. *Left*, positions and molecular masses (in kDa) of protein standards (*Lane M*). *Arrowhead*, protein band corresponding in size to glycoforms of tyrosinase.

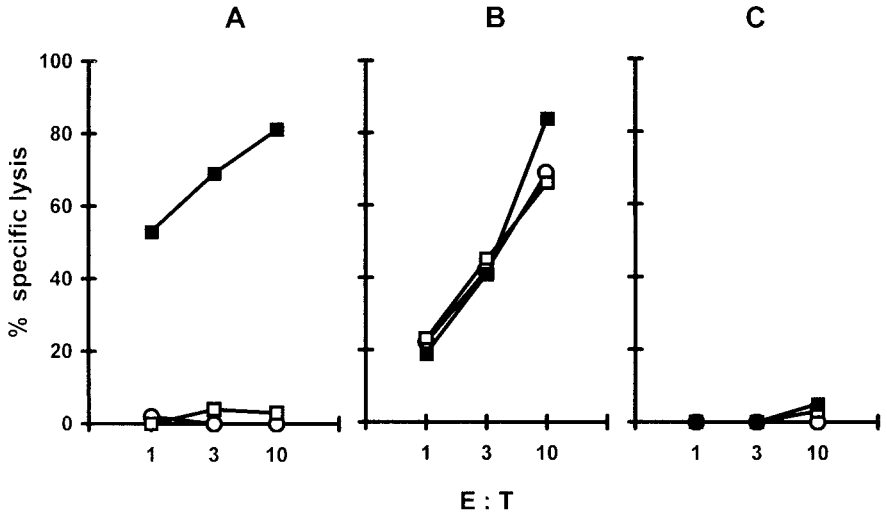


Fig. 3. Recognition of MVA-hTyr-infected primary human cells and human cell lines by the tyrosinase 369–377, peptide-specific CD8$^+$ CTL line IVSB. Specific $^{51}$Cr release is shown at the indicated E:T ratios. Target cells infected with either MVA-hTyr (■), MVA-wt (□), or mock (○) included tyrosinase-negative, A*0201-positive, monocyte-derived DCs (*A*), tyrosinase positive, A*0201-positive Malme-3 (*B*), and tyrosinase-positive, A*0201-negative MZ7-Mel (*C*).

effector T-cell preparations also exhibited lytic activity against MVA-wt-infected DCs, which is likely to be vaccinia virus specific, because all patients were vaccinated against smallpox during childhood. However, the amount of lysis was significantly lower as compared with that seen after infection with MVA-hTyr. Lysis of MVA-hTyr-infected DCs obtained with CTLs derived from two other melanoma patients did not differ from that observed with MVA-wt-infected DCs (data not shown).

To further characterize the effector CTLs from patients W. E. and L. E., we investigated their ability to recognize tumor cells in a tyrosinase-specific and HLA-A*0201-restricted manner. In these experiments, allogeneic A*0201-positive melanoma cells, naturally processing and presenting the tyrosinase 369–377 peptide, or LCL cells exogenously loaded with the synthetic peptide, served as target cells (Fig. 4*B*). Coculture of melanoma or peptide pulsed LCL cells with CTLs from both patients resulted in the synthesis of high levels of TNF-$\alpha$, demonstrating the substantial recognition of human melanoma cells. Omission of CTLs or cocultivation of CTLs with unpulsed LCL cells resulted in TNF-$\alpha$ production at background levels, confirming the antigen specificity of the effector T cells. Furthermore, the presence of the HLA-A*0201 blocking mAb MA2.1 significantly reduced the TNF-$\alpha$ amount secreted by the CTLs, indicating the HLA-A*0201 restriction of the effector CTL. These results demonstrate that MVA-hTyr transduced autologous DCs may function as strong activators for an antitumor T-cell response.

**Specific Immunogenicity of MVA-hTyr as a Live Vaccine in A*0201/K$^b$-Tg Mice.** To evaluate the efficacy of MVA-hTyr for melanoma immunotherapy, we tested whether a tyrosinase-specific CTL response could be induced by the vaccine *in vivo* after immunization of A*0201/K$^b$-Tg mice. A*0201/K$^b$-Tg mice were primed i.p. with either MVA-hTyr or MVA-wt. Mice were not boosted or boosted twice. Spleen cells from immunized mice were restimulated with the tyrosinase peptide 369–377 *in vitro* and tested for an HLA-A*0201-restricted, tyrosinase peptide-specific CTL response. Tyrosinase-specific CTLs were only generated after *in vivo* priming with MVA-hTyr as opposed to MVA-wt, indicating that the tyrosinase peptide 369–377 has been endogenously processed and presented by murine APCs *in vivo* (Figs. 5 and 6). No tyrosinase-specific lytic activity was detectable after restimulation of spleen cells from MVA-wt primed Tg mice, thereby precluding the possibility of a primary *in vitro* induction of murine effector cells by tyrosinase peptide-pulsed stimulator cells

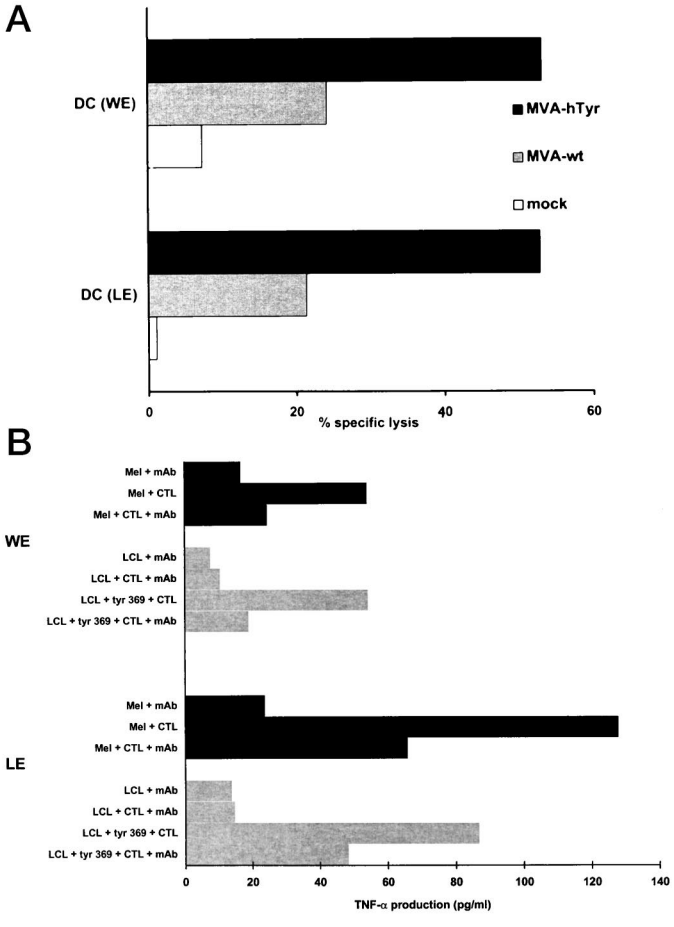


Fig. 4. MVA-hTyr-induced *in vitro* generation of tyrosinase-specific CTLs from PBMCs of two A*0201-positive melanoma patients (W. E. and L. E.). Autologous DCs (*A*) and allogeneic HLA-A*0201 expressing human cell lines (*B*) served as targets presenting the melanoma-associated antigen tyrosinase. *A*, specific lysis of MVA-hTyr-infected autologous DCs. DCs were infected either with MVA-hTyr (■), MVA-wt (▨), or mock (□) and cocultured with bulk culture CTLs at an E:T ratio of 20:1 in a 4-h $^{51}$Cr release assay. *B*, SK29-Mel-1 (*Mel*) naturally expressing tyrosinase and tyrosinase-negative SY9287 (*LCL*) cells pulsed with (+ $tyr_{369-377}$) or without peptide were incubated with bulk culture T cells (+ *CTL*) at an E:T ratio of 0.4:1. Melanoma or LCL cells in the absence of CTLs or in the presence of the anti-HLA-A*0201 blocking mAb MA2.1 (+ *mAb*) served as additional controls. Supernatants were harvested after 40 h and assayed for TNF-$\alpha$ by ELISA.

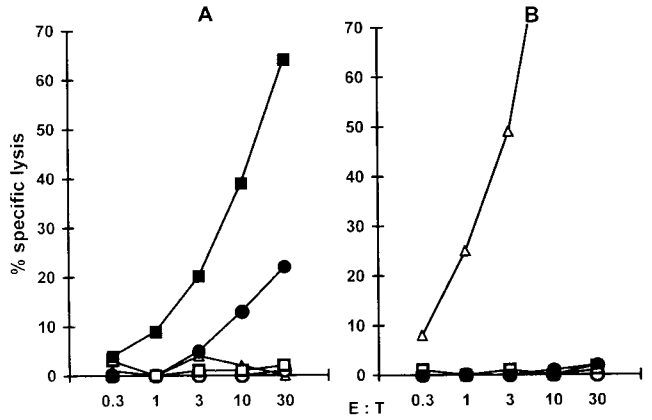


Fig. 5. Specific lysis of peptide-pulsed human target cells by tyrosinase 369–377 peptide-specific CTLs derived from MVA-hTyr-vaccinated A*0201/$K^b$-Tg mice. Effector CTLs were generated by i.p. immunization of Tg mice with either MVA-hTyr given once (●) or three times (■) or MVA-wt given once (○) or three times (□). A CTL line recognizing the A*0201-binding peptide 58–66 of the A/PR/8/34 influenza virus matrix protein M1 (△) was used as a control. CTLs were assayed for cytotoxicity at the indicated E:T ratio in a 4-h $^{51}$Cr release assay against T2 targets pulsed with either the tyrosinase 369–377 peptide (*A*) or the influenza M1 58–66 peptide (*B*) at 1 μM.

(Fig. 5*A* and 6*D*). A*0201-restricted CTLs derived from MVA-hTyr-infected Tg mice recognized tyrosinase 369–377 on peptide-pulsed human T2 targets (Fig. 5*A*). No lysis was observed with nonpeptide-pulsed T2 targets (data not shown) and T2 cells pulsed with the A*0201-binding influenza virus matrix M1 58–66 peptide, although the latter target cells were efficiently recognized by an M1 peptide-specific CTL line (Fig. 5*B*). The magnitude of lytic activity against peptide-pulsed T2 targets observed with responder CTLs from mice boosted twice with MVA-hTyr was more than 3-fold higher as compared with that obtained with CTLs derived after a single immunization (Fig. 5*A*). Moreover, the *in vivo*-generated CTLs were able to recognize the endogenously processed tyrosinase 369–377 peptide presented by HLA-A*0201 on human melanoma and nonmelanoma target cells. As shown in Fig. 6, substantial lysis was detected against MVA-hTyr-infected SY9287 cells (Fig. 6*A*), NA8-Mel melanoma cells stably transfected with the human *tyrosinase* gene (Fig. 6*B*), and Malme-3 cells, which naturally express tyrosinase (Fig. 6*C*). Specific lysis of Malme-3 targets was not enhanced after pretreatment with recombinant IFN-γ (Fig. 6*C*). Recognition of endogenously presented tyrosinase peptide, however, was only observed with CTLs derived from MVA-hTyr-boosted mice as opposed to responder cells generated after a single vaccination (data not shown). A*0201/$K^b$-Tg mice do express endogenous MHC class I (Kb/Db). Therefore, we investigated the possible presentation of the model peptide epitope tyrosinase 369–377 in the context of mouse class I. Murine Tg-CTL restimulated once *in vitro* with this peptide did not recognize syngeneic A*0201-negative EL4 target cells but efficiently lysed EL4-A*0201/Kb target cells transfected with the A*0201/Kb transgene (data not shown). The A*0201 restriction of the MVA-hTyr primed CTLs was further demonstrated by their failure to respond to A*0201-negative human targets pulsed with the tyrosinase 369–377 peptide (data not shown).

## DISCUSSION

High-level recombinant gene expression, together with stability, safety, and immunogenicity of viral vectors, are appealing prerequisites for clinical application of live recombinant vaccines. The particular genotype of MVA with regard to host-range and immunomodulatory genes qualifies MVA-based vectors as a promising gene delivery system that provides high immunogenicity of the target antigen and an excellent safety profile (33, 35, 37). Here we describe the construction and characterization of the replication-deficient vaccinia virus MVA-hTyr that expresses the human *tyrosinase* gene.

Professional APCs, such as DCs, are required for the induction of primary T-cell responses (55). Clinical efficacy of peptide- or tumor lysate-pulsed DCs has already been reported in melanoma patients (56). APCs infected *ex vivo* by recombinant MVA vectors encoding TAAs could provide another attractive vaccination approach and could either be directly retransferred *in vivo* or used to generate CTLs *in vitro* for adoptive transfer. Therefore, we used monocyte-derived DCs as APCs in this study. After infection with MVA-hTyr, these APCs demonstrated efficient antigen processing and MHC class I-re-

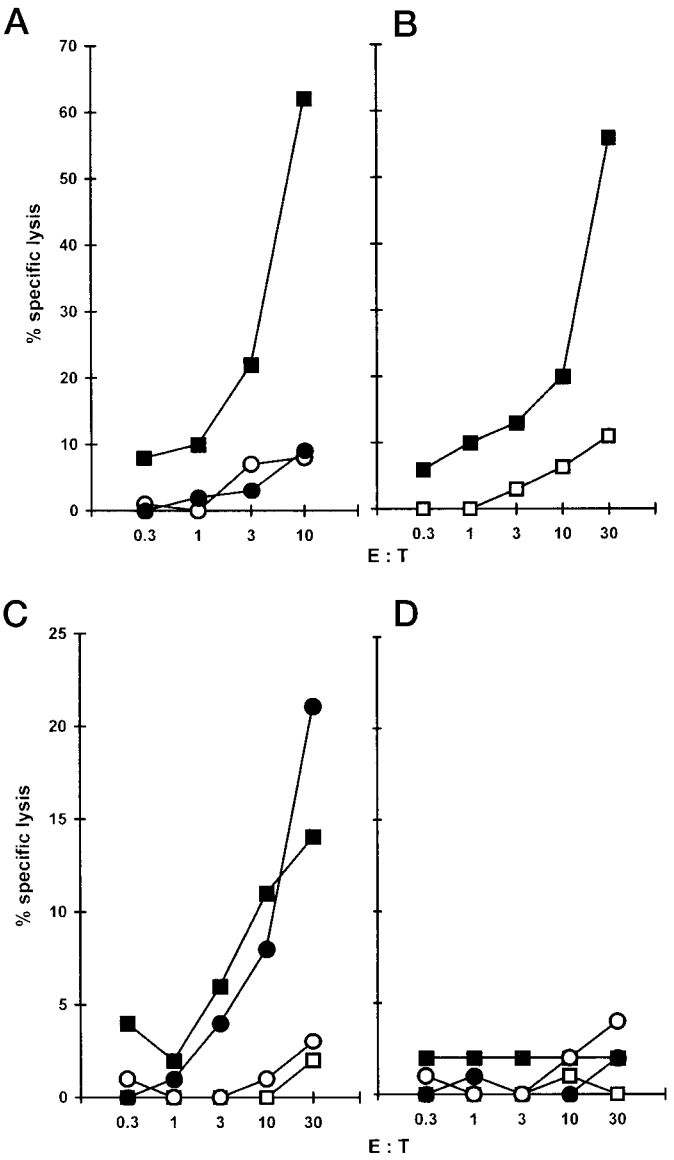


Fig. 6. Recognition of the endogenously processed tyrosinase peptide 369–377 by CTLs derived from MVA-hTyr-infected A*0201/$K^b$-Tg mice. Responder CTLs were generated after three subsequent immunizations of A*0201/$K^b$-Tg mice with either MVA-hTyr (*A–C*) or MVA-wt (*D*). Spleen cells were restimulated with peptide *in vitro*, and A*0201-restricted and tyrosinase-specific CTLs were tested for cytotoxicity at the indicated E:T ratios in a 5-h $^{51}$Cr release assay. The targets were: *A*, uninfected SY9287 cells (○); SY9287 cells infected with either MVA-hTyr (■) or MVA-wt (●); *B*, tyrosinase-negative melanoma cells NA8-Mel transfected with either the full-length *tyrosinase* gene (■) or a mutated *CDK4* gene (□); and *C* and *D*, tyrosinase-positive Malme-3 (■) or tyrosinase-negative NA8-Mel cells (□), which have also been pretreated with recombinant IFN-γ at 20 ng/ml for 15 h: ●, Malme; ○, NA8-Mel.

stricted peptide presentation. In addition, stimulation of melanoma patient-derived PBMCs by autologous DCs infected with MVA-hTyr induced a strong tyrosinase- and melanoma-specific CTL response *in vitro*.

Vaccinia virus-specific T-cell immunity has been shown to be long lived in smallpox vaccinated individuals (57). It has been anticipated that these memory T cells may impair the immunogenicity of recombinant vaccinia viruses because it has been suggested for adenoviral vectors that failed to immunize patients against melanoma antigens due to high levels of neutralizing antibody present in pretreatment sera of these patients (58). Because in this study all patients were vaccinated previously against smallpox during childhood, the vaccinia virus-specific response may have been due to the amplification of preexisting vaccinia virus-reactive memory T cells and *in vitro* priming of naive T cells. Nevertheless, in two of four patients, the anti-vaccinia background response was significantly lower as compared with the tyrosinase- and melanoma-specific CTL response that could be induced. These results emphasize that MVA-hTyr-transduced APCs may indeed function as activators for a tumor killing CTL response in melanoma patients, even after earlier immunization against smallpox during childhood.

The recent finding that MVA fails to produce soluble receptors for IFN-$\gamma$, IFN-$\alpha/\beta$, and TNF-$\alpha$ but strongly induces type I IFN in primary human cells (36) is in line with our results demonstrating high immunogenicity of MVA-expressed antigens *in vitro* and *in vivo*. Previous vaccination experiments in other animal models also illustrated the remarkable efficacy of recombinant MVA vaccines (33–35, 37). Because MVA-based vaccines seem particularly suitable for direct application *in vivo*, the immunogenicity of MVA-hTyr was tested in A*0201/$K^b$-Tg mice. After immunization with influenza virus, these Tg mice have already been reported to generate A*0201-restricted, virus-specific CTL responses (38) and considered an attractive animal model to evaluate MHC class I-restricted T cell responses in vaccination studies. Here, we demonstrate that HLA-A*0201-restricted CTLs can be induced against the tyrosinase 369–377 epitope *in vivo* by vaccination with MVA-hTyr. The presentation of the converted tyrosinase 369–377 peptide by HLA-A*0201 has been shown to be transporter associated with antigen processing and proteasome dependent (54). Our results indicate that tyrosinase expressed by MVA-hTyr in A*0201/$K^b$-Tg mice is being processed by murine APCs, and the tyrosinase peptide 369–377 is presented by A*0201/$K^b$ molecules *in vivo*. Because MVA-hTyr does express the full-length *tyrosinase* gene, it is anticipated that this vector induces CTL, possibly specific for a variety of known tyrosinase epitopes restricted by MHC class I and II (2–6). Here, we demonstrated the immunogenicity of the vector virus MVA-hTyr using the HLA-A*0201-restricted tyrosinase peptide 369–377 as a defined model epitope. Future experiments will be of high interest to analyze the presentation of further peptide epitopes by HLA-A*0201 or other HLA molecules. A vaccine delivering various epitopes may be extremely valuable for widening the range of patients eligible for immunotherapy, despite the expression of different MHC class I and II alleles.

We also found a clear beneficial effect of repeated MVA-hTyr inoculations. These booster vaccinations with the same vector vehicle did not abolish the immunogenicity of the target antigen tyrosinase, as could have been anticipated from other reports (59). In contrast, boosting was a prerequisite to obtain recognition and destruction of human melanoma cells by tyrosinase-specific CTLs with sufficient avidity for endogenous peptide presentation. As we understand the term avidity as functional term for the sum of various different ligand/receptor pair interactions, in particular however, the interaction of CD8 and T-cell receptor with MHC class I/peptide complexes, CTLs from A*0201/$K^b$-Tg mice are usually at a disadvantage in recognizing cells expressing A*0201 as opposed to A*0201/$K^b$ because of the inability of murine CD8 to interact with the $\alpha$-3 domain of the human A*0201 molecule (38). Therefore, the murine CTLs derived from A*0201/Kb-Tg mice can only use their T-cell receptors to interact with the MHC class I molecule of human target cells, whereas human CTLs can use both CD8 and TCR to bind this ligand. This is particularly significant for cells that express the relevant class I MHC-peptide complexes at low level copy numbers (50). Because Malme-3 cells have high expression of A*0201 (data not shown), the lower magnitude of lysis by A*0201/$K^b$-restricted CTLs may, therefore, be the result of their lower level of tyrosinase expression that has not been overcome by preincubation with IFN-$\gamma$ (Fig. 6*C*). In contrast, NA8-Mel or SY9287 cells, which show high-level expression of both, the A*0201 molecules (data not shown) and the transfected or MVA-hTyr-transduced human *tyrosinase* gene (Fig. 2*A*) were efficiently lysed by tyrosinase-specific CTLs derived from A*0201/$K^b$-Tg mice. In view of a clinical application, it is of interest to note that we did not detect signs of autoimmune disease, such as depigmentation or neurological disorders, possibly related to the anti-tyrosinase directed immune response, despite the fact that some immunized mice were kept for almost 3 months because of repeated vaccinations.

MVA-hTyr expresses a human gene and is tested in a humanized murine model. Therefore, the immune response against the human tyrosinase peptide 369–377 in Tg mice may not reflect a potential self-tolerance situation in humans. However, the homology between human and murine tyrosinase is >85% on the gene and protein level. The same is true for the sequence homology of human tyrosinase peptide 369–377 YMDGTMSQV as compared with murine tyrosinase peptide 369–377 FMDGTMSQV, which only differs in position one due to a conservative exchange of tyrosine to phenylalanine. We found that both peptides had an identical binding affinity to HLA-A*0201. In addition, the A*0201-restricted murine CTLs induced by MVA-hTyr did efficiently cross-recognize the exogenously as well as endogenously processed and presented murine tyrosinase peptide 369–377.[5] This indicates that a T-cell repertoire could be activated *in vivo* by immunization with MVA-hTyr that recognizes the murine tyrosinase peptide 369–377, although it represents a self-epitope in this Tg mouse model. Furthermore, the generation of CTL responses against the human tyrosinase peptide 369–377 *in vitro* by stimulating autologous PBMCs derived from two melanoma patients using DCs transduced with MVA-hTyr demonstrates the efficacy of the vaccine in another situation, where such a response may possibly require breaking of self-tolerance.

In summary, we demonstrate an effective HLA-restricted cellular immune response against a human TAA being induced *in vitro* and *in vivo* with a recombinant MVA vaccine. Important results that make MVA-hTyr particularly attractive for a CTL-based approach for melanoma immunotherapy include: (*a*) unimpaired synthesis of recombinant tyrosinase in transformed as well as primary human cells; (*b*) endogenous processing and presentation of the tyrosinase peptide epitope 369–377 by HLA-A*0201 in MVA-hTyr-infected human DCs; and (*c*) high immunogenicity of the TAA delivered by MVA, as demonstrated by the induction of a strong tyrosinase- and melanoma-specific A*0201-restricted CTL response after both transduction of autologous human DCs *ex vivo* and vaccination of A*0201/$K^b$-Tg mice *in vivo*.

[5] I. Drexler, E. Antunes, M. Theobald, and G. Sutter, unpublished observations.

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