# Adjuvant effect of dendritic cells transduced with recombinant vaccinia virus expressing HPV16-E7 is inhibited by co-expression of IL12

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**Abstract.** Dendritic cells (DC) enhanced the immunogenicity of recombinant vaccinia viruses (rVV) expressing the E7 protein of HPV16, a tumor-associated antigen (TAA). Immunization with DC transduced by rVV generated from strain Praha or MVA induced better protection against the growth of transplanted TC-1 tumors in C57Bl/6 mice than did immunization with either of these rVVs administered alone by the same route. Interestingly, DC transduced with a double recombinant vaccinia virus expressing E7 protein together with the Th1-polarizing cytokine IL12, which has been shown to enhance the cellular response in several other systems, induced lower anti-tumor immunity than DC transduced with rVV expressing E7 protein alone. The inhibitory effect mediated by IL12 on immunization with rVV-infected DC was dose-dependent and was observed after immunization with DC transduced with IL12-expressing rVV even at low multiplicity.

## Introduction

Papilloma viruses are often associated with benign and malignant cutaneous or mucosal neoplastic lesions in many species including humans. The DNA genome of human papilloma virus (HPV) genotype 16, 18, or 45 can be detected in most cases of cervical carcinoma (CC) (1) and there is a very strong evidence that these viruses are the etiological agents of this malignancy. High-grade precancerous cervical lesions and tumor cells generally express two viral early proteins known as E6 and E7, both of which are located in the nucleus and the cytoplasm, and whose expression is a necessary condition for the transformation and maintenance of malignant phenotype of the cell (2). The E6 and E7 oncoproteins represent natural targets for anti-tumor immune response, and therefore are considered tumor-associated antigens (TAA). Cytotoxic T lymphocyte (CTL)-specific epitopes have been identified within the E7 protein of HPV16 in humans (3) and also in the H-$2^b$ mice (4,5). The E7 protein is an important model antigen for a study of the induction of HPV-specific CTL responses in mice and a promising target for the development of human vaccine against HPV (6). The E7 oncoprotein is poorly immunogenic, but fusing the molecule to certain proteins can change its processing efficiency, intracellular location, or stability, thereby its immunogenicity. For instance, fusion molecules of the E7 with the lysosome associated membrane protein 1 (LAMP1) (7,8), Listeriolysin O (LLO) (8) or glucuronidase of *E. coli* (GUS) (9) have been successfully used for vaccination in the form of proteins, or expressed by DNA vaccines or viral vectors.

Several lines of evidence show that the priming of the cytotoxic T cell lymphocytes (CTL) requires MHC class I-restricted presentation of antigenic peptides by the professional antigen presenting cells (APC) and that the character of $CD4^+$ T cell helper immune response mainly depends on the balance between cytokines in the early phase of CTL priming. The generation of CTL and T-helper cell type 1 response (Th1) is potentiated by interleukin 12 (IL12) (10,11) which simultaneously inhibits the development of T-helper cell type 2 (Th2) specific response. The immunomodulatory activity of IL12 found *in vivo* can be assigned to several biological activities that have been observed *in vitro*. IFNγ has been shown to mediate the key effects induced by IL12. Interleukin 12 upregulates the cell surface expression of adhesion/activation molecules, cytokine receptors and MHC class II molecules and activates perforin and granzyme B gene transcription in effector T cells. In a primed Th1 subset, IL12 can induce IFNγ secretion and interact with the CD80-CD28 pathway in order to fully activate differentiated Th1 cells (12). Several types of APC produce IL12, but dendritic cells (DC) are its major producers (13). After stimulation, mature DC transiently produce IL12 (14) and during this period they are able to initiate a priming of Th1 cells (15). Later, IL12 synthesis is restrained and DC preferentially prime a response of Th2 and non-polarized T cells.

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Recombinant IL12 administered as a protein or produced from expression vectors can modulate immune responses after vaccination. Interleukin 12 co-expressed with an antigen from plasmids in various DNA vaccines can substantially enhance the development of Th1 cells (14,16,17). In several cases, DCs engineered to secrete IL12 induced antigen-specific IFNγ responses and protection against parasite infection and tumors (18,19).

Vaccines based on DC pulsed *ex vivo* with TAA are considered to be a promising approach in cancer immunotherapy (20). An attractive alternative, designed to achieve an MHC class I- and MHC class II-restricted presentation of tumor antigens, employs the *ex vivo* transduction of DC with expression viral vectors, which enables prolonged presentation of multiple TAA peptides (21) and results in eliciting potent CTL and Th1 responses against extrinsic antigens.

Recombinant poxvirus vectors expressing a variety of extrinsic antigens have proved to be potent inducers of both cell-mediated immunity (CMI) and antibodies (22). They can be also employed for the *ex vivo* transduction of DC (18,23-26). Although the analysis of the interaction of poxviruses with DC has revealed several viral-mediated mechanisms that significantly interfere with the main functions of DC (27), it has been demonstrated that DC infected with the recombinant poxviruses present antigens to the T cells both directly (28) and through cross priming (29). The prevailing mechanism of antigen presentation depends on the route of administration of the transduced DC (30).

In our study, we investigated the immunogenicity of DC transduced with vectors derived from the conventional vaccinia virus (VV) strain Praha or from the highly attenuated MVA strain (31) which is unable to replicate in human cells and is therefore considered to be a very safe vector. We demonstrated that DC transduced by these vectors induced anti-tumor immunity and provided protection against the growth of transplanted, E7-expressing tumors. We found that the co-expression of IL12 had an inhibitory effect on the immunogenicity of VV-transduced DC even when low multiplicities of IL12-expressing virus were used for the transduction.

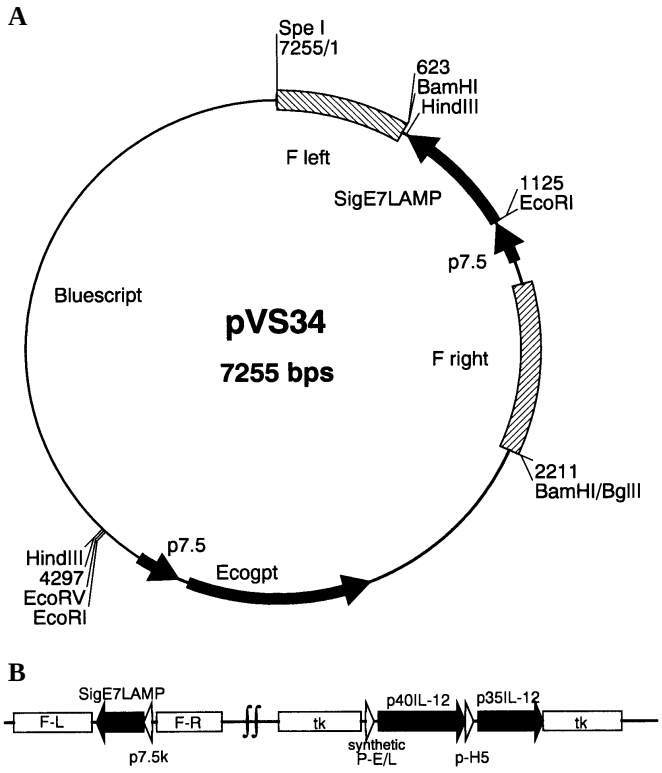


Figure 1. Structure of rVV expressing SigE7LAMP and IL12. (A), Plasmid pVS34 used for integration of SigE7LAMP fusion gene into *Hin*dIII-F fragment of VV genome. F left and F right have the role of recombination arms. They are formed by joining subfragments of *Hin*dIII-F which were excised by *Spe*I-*Bam*HI (nt. 1639-2262) and *Bam*HI-*Bgl*II (nt. 2262-3048, numbers denote position inside the *Hin*dIII-F fragment). The SigE7LAMP fusion gene was isolated from pCMVneo-SigE7LAMP plasmid (51) as the *Eco*RI-*Bam*HI fragment. Expression of SigE7LAMP gene is under a control of the early-late 7.5 promoter. The sequence between nt. 2211 and 4297 contains *E. coli* gpt gene and 7.5 promoter and was excised from pGpt07 (38). Plasmid pVS34 contains the entire pBluescript sequence (nt. 4297-7255). (B), Scheme of the genome of double recombinants. SigE7LAMP fusion gene was inserted in the non-essential F7L locus. The gene cassette containing IL12 genes was inserted into the thymidine kinase locus (TK).

## Materials and methods

*Viruses and cells.* Recombinant viruses were prepared from MVA clone 2 derived in our laboratory from parental MVA virus strain, which had been kindly provided by W. Altenburger (Basel), or from clone 13 of VV strain Praha (P13) (32). The generation of P13-IL12 and MVA-IL12 viruses was described previously (33). The P13-preS2S virus, which expresses the middle envelope protein (preS2 + S) of hepatitis B virus, was prepared using plasmid pM3 (34). Recombinant P13 viruses were propagated in human-embryo diploid cells (LEP) or monkey-kidney cells (CV-1). MVA recombinant viruses were grown in primary chicken fibroblast cultures. HPV16 E6E7-expressing TC-1 cells, derived from C57BL/6 mouse (7), were kindly provided by T.C. Wu (Baltimore). All cell lines were cultivated in modified E-MEM (EPL) medium containing bovine serum growth-active proteins but no complete serum (35). The VV recombinants used for *in vivo* experiments were grown in the chorioallantoic membranes of 11-day chicken embryos and were partially purified (36,37). Viruses for the infection of DC were grown in BSC40 cells and were purified by sucrose-gradient centrifugation (36).

*Generation of VV-SigE7LAMP and VV-SigE7LAMP + IL12 recombinants.* Viruses carrying the SigE7LAMP fusion gene inserted in the F7L gene were generated by homologous recombination using plasmid pVS34 (Fig. 1A). The recombination and the selection of recombinants were performed by standard procedures (38-40). First, recombinant viruses carrying the guanine phosphoribosyl transferase (gpt) gene were selected and double plaque purified under selection pressure (mycophenolic acid 25 μg/ml, xanthine 250 μg/ml, hypoxanthine 14 μg/ml). Then another double plaque purification procedure without selection pressure enabled reversion to the gpt$^-$ phenotype. Viruses that contained the SigE7LAMP fusion gene were detected by PCR using primers homologous with the E7 region. To obtain double recombinants, the SigE7LAMP gene was inserted as a second extrinsic gene into P13-IL12/EL and MVA-IL12/EL viruses. The production of the E7LAMP fusion protein was detected by immunoblotting, as described previously (41). The amount of E7LAMP

produced by single and double recombinants was similar (data not shown). The construction and properties of the IL12-expressing viruses were described previously (33). The P13-IL12/EL and MVA-IL12/EL recombinants produced, respectively, 22.8 and 10.0 pg p70-IL12 per one CV-1 cell infected at m.o.i. = 3 for 48 h. In all recombinants, the expression of the IL12-chain p35 was controlled by the early-late VV-H5 promoter and p40 was expressed from the synthetic early-late E/L promoter.

*Preparation of transduced dendritic cells for immunization.* Dendritic cells were prepared essentially as described elsewhere (42). Briefly, bone marrow leukocytes from C57Bl/6 mouse femurs were seeded at $4x10^6$ cells per 100 mm bacterial Petri dish in 10 ml R10 medium [RPMI 1640, 2 mM L-glutamine (Sigma), 50 μM 2-mercaptoethanol] supplemented with 10% FBS (Gibco) and 10% AG3 hybridoma culture supernatant (R10-GM). The AG3 cell line expressing GM-CSF, was obtained through Dr M. Lutz (Erlangen, Germany) with a kind permission of Dr B. Stockinger (London, UK). On day 3, another 10 ml R10-GM medium was added per plate. On day 6, a half of the culture supernatant from each plate was collected, centrifuged, and the cell pellet was resuspended in 10 ml of fresh R10-GM and returned into the original plate. On day 9, cells were harvested by gentle pipetting, centrifuged and resuspended at a concentration $1x10^6$/ml in R10-GM containing 50% of fresh medium and 10 ng/ml TNFα (Sigma) followed by a transfer to a new culture dish. On day 10, cells were harvested, and resuspended in RPMI containing 10% mouse serum. The DC were then transduced with purified rVV at $1x10^7$ cells per ml, m.o.i. = 1. After 1 h at 37˚C, 5% $CO_2$, infected DC were washed with RPMI containing 2% of mouse serum, centrifuged and resuspended in RPMI containing 10% mouse serum and used for immunization within 1-2 h. The stage of cell maturation was determined by analysis of the surface expression of MHC class II, CD80, CD86 and CD11c molecules by flow cytometry. Viability was determined by staining with propidium iodide.

*Immunization of mice*. Groups of eight 6-week-old C57BL/6 (H-$2^b$) female mice (Charles River) received intramuscularly (i.m.) $1x10^6$ transduced DC in 100 μl, separately into two sites of the musculus quadriceps femoris and the musculus triceps surae of the right leg. Control groups were inoculated with medium or with uninfected DC. Alternatively, mice received i.m. injections of gradient-purified virus only, or were inoculated intraperitoneally (i.p.) with VV propagated on chicken-egg chorioallantoic membranes and diluted in 0.5 ml PBS.

*Assay of E7-specific cellular immune response*

*Restimulation of splenocytes.* Fourteen days after immunization, mouse spleens were homogenized with cell dissociation sieve (Sigma) in R10 medium. Lymphocytes were separated on Histopaque-1083 (Sigma) and cultivated at a concentration $5x10^6$ cells/ml in R10, with or without 0.1 μg $E7_{49-57}$ (RAHYNIVTF) peptide (4), for 6 days.

*ELISPOT*. The response of $CD8^+$ T cells specific for $E7_{49-57}$ peptide was determined from the number of IFNγ-producing splenocytes isolated from immunized animals (43). Ninety-six-well MAHA 45 plates (Millipore) were coated with 5 μg/ml anti-mouse IFNγ monoclonal antibody R4-6A2 (BD PharMingen) in 0.1 M Na-phosphate buffer (pH 9.0) at room temperature overnight. The antibody-coated plates were washed four times with PBS and blocked with R10 medium at room temperature for 1 h. Aliquots of 100 μl of *in vitro* restimulated splenocytes were added to the wells and incubated in the presence or absence of the peptide at 37˚C in 5% $CO_2$ for 20 h. The wells were washed three times with PBS and three times with PBS containing 0.05% Tween-20 (PBS-T) and this was followed by overnight incubation at 4˚C with 2 μg/ml of biotinylated anti-mouse IFNγ monoclonal antibody XMG 1.2 (BD PharMingen) in PBS. The wells were then washed with PBS-T. Avidin-horseradish peroxidase conjugate (BD PharMingen) diluted 1:1,000 in PBS-T was added to the wells followed by incubation at 37˚C for 3 h. After washing with PBS, the spots were visualized using 3-amino-9-ethyl carbazol substrate (Sigma).

*Tetramer staining and FACS analysis*. MHC-I tetramers were prepared as described (44), in collaboration with the Tetramer Facility, Emory University, Atlanta. In brief, heavy and light chains were expressed separately in *E. coli* and were used in the form of inclusion bodies for the folding reaction. Mouse H-$2D^b$, human ß2-microglobulin, and the $E7_{49-57}$ peptide were folded *in vitro* to preform MHC-I monomers. After concentration of the reaction mixtures and the exchange of buffer, the preformed monomers were enzymatically biotinylated by BirA biotin synthetase and then purified by S300 column chromatography and Mono Q ion-exchange column chromatography. Tetramers were obtained by mixing the biotinylated protein complex with streptavidin-R-phycoerythrin conjugate (Molecular Probes) at a molar ratio 4:1.

Tetramer staining was performed on lymphocytes from $E7_{49-57}$ stimulated bulk cultures. Cells (1 ml) were centrifuged and resuspended in 50 μl FACS buffer (PBS supplemented with 1% fetal bovine serum and 0.065% sodium azide) containing 2 μg rat anti-mouse CD16/CD32 (Fc-block; BD PharMingen; 0.05 mg/ml) and incubated on ice for 20 min. Washed cells were stained in 50 μl with a mixture of PE-labeled tetramer and CD8a-FITC antibody (clone 53-6.7, BD PharMingen) on ice for 1 h. After washing, the samples were analysed with FACScan instrument and data were processed with CellQuest software (Becton Dickinson).

*Tumor growth inhibition*. Immunized mice were challenged with TC-1 cells expressing HPV16 E6 and E7 antigen. In all experiments, $2x10^4$ TC-1 cells in 100 μl were injected s.c. into the backs of mice, 2 weeks after immunization. Tumor growth was measured weekly. Significance of the differences between tumor growth curves was calculated using t-test, statistical analysis of survival curves was performed by the log-rank test using the GraphPad Prism 3.0 program. All experiments on laboratory animals were performed in accordance with the Czech Law No. 246/92 (Law Gazette), 'Breeding and Utilization of Experimental Animals'.

*Interleukin 12 detection*. Mature DC were either infected with rVV (m.o.i. = 1) or treated with 0.5 μg/ml LPS *E. coli*, sero-

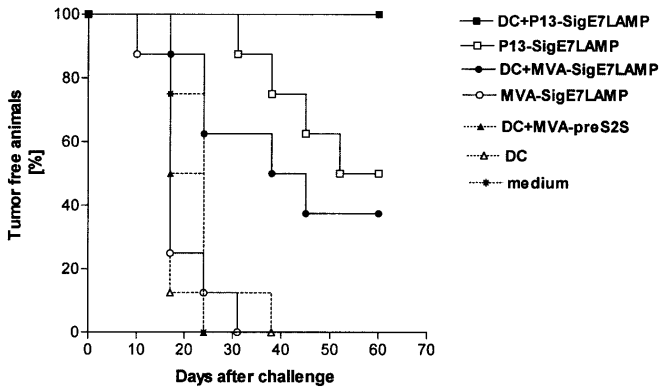


Figure 2. Transduction of DCs enhanced immunogenicity of VV-SigE7LAMP. Groups of mice (n=8) were injected intramuscularly with $1x10^6$ pfu of rVV or with $1x10^6$ dendritic cells infected with virus at m.o.i. = 1. Exceptionally in this experiment, the cells were not washed after infection and were injected together with the virus in the medium. Two weeks after immunization, the animals were challenged with $2x10^4$ of TC-1 tumor cells administered s.c. The survival curves represent numbers of mice with tumors <4 $mm^2$.

type 055:B5 (Sigma). After 24 h, the concentration of p70-m IL12 was quantified by ELISA (mouse IL12 BD OptEIA ELISA Set, BD PharMingen).

## Results

*Dendritic cells enhanced the immunogenicity of the replicating and non-replicating HPV16-E7-expressing rVV.* We directly analysed bone marrow-derived DC transduced with different rVV-SigE7LAMP for their immunogenicity and capacity to induce a protection against the growth of the HPV16-E7-expressing syngeneic TC-1 tumor cells. After intramuscular immunization with medium alone, with uninfected DC, or with DC infected with the control virus, MVA-preS2S, all animals developed tumors within 3-4 weeks after challenge (Fig. 2). In contrast, intramuscular immunization with $1x10^6$ pfu of the replicating virus P13-SigE7LAMP provided a partial protective effect against the tumor growth, while immunization with DC infected with the same virus resulted in a complete inhibition of the tumor growth. Similarly, immunization with the non-replicating virus MVA-SigE7LAMP alone did not provide any significant protection against the tumor growth while immunization with MVA-SigE7LAMP-infected DCs significantly inhibited the growth of transplanted TC-1 cells. (Fig. 2, p=0.034).

*Co-expression of IL12 decreased the immunogenicity of HPV16-E7-expressing rVV.* To explore whether the tumor-specific protective immunity induced by immunization with the rVVs expressing the SigE7LAMP fusion gene could be further enhanced by a co-expression of IL12 in the same vector, we prepared the double recombinant viruses P13-IL12-SigE7LAMP and MVA-IL12-SigE7LAMP. Groups of mice were immunized i.p. with rVV and the immune response was determined 2 weeks later. A portion of the animals was tested for the cellular immune response by the ELISPOT (Fig. 3A) and by the tetramer assay (Fig. 3B), and the remaining animals were challenged with the TC-1 cells

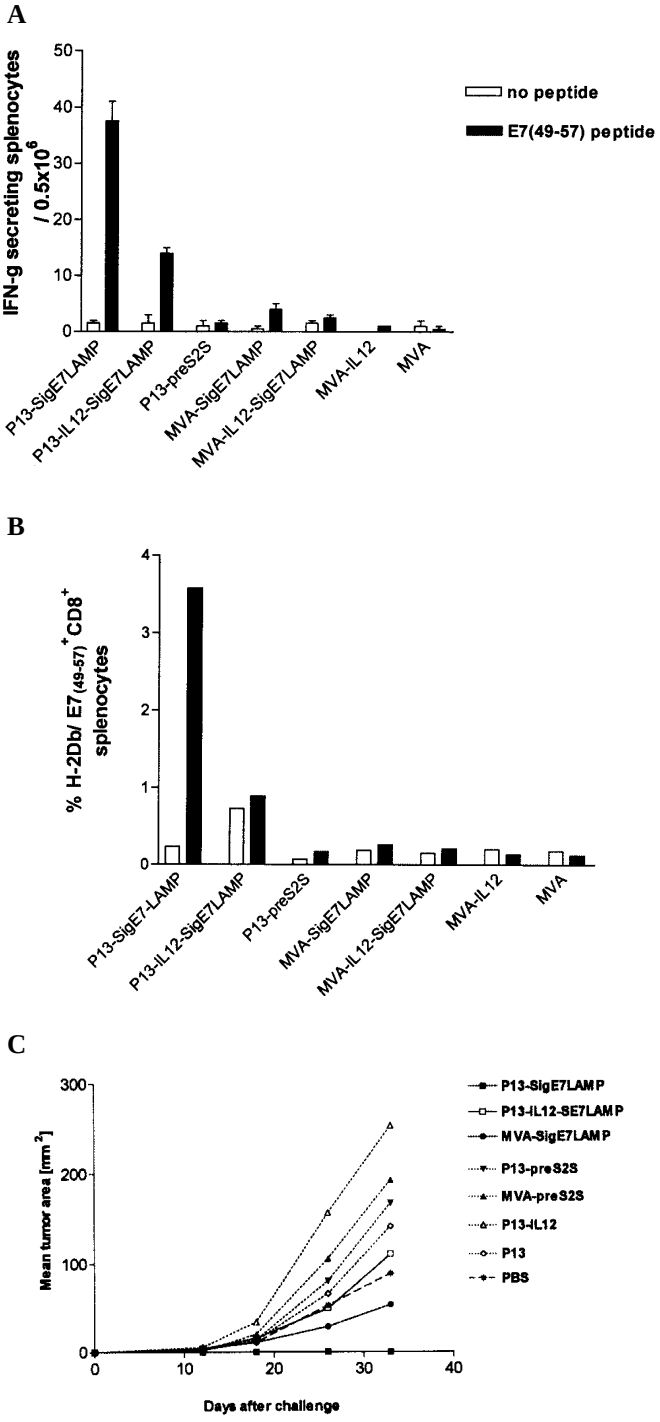


Figure 3. Co-expression of IL12 decreased E7-specific cell mediated and tumor growth protection induced by immunization with rVVs. Groups of mice (n=2) were injected i.p. with $3x10^6$ pfu of virus. Fourteen days after immunization, pooled splenocytes were used for detection of E7 specific cell mediated immune response by ELISPOT (A) and tetramer assay (B). Other groups of mice (n=8) were immunized i.p. with $5x10^5$ pfu of virus. Fourteen days after immunization the animals were challenged with $2x10^4$ of TC-1 tumor cells administered s.c. The tumor growth curves are shown (C).

(Fig. 3C). Since only the immunization with the replicating virus, P13-SigE7LAMP, but not with the attenuated virus, MVA-SigE7LAMP, induced a response detectable by the ELISPOT in the freshly isolated splenocytes, all tests employed spleen cells restimulated *in vitro* with the $E7_{49-57}$ peptide. All

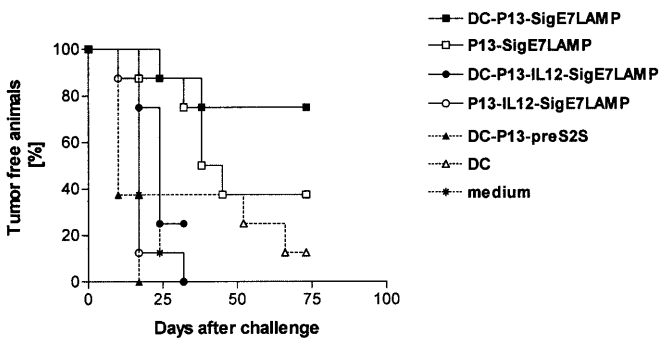


Figure 4. Co-expression of IL12 inhibited immunity induced by immunization with dendritic cells infected with recombinant P13 virus expressing SigE7LAMP gene. Groups of mice (n=8) were injected intramuscularly with $1x10^6$ pfu of virus or with $1x10^6$ dendritic cells infected with VV at m.o.i. = 1. The animals were challenged with TC-1 cells as described in Fig. 2.

assays demonstrated a lower immunogenicity for the P13-IL12-SigE7LAMP than for the single recombinant P13-SigE7LAMP. The decrease in a protection against the tumor growth was statistically significant (Fig. 3C, p=0.038). The immunogenicity of the attenuated MVA-SigE7LAMP virus was lower than that of the P13-SigE7LAMP when administered i.p. without DC (Fig. 3A and B) and immunization with the former provided only partial protection (Fig. 3C). Since the immunogenicity of MVA-SigE7LAMP was not increased by the co-expression of IL12 (Fig. 3A and B), the tumor growth protection induced with the MVA-IL12-SigE7LAMP was not tested. The size of tumors in mice that received P13-IL12 was larger than in animals inoculated with control viruses P13-preS2S (p=0.086) and P13 (p=0.026) or with PBS (p=0.003). However the differences between the curves of tumor-free animals were not significant (data not shown).

*Co-expression of IL12 inhibited the immunogenicity of DC vaccine against HPV16-E7.* We have shown previously that the expression of IL12 substantially reduced the multiplication of P13-IL12 *in vivo* (33). Decreased virus replication was most likely responsible for the low immune response induced by the double recombinant P13-IL12-SigE7LAMP, as shown in Fig. 3. We assumed that besides the attenuation of P13-IL12 due to the IL12 production *in vivo*, IL12 might directly activate Th1 cells thereby enhancing the E7-specific cellular immune response. Therefore, we decided to determine the effect of IL12 on the development of protective immunity against the challenge with the TC-1 cells under conditions in which the virus multiplication *in vivo* was limited.

Dendritic cells, which are naturally non-permissive for poxviruses, were infected with P13-SigE7LAMP or P13-IL12-SigE7LAMP for1 h, washed to remove the unadsorbed virus, and injected i.m. into mice. To evaluate the induced immunity, mice were challenged with the TC-1 cells 2 weeks later (Fig. 4). We found that the co-expression of IL12 by the double recombinant P13-IL12-SigE7LAMP eliminated the adjuvant activity of DC. The increase in the tumor incidence was statistically significant when compared with the group immunized with DC transduced with P13-SigE7LAMP (p=0.0127, Fig. 4). Specificity of the protection was inferred from multiple control groups (Fig. 4, dotted lines).

*Co-expression of IL12 decreased the immunogenicity of DC infected with the MVA at a wide range of m.o.i.* To eliminate the possible effect of multiplication of P13 virus in other than dendritic cells, e.g. granulocytes, which also diferentiate in the bone marrow cultures, we used in the following experiments the MVA-SigE7LAMP virus. Since the inhibitory effect of IL12 shown in the previous experiments could have been dose-dependent, we first compared the amount of IL12 secreted *in vitro* from MVA-infected DC and from LPS-activated DC. Mature DC were infected with the MVA-SigE7LAMP at m.o.i. = 1 and MVA-IL12-SigE7LAMP at m.o.i. = $1x10^{-0}$-$1x10^{-5}$. The production of IL12 by DC after 24 h is shown in Table I. The results show that DC infected with VV-IL12 at m.o.i. = 1 released approximately 2,000 times more IL12 than did DC in response to LPS.

In previous experiments with a green fluorescent protein-producing MVA virus (MVA-GFP, data not shown) we found that infection of mature DC with the MVA-GFP at m.o.i. = 1

Table I. Production of IL12 by dendritic cells activated with LPS or infected with recombinant MVA-IL12.

| Treatment of dendritic cells | Concentration of IL12 produced by DCs[a] (ng/ml) |
|---|---|
| - | 0.0 |
| LPS[b] | 0.7 |
| $10^6$ pfu MVA-SigE7LAMP[c] | 0.0 |
| $10^6$ pfu MVA-SigE7LAMP + $10^6$ pfu MVA-SigE7LAMP-IL12 | 1200.0 |
| $10^6$ pfu MVA-SigE7LAMP + $10^5$ pfu MVA-SigE7LAMP-IL12 | 135.7 |
| $10^6$ pfu MVA-SigE7LAMP + $10^4$ pfu MVA-SigE7LAMP-IL12 | 8.5 |
| $10^6$ pfu MVA-SigE7LAMP + $10^3$ pfu MVA-SigE7LAMP-IL12 | 0.9 |
| $10^6$ pfu MVA-SigE7LAMP + $10^2$ pfu MVA-SigE7LAMP-IL12 | 0.0 |
| $10^6$ pfu MVA-SigE7LAMP + $10^1$ pfu MVA-SigE7LAMP-IL12 | 0.0 |

[a]Cell culture was frozen and thawed once and clarified at 2,000 rpm/10 min. IL12 was determined in supernates. [b]$10^6$ mature DCs were cultivated with LPS (0.5 μg/ml) for 20 h. [c]$10^6$ mature DCs were infected with rVV and cultivated for 24 h.

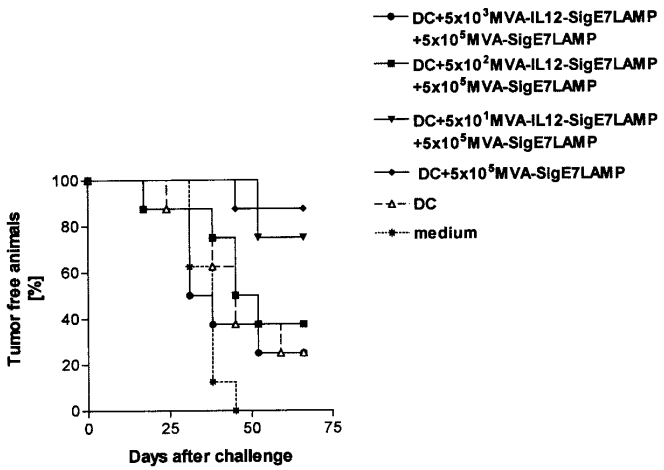


Figure 5. Effect of low levels of IL12 co-expression on immunity induced by immunization with dendritic cells infected with recombinant MVA virus expressing SigE7LAMP gene. Groups of mice (n=8) were injected intramuscularly with $5x10^5$ dendritic cells infected with MVA-SigE7LAMP at m.o.i. = 1 and MVA-IL12-SigE7LAMP at m.o.i. = $1x10^{-2}$-$1x10^{-4}$. The animals were challenged with TC-1 cells as described in Fig. 2.

resulted in approximately 30% of $GFP^+$ cells. Supposing this, we calculated from data in Table I that each DC infected with MVA-IL12-SigE7LAMP produced about 3.6 pg IL12 in 24 h. However, the total amount of IL12 produced by DC decreased after infection with the MVA-IL12-SigE7LAMP at a lower m.o.i.

The next goal was to determine whether a lower concentration of co-expressed IL12 would increase the immunogenicity of DCs. Mice were immunized with $5x10^5$ DC infected at m.o.i. = 1 with MVA-SigE7LAMP admixed at ratios 1:100, 1:1,000 and 1:10,000 with MVA-IL12-SigE7LAMP. Anti-tumor immunity was determined by the tumor growth inhibition test (Fig. 5). A suppressive effect on the protection against tumors was detected still at a ratio 1:100 in comparison with administration of MVA-SigE7LAMP-infected DC, the difference between survival curves was statistically significant ($p=0.0294$).

## Discussion

Recombinant vaccinia viruses derived from two vaccination strains, Praha and MVA, which both encode the E7 protein, the main HPV16-associated tumor antigen, were tested for their immunogenicity in C57Bl/6 mice. The SigE7LAMP transgene was inserted in the F7L locus of VV genomic DNA. It has been reported that the expression of genes inserted in this locus is higher in comparison with the insertion in the TK gene (45), because the activity of the inserted promoter sequence is enhanced by the F7L promoter if both promoters are in the same orientation. The main advantage of this construct is that the TK gene can be used for the insertion of another gene, e.g. a cytokine. A recombinant virus prepared from a strain Praha was immunogenic in mice and induced E7-specific cellular immunity and protection against tumor growth. Since immunization with a significantly attenuated MVA recombinant virus was much less effective, we attempted to increase the immunogenicity of rVVs. We found that the protection against tumors could be increased if DC transduced *ex vivo* with rVV were used for immunization instead of inoculation with the rVV only. Dendritic cells derived from the bone marrow cells and transduced with rVVs were injected i.m. We compared different administration routes (data not shown) and found, that immunization with rVV-transduced DC by the i.m. route was superior to s.c. administration, similarly as has been previously reported for an immortalized DC line expressing HPV16-E7 (46). An advantage of the experimental modification in which the E7 protein is fused to LAMP1 protein, is that the protein is directed into the endosomal compartment of the cell. This cellular targeting facilitates in professional APCs the presentation of E7 protein in the context of MHC class II and better stimulates $CD4^+$ helper T lymphocytes.

In order to achieve a better protective anti-tumor immunity by immunization with rVV-transduced DC, we decided to enhance the $CD4^+$ Th1 response by the co-expression of IL12 with E7 protein. It had been previously shown that a specific cellular immune response elicited by a poxvirus vector could be increased by the co-expression of this cytokine. However, IL12 significantly attenuated the recombinant virus and enhanced the Th1-IFNγ response to the HIV Env glycoprotein and to the vector itself. The dose of rVV used for immunization determined the resulting increase or decrease of cellular response (17).

Our data demonstrate that the multiplication of rVV used for immunization was the most important factor for the magnitude of cellular response. Induction of E7-specific response and anti-tumor immunity by rVV inoculation could only be achieved with replicating recombinants, while the non-replicating virus MVA-SigE7LAMP did not induce any significant anti-tumor immunity. As compared with P13-VV, the *in vivo* replication of P13-IL12 virus was significantly impaired, as shown previously (33). Therefore, limited *in vivo* replication might explain the low anti-tumor protection induced by immunization with P13-IL12-SigE7LAMP virus. Another cause of the low protectivity after immunization with this virus might be a non-specific immunosuppressive effect due to the high level expression of IL12 by recombinant viruses. Perhaps the enhancement of tumor growth following the inoculation of a control rVV expressing IL12 but not SigE7LAMP 2 weeks before challenge, could also be attributed to the immunosuppression (Fig. 3C). This effect was not statistically significant in comparison with the control P13-preS2S virus, but it appeared after the inoculation of P13-IL12 as well as after MVA-IL12 (data not shown). The interval between the inoculation of IL12-expressing virus and TC-1 challenge seems to be of great importance, because anti-tumor protection rather than an enhancement of tumor growth activity was observed if P13-IL12 virus was used for the treatment of established tumors (33). Our findings obtained with the HPV16-E7 model differ considerably from the results repeatedly reported for the mouse CT26-CL25 tumor, which expresses lacZ as surrogate TAA (47,48) and in which the co-expression of IL12 enhanced the therapeutic effect of the rVV expressing the TAA. These viruses were inoculated i.v. following tumor cells inoculation 3 days earlier by the same route. In this case, the anti-tumor effect of the IL12-

expressing recombinants might be a result of a combination of the non-specific activity of IL12 toward established tumors and the specific anti-TAA immune response. This response differs from our preventive model, in which the attenuation of the virus and the immunotoxicity of IL12 might be responsible for lower protection induced by immunization.

Since it is well known that activated DC release IL12 in the course of stimulation of Th1 lymphocytes and that infection with poxvirus inhibits the maturation and differentiation of DC (27), we hypothesised that a substitution of the DC-derived IL12 by infection with an IL12-expressing virus could enhance the Th1 cell response. However, this presumption was not confirmed: when we tested DC transduced by the replicating P13 (Fig. 4) and the non-replicating MVA virus (Fig. 5) we found for each virus that IL12 suppressed the induction of protection against E7-expressing tumor cells. Since we were aware of the previously reported observations that the effects of IL12-expressing poxviruses are greatly dose-dependent (17,49), we used in the immunization scheme DC transduced with the MVA-SigE7LAMP admixed with the MVA-IL12-SigE7LAMP. The use of the MVA guaranteed that IL12 would not be expressed from other cells than the infected DC, because mouse DC cultures do not support MVA replication (unpublished data). The finding that IL12-producing DC were able to significantly suppress the anti-tumor effect of transduced DC even at a 1:100 ratio of the IL12-expressing virus to the virus without the IL12 gene indicated that the inhibitory effect of the cytokine is not restricted to the MVA-IL12-SigE7LAMP-transduced DC. Instead, this effect can be transmitted to other MVA-SigE7LAMP-infected DC. The dominance of the suppressive effect of IL12 rather suggests that IL12, or its mediator, may affect the bystanding cells, which are essential for a protection against tumors, and that suppression of the anti-tumor effect can be induced by the cytokine at a low concentration.

Similar immunosuppressive effect of IL12 has been observed in the mouse B16 melanoma model after immunization with DC co-transduced with adenoviruses expressing MART-1 and IL12 (50). The mechanism responsible for the effect of IL12 in this system was not determined, but the NK cell cytotoxicity against DC and increased nitric oxide concentration were experimentally excluded. Together with these data, our experiments indicate that the immunosuppressive effect of IL12 is directed at those arms of specific immunity which are important for protection against tumors.

To conclude, we have shown that the attenuated MVA virus was able to elicit immunity against tumors induced by HPV16 if vaccination was performed with DC transduced with MVA expressing the E7 protein as a TAA. We also demonstrated that DC modified to produce high amounts of IL12 had a suppressive effect on the induction of immunity against tumors.

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ORIGINAL ARTICLE

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# Prime/boost immunotherapy of HPV16-induced tumors with E7 protein delivered by *Bordetella* adenylate cyclase and modified vaccinia virus Ankara

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**Abstract** The *Bordetella* adenylate cyclase toxoid (CyaA) targets cells expressing the $\alpha_M\beta_2$ integrin receptor CD11b/CD18 (CR3 or Mac-1) and can penetrate into cytosol of professional antigen-presenting cells, such as dendritic cells. This allows us to use CyaA for delivery of passenger antigens into the cytosolic pathway of processing and MHC class I-restricted presentation, which can promote induction of antigen-specific $CD8^+$ cytotoxic T-lymphocyte immune responses. We show here that vaccination with a genetically detoxified CyaA336/E7 protein, carrying the full-length oncoprotein E7 of the human papilloma virus 16 inserted at position 336 of the cell-invasive AC domain of CyaA, induces an E7-specific $CD8^+$ T-cell immune response and confers on mice protective, as well as therapeutic immunity against challenge with TC-1 tumor cells expressing the E7 oncoprotein. The therapeutic efficacy of priming with the CyaA336/E7 vaccine could further be enhanced by a heterologous booster immunization with a highly attenuated modified vaccinia virus Ankara (MVA) expressing the E7 protein fused to the lysosome-associated membrane protein (LAMP1). These results establish the potential of CyaA as a new antigen delivery tool for prime/boost immunotherapy of tumors.

**Keywords** Vaccine · HPV-E7 · Vaccinia virus · MVA · *Bordetella* adenylate cyclase

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## Introduction

Human papilloma viruses (HPV) have been identified as the etiological agent of cervical carcinoma, the second most common malignancy in women worldwide. Moreover, HPVs are suspected to be also involved in induction of other tumors [25] and development of prophylactic and therapeutic vaccines against HPV remains of utmost priority. Efficient therapeutic vaccination against HPV-associated disease will most likely depend on induction of specific cellular immune responses, since regression of lesions in HPV16-infected humans appears to be associated with $Th_1$ and $CD8^+$ T-cell responses to the early proteins E2, E6 and E7 [18]. The proteins E6 and E7 are expressed in high-grade precancerous lesions and invasive cancers and are essential for induction and maintenance of oncogenic transformation of HPV-infected cells [24]. The E6 and E7 proteins appear, hence, as the most suitable antigenic targets for immunotherapy of HPV-induced lesions and tumors.

A generally promising vaccination strategy for induction of cellular immune responses is the heterologous 'prime/boost' immunization [10] with two different vaccines being administered subsequently. Several recent studies, employing heterologous prime/boost strategies to prevent and cure infectious and/or cancer pathologies, have shown that this type of immunization protocol can lead to induction of cytotoxic T-lymphocytes (CTLs) specific for tumor antigens [11]. Many effective prime/boost protocols consist in priming with a DNA

vaccine followed by a booster immunization with a live attenuated virus vector, such as the modified vaccinia virus Ankara (MVA). Due to at least six major deletions, about 15% of the original DNA of the parental Ankara strain is missing in MVA and the virus could be developed into a safe attenuated live vaccine against smallpox. Since MVA has a limited capability of replication in various human cell lines and is nonpathogenic even to immunocompromised animals [17], it is considered as an exceptionally safe viral vector. Indeed, MVA is currently used in advanced stage clinical trials of several prophylactic and therapeutic vaccines against infectious diseases and cancer [23].

In this study, we evaluated the potency of a heterologous prime/boost immunization protocol involving MVA expressing the E7 antigen of HPV16 in combination with a CyaA vaccine carrier derived from the adenylate cyclase (AC) toxoid of *Bordetella pertussis* [22]. This novel antigen carrier has the capacity to target primarily the myeloid phagocytic leukocytes carrying the CD11b/CD18 receptor and in particular the dendritic cells [9], which are the key cells capable of antigen processing and presentation. Moreover, the cell-invasive AC domain of CyaA was previously shown to penetrate into the cytosol of eukaryotic cells and has already been successfully used for delivery of a variety of heterologous passenger epitopes into professional antigen presenting cells in vivo [22, 3]. This capacity of CyaA could be used to induce potent $CD8^+$ CTL responses that are effective in protection of experimental animals against both viral and tumoral challenges (see reviews [22] and [3]). In this report, we show that a CyaA/E7 construct delivering the full-length E7 antigen of HPV16, induces protective as well as therapeutic immunity against TC-1 tumors in mice and that its immunotherapeutic efficacy can be further enhanced when used in prime/boost regimens combining delivery of the E7 antigen by CyaA and the attenuated MVA vector.

## Materials and methods

### Recombinant CyaA and MVA vaccines delivering the HPV16 E7 antigen

Using primers Cya-E7-1 (5′- G CTT GTA CAC ATG CAT GGA GAT ACA CCT) and Cya-E7-2 (5′- CT GTG TAC AGG TTT CTG AGA ACA GAT GG), the sequence encoding the entire E7 protein of HPV16 was amplified from the pEA16-E7 template DNA (obtained from I. Jochmus, Heidelberg, Germany). The purified PCR product was then inserted into the unique *Bsr*GI sites located at different positions within the CyaA gene (Table 1) on a set of pT7CACT1 *Bsr*GI plasmids [19], and the orientation and sequence of E7 inserts were verified by DNA sequencing. Recombinant CyaA/E7 proteins were produced in *Escherichia coli*, purified close to homogeneity and characterized for enzymatic AC and cell-invasive activity on sheep erythrocytes used in the surrogate assay for determination of membrane penetration capacity of the CyaA vector, as described in detail previously [19]. The LPS content of CyaA preparations was between 50 ng and 250 ng per vaccine dose, as determined by Limulus Amebocyte Lysate Assay (BioWhittaker).

Recombinant MVA virus was prepared from clone 2 isolated by plaque purification in our laboratory from the MVA vaccine provided kindly by W. Altenburger, Basel, Switzerland. Generation of the MVA-SigE7-LAMP virus expressing E7 in fusion to the lysosome-associated membrane protein (LAMP1) was described elsewhere [16]. MVA-SigE7LAMP virus was multiplied in primary chicken fibroblast cultures and the virus used for in vivo experiments was grown in chorioallantoic membranes of 11-day-old chicken embryos and partially purified as previously described [12].

### Antibodies

Monoclonal antibodies specific for mouse CD11b (M1/70), CD11c (HL3), I-$A^b$ (AF6-120.1), CD86 (GL1) and CD8a (53–6.7) labeled with FITC or R-phycoerythrin were purchased from BD Pharmingen. Anti-CyaA monoclonal antibody 9D4 [13] was a kind gift of E.L. Hewlett (University of Virginia). Peroxidase-labeled sheep anti-mouse Ig was from AP Biotech (Vienna, Austria).

### Cells

Dendritic cells (DC) were prepared from precursors isolated from bone marrow of C57Bl/6 (H-$2^b$) mice by in vitro cultivation in the presence of GM-CSF for 9 days

**Table 1** Characteristics of CyaA constructs with HPV16 E7 protein inserts

| CyaA protein | Insert/flanking sequences[a] | AC enzyme activity[b] (%) | Binding to dendritic cells[c] |
|---|---|---|---|
| CyaA wt | none | **100** | + |
| **CyaA336/E7** | $G^{335}$ . *VH* **E7(1-98)** *VH*.$Q^{336}$ | ***ND*** | + |
| CyaA1334/E7 | $G^{1333}$ . *VH* **E7(1-98)** *VH*.$Q^{1334}$ | **100** | - |

[a] The HPV16 protein E7 residues 1 to 98 (*underlined in bold*) were inserted at various permissive positions of CyaA [19]. The VH dipeptides flanking the E7 insert are shown in *italics*

[b] Specific enzymatic AC activity was determined as described previously [19]. *ND*, not detectable

[c] Binding of CyaA to mouse dendritic cells was assessed by immunodetection of CyaA as described under Materials and methods

and matured with TNF-α for additional 20 h [16]. DC were examined for surface antigens by fluorescent staining and subsequent FACS analysis using a FACScan instrument and Cell Quest software (Becton Dickinson). The population of dendritic cells contained 85% $CD11c^+$, >95% $CD11b^+$, 30% $MHCII^{hi}$ and 50% $CD86^+$ cells. TC-1 tumor-inducing cells derived from lung cells of C57BL/6 mouse [14] and expressing the HPV16 E6 and E7 antigens were provided by T.C. Wu (Johns Hopkins Medical Institutions, Baltimore).

### Binding of CyaA to DC

One microgram of CyaA was incubated with $1\times10^6$ dendritic cells/ml at 37°C for 2 h in RPMI 1640 medium conditioned for cultivation of DC [16]. The supernates were removed and cells were washed three times, resuspended in 50 µl of RPMI 1640 medium containing protease inhibitor cocktail (Sigma) in a new Eppendorf tube and lysed by addition of Laemmli SDS-PAGE loading buffer. The samples were separated by 8% SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using the CyaA-specific antibody 9D4, the sheep anti-mouse Ig-peroxidase conjugate and the ECL detection system (APBiotech).

### Animal experiments

Groups of 6-week-old C57Bl/6 ($H\text{-}2^b$) female mice (Charles River, Germany) were immunized intraperitoneally with 0.5 ml PBS containing 50 µg of CyaA proteins or $1\times10^7$ PFU of MVA-SigE7LAMP virus. Control groups were inoculated with PBS only. For detection of protective immunity in preventive vaccination, the immunized animals were challenged with $3\times10^4$ TC-1 tumor cells administered s.c. 2 weeks after the last immunization. In immunotherapeutic experiments, mice were first inoculated with $3\times10^4$ or $6\times10^4$ TC-1 cells s.c. and then immunized on day 1 and 8 after tumor cell transplantation. All immunization experiments were reproduced at least twice. Tumor formation was statistically analyzed by comparison of survival curves of tumor-free animals (graphs not shown) with the controls injected with CyaA wt or PBS, by the logrank test. The data were plotted and analyzed using Prism software, version 3.0 (GraphPad Software, Inc., San Diego, CA, USA). The experiments on laboratory animals were performed in compliance with Act No. 246/92, on animal protection against cruelty and Decree No. 311/97 of the Ministry of Health of the Czech Republic, on the care and use of experimental animals.

### Assays of E7-specific T-cell responses

Spleens were removed from mice 12 days after the last immunization and the response of IFN-γ-producing cells was determined by ELISPOT assay upon restimulation with $H\text{-}2D^b$ restricted $HPV16\ E7_{(49–57)}$ (RAHYNIVTF) peptide for 6 days in vitro, as described recently [16]. E7-specific $CD8^+$ CTLs were detected by tetramer-staining assay in lymphocyte bulk cultures, restimulated in vitro for 6 days with $HPV16\ E7_{(49–57)}$ peptide, using the R-phycoerythrin labeled $H\text{-}2D^b$ / $E7_{(49–57)}$ tetramer reagent, as described previously [16]. The stained cells were analyzed using a FACScan instrument and Cell Quest software (Becton Dickinson). The mean fluorescence values obtained for peptide-restimulated and control cells were analyzed by the unpaired *t*-test.

## Results

### Characteristics of the CyaA/E7 vaccines

To assess the capacity of CyaA to deliver the E7 protein of HPV16 for antigenic presentation, two CyaA toxoids were constructed by inserting the E7 polypeptide (98aa) at previously identified permissive sites of CyaA [19]. One insertion was made at position 336 of the cell-invasive AC domain, shown previously to be suitable for delivery of rather long polypeptides to the cytosolic entry of the MHC class I-restricted antigen presentation pathway [19, 5]. As observed previously with other antigens placed at these sites and as documented here in Table 1, insertion of the 98-residues-long E7 antigen at position 336 resulted in ablation of the enzymatic AC activity and yielded, hence, a genetically detoxified CyaA336/E7 protein. More importantly, the CyaA336/E7 construct bound to the CD11b-expressing mouse bone marrow-derived dendritic cells (DCs) to similar levels as the intact Cyawt protein (Table 1 and Fig.1). As a control for specificity of the CyaA336/E7-mediated

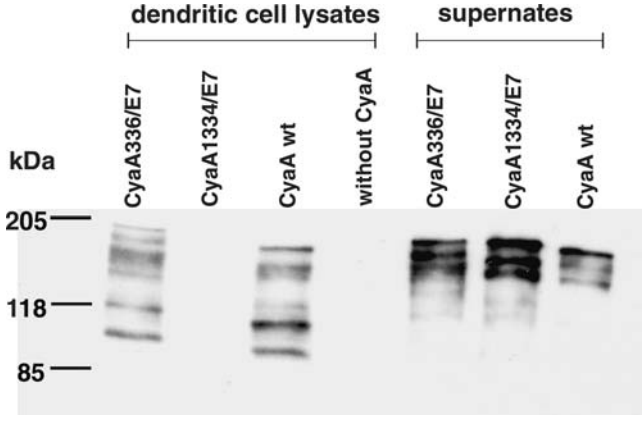


**Fig. 1** Binding of CyaA proteins to dendritic cells. CyaA proteins were incubated with DC (1 µg protein/$1\times10^6$ cells/ml) at 37°C for 2 h. The cells were washed three times and lysed in the presence of protease inhibitor cocktail (Sigma). Lysate aliquots corresponding to $3\times10^5$ toxoid-treated dendritic cells and to 10 µl of culture supernatants containing unbound CyaA were analyzed on Western blot, using the monoclonal antibody 9D4 recognizing the RTX domain of CyaA [13]

delivery of the E7 antigen to MHC I-restricted presentation, a second toxoid (CyaA1334/E7) was constructed carrying the E7 insert at position 1334 within the RTX repeat domain, which was previously found unsuitable to deliver passenger antigens to the MHC I-restricted pathway [19]. As expected, insertion of the E7 polypeptide at position 1334 had no discernible effect on the AC activity of CyaA, while the capacity of the CyaA1334/E7 construct to bind to dendritic cells was ablated, most likely due to disruption of the integrin-binding domain of CyaA by the E7 insert [4].

## The CyaA336/E7 vaccine elicits E7-specific cellular immune responses protecting mice against growth of HPV16-induced TC-1 tumors

It was important to examine whether immunization with the CyaA336/E7 vaccine induces an E7specific cellular immune response. As documented in Fig. 2A, the intraperitoneal administration of two doses of 50 μg of CyaA336/E7 in 3-week intervals elicited an E7-specific $CD8^+$ T-cell response characterized by significant expansion of E7-specific T-cell precursors, as detected in the ELISPOT assay for IFN-γ-secreting cells following in vitro restimulation of total mouse splenocytes with the $E7_{(49–57)}$ $CD8^+$ CTL epitope peptide. The direct assay for expansion of $CD8^+$ T cells binding the $H\text{-}2D^b$ MHC I tetramers loaded by the $E7_{(49–57)}$ peptide yielded a very similar result, as shown in Fig. 2b, demonstrating the capacity of CyaA336/E7 to induce E7-specific $CD8^+$ T-lymphocyte responses. As expected, no such T-cell response was detected upon immunization by mock CyaA, or the control protein CyaA1334/E7, unable to target dendritic cells.

Next, we determined whether immunization by CyaA336/E7 could confer on the animals any protection against the growth of TC-1 tumors expressing the E7 oncoprotein. As illustrated in Fig. 2c, all eight control mice that received buffer (PBS), and six out of eight mice immunized by the control CyaA1334/E7 or mock CyaA protein, respectively, had already developed discernible TC-1 tumors 15 days following injection of $3\times10^4$ TC-1 cells at two weeks after the last immunization. In contrast, upon vaccination with two doses of the CyaA336/E7 protein (50 μg) administered i.p. at a 3-week interval, six out of the eight immunized mice ($P=0.002$) were completely protected from growth of TC-1 tumors under identical challenge conditions. This result was well reproducible in repeated experiments and the protected mice did not develop any tumors over a period of 50 days.

## Prime/boost combination with the MVA-SigE7LAMP virus enhances efficacy of the CyaA336/E7 vaccine in immunotherapy of growing TC-1 tumors

The cellular response and prevention of TC-1 tumors growth induced by vaccination with CyaA336/E7 suggested that treatment with this vaccine might also allow immunotherapy of already growing tumors. We further reasoned that efficacy of the therapeutic vaccination could be enhanced in a heterologous prime/boost regimen with a different second vaccine delivering the E7 protein, such as the strongly attenuated MVA-SigE7LAMP virus expressing the E7 antigen in fusion with the LAMP1.

Indeed, as shown in Fig. 3a, priming immunization with 50 μg of the CyaA336/E7 vaccine followed at a 3-week interval by booster vaccination with $10^7$ PFU of MVA-SigE7LAMP induced higher numbers of precursors of E7-specific IFN-γ producing cells ($P=0{,}0067$) than the immunization with two doses of the CyaA336/E7 vaccine alone. A stronger response induced by heterologous prime/boost immunization was also detected by the tetramer assay, as shown in Fig. 3b, albeit this result was statistically insignificant. To compare the therapeutic efficacy of the induced immune responses, animals were challenged s.c. with $3\times10^4$ TC-1 cells and treated 1 and 8 days later by prime/boost immunization with the two vaccines. As documented in Fig. 3c, immunization with a priming dose of CyaA336/E7 followed by a booster dose of the MVASigE7LAMP vaccine as well as immunization with two doses of the CyaA336/E7 vaccine resulted in complete protection of animals against growth of transplanted TC-1 tumors. This high therapeutic efficacy was, however, achieved only in regimens comprising at least one dose of the CyaA336/E7, where among the 24 mice from the three groups treated by this vaccine only two animals developed any TC-1 tumors. In contrast, a significantly lower therapeutic efficacy was observed for immunization with two doses of the MVA-SigE7LAMP vaccine, which yielded only three tumor-free mice out of eight challenged animals and repeatedly resulted in a comparably poor therapy as that induced by a single immunization by MVA-SigE7LAMP and followed by administration of mock CyaA wt (Fig. 3c).

As shown in Fig. 3d, however, a certain superiority of the therapeutic efficacy of the heterologous prime/boost immunization with the CyaA336/E7 and MVA-SigE7-LAMP vaccines, over the effect of homogeneous vaccination with two doses of CyaA336/E7 alone, was apparent when the immunotherapy conditions were made more stringent by using a doubled challenge dose of $6\times10^4$ TC-1. While two out of eight mice developed tumors following treatment with two doses of the CyaA336/E7 vaccine alone, all mice treated with the combination of the CyaA336/E7 and MVA-SigE7-LAMP vaccines remained free of tumors by day 25 post challenge and later. This difference was, however, not statistically significant on the level of a single experiment with eight animals per group ($P=0.14$). Only upon logrank test analysis of cumulated data from two repeated experiments involving a larger number of animals ($n=14$), the heterologous prime/boost protocol exhibited a somewhat higher therapeutic efficacy than the immunization with two doses of the same vaccine ($P=0.015$).

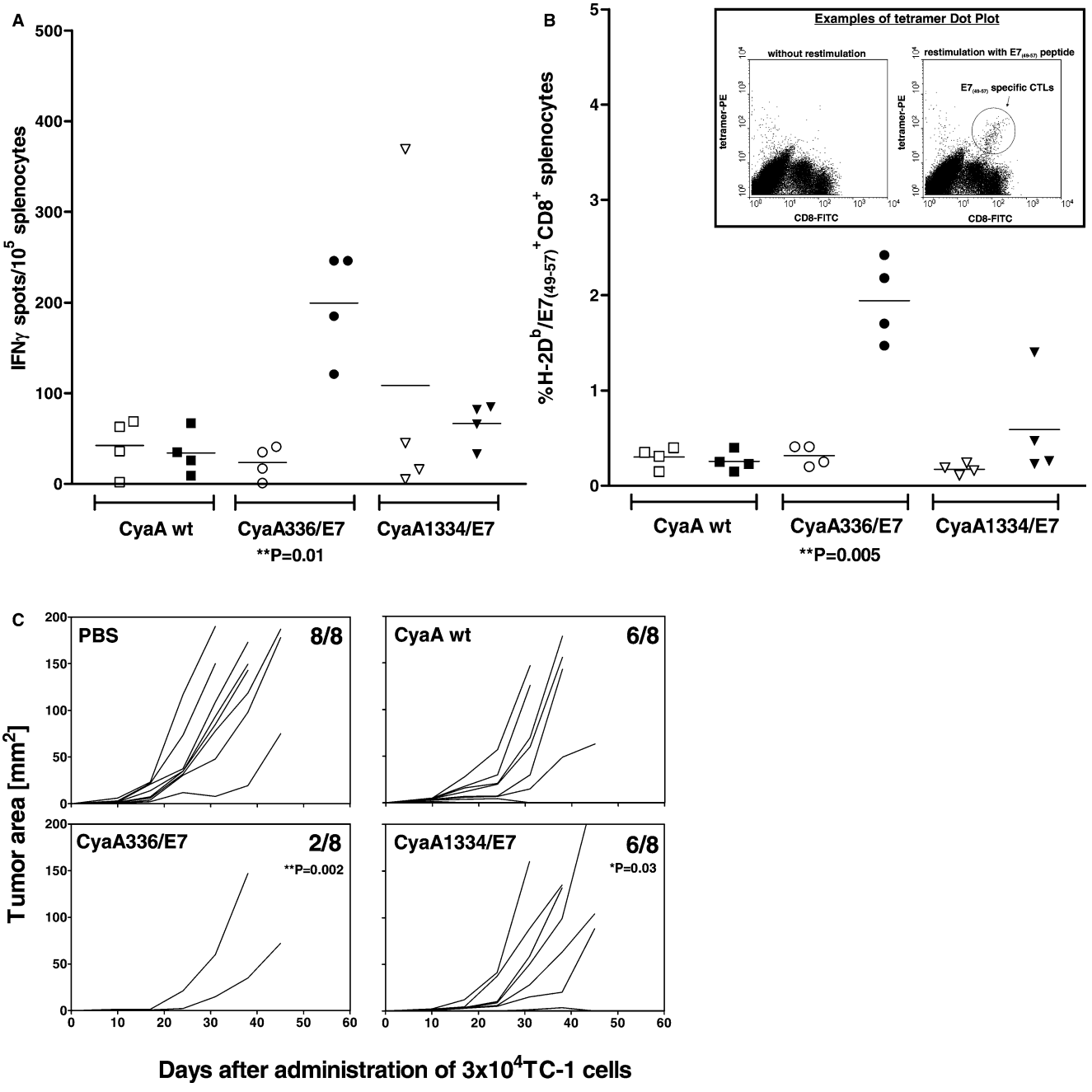


**Fig. 2** Immunization with the CyaA336/E7 construct induces E7-specific $CD8^+$ T-cell response and protection against growth of TC-1 tumors. **a, b** Six-week-old C57Bl/6 ($H\text{-}2^b$) female mice ($n=4$) were injected i.p. with two doses of 50 μg of CyaA toxoid in 3-week intervals and 12 days after the last immunization the mice were killed. Induction of E7-specific $CD8^+$ T-cell lymphocytes was determined in individual mice by IFN-γ ELISPOT (**a**) and tetramer (**b**) assays. The mean values obtained in the presence (*full symbols*) and absence (*empty symbols*) of the $E7_{(49–57)}$ peptide were compared and analyzed by the unpaired *t*-test. Inset in **b** shows an example of detection of tetramer$^+$ $CD8^+$ cells. **c** Two weeks after the second immunization, the animals ($n=8$) were challenged s.c. with $3\times10^4$ TC-1 tumor cells and the growth of tumors was monitored 45 days. Tumor growth in individual animals is shown on separate graphs for each group and the fraction of mice that developed tumors is indicated in the upper right corner of each graph. The data were statistically analyzed by comparison of survival curves of tumor-free animals in individual groups with the control group injected with CyaA wt using logrank test. Representative results from one out of two independent challenge experiments are shown

Furthermore, the order of administration of the two different vaccines also appeared of some importance. Consistently, higher $CD8^+$ T-cell immune response (Fig. 3a, b) and a more efficient therapy, with no transiently observable tumor growth, was reproducibly observed upon a priming immunization with the CyaA336/E7 vaccine followed by a booster with MVASig-E7LAMP, than immunization with the same vaccines given in the reverse order (Fig. 3c, d).

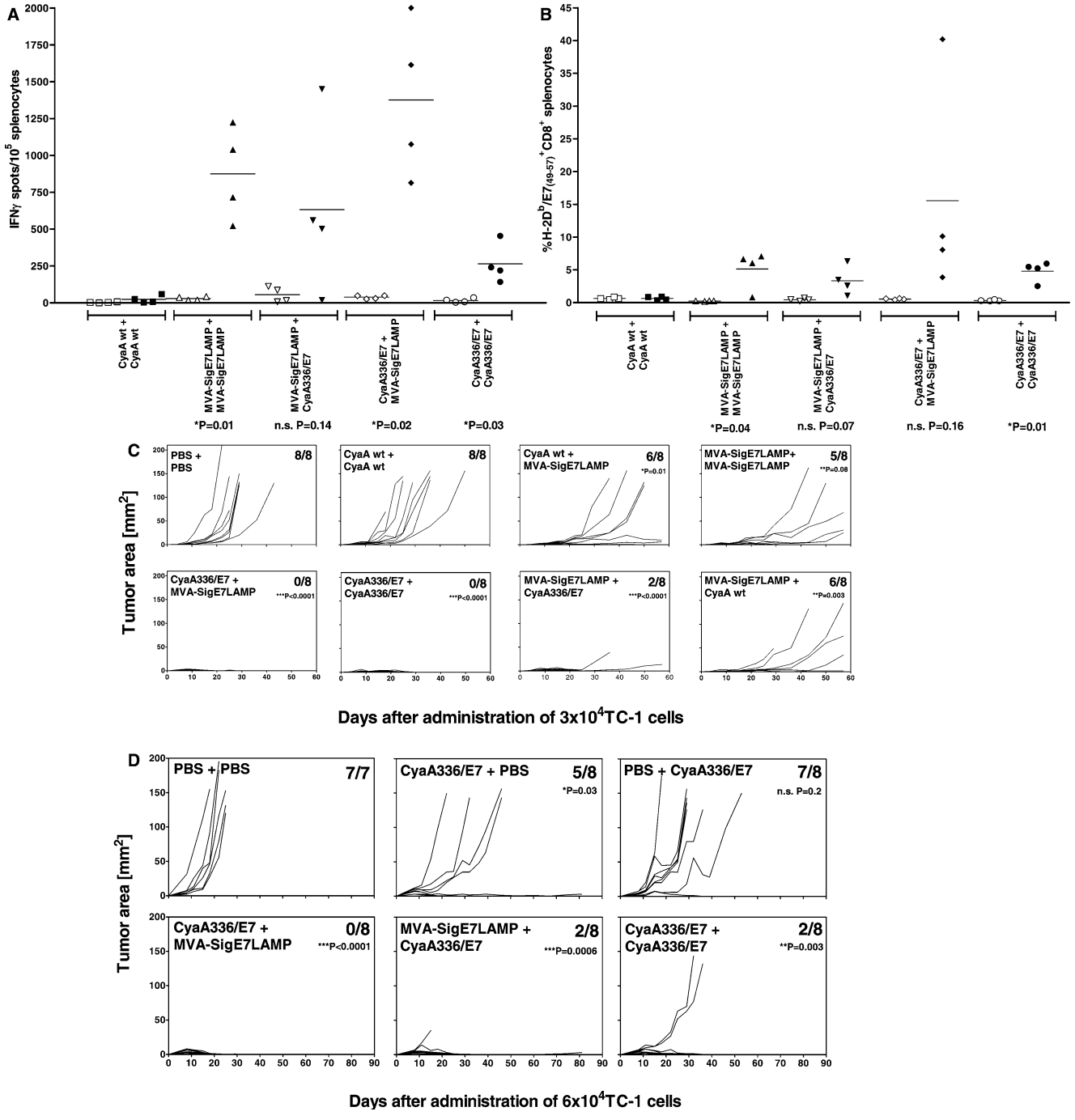


**Fig. 3** Heterologous prime boost vaccination with the combination of CyaA336/E7 and MVA-SigE7LAMP vaccines induces strong cellular immune response and results in more efficient immunotherapy of TC-1 tumors. **a, b** Mice ($n=4$) were immunized in 3-week intervals by two intraperitoneal injections of the indicated vaccine combinations (50 μg CyaA or $10^7$ PFU MVA-SigE7LAMP per dose) and T-cell immune responses were determined by IFN-γ ELISPOT (**a**) and tetramer (**b**) assays, as described in Fig. 2a, b. For evaluation of the therapeutic efficacy of the combinations of the CyaA336/E7 and MVA-SigE7LAMP vaccines, mice ($n=8$) were s.c. injected with $3\times10^4$ TC1 cells (**c**) or $6\times10^4$ TC-1 cells (**d**), respectively. One and 8 days later, the animals were treated by prime boost immunizations with the indicated vaccine combinations of 50 μg of CyaA336/E7 and $10^7$ PFU of the MVA-SigE7LAMP virus. The growth of tumors is shown on separate graphs for each of the tested groups, with the fraction of mice that developed tumors indicated in the upper right corner. The data were statistically analyzed by comparison of survival curves of tumor-free animals in individual groups with the group of control animals injected with CyaA wt (**c**) or PBS (**d**) by using logrank test. Representative results from one out of two independent therapeutic experiments are shown

## Discussion

In this report, we show that immunization with the CyaA336/E7 protein carrying the entire E7 antigen of HPV16 can induce a sufficiently potent and specific cellular immune response against the E7 antigen that can protect mice against growth of HPV16-induced experimental tumors. The CyaA toxoid could efficiently deliver an entire medically relevant antigen, of up to 98 amino acid residues in size, for processing and presentation on the MHC class-I molecules, which allowed induction of specific $CD8^+$ T-cell responses efficient in both prophylactic, as well as therapeutic vaccination.

Extending our previous work with model epitopes of defined sequence, these results represent an important progress towards the practical use of CyaA as an antigen carrier. Therapeutic CyaA vaccines delivering larger portions of antigens could be potentially used to cover a broader spectrum of MHC haplotypes by allowing the immune system of the vaccinee to select the appropriate epitope from the delivered polypeptide.

The results obtained here with the CyaA/E7 and MVA vaccines go well with previous reports demonstrating the feasibility of specific immunotherapy of HPV E6- and E7-expressing tumors in rodent models [7]. Compared to priming with DNA vaccines, however, the chemically defined, highly purified and genetically detoxified CyaA-derived vaccines are expected to raise less concerns about safety. While with DNA vaccines or live virus vectors, the activation of antigen-presenting cells (APC) leading to CTL stimulation is accomplished by nonspecific mediators, such as CpG oligodeoxynucleotides (ODN), dsRNA and/or heat-shock proteins (Hsp), the efficacy of cellular immune response induction by the CyaA appears to be due to its capacity to target specifically the professional antigen-presenting cells expressing the CD11b/CD18 ($\alpha_M\beta_2$) integrin receptor for CyaA.

CyaA is capable of delivering the antigenic cargo into both the APC cytosol and endosomes to allow processing and presentation of the passenger antigens along both the MHC class I- and class II-restricted pathways, thereby promoting induction of both $Th_1$-polarized $CD4^+$ and $CD8^+$ cytotoxic T-cell responses [19, 15, 21]. Such a capacity would also be expected to foster the efficacy of the CyaA-derived vaccines in immunotherapy of HPV16-derived tumors, where simultaneous stimulation of both $CD4^+$ $Th_1$ and $CD8^+$ CTL responses appears to be of importance.

It has, indeed, been previously reported that the low immunogenicity of the E7 protein can be increased when it is fused to various carrier molecules. For DNA vaccines or viral vector-mediated delivery of E7, the carrier moieties fused to E7 were found to account for relocalization of E7 from the nucleus to the antigen-processing compartments of APC, such as to the endosome [14] and/or the endoplasmic reticulum [1]. Moreover, immunogenicity of the exogenously produced E7 protein could also be improved by linking it to carriers targeting surface receptors of APC and activating their maturation. Thus far, the best results were obtained when E7 was delivered in the form of chimeric virus-like particles [8, 20], or in fusion to *Mycobacterium tuberculosis* heat shock protein as Hsp-E7 [2].

We repeatedly observed a better protection against the TC-1 challenge in therapeutic immunization with CyaA/E7 vectors than upon preventive immunization. In the preventive vaccination scheme, an antibody response against the CyaA protein induced upon the first immunization with CyaA336/E7 could limit cellular immune response at the second CyaA336/E7 vaccine dose. However, no such inhibitory effect of even high levels of antibodies against the CyaA carrier could be previously observed in model experiments, when animals were repeatedly immunized by mock CyaA prior to assessment of efficacy of induction of CTL responses against model epitopes delivered by CyaA [6, 5]. However, it could not be excluded here that antibodies induced against the relatively large E7 antigen moiety of the CyaA336/E7 fusion protein (data not shown) might be somehow limiting the delivery of the E7 polypeptide for MHC class I-restricted presentation upon booster immunization with the CyaA336/E7 vaccine.

Indeed, priming with CyaA336/E7 and boosting with MVA-SigE7LAMP induced a higher response of $CD8^+$ T lymphocytes than repeated doses of CyaA 336/E7 or MVA-SigE7LAMP. A plausible interpretation of this result would be that differences in processing of the E7 antigen and/or signaling events accompanying delivery of the E7 antigen by the two different vaccines in the prime/boost regimen may be synergizing to bring about a broader or more potent immune response to the E7 antigen than each vaccine individually. In line with that, a somewhat higher efficiency of immunotherapy of HPV16-derived tumors at high tumor challenge doses was achieved in the heterologous prime boost regimens involving the different CyaA336/E7 and MVA-SigE7-LAMP vaccines. Therapeutic intervention against chronic viral infections and/or tumors expressing poorly immunogenic antigens is expected to require repeated vaccine administration regimens in which development of immunity against the vaccine carriers and especially the viral vectors can become rather limiting. Broadening the spectrum of available vaccination tools for antigen delivery and induction of cellular immune response is, therefore, sorely needed. The results obtained here suggest that vaccines exploiting the capacity of CyaA to target antigen-presenting cells may represent such a novel tool for design of heterologous prime/boost immunotherapeutic protocols. It will, hence, be important to assess how well the CyaA E7 vaccine would perform in patients with cervical carcinoma.

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ORIGINAL ARTICLE

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# Immune response to E7 protein of human papillomavirus type 16 anchored on the cell surface

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**Abstract** To target the E7 protein of human papilloma virus 16 to the cell surface, a fusion gene was constructed. It encodes the signal peptide, part of the immunoglobulin (IgG)-like domain, the transmembrane anchor of vaccinia virus (VV) hemagglutinin (HA), and the complete E7-coding sequence. The fusion gene was expressed under the HA late promoter by a recombinant VV, designated VV-E7-HA. The E7-HA protein was displayed on the surface of cells infected with the recombinant virus and was more stable than unmodified E7. The biological properties of the VV-E7-HA virus were compared with those of a VV-E7 virus that expressed the unmodified E7 and with a VV expressing the Sig-E7-LAMP fusion protein. While the first two of these recombinants were based on VV strain Praha, the third was derived from the WR strain of VV. Infection of mice with the VV-E7-HA virus induced the formation of E7-specific antibodies with the predominance of the IgG2a isotype, whereas the other two viruses did not induce the formation of E7-specific antibodies. Unlike the other two viruses, VV-E7-HA did not induce a response of cytotoxic T lymphocytes or Th1 cells and did not protect mice against the growth of E7-expressing tumors. Thus, VV-E7-HA induced a differently polarized immune response to the E7 protein than the other two viruses.

**Keywords** Antibody · Cellular immunity · E7 · Hemagglutinin · HPV16 · Protein targeting · Vaccinia virus

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## Introduction

Recombinant vaccinia virus (VV) vectors have been used to induce immune responses to many different antigens derived from viruses, bacteria, parasites and mammals (for a review, see [23]). It has been shown that the anchoring of an antigen on the surface of cells infected with a recombinant virus encoding this antigen can increase its immunogenicity. For example, appending the transmembrane domain of membrane immunoglobulin (IgG1) to the carboxy terminus of a secreted *Plasmodium* antigen results in a change in the subcellular location of the S-antigen and increases the immune response to the otherwise weak immunogen [18]. Enhanced immune responses have been observed after fusing the MSA1 signal and anchor sequences with malaria merozoite surface antigen 1 [32]. Similarly, the addition of the membrane anchor to the C terminus of rotavirus VP7 glycoprotein has enhanced the immunogenicity of this protein when expressed by recombinant VV [2].

It has been demonstrated that the hemagglutinin (HA; A56R gene) of VV is not required for infection and replication of this virus [10]. The substitution of the external IgG-like domain of HA (AA 34–103) with a single-chain antibody results in the synthesis of a fusion protein which is exposed on the envelope of extracellular virus (EEV) and on the surface of virus-infected cells. The specificity (anti-ErbB2) of the single-chain antibody is retained, and the surface of EEV can bind the corresponding antigen [7].

An etiological association between human papillomavirus type 16 (HPV16) infection and cervical neoplasia has been firmly established [22]. The viral E7

oncoprotein, which is localized in the nucleus and cytoplasm of HPV16-infected and transformed cells, represents an attractive target for therapeutic vaccines.

In this study we report on our attempts to increase the immunogenicity of the E7 protein by changing its subcellular location via fusing it to the transmembrane sequence of VV HA. The immunogenicity of the recombinant VV expressing this fusion protein has been compared with that of two other VV-E7 recombinants.

## Material and methods

### Plasmid vectors

Plasmid pRS3 was prepared as follows. An internal portion of the VV HA, between its 64th and 275th amino-acid residues, was replaced by the entire E7 polypeptide. The E7 gene of HPV16 was amplified with the oligonucleotide primers E7–1: 5'-GCTGACGTCATGCATGGAGATACACC-3' and E7–2: 5'-AGACCAAGGTTTCTGAGAACAGATGGG-3' by PCR, with plasmid pEA16E7 (prepared by Ingrid Jochmus, Heidelberg, Germany) as the DNA template. The resulting 300-bp *Aat*II-StyI fragment containing the E7 gene was inserted between the *Aat*II and *Sty*I sites of plasmid pHA. To ensure translation into the complete E7-HA fusion protein in frame, the *Sty*I ends were ligated as blunt ends. The resulting plasmid was denoted pHA-E7. Plasmid pHA was prepared by cleaving the G fragment of a *Sal*I genomic library of VV strain Praha with restriction enzymes *Hin*dIII and *Sal*I, and then cloning the 1,796-bp fragment obtained which contained the HA gene, into pUC18. To obtain a plasmid that would allow "transient dominant selection" of recombinant viruses, plasmid pHA-E7 was cleaved with *Bam*HI and ligated with a *Bam*HI fragment, which contains the $\beta$-galactosidase gene of *Escherichia coli* and the 7.5-promoter of VV. The resulting plasmid was denoted pRS3. Plasmid pH5-E7 was prepared by amplifying the E7 gene with primers E7-B: 5'-ATAGGATCCCTGTAATCATGCATG-GAG-3' and E7-E: 5'-GGCGAATTCGATTATGGTTTCTGA-GAACAG-3' by PCR, using plasmid pEAE7 as the DNA template. The amplified 325-bp fragment was cleaved with *Bam*HI and *Eco*RI and ligated with plasmid pSC59-H5 cut with the same enzymes. Plasmid pSC59-H5 had been prepared by insertion of the VV H5 promoter into plasmid pSC59 (obtained through the courtesy of B. Moss, Bethesda, Md.). The 167-bp fragment containing the H5 promoter [8] had been prepared by PCR with primer H5–1: 5'-GCCAGATCTGACACTGTCTTTATTCTATACTTAAAAA-GTGAAAATAAATAC-3' and primer H5–2:5'- GCTGTCGACG-AGCTCCTAGGATCCTATTTACGATACAAACTTAACGG-ATATCG-3', with the H fragment of the *Hin*dIII VV genomic library used as the DNA template. The resulting fragment was cleaved with *Bgl*II and *Sal*I, and was ligated with pSC59 cut with *Bam*HI and *Sal*I.

### Viruses and cells

Vaccinia virus clone P13, generated from the Sevac VARIE smallpox vaccine (strain Praha) [16], was used as the parental virus for the construction of recombinants. The recombinant viruses were grown in human-embryo diploid cells (LEP) or monkey-kidney cells (CV-1). Thymidine kinase-deficient ($TK^-$) RAT 2 rat cells [30] were used for the selection of $TK^-$ VV recombinants. HPV16 E6E7-expressing TC-1 cells, derived from C57BL/6 mice [19], were kindly provided by T.C. Wu (Baltimore, Md.). All cells were cultivated in modified E-MEM (EPL) medium containing bovine serum growth-active proteins, but no complete serum [21]. The VV recombinants used for immunization experiments were grown in chorioallantoic membranes of 11-day-old chicken embryos and were partially purified by the modified method of Joklik [13, 17].

### Construction of VV recombinants

Vaccinia virus recombination and selection of TK- recombinants were performed by standard procedures [24]. VV-HA-E7 was prepared using plasmid pRS3. Recombinant viruses produced by a single crossing over were selected by their co-expression of $\beta$-galactosidase [4]. "Blue" virus was plaque-purified. After the second plaque purification, "colorless plaque" viruses were isolated and recombinants with double crossing over were identified as carriers of the E7 insert by dot-blot hybridization and PCR. Expression of the E7 fusion protein and absence of HA in selected virus clones were confirmed by immunoblotting using VV-specific antisera. The E7-HA fusion gene was expressed from the late promoter of HA. A schematic view of the protein produced by VV-E7-HA is shown in Fig. 1.

A VV expressing the unmodified E7 protein was prepared by recombination with plasmid pH5-E7. The E7-coding sequence was inserted into the thymidine kinase (TK) gene, and its expression was controlled by the H5 early-late promoter. The third virus used in this study, VV-SigE7LAMP, expresses a fusion molecule consisting of the E7 protein with signal and a transmembrane sequence of the lysosome-associated membrane protein (LAMP1). This recombinant virus, originally prepared with the WR strain, was obtained through the courtesy of T.C. Wu (Baltimore, Md.) [19]. The viruses VV-pS2S ($TK^-$) and WR-pS2S ($TK^-$), which express the middle envelope protein (preS2 + S) of hepatitis B virus, had been prepared using plasmid pM3 [15]. The VV-gE ($HA^-$, $TK^+$) virus had also been prepared previously [17].

### Peptides

The synthesis of peptides $E7–1_{(1–20)}$, $E7–2_{(10–30)}$, $E7–3_{(20–40)}$, $E7–4_{(30–50)}$ and $E7–5_{(40–60)}$ derived from the sequence of HPV16 E7 used in ELISA has been described earlier [14]. The peptide HPV16 $E7_{(49–57)}$ (RAHYNIVTF) [6] was used for the production of MHCI tetramer; the peptide $E7_{(49–57)}$ and E7 peptide-8Q $_{(44–62)}$ (QAEPDRAHYNIVTFCCKCD) [28] were used for restimulation of splenocyte bulk cultures and in ELISPOT.

### MS2E7 protein

MS2E7 protein was produced in *Escherichia coli* transformed with plasmid pEX 8mer-HPV16 E7 and purified according to Jochmus-Kudielka et al. [12]. MS2E7 molecule contains E7 protein (encoded by HPV16 nt 585–855) fused to the first 100 amino acids of the bacteriophage MS2 polymerase.


**Fig. 1** Schematic diagram showing structure of E7-HA fusion protein. Indicated amino-acid positions refer to the wild-type HA sequence. HA-Sig: HA signal peptide; HA-Ig-domain: part of the IgG-like domain; HA-TM: transmembrane domain; E7: full-length E7 polypeptide (98 AA)

## Antibodies

Rabbit antiserum against E7, which was used in immunoblot and immunofluorescence tests, was prepared by immunization with four i.m. doses, each containing 2.5 mg MS2E7 fusion protein [12] in Freund's adjuvant. The first dose was administered in complete adjuvant, the subsequent three doses in incomplete adjuvant. Mouse anti-MS2E7 serum was prepared by the same procedure. Each dose contained 0.5 mg protein.

## Preparation of H-2D$^b$/E7$_{(49–57)}$ tetramers

Major histocompatibility complex (MHC)-I tetramers were prepared as described by Altmann et al. [1]. In brief, heavy and light chains were expressed separately in *E. coli* and used in form of inclusion bodies for folding reaction. The mouse H-2D$^b$ (MHC-I heavy chain), human $\beta$2-microglobulin (light chain) and E7$_{(49–57)}$ peptide were folded in vitro to preform MHC-1 monomers. After concentrating the reaction mixtures and buffer exchange, the preformed monomers were subjected to enzymatic biotinylation by BirA biotin synthetase, and then purified with S300 column chromatography and Mono Q ion exchange column chromatography. Tetramers were obtained by mixing the biotinylated protein complex with streptavidin-R–phycoerythrin conjugate (Molecular Probes) at a molar ratio of 4:1.

## Immunoblotting

Infected cell cultures were washed twice with PBS and lysed on ice with RIPA buffer (1% NP40, 150 mM NaCl, 50 mM Tris–HCl, pH 8.0) for 30 min. Cell lysates were clarified by centrifugation at 19,000 *g* for 10 min at 4°C. Supernatants were mixed with Laemmli buffer containing 2-mercaptoethanol. Samples were heated for 5 min at 95°C. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 15% gel. The proteins separated were then transferred onto a nitrocellulose membrane using a semi-dry electrophoretic blotting method. Membrane, preincubated with 10% skimmed milk in phosphate-buffered saline (PBS) for 1 h, was incubated with E7 specific rabbit antiserum diluted 1:1,000 in PBS containing 5% milk at 4°C overnight. After being washed (PBS–0.2% Tween, five times for 5 min) the membrane was incubated for 1 h with goat anti-rabbit IgG labeled with horseradish peroxidase (HRP; Sigma) diluted 1:5,000 in PBS–5% milk. Finally, the membrane was washed again as above, developed by ECL (Amersham) and exposed to autoradiography film.

## Immunofluorescence

CV1 cells ($4{\cdot}10^3$) were grown in 16-well chamber slides (Nunc). Confluent monolayers were infected with recombinant VV at MOI 0.1. After overnight incubation, infected cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and washed with PBS 3×5 min. Then 0.1% Triton X-100 was added for 2 min and cells were washed with PBS 3×5 min and blocked with 10% skimmed milk in PBS for 1 h. Next, rabbit E7 antiserum diluted 1:50 in 5% milk–PBS was added for 1 h at room temperature. The monolayers were washed as above and incubated with a FITC-conjugated swine anti-rabbit secondary antibody (diluted 1:200) at room temperature for 1 h. Finally the cells were washed with PBS, overlaid with 50% glycerol in PBS, and examined with a Nikon 600 Eclipse microscope.

The expression of E7 on the surface of infected cells was examined by FACS analysis. Briefly, monolayers of CV1 cells in Petri dishes were infected at MOI 0.2. After overnight incubation, when the CPE in all cultures was about 50%, cells were harvested by trypsinization and resuspended in FACS buffer (PBS, 3% bovine serum albumin (BSA), 0.1% $NaN_3$). The cells were incubated with rabbit E7 antiserum diluted 1:50 in FACS buffer for 1 h at 0°C, then washed three times with the same buffer and incubated with a FITC-conjugated swine anti-rabbit secondary antibody (diluted 1:200 in FACS buffer) for 1 h at 0°C. Samples were analyzed on a Becton Dickinson FACScan instrument using WinMDI 2.8 software.

## Subcellular fractionation

Preparation of subcellular fractions was performed as described by Jin et al. [11]. Briefly, CV-1 cells ($8{\cdot}10^6$) were infected with viruses at MOI 2 at 37°C. After 1 h, unadsorbed virus was removed and cells washed with medium were incubated for another 8 h. The cells were scraped into medium, centrifuged, washed once with PBS and resuspended in 1 ml of hypotonic buffer (42 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, pH 7.4) with protease inhibitor cocktail (Sigma) diluted 1:100 and incubated on ice for 15 min. Cells were then passed through a 29-G needle six times; and cell disruption was verified under a microscope. The extract was centrifuged at 200 *g* for 10 min at 4°C, and sediment containing the nuclear fraction was further purified (see below). The supernatant was centrifuged at 10,000 *g* for 10 min at 4°C to separate the heavy membrane fraction and then at 150,000 *g* for 90 min to collect the light membrane fraction. The remaining supernatant was used as the cytosolic fraction. Nucleus purification was performed according to the published protocol [9]. To nuclear fraction resuspended in 4 ml of sucrose buffer I [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris–Cl, pH 8.0, 1 mM DTT, 0.5% (v/v) NP-40], another 4 ml of sucrose buffer II (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris–Cl, pH 8.0, 1 mM DTT) were added and thoroughly mixed. The extracts were layered over 4.4 ml of sucrose buffer II in Beckman polyallomer SW 40.1 tubes and centrifuged at 30,000 *g* for 45 min at 4°C to prepare sediment containing nuclei. The sediments were resuspended in 100 µl of Laemmli buffer containing 2-mercaptoethanol. 0.8 ml of cytosolic fraction was mixed with 200 µl of 5×Laemmli buffer. All samples were denatured in boiling water for 3 min, separated on 12% SDS–PAGE and analyzed by immunoblot.

## Immunization of mice

Six-week-old inbred mice, strain C57BL/6 (H-2$^b$; Charles River) were injected intraperitoneally with 0.5 ml PBS containing $10^7$ PFU of recombinant VVs. All experiments on laboratory animals were conducted maintaining the principles of the Czech law 246/92 Sb. on "Breeding and Utilization of Experimental Animals."

## Restimulation of splenocytes in vitro

HPV16E7-specific lymfocyte bulk cultures were generated from splenocytes obtained from immunized mice 12 days after virus inoculation. In brief, mouse spleens were homogenized with cell dissociation (Sigma) in complete RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco), $5{\times}10^{-5}$ M 2-mercaptoethanol, 1.5 mM glutamine, and antibiotics. Lymphocytes were separated on Histopaque-1083 (Sigma) and cultivated at a concentration of $5{\times}10^6$ cells/ml in complete RPMI 1640 with addition of 0.3 µg or 15 µg E7$_{(49–57)}$ peptide or 1 µg (8Q) peptide for 6 days.

## Tetramer-staining and FACS analysis

Splenocytes were used for analysis immediately after their isolation or after 6-day in vitro restimulation. Lymphocytes for tetramer-staining were adjusted onto $2{\times}10^7$ cells per ml with FACS buffer (PBS supplemented with 2% FBS and 10 mM sodium azide), incubated with 2 µl of rat anti-mouse CD16/CD32 (Fc-block; Pharmingen; 0.05 mg/ml) for 20 min on ice, washed and an aliquot of $2{\times}10^6$ cells was resuspended in 80 µl of ice-cold FACS buffer. The samples were stained on ice each with 20 µl of a mixture of 2 µl of tetramer–PE and 2 µl of rat anti-mouse CD8a-FITC antibody (Pharmingen; stock concentration 0.5 mg/ml) in FACS buffer for at least 1 h in the dark. The lymphocytes were washed and resuspended

in 200 µl of FACS buffer. The stained cells were analyzed on a FACScan instrument, using CellQuest software (Becton Dickinson).

### ELISPOT assay

Ninety-six well plates MAHA 45 (Millipore) were coated with 5 µg/ml of the anti-mouse IFN$\gamma$ monoclonal antibody R4–6A2 (Pharmingen) or anti-mouse IL4 monoclonal antibody BVD4–1D11 (Pharmingen) in 0.1 M Na–phosphate buffer (pH 9.0) at room temperature overnight. The antibody-coated plates were washed four times with PBS and blocked with RPMI 1640 with 10% FBS for 1 h at room temperature. 100 µl of in vitro restimulated splenocytes in culture medium were added to the wells and incubated for 20 h at 37°C in 5% $CO_2$ in the presence or absence of peptides. Wells were washed three times with PBS and three times with PBS containing 0.05% Tween 20, followed by overnight incubation at 4°C with 2 µg/ml of the biotinylated anti-mouse IFN$\gamma$ monoclonal antibody XMG 1.2 (Pharmingen) or the biotinylated anti-mouse IL4 monoclonal antibody BVD6–2462 (Pharmingen) in PBS, respectively. The wells were washed with PBS, 0.05% Tween 20. Avidin–horseradish peroxidase (HRP) conjugate (Pharmingen) diluted 1:1,000 in PBS, 0.05% Tween 20 was added to the wells for 3 h at 37°C. After washing the plates with PBS, the spots were stained with 3-amino-9-ethyl carbazol.

### Serological tests for E7 antibodies

Sera of immunized mice were individually tested by ELISA for the presence of specific anti-E7 antibody. Wells of microtiter plates (Maxisorb, Nunc, Denmark) were coated with 2 µg of the oligopeptides E7–1 to E7–5 in 100 µl carbonate buffer, pH 9.6, at 37°C. Unbound antigen was removed and free potential binding sites were blocked with 1% bovine serum albumin (BSA). After threefold washing, the wells were incubated with 100 µl of 1:25 dilution of serum for 1 h. The plates were then repeatedly washed and 100 µl of 1:2,000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Sigma, USA) was added for 1 h. The plates were washed again, stained with *o*-phenylene-diamine and absorbance at 492 nm was measured. Control sera known to be positive and negative were tested on each plate. Antibody isotypes were determined in plates coated with the E7–1 peptide, using HRP-conjugated rat anti-mouse IgG2a or IgG1 (Pharmingen) diluted 1:500.

## Results

### The VV-E7-HA recombinant virus expresses the E7 polypeptide fused into VV hemagglutinin

The VV-E7-HA construct was designed to express the E7-HA fusion protein. This protein contained the N-terminal portion of the VV hemagglutinin polypeptide chain including the signal sequence and part of the IgG-like domain (AA 1–62), the entire HPV16 E7 sequence (98 AA) in place of the internal part of the hemagglutinin polypeptide chain (211 AA), and the C-terminal portion of hemagglutinin including the transmembrane anchor (AA 273–315) (Fig. 1).

### E7-HA is a glycoprotein targeted to the cell surface

#### *In vitro expression of the E7-HA protein*

To determine whether the VV recombinants induced synthesis of the E7 protein in CV1 cells, lysates of infected cells were analyzed by immunoblotting using E7-specific antibody. The lysates analyzed in Fig. 2A were prepared from cells harvested 18 h after infection. Fusion proteins produced by VV-E7-HA virus were found in two main bands of m.w. 33 and 35 kDa (lane 3) and in two minor bands of smaller size that probably represented fragments of the longer polypeptides. All polypeptides must have been glycosylated, because neither of the bands was found if the infected cells were kept in the presence of tunicamycin (5 µg/ml). The m.w. of the unglycosylated E7-HA polypeptide was about 30 kDa (lane 4). The upward shifts of 5 and 3 kDa might indicate the presence of one or two carbohydrate residues. With the use of the PCGENE program, two potential N-linked glycosylation sites were indeed predicted in the E7-HA protein: in the 34th position of HA and in the 29th AA of E7. The VV-E7 virus produced a 19 kDa E7 protein (lane 5), which was of the same size as that of the E7 protein produced in cells transfected with expression plasmid pBK-E7 or in Caski cells (not shown). The size of the E7 protein was not influenced by the addition of tunicamycin; however, the latter apparently reduced the amount of E7 produced (lane 6). A faint E7 band was discernible in lane 6 after longer film exposure (not shown). The

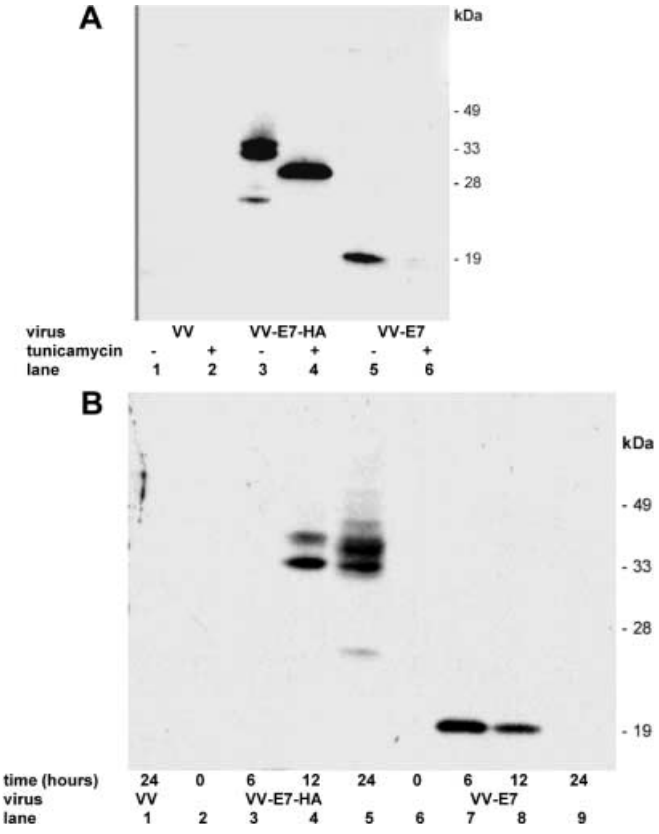


**Fig. 2** Immunoblot analysis of E7 proteins expressed by recombinant VV. **A** CV1 cells were infected with parental VV, VV-E7-HA or VV-E7, cultivated in the absence or presence of tunicamycin of 5 µg/ml and harvested after 18 h. **B** Cells were harvested at different times after infection. The samples were analyzed on SDS–15% PAGE and by immunoblot using E7-specific rabbit antiserum

kinetics of production of the E7-HA glycoprotein and the unmodified E7 in cells infected with the respective recombinant viruses appeared to be different (Fig. 2B); E7-HA was detected at 12 h after infection and its amount was higher at 24 h (lanes 4 and 5), whereas the E7 molecule was detected already at 6 h (lane 7) after infection and thereafter its amount kept decreasing; a low amount of E7 present in cells at 24 h after infection (lane 9) was only visible on autoradiograms after a longer exposure time (not shown). Cells infected with non-recombinant VV did not express any E7-specific bands (Fig. 2A and B; lanes 1 and 2 and 1, respectively).

*Subcellular location of the E7 antigen*

The presence of the E7 antigen inside permeabilized cells was detected by immunofluorescent staining with specific antibodies (Fig. 3A). The cell infection with VVE7-HA was accompanied by formation of large syncytia. Their presence is typical of cultures infected with $HA^-$ mutants [10]. The E7 antigen was found at the nuclear membranes and in cytoplasmic structures of VV-E7-HA-infected cells (a). Unmodified E7 expressed by VV-E7 was mainly present in the nuclei of infected cells (b). Cells infected with parental VV exhibited some fluorescence of very low intensity (c). E7 antigen on the surface of non-permeabilized infected cells was determined by flow cytometry. Fluorescence of high intensity was found on the surface of cells infected with VV-E7-HA only, whereas the FACS profile of VV-E7 infected cells showed no difference from cells infected with parental VV (Fig. 3B). To determine the amounts of E7 proteins in subcellular fractions, CV1 cells, infected with recombinant VVs for 8 h, were lysed in hypotonic solution and fractionated by centrifugation into nuclear,

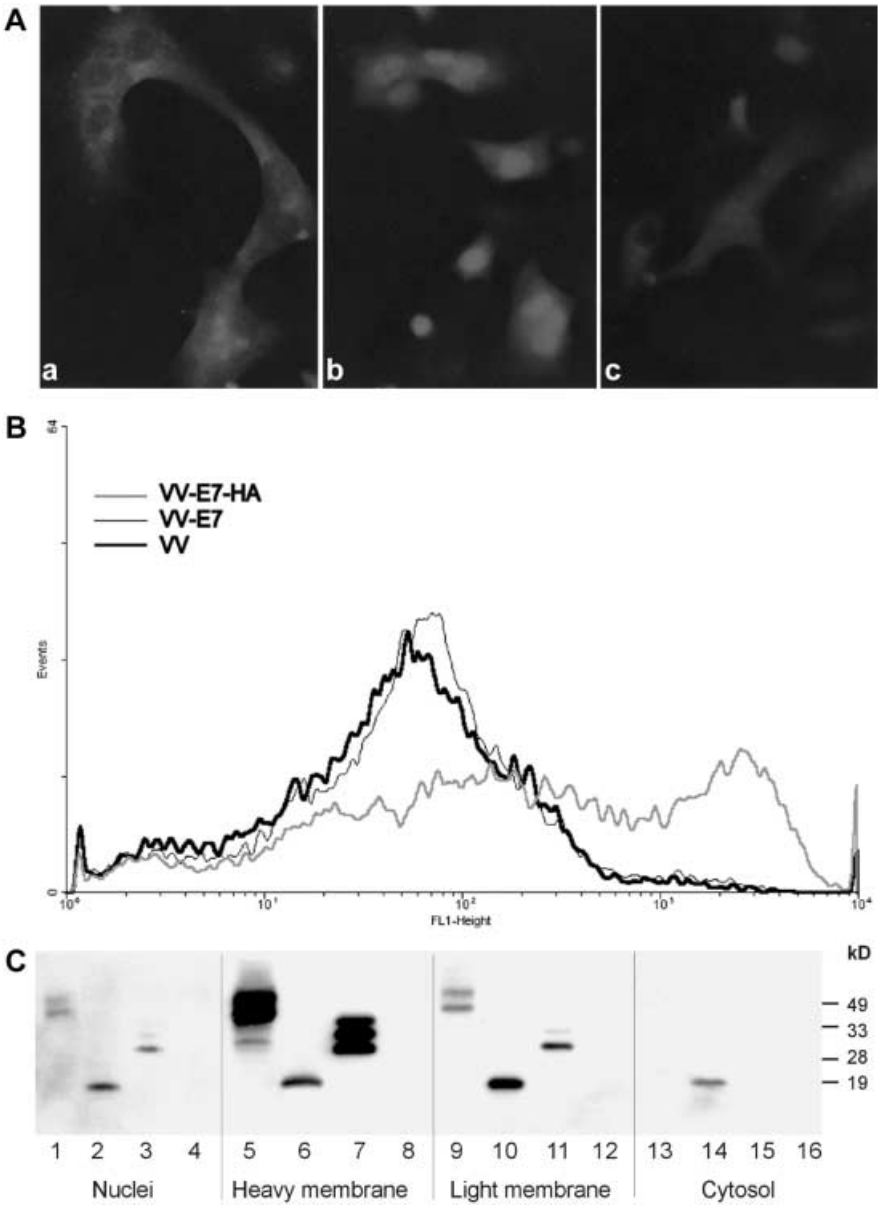


**Fig. 3** Location of E7 proteins in CV1 cells after infection with recombinant VVs. **A** Immunofluorescence of E7 antigen in cells infected with: (a) VV-E7-HA; (b) VV-E7; or (c) VV. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and subsequently stained with E7 specific antibodies. **B** Detection of E7 on the surface of cells infected with VV-E7-HA (*grey line*), VV-E7 (*hair line*) or parental VV (*thick line*) by cytofluorometry. **C** Location of recombinant E7 proteins in subcellular fractions by immunoblot. The cells were infected with VV-E7-HA (*lanes 1, 5, 9, 13*), VV-E7 (*lanes 2, 6, 10, 14*), WR-SigE7LAMP (*lanes 3, 7, 11, 15*), parental VV (*lanes 4, 8, 12, 16*) and fractionated. Subcellular fractions were analyzed on SDS–12% PAGE and by immunoblot using E7 specific rabbit antiserum

heavy membrane, light membrane and cytosolic fractions. The amounts of E7 proteins in these fractions were analyzed by immunoblot (Fig. 3C). Most HA-E7 and E7-LAMP proteins were found in the heavy membrane fraction (lane 5 and 7, respectively) which included the cellular, mitochondrial and lysozomal membranes. Nuclear (lanes 1 and 3, respectively) and light membrane fractions (lanes 9 and 11, respectively) contained only low amounts of these proteins. Neither E7-HA nor E7-LAMP proteins were detected in the cytosolic fraction (lanes 13 and 15, respectively). Non-modified E7 protein predominated in the light membrane fraction (lane 10), though a substantial amount of E7 was found in all the other subcellular fractions (lanes 2, 6 and 14). The presence of E7 in membrane fractions could be explained by its capacity to bind to viral DNA, which is abundant in areas in the cytoplasm where VV maturation occurs. In agreement with published data, non-modified E7 was found in the nuclear and cytosolic fractions (lanes 2 and 14).

## The E7-HA fusion protein induces an antibody response but not cell-mediated immunity

To determine how the fusion of the E7 protein with VV HA influenced the immunogenic properties of E7, we immunized C57BL/6 mice with one dose of $10^7$ PFU of VV-E7-HA or VV-E7 virus by intraperitoneal route. Besides using these viruses prepared in our laboratory, we employed the WR-SigE7LAMP virus, which had been shown to induce a cell-mediated response and protection against tumor growth. This virus was included as positive control in immunity tests in spite of the fact that it was prepared from another parent virus strain. After the vaccinations, both humoral and cellular immune responses were examined in individual mice.

### *Induction of E7-specific antibodies*

Groups of eight mice each were killed 6 weeks after virus inoculation. Their sera were individually examined for the presence of anti-E7 and anti-VV antibodies. The animals immunized with VV-E7-HA produced antibodies reactive with synthetic oligopeptides derived from the E7 amino-acid sequences 1–20 and 40–60 (Fig. 4A). Antibodies specific for the amino-acid sequence 20–40 were not detected in mouse sera (not shown). E7-Specific antibodies were not detected in sera of mice immunized with the VV-E7, WR-SigE7LAMP, or control viruses. To characterize the antibody response to VV-E7-HA more closely, the isotypes of anti-E7 IgGs were determined. Anti-E7 IgG2 prevailed in mice inoculated with VV-E7-HA, whereas the IgG1 isotype predominated after immunization with the purified MS2E7 fusion protein (Fig. 4B). All recombinant viruses induced anti-VV antibodies in all of the mice inoculated; however, the responses elicited by VV-E7-HA and WR-SigE7LAMP

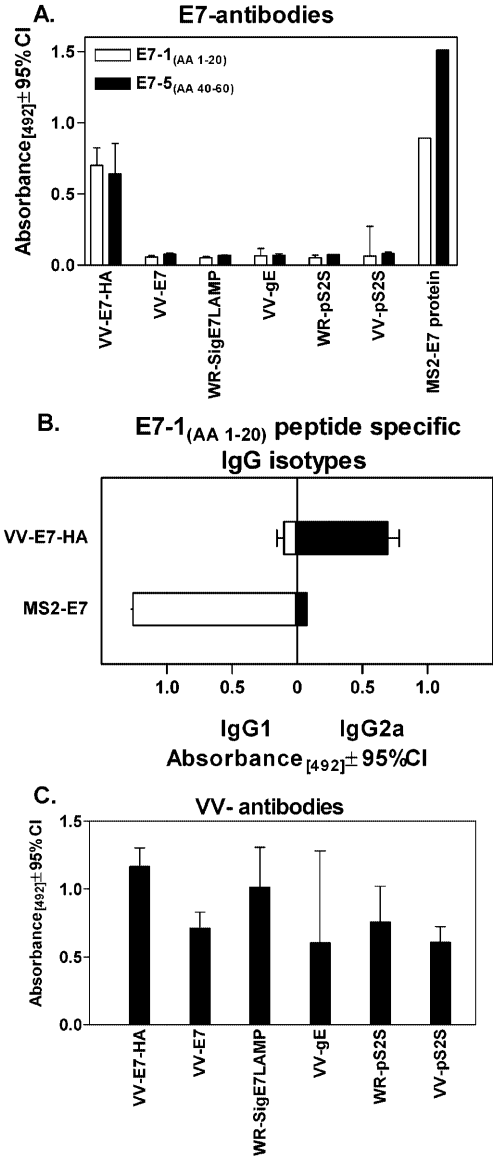


**Fig. 4** E7-specific antibody response of mice inoculated with recombinant VVs. **A** Individual sera of six immunized mice were assayed by ELISA for antibodies specific for E7 oligopeptides E7-1 and E7-2. **B** Anti-E7-1 specific IgG1 and IgG2a isotypes were determined in individual positive sera of animals inoculated with VV-E7-HA. Anti MS2-E7 was a pool of sera of mice immunized with recombinant protein. **C** The same mouse sera as in **A** were examined for the presence of VV-specific antibodies

were the highest. The similar anti-VV antibody responses to these two recombinants suggest that the viruses multiplied at a similar rate, and that consequently the differences in anti-E7 antibody response to them cannot be ascribed to a difference in their replication.

### *Cell-mediated immunity*

In testing the E7-specific cellular response to the different viruses, groups of two mice were used. Each spleen

was processed separately. Immunization with either of the E7-expressing viruses resulted in a primary response undetectable by ELISPOT or the tetramer assay in freshly isolated splenocytes (not shown). However, when the splenocytes were restimulated in vitro by E7 peptides for 6 days, IFN$\gamma$- and IL4-secreting splenocytes were detected (Fig. 5A and B, respectively). The binding of the $2D^b/E7_{(49–57)}$ tetramer by $CD8^+$ lymphocytes (Fig. 5C) was also found. Specific responses in IFN$\gamma$-ELISPOT and the tetramer assay were detected in mice immunized with VV-E7 or WR-SigE7LAMP but not with VV-E7-HA or the control virus (VV). In contrast, immunization with VV-E7-HA but not with VV-E7 or WR-SigE7LAMP resulted in the presence of IL4-secreting splenocytes. Similar results were obtained in three independent experiments.

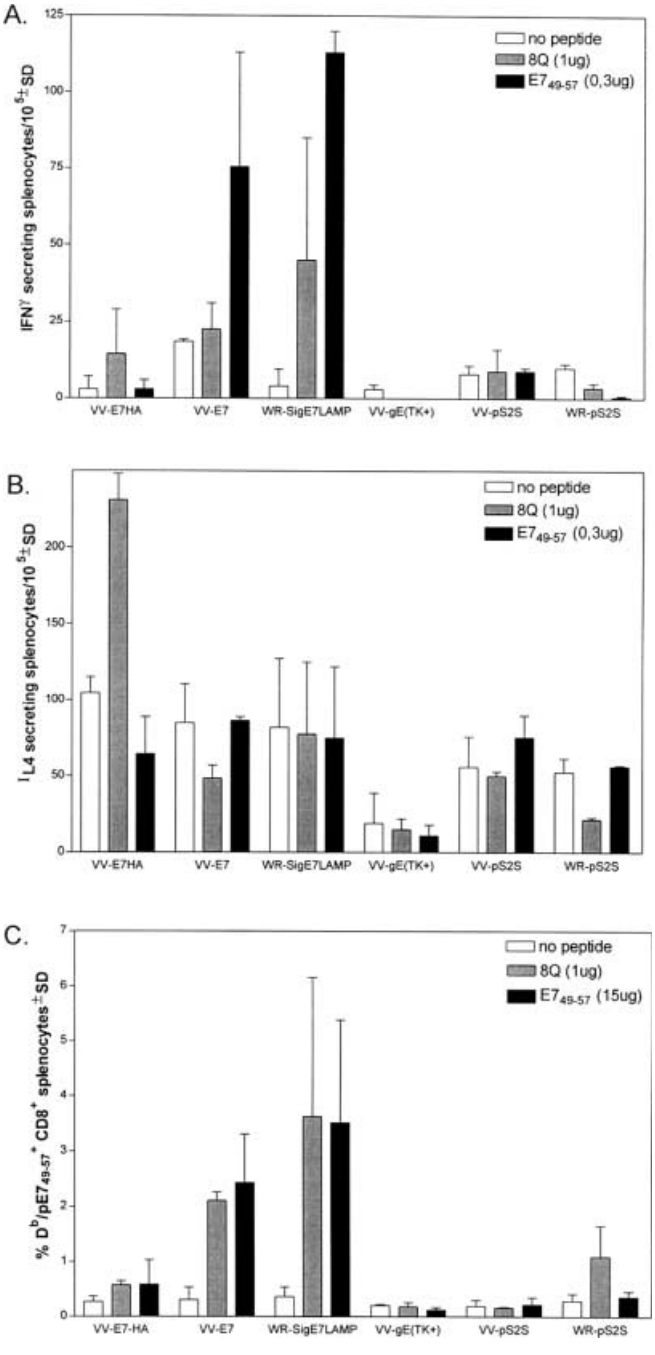


**Fig. 5** Cell-mediated response of mice inoculated with recombinant VVs. Splenocytes isolated 12 days after virus inoculation were restimulated with indicated oligopeptides for 6 days and then analyzed by ELISPOT for cytokine production. Frequency of E7 specific cells secreting: **A** IFN$\gamma$, **B** IL4. **C** Frequency of $D^b/pE7_{(49–57)}{}^+$ $CD8^+$ cells among restimulated splenocytes detected by flow cytometry

## Vaccination with VV-E7 but not with VV-E7-HA protects against E7-expressing tumor challenge

In order to determine whether the recombinant E7-expressing viruses induced antitumor immunity, vaccinated mice (eight per group) were challenged with a dose of $2{\cdot}10^4$ TC-1 cells/animal. Statistically significant protection against tumor growth was found after immunization with VV-E7 or WR-SigE7LAMP. Vaccination with VV-E7-HA, similarly to control viruses, did not induce any significant delay of tumor growth as compared with a group of animals inoculated with PBS only (Fig. 6A). Comparison of the growing tumors by size (Fig. 6B) showed that their mean size in mice vaccinated with VV-E7-HA and in animals given no VV (PBS controls) was similar. The inoculation of control viruses (VV-gE, VV-pS2S or WR-pS2S) enhanced the growth of tumors, which was in line with our previous unpublished results.

## Discussion

In the present study we changed the subcellular location of the E7 protein expressed by human papillomavirus type 16 (HPV16) to the surface of infected cells, intending thereby to increase the immunogenicity of this tumor antigen. For this purpose, a recombinant VV with the E7 gene fused into the transmembrane sequence of the VV HA gene was prepared. The E7-HA fusion protein, as shown by immunofluorescence and subcellular fractionation, is found in the heavy membrane fraction and is localized on the surface of VV-E7-HA infected cells. Our approach was motivated by a previous report by Galmiche et al. [7], but our method of preparation of recombinant viruses differed in certain details. We inserted the E7 DNA fragment in a different region of HA and removed part of the HA chain. Consequently, our virus was $HA^-$. Still, the construct induced a high expression of the fusion protein on the cell surface. We were interested in finding out whether this targeting of the antigen would influence its immunogenicity, as reported previously by Andrew et al. and Langford et al. [2, 18]. In these authors' experiments, the cell-surface anchoring of antigen expressed by recombinant VV increased both the cellular and humoral immune responses to foreign antigen. Moreover, we presumed that vaccination with the E7-HA fusion

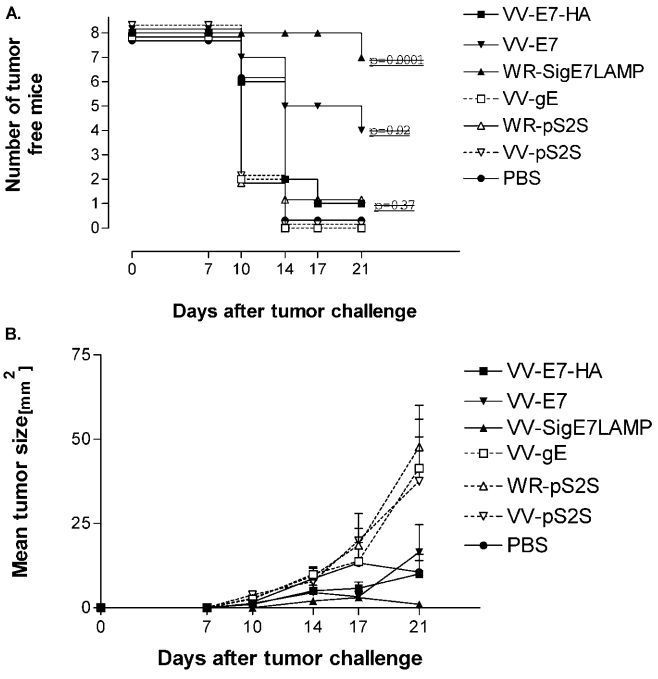


**Fig. 6** In vivo tumor protection and growth of tumors in mice inoculated with recombinant VVs. Eleven days after virus inoculation groups of mice were challenged with $2{\cdot}10^4$ of TC1 tumor cells administered s.c. *Graph A* shows numbers of tumor-free animals; *graph B* demonstrates the mean size of growing tumors $>1$ mm$^2$. The results indicate that VV-E7-HA was not able to protect mice from the growth of E7 expressing tumor. Immunization with VV-E7 induced protection of a portion of animals. WR-SigE7LAMP was included as a positive control virus that is able to induce full protection of animals. The in vivo protection test was repeated three times with similar results. Statistical significance was determined using the log-rank test. All groups were compared with the group that received PBS only

protein expressed under the HA (A56R) late promoter might protect mice against challenge with HPV16-induced-tumor cells. Bronte et al. [3] have shown that vaccination with viruses expressing tumor-associated antigen under late promoters mediates protection against tumor growth, albeit a combination of early/late promoters was more effective.

In our model, immunization with VV-E7-HA provided no protection against the growth of E7-expressing tumors. Analysis of the immune responses showed that E7-HA did not induce a response of $CD8^+$ T lymphocytes or Th1 cells, which are the effectors of protection against HPV-induced tumor [6]. On the other hand, our VV-E7-HA recombinant was a good inducer of anti-E7 antibodies, as well as of Th2 cells, the producers of IL4. An analysis of the IgG isotypes revealed a Th1-like response, with predominance of the IgG2a isotype. Presumably, the switch to IgG2a might be related to an increased production of IFN$\gamma$ as a result of the infection with VV and is not a consequence of the E7-specific Th1 response [20]. We demonstrated that the fusion of E7 with the signal peptide and transmembrane domain of HA increased the stability of the E7 protein. Unmodified E7 is a short-lived molecule [25] and its rapid degradation by the ubiquitine-proteasome pathway may contribute to its ability to induce CTLs [29]. However, the increased stability of E7-HA cannot wholly explain its different immunogenicity, because WB analysis indicated that the SigE7LAMP fusion protein accumulated in recombinant VV-infected cells at similar levels to E7-HA (not shown).

The predominance of the antibody response over CTL might be ascribed to the concurrent activity of several factors. It is possible that soon after the inoculation of VV-E7-HA, the infected cells were able to present E7 on their surface mainly to B cells but not to T lymphocytes, because the expression of MHC molecules might at this time (late phase) be downregulated owing to the viral infection [31]. Activated B cells soon start to play the role of antigen-presenting cells, and their interaction with T cells results in a proliferation of Th2 cells. Under these conditions the activation of type 1 helper lymphocytes by cross-priming in secondary lymphoid organs might be suppressed by an already established response of Th2 lymphocytes [26]. The viruses VV-E7 and VV-E7-HA induced in mice opposite polarized type 1 and type 2 responses, characterized by proliferation of IFN$\gamma$- and IL4-producing lymphocytes, respectively. It is well known that IL4 has the potential to modulate the function of cytotoxic and IFN$\gamma$-producing T cells, and that polarization of the immune response is usually established soon after exposure to antigen and is sustained by immunological memory. Since polarization of the immune response is a critical parameter for the outcome of virus infection [27] and protective antitumor immunity [5], we plan to use the recombinants described in this paper for a study of the detrimental effect of the type 2 response on protective immunity to HPV-associated tumors.

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**RESEARCH ARTICLE**

# Combined immunization with fusion genes of mutated E7 gene of human papillomavirus type 16 did not enhance antitumor effect

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## Abstract

**Background** The E7 oncoprotein of human papillomavirus type 16 (HPV16) is frequently used as a model tumor-associated antigen. Its immunogenicity has been substantially enhanced by fusion with several proteins of various origins and functions. Different mechanisms have been responsible for increased vaccination efficacy of fusion proteins.

**Methods and results** We linked E7 and its mutated form (E7GGG) with the mouse heat-shock protein 70.1 (HSP70.1). Enhanced immunogenicity of both fusion genes administered via a gene gun was demonstrated by protection of C57BL/6 mice against oncogenic MHC class I positive TC-1 cells producing the HPV16 E7 oncoprotein but not against the MHC class I negative TC-1/A9 subline. To assess if the efficacy of E7-based DNA vaccines could be increased by combination of various fusion genes, we combined the HSP70.1 fusion genes (i.e. E7HSP or E7GGGHSP) with the fusion construct linking E7GGG with targeting signals of lysosome-associated membrane protein 1 (Sig/E7GGG/LAMP-1). Treatment of mice 4 days after TC-1 cell inoculation showed moderately higher immunization potency of HSP70.1 fusion genes in comparison with the Sig/E7GGG/LAMP-1 gene. Any combination of two fusion genes given in the same gene gun shot neither was more effective compared with single genes nor protected mice against TC-1/A9 cells. As fusion of E7GGG with *E. coli* glucuronidase (E7GGG.GUS) had been previously proven to provide partial protection from TC-1/A9-induced tumors, we also combined E7GGGHSP with E7GGG.GUS. The genes were inoculated either in mix in two gene gun shots or separately each gene in one shot into opposite sides of the abdomen. Neither mode of combined immunization induced higher protection than E7GGG.GUS alone. However, doubling the DNA dose considerably enhanced the antitumor efficacy of E7GGG.GUS.

**Conclusions** We constructed highly immunogenic fusions of HPV16 E7 and E7GGG with mouse HSP70.1. Furthermore, we substantially enhanced protection against TC-1/A9 cells with downregulated MHC class I expression by doubling the pBSC/E7GGG.GUS dose, but we failed to demonstrate a beneficial effect of any combination of two fusion genes with different mechanisms causing enhancement of HPV16 E7 immunogenicity. 

**Keywords** HPV; E7 oncogene; HSP; gene gun; fusion genes; MHC class I

## Introduction

Heat shock proteins (HSPs) are a large group of proteins classified into a number of families that are named principally for the molecular weight

(kDa): HSP100, HSP90, HSP70, HSP60 and the small molecular weight HSPs [11]. The fact that HSPs covalently linked to an antigen enhance humoral and cellular immune responses to the fused antigen has been demonstrated with members of the HSP70 [12–16], HSP60 [17] and HSP90 families [18]. Similar effects have also been observed with another stress-induced protein, calreticulin [20]. The antigen-HSP fusion proteins have been shown to elicit strong $CD8^+$ cytotoxic T lymphocyte (CTL) responses that have been sufficient to mediate $CD4^+$ T-cell-independent rejection of antigen-expressing tumors [15,16,22,24]. The mechanisms by which antigen-HSP fusion proteins can elicit antigen-specific $CD8^+$ CTLs in the absence of $CD4^+$ T cells is not yet fully understood. However, the published data indicate that HSPs can induce maturation of antigen-presenting cells (APCs) and upregulate their surface expression of major histocompatibility complex (MHC) molecules [27,28,35].

The strategy of an antigen-HSP fusion has been successfully used to enhance the immunogenicity of vaccines based on recombinant proteins [13–18,22,24], RNA [26], DNA [12,25,30], and recombinant adeno-associated [29] or vaccinia viruses [34]. Encouraging results from preclinical studies have led to the first clinical trials [33,37]. Recently, the fusion protein vaccine based on *Mycobacterium bovis* strain BCG HSP65 linked to the human papillomavirus type 16 (HPV16) E7 oncoprotein has induced complete or partial response in 14 out of 22 patients with anogenital warts [32].

One of the most frequently studied HSP families is the highly conserved HSP70 family with a 40–78% base identity among different species [19]. Proteins of the HSP70 family contain two well-defined parts, an N-terminal ATP-binding domain and a C-terminal peptide-binding domain [11]. The most important region for induction of CTL responses has been identified in the second half of the ATP-binding domain, suggesting that no HSP chaperone properties are necessary for this effect and thus supporting a model in which HSP bypasses the need for $CD4^+$ help by activation of the maturation state of APCs [15,16]. It has also been demonstrated that CTL responses elicited by *Mycobacterium tuberculosis* HSP70- and mouse HSP70.1-ovalbumin fusion proteins in mice have been equally efficient, indicating that the strong CTL responses are not associated with the mycobacterial origin of HSP70 [16].

HPV16 E7 is a model antigen for the development of therapeutic vaccines against HPV-associated diseases, particularly cervical cancer, the second most common malignancy in women worldwide. The immunogenicity of HPV16 E7 has been enhanced by fusion with many different proteins, e.g. lysosome-associated membrane protein 1 (Sig/E7/LAMP-1) [10,31]. To increase the safety of the HPV16 E7 oncogene, we have introduced three point mutations into the sequence coding for the pRb-binding site resulting in E7GGG [3] and Sig/E7GGG/LAMP-1 [2]. It has been shown in mice that the antitumor effect induced by the E7GGG gene is enhanced in comparison with that of the E7 gene [3], whereas the Sig/E7GGG/LAMP-1 and Sig/E7/LAMP-1 genes are equal in immunogenicity [2]. Furthermore, we have constructed the E7GGG.GUS fusion gene containing the mutated E7GGG gene linked to the 5′-end of the *E. coli* $\beta$-glucuronidase gene and have shown the increased immunogenicity of this fusion gene in comparison with Sig/E7GGG/LAMP-1 [40].

In this study, we investigated the capability of fusion genes composed of mouse HSP70.1 and HPV16 E7 or E7GGG and applied either separately or in combination with Sig/E7GGG/LAMP-1 or E7GGG.GUS, to protect mice against the induction of tumors by the HPV16 E7-positive TC-1 cells and their derivative TC-1/A9 cells with downregulated MHC class I expression resulting in enhanced resistance to DNA immunization [2].

# Materials and methods

## Animals

Female C57BL/6 mice aged 6–8 weeks ($H\text{-}2^b$; Charles River, Germany) were used in the immunization experiments. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals.

## Cell lines

The efficacy of DNA vaccines was evaluated using two tumor cell lines with different levels of MHC class I surface expression: TC-1 cells [1] prepared by transformation of primary C57BL/6 mouse lung cells with the HPV16 E6/E7 oncogenes and activated *H-ras* (kindly provided by T.-C. Wu, Johns Hopkins University, Baltimore, MD, USA), that are MHC class I positive, and their derivative, the TC-1/A9 clone [2], with reduced surface expression of MHC class I molecules. The loss of surface MHC class I expression has been accompanied by increased resistance of TC-1/A9 cells to DNA vaccination against the E7 antigen [2]. To verify the expression of the E7 and HSP70.1 antigens from constructed plasmids, mouse NIH 3T3 fibroblasts and human embryonic kidney 293T cells (kindly provided by J. A. Kleinschmidt, DKFZ, Heidelberg, Germany) were transfected. All cells were grown in Dulbecco's modified Eagle's medium (PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (FCS, PAA Laboratories), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

## Plasmids

The construction of pBSC, pBSC/E7, pBSC/E7GGG, pBSC/E7LAMP [3] and pBSC/E7GGGLAMP [2] has been described previously.

The plasmid pCR3/HSP containing the mouse HSP70.1 gene was kindly provided by R. G. Vile (Mayo Clinic, Rochester, MN, USA). For the generation of pBSC/HSP, the fragment carrying the HSP70.1 gene was isolated after digestion of pCR3/HSP with the *Eco*RI restriction enzyme and cloned into the *Eco*RI restriction sites of the pBSC expression vector downstream of the cytomegalovirus promoter and the rabbit $\beta$-globin intron.

For the generation of pBSC/E7HSP and pBSC/E7GGGHSP, fragments encoding two parts of HSP70.1 were first isolated after cutting pCR3/HSP with *Nco*I + *Bgl*II or *Bgl*II + *Nsi*I and cloned into the corresponding restriction sites of pUC131 (kindly provided by J. A. Kleinschmidt, DKFZ, Heidelberg, Germany). The resultant plasmids, pUC131/HSP(*Nco*I*Bgl*IIfragment) and pUC131/HSP(*Bgl*II*Nsi*Ifragment), were used for further cloning. The DNA fragments encoding E7 or E7GGG were amplified by polymerase chain reaction (PCR) from pBSC/E7 and pBSC/E7GGG, respectively, using primers 5′-ccccaagcttgccgccatgcatggagatacacc-3′ (forward) and 5′-catgccatgggtttctgagaacagatg-3′ (reverse). *Hin*dIII and *Nco*I sites are underlined. The amplified products were cloned between the *Hin*dIII and *Nco*I cloning sites of pUC131/HSP(*Nco*I*Bgl*IIfragment). The accuracy of pUC131/E7HSP(*Nco*I*Bgl*IIfragment) and pUC131/E7GGGHSP(*Nco*I*Bgl*IIfragment) was confirmed by DNA sequencing. The cloning sites prior to coding sequences were modified by ligation of the 22 bp long fragment isolated after cutting pBluescript-SK+ (Stratagene, La Jolla, CA, USA) with *Xho*I and *Hin*dIII restriction enzymes between the *Sal*I and *Hin*dIII sites of the pUC131/E7HSP(*Nco*I*Bgl*IIfragment) and pUC131/E7GGGHSP(*Nco*I*Bgl*IIfragment). The fragment encoding the second part of HSP70.1 isolated after cutting pUC131/HSP(*Bgl*II*Nsi*I) with *Bgl*II and *Bam*HI restriction enzymes was cloned into the *Bgl*II sites of these two constructs resulting in pUC131/E7HSP and pUC131/E7GGGHSP. Finally, fragments encoding E7HSP and E7GGGHSP were cloned into the *Eco*RI sites of the pBSC plasmid to generate pBSC/E7HSP and pBSC/E7GGGHSP.

The plasmids were propagated in *E. coli* XL1-blue or DH5$\alpha$ strains, cultured in Luria broth medium with 100 μg/ml ampicillin added, and purified with a Qiagen Plasmid Maxi kit (Qiagen, Hilden, Germany).

## Detection of E7 and HSP antigens by immunoblotting

293T cells were grown on 6-cm dishes and transfected with 15 μg plasmids by modified calcium phosphate precipitation [4]. After 2 days, the cells were collected and spun at 1200 rpm for 5 min at 4 °C. The cell pellets were washed with 1 ml ice-cold phosphate-buffered saline (PBS) and resuspended in 100 μl lysis buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 2 mM EDTA, 100 mM Tris-HCl, pH 8) [5] with 1 : 100 diluted protease inhibitor cocktail (Sigma) added. Cell lysates were then passed five times through a 21-G needle and centrifuged at 13 000 rpm for 3 min at 4 °C. The last two steps (i.e. passing through a needle and centrifugation) were repeated. The cell lysates were mixed 1 : 1 with 0.02% bromphenol blue solution and denaturated by incubation at 98 °C for 3 min. Proteins were further separated by 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto a nitrocellulose or poly(vinylidene difluoride) (PVDF) membrane, incubated with mouse anti-E7 monoclonal antibody (clone 8C9; Zymed, San Francisco, CA, USA) or mouse anti-HSP70 monoclonal antibody (clone C92F3A-5; StressGen Biotechnologies Corp., Victoria, Canada) and secondary peroxidase-labelled anti-mouse IgG antibodies. The membranes were stained using the ECL Plus kit (Amersham Biosciences, Little Chalfont, UK).

## Detection of E7 and HSP antigens by immunofluorescence

NIH 3T3 cells were grown on slides in 24-well plates and transfected with 4 μg plasmids by modified calcium phosphate precipitation [4]. Two days after transfection, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 containing 5 μg/ml DAPI for nuclear staining for 3 min. The E7 antigen was stained with mouse anti-E7 monoclonal antibody (clone 8C9, Zymed) and secondary anti-mouse IgG antibodies labelled with FITC (Sigma). The HSP70.1 antigen was stained with mouse anti-HSP70 monoclonal antibody (clone C92F3A-5, StressGen Biotechnologies Corp.) and FITC-labelled anti-mouse IgG antibodies (Sigma). The slides were examined by sequential scanning with a Leica TCS SP2 AOBS confocal microscope.

## Preparation of gene gun cartridges

Plasmid DNA was coated onto 1-μm gold particles (Bio-Rad, Hercules, CA, USA) as described previously [3]. Each cartridge contained 1 μg DNA coated onto 0.5 mg gold particles. For combined immunizations, gold particles were simultaneously coated with equal amounts of two plasmids, so that cartridges contained 0.5 μg of each plasmid.

## Stimulation of splenocytes for *in vitro* assays

For *in vitro* assays, mice (3 per group) were vaccinated via a gene gun with 1 μg plasmid DNA into the shaven abdomen, at a discharge pressure of 400 psi, and 2 weeks later boosted with the same dose. Splenocytes from vaccinated mice were isolated 2 weeks after the last vaccination

and restimulated with 0.001 μg/ml E7-specific H-2D$^b$ CTL epitope RAHYNIVTF (aa 49–57) [6] or with 10 μg/ml E7-derived peptide QAEPDRAHYNIVTFCCKCD (aa 44–62) carrying H-2D$^b$ CTL epitope, B cell epitope and T helper cell epitope [7]. Control splenocytes were incubated without the peptides.

## ELISPOT assay

The ELISPOT assay described by Miyahira *et al.* [8] and Murali-Krishna *et al.* [9] was modified to detect HPV16 E7-specific T cells. The 96-well filtration plates (Millipore Corp., Bedford, MA, USA) were coated with 10 μg/ml rat anti-mouse IFN-$\gamma$ or IL-4 antibody (BD Biosciences Pharmingen, San Diego, CA, USA) in 50 μl of PBS. After overnight incubation at 4 °C, the wells were washed and then blocked with culture medium containing 10% FCS. Different concentrations of non-restimulated or restimulated splenocytes from each vaccinated group of mice, starting from $1 \times 10^6$/well, were added to the wells. Cells were incubated at 37 °C for 24 h either with or without 0.001 μg/ml $E7_{49-57}$ peptide or 10 μg/ml $E7_{44-62}$ peptide. The plates were washed and incubated with 5 μg/ml biotinylated anti-IFN-$\gamma$ or anti-IL-4 antibody (BD Pharmingen) in 50 μl of PBS at 4 °C overnight. After washing, the avidin-horseradish peroxidase conjugate (BD Pharmingen) was added and the plates were incubated for 2 h at room temperature. After washing, spots were developed by adding 50 μl of 0.5 mg/ml aminoethylcarbazole solution (Fermentas Inc., Hanover, MD, USA) and 0.03% $H_2O_2$ and incubation at room temperature for 1 h. The spots were counted using a dissecting microscope.

## Detection of E7-specific CD8$^+$ cells by tetramer staining

Splenocytes restimulated with the $E7_{49-57}$ peptide for 6 days were adjusted to $2 \times 10^7$/ml with FACS buffer (PBS supplemented with 2% FCS and 10 mM sodium azide), incubated with 2 μl of rat anti-mouse CD16/CD32 antibody (Fc-block, BD Pharmingen, stock concentration 0.5 mg/ml) for 20 min on ice and washed. An aliquot of $2 \times 10^6$ cells was resuspended in 80 μl of ice-cold FACS buffer and stained on ice with 20 μl of a mixture of 2 μl of tetramer-PE and 2 μl of rat anti-mouse CD8a-FITC antibody (BD Pharmingen, stock concentration 0.5 mg/ml) in FACS buffer for at least 1 h in the dark. The splenocytes were washed and resuspended in 200 μl of FACS buffer. The stained cells were analyzed with a FACScan instrument using CellQuest software (Becton Dickinson).

## Detection of anti-E7-specific antibodies by GST capture ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed as described previously [38,39]. Ninety-six-well plates (Dynatech, Chantilly, VA, USA) were coated overnight at 4 °C with 200 ng/well of glutathione casein in 50 mM carbonate buffer, pH 9.6. Thereafter, the wells were incubated for 1 h at 37 °C with 100 μl of blocking buffer (0.2% casein in PBS with 0.05% Tween 20) and washed three times with washing buffer (PBS with 0.05% Tween 20). The cleared lysate from *E. coli* expressing the glutathione S-transferase (GST)-E7-tag protein diluted in blocking buffer to 25 μg/100 μl was added to each well for 1 h at 37 °C. Unbound material was washed away five times with washing buffer. Mouse sera assayed for anti-E7 antibodies were diluted 1 : 50 in blocking buffer containing 0.25 μg/μl total lysate proteins from the GST-tag-transformed *E. coli* (to block reactivities of the sera with contaminating *E. coli* proteins) and incubated for 1 h at 37 °C. After washing the plates five times, bound mouse antibodies were detected by sheep anti-mouse IgG antibodies conjugated to horse radish peroxidase diluted 1 : 2000 in blocking buffer for 1 h at 37 °C. The plates were washed five times and stained with 100 μl of 10 μg/ml tetramethylbenzidine (Sigma) and 0.003% $H_2O_2$ for 5–10 min. The reaction was stopped by adding 50 μl of 1 M sulfuric acid and the absorbance was measured at 450 nm.

## Tumor protection experiments

Mice (5 or 8 per group) were twice vaccinated by a gene gun with 1 or 2 μg (see the Results section) of plasmids at a 2-week interval. Two weeks after the last vaccination, mice were subcutaneously (s.c.) challenged in the back with $3 \times 10^4$ TC-1 or TC-1/A9 tumor cells suspended in 0.15 ml PBS and then monitored twice a week for tumor growth.

## Tumor treatment experiment

Mice (8 per group) were s.c. inoculated in the back with $3 \times 10^4$ TC-1 tumor cells suspended in 0.15 ml PBS. Then, 4 and 10 days later, mice were given 1 μg plasmids via a gene gun. The mice were monitored twice a week for tumor growth.

## Statistical analysis

Tumor formation after DNA immunization was analyzed by the log-rank test. A difference between groups was considered significant if $P < 0.05$.

# Results

## Fusion with mouse HSP70.1 increased the steady-state level of the E7 antigen

The fusion genes E7HSP and E7GGGHSP were constructed by linking the HPV16 E7 oncogene or its

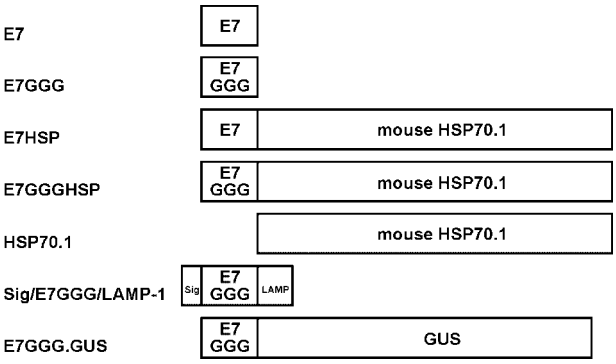


**Figure 1. Schema of genes used for immunization**

mutated form E7GGG, respectively, to the 5′ end of the mouse HSP70.1 gene as described in Materials and methods (Figure 1). Then, the fusion genes and HSP70.1 alone were cloned into the mammalian expression plasmid pBSC downstream of an immediate early cytomegalovirus promoter. The expression of the E7 antigen was detected in transfected 293T cells by immunoblotting. We found that the steady-state level of the E7 antigen in pBSC/E7HSP- or pBSC/E7GGGHSP-transfected cells was 10–20-fold higher in comparison with pBSC/E7- or pBSC/E7GGG-transfected cells (Figure 2A), approximately 2-fold higher than that in pBSC/E7GGGLAMP-transfected cells, and comparable to that in pBSC/E7GGG.GUS-transfected cells (Figure 2B).

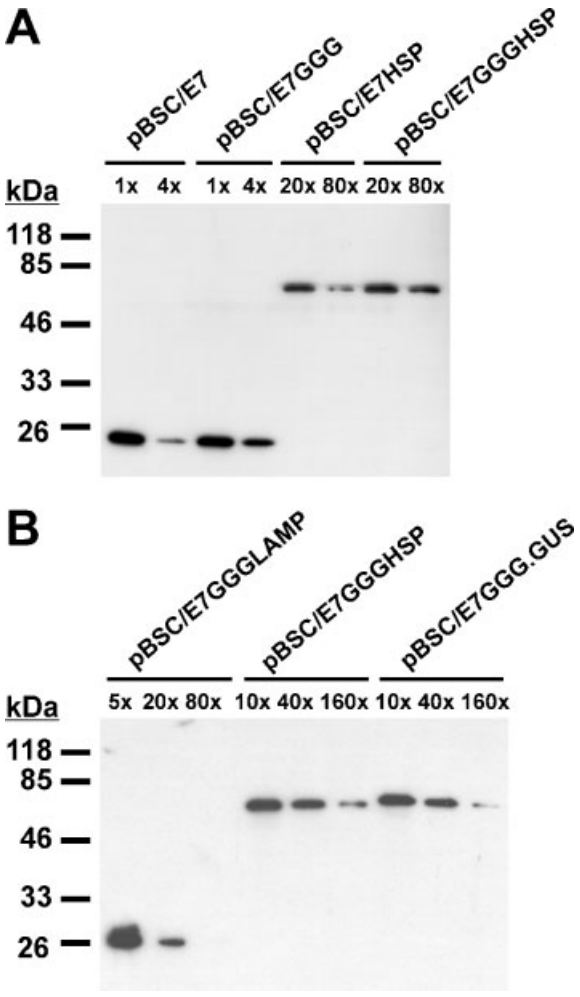


**Figure 2. Immunoblotting detection of the E7 antigen in 293T cells transfected with pBSC-derived plasmids. Proteins separated by 10% SDS-PAGE were transferred onto a PVDF membrane, detected with the HPV16 E7-specific antibody, and visualized with ECL. Dilutions of cellular extracts are indicated. The experiments were repeated with similar results**

## Fusion with E7 or E7GGG slightly decreased the steady-state level of the HSP70.1 antigen

The expression of the HSP70.1 antigen in transfected 293T and NIH 3T3 cells was compared by immunoblotting. The 293T cells produced a high level of the endogenous HSP70.1 protein and their transfection with pCR3/HSP or pBSC/HSP resulted in an increased expression of the HSP70.1 protein in comparison with non-transfected cells. The 293T cells transfected with pBSC/E7HSP or pBSC/E7GGGHSP produced both their endogenous HSP70.1 and more weighty E7HSP or E7GGGHSP fusion proteins, respectively (Figure 3A). However, these results did not enable us to compare the expression of the HSP70.1 fusion proteins with that of the HSP70.1 protein alone in the transfected 293T cells. Therefore, we also analyzed the steady-state levels of the HSP70.1 antigen in transfected NIH 3T3 cells, which exhibit low spontaneous production of the endogenous HSP70.1 protein. Similar amounts of the HSP70.1 protein were expressed in NIH 3T3 cells transfected with the original plasmid pCR3/HSP and with pBSC/HSP, while cells transfected with pBSC/E7HSP or pBSC/E7GGGHSP showed slightly lower production of HSP70.1 fusion proteins (Figure 3B).

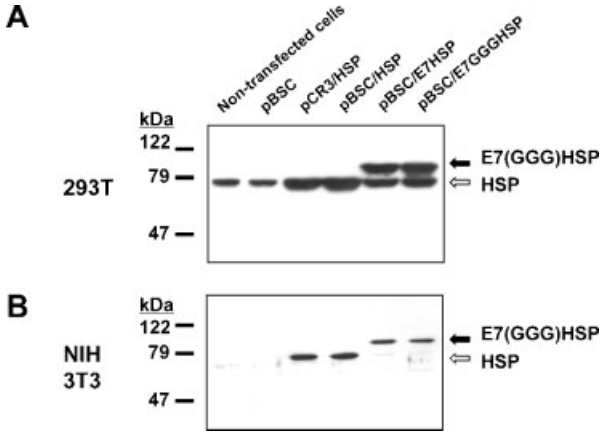


**Figure 3. Immunoblotting detection of the HSP70.1 antigen in transfected 293T (A) or NIH 3T3 cells (B). Proteins separated by 10% SDS-PAGE were transferred onto a PVDF membrane, detected with the HSP70-specific antibody, and visualized with ECL. The experiments were repeated with similar results**

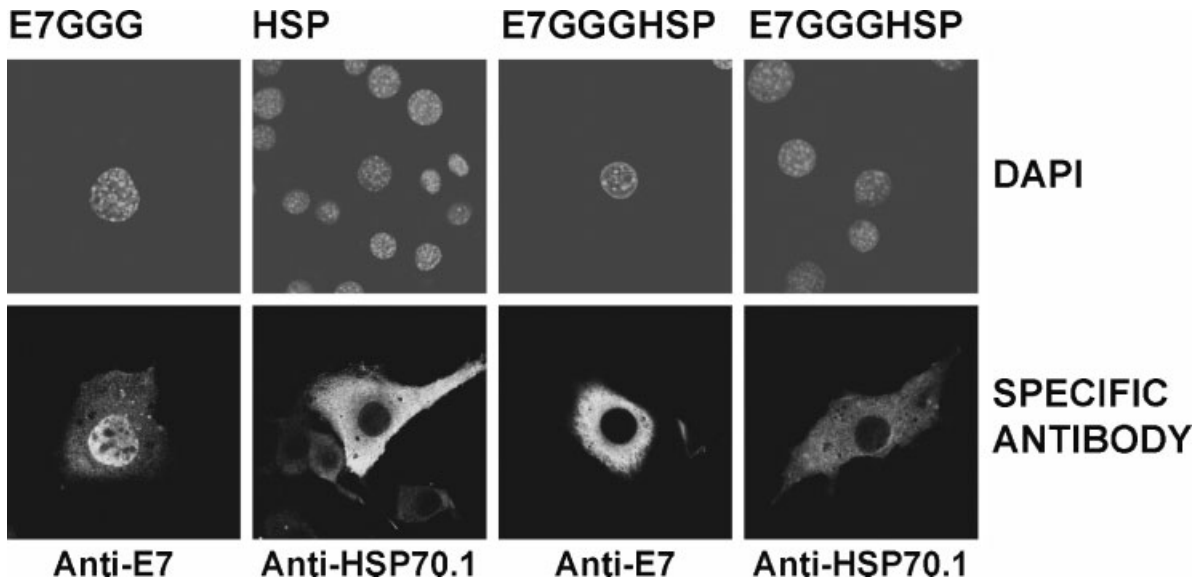


**Figure 4. Immunofluorescence detection of the E7GGG and HSP70.1 antigens in NIH 3T3 cells transfected with pBSC-derived plasmids. The paraformaldehyde-fixed cells were stained with specific monoclonal antibodies and DAPI 2 days after transfection and examined by a confocal microscope. Similar results were obtained for plasmids with wild-type E7 (pBSC/E7 and pBSC/E7HSP)**

## Fusion with mouse HSP70.1 targeted the E7 antigen into the cytoplasmic compartment

Transfection of NIH 3T3 cells with subsequent immunofluorescent staining was used to determine the cellular localization of the HSP70.1 fusion proteins. As expected, the cells transfected with wild-type E7 or E7GGG showed both cytoplasmic and nuclear staining and, in cells transfected with HSP70.1, cytoplasmic staining was recorded (Figure 4). In cells transfected with pBSC/E7HSP or pBSC/E7GGGHSP, localization of the E7 antigen was limited to the cytoplasm. We also stained these cells with antibody against HSP70. The results showed the same cytoplasmic localization of the HSP70.1 antigen (Figure 4). In non-transfected cells no specific staining was detected (data not shown).

## Vaccination with pBSC/E7HSP or pBSC/E7GGGHSP protected mice against the growth of TC-1-induced tumors

To determine whether vaccination with the E7HSP or E7GGGHSP fusion genes protects mice against E7-expressing tumors, animals were immunized twice with plasmids and then challenged with TC-1 cells (Figure 5). All mice that received control plasmids pBSC or pBSC/HSP developed tumors within 17 days. In contrast, all of the mice immunized with pBSC/E7HSP or pBSC/E7GGGHSP remained tumor-free for 70 days after TC-1 challenge ($P < 0.005$). The vaccination with the E7HSP fusion gene protected mice against formation of TC-1 tumors significantly more efficiently than E7 or combination of E7

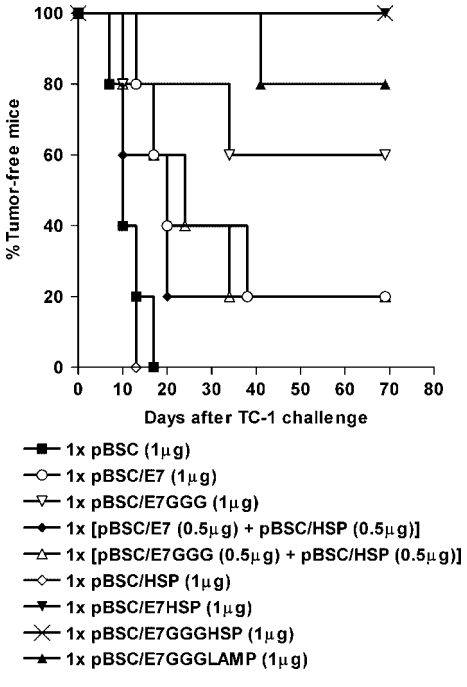


**Figure 5. Tumor formation after preventive immunization against TC-1-induced tumors. Mice (5 per group) were twice immunized with 1 μg of E7-based plasmids at a 2-week interval and challenged s.c. with TC-1 cells 2 weeks after the second immunization dose. In combined immunizations, plasmids were delivered in a 1 : 1 mix in one shot. Number of shots per each immunization and the amount of plasmid DNA delivered by one shot are indicated**

with HSP (delivered in mix in one gene gun shot) – in both groups only one out of five mice did not develop tumor ($P < 0.05$). The vaccination with the E7GGGHSP fusion gene also showed enhanced protection against formation of TC-1-induced tumors in comparison with E7GGG

(non-significant) or combination of E7GGG with HSP ($P < 0.05$). Thus, both E7HSP and E7GGGHSP generated potent protection against the formation of TC-1-induced tumors, comparable with that of Sig/E7GGG/LAMP-1. Covalent linkage with the antigens appears to be necessary for the enhancing effect of HSP70.1.

## Therapeutic vaccination with pBSC/E7HSP or pBSC/E7GGGHSP reduced the growth of TC-1 cells

The potential of DNA vaccines was also assessed by therapeutic immunization that was initiated 4 days after TC-1 inoculation (Figure 6A). While all of the control mice vaccinated with the pBSC plasmid developed tumors within 17 days, about half of mice immunized with the pBSC/E7HSP or pBSC/E7GGGHSP plasmids remained tumor-free for 80 days after inoculation of tumor cells ($P < 0.001$ and $<0.05$, respectively). Only two out of eight mice immunized with the pBSC/E7GGGLAMP vaccine remained tumor-free ($P < 0.01$), which is suggestive of a higher immunization effect of E7HSP or E7GGGHSP compared with Sig/E7GGG/LAMP-1. The immunization with combinations of pBSC/E7HSP or pBSC/E7GGGHSP with pBSC/E7GGGLAMP delivered in mix in one gene gun shot cured about 50% of mice ($P < 0.05$ and $<0.01$, respectively) which is comparable to the effects of either pBSC/E7HSP or pBSC/E7GGGHSP alone (Figure 6A).

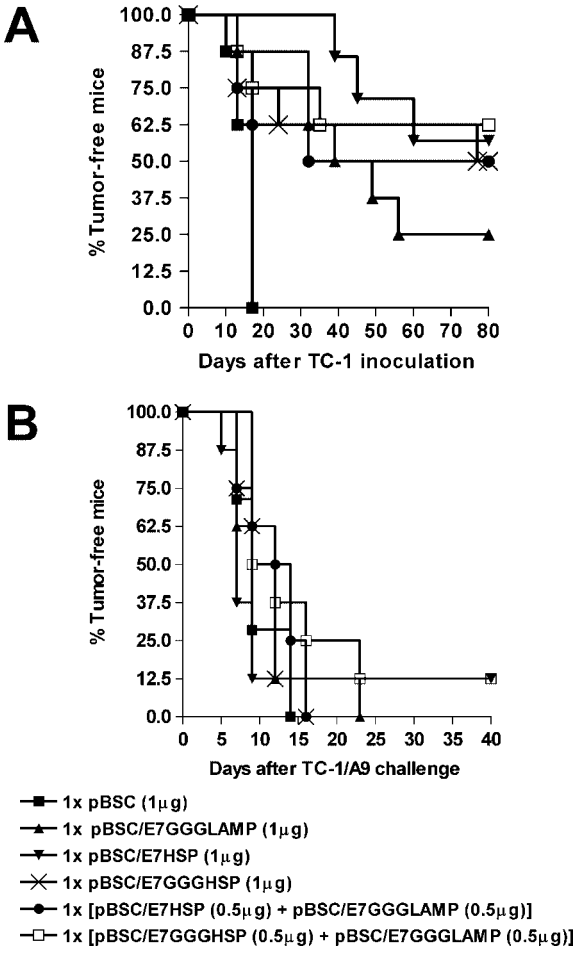


**Figure 6. Tumor formation after immunization with 1 μg of E7-based plasmids. Mice (8 per group) were either inoculated with TC-1 cells and vaccinated on days 4 and 10 (A) or immunized twice at a 2-week interval and challenged 2 weeks later with TC-1/A9 cells (B). In combined immunizations, plasmids were delivered in a 1 : 1 mix in one shot. Number of shots per each immunization and the amount of plasmid DNA delivered by one shot are indicated**

## Vaccination with a regular dose of pBSC/E7HSP or pBSC/E7GGGHSP did not protect mice against the growth of TC-1/A9-induced tumors

Furthermore, the antitumor effect of the HSP70.1 fusion genes was tested in a more stringent model using TC-1/A9 cells with downregulated expression of MHC class I molecules. Mice were twice immunized with a regular dose (1 μg) of plasmids and challenged with TC-1/A9 cells. Only one out of eight mice immunized with pBSC/E7HSP or combination of pBSC/E7GGGHSP with pBSC/E7GGGLAMP was protected against tumor formation within 40 days of observation (Figure 6B). In the other groups immunized with pBSC, pBSC/E7GGGLAMP, pBSC/E7GGGHSP or combination of pBSC/E7HSP with pBSC/E7GGGLAMP, all of the mice developed tumors within 23 days (Figure 6B). Immunization did not inhibit tumor growth either.

## Vaccination with pBSC/E7HSP or pBSC/E7GGGHSP enhanced E7-specific $CD8^+$ T-cell-mediated immune responses

We performed ELISPOT assay to detect the E7-specific IFN-$\gamma$-secreting $CD8^+$ T cells in splenocytes from vaccinated mice. The results of one representative experiment are shown in Figure 7A. For this particular ELISPOT assay, splenocytes were restimulated for 5 days with the $E7_{49-57}$ peptide. While only weak stimulation of E7-specific $CD8^+$ T cells was recorded after immunization with pBSC/E7 or pBSC/E7GGG (about 10 and 20 IFN-$\gamma$-producing cells per $10^5$ splenocytes, respectively), fusion with HSP70.1 and LAMP-1 induced 30–40-fold higher immune response. Combinations of fusion genes administered in mix in one shot did not further enhance the number of IFN-$\gamma$-producing cells. Tetramer staining of E7-specific $CD8^+$ T lymphocytes showed analogical proportions among the tested groups (data not shown). These data correspond well to the effects of immunization against TC-1 cells (see above).

In parallel with IFN-$\gamma$ ELISPOT, we also detected IL-4-secreting splenocytes by ELISPOT. However, the numbers of positive cells were low in all groups

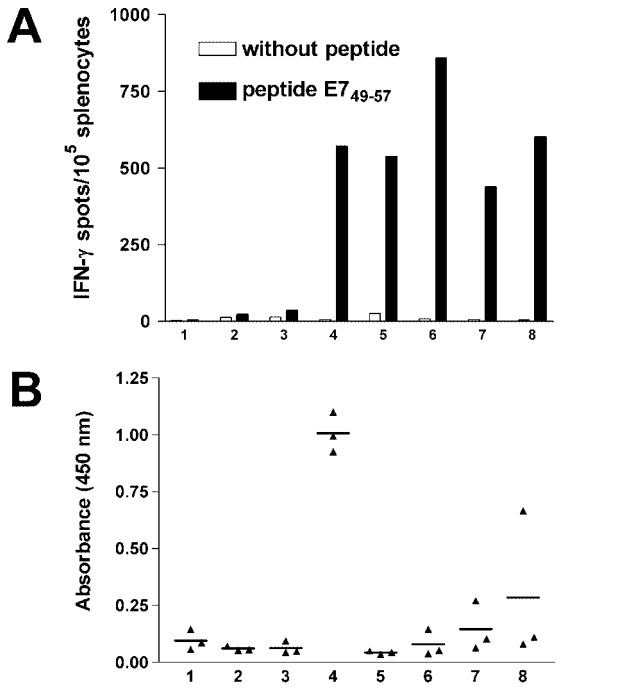


**Figure 7.** ***In vitro*** **characterization of immune responses elicited in mice by vaccination with E7-based DNA vaccines. Mice (3 per group) were immunized twice via a gene gun with 1 μg of plasmids at a 2-week interval. In combined immunizations, plasmids were delivered in a 1 : 1 mix in one shot. Sera and splenocytes were obtained 2 weeks after the second immunization. Splenocytes were restimulated for 5 days with the $E7_{49-57}$ peptide and IFN-γ-secreting cells were detected by ELISPOT (A). Anti-E7 antibodies were detected by ELISA in 1 : 50 diluted sera (B)**

(8–34 IL-4-producing cells per $10^5$ splenocytes), suggesting the absence of relevant differences (data not shown).

## Vaccination with pBSC/E7HSP or pBSC/E7GGGHSP did not induce E7-specific antibodies

The quantity of anti-E7 antibodies in sera of vaccinated mice was determined by ELISA at a dilution of 1 : 50. Blood samples were obtained simultaneously with splenocytes for ELISPOT assay from the same mice. No anti-E7 antibodies were detected in sera of mice vaccinated with pBSC plasmids containing E7, E7GGG, E7HSP or E7GGGHSP genes. The pBSC/E7GGGLAMP plasmid induced the production of anti-E7 antibodies in all three immunized mice. It is noteworthy that DNA vaccines based on the combinations of half amount of the pBSC/E7GGGLAMP plasmid with pBSC/E7HSP or pBSC/E7GGGHSP only generated anti-E7 antibodies in one out of three mice (Figure 7B).

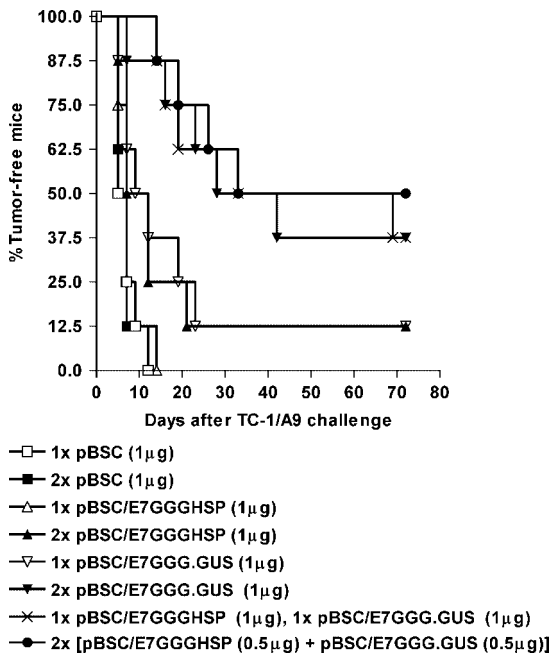


**Figure 8. Tumor formation after preventive immunization against TC-1/A9-induced tumors. Mice (8 per group) were twice immunized at a 2-week interval with a regular (1 μg) or double dose (2 μg) of E7-based plasmids and challenged with TC-1/A9 cells 2 weeks after the second immunization. In combined immunizations, plasmids were delivered either in a 1 : 1 mix in two shots (marked with filled circles) or separately into opposite sides of the abdomen each plasmid in one shot (marked with cross signs). Number of shots per each immunization and the amount of plasmid DNA delivered by one shot are indicated**

## Vaccination with the double dose of pBSC/E7GGGHSP or its combination with pBSC/E7GGG.GUS did not substantially enhance the protection against TC-1/A9 tumor cells

To improve the protection of mice against the formation and growth of TC-1/A9-induced tumors, we doubled the dose of pBSC/E7GGGHSP and combined this plasmid with pBSC/E7GGG.GUS that had been previously shown to be efficient in this model [40]. Doubling the dose of pBSC/E7GGGHSP slightly enhanced the protection against tumor formation, with some tumors appearing later in mice vaccinated by the double dose compared with regular dose and one out of eight mice remaining tumor-free (Figure 8). A substantially higher effect was observed after vaccination with pBSC/E7GGG.GUS. While one out of eight mice vaccinated with the regular dose of pBSC/E7GGG.GUS did not develop a tumor ($P < 0.05$, compared with the regular pBSC dose), the double dose protected three mice and delayed tumor formation in most animals ($P < 0.001$, compared with the double pBSC dose).

Since the site of delivery might play a role in the induction of immune responses, we tested the combined vaccines of pBSC/E7GGGHSP and pBSC/E7GGG.GUS in two different modes of application. The plasmids were delivered either mixed or separately to two distinct immunization sites in opposite sides of the murine abdomen. One microgram of each plasmid was administered in either vaccination. Both modes of application resulted in significant protection against TC-1/A9 cells ($P < 0.001$), comparable to that induced by the double dose of pBSC/E7GGG.GUS.

## Vaccination with the double dose of pBSC/E7GGGHSP augmented E7-specific CD8$^+$ T-cell-mediated immune responses

IFN-$\gamma$ ELISPOT with splenocytes incubated with the $E7_{49-57}$ peptide for 1 day directly on the ELISPOT plate (Figure 9) showed that doubling the dose of pBSC/E7GGGHSP highly increased the number of E7-specific splenocytes producing IFN-$\gamma$ while doubling the dose of pBSC/E7GGG.GUS only had a slight effect. Moreover, both combinations of plasmids did not exceed the efficacy of the double dose of pBSC/E7GGG.GUS.

Sera obtained from mice immunized for the ELISPOT assay were used for the quantification of anti-E7 antibodies by ELISA. In accordance with the data reported above, no anti-E7 antibodies were detected in sera of mice vaccinated with pBSC or pBSC-derived plasmids containing E7GGGHSP or E7GGG.GUS or with combinations of these plasmids (data not shown).

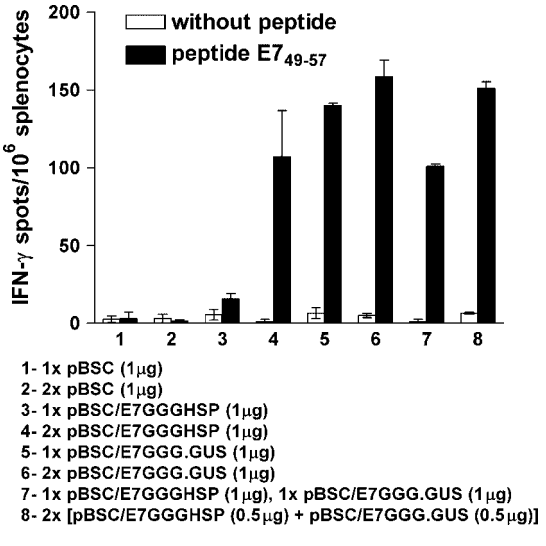


**Figure 9. Detection of immune responses by IFN-$\gamma$ ELISPOT. Mice (3 per group) were immunized twice with 1 or 2 μg of plasmids at a 2-week interval. The ELISPOT assay was performed 2 weeks after the second vaccination. IFN-$\gamma$-producing E7-specific CD8$^+$ lymphocytes were detected in splenocytes incubated with the $E7_{49-57}$ peptide for 1 day directly on the ELISPOT plate**

# Discussion

In this study, we constructed fusion genes of the HPV16 E7 oncogene and its mutated form (E7GGG) with mouse HSP70.1 (E7HSP and E7GGGHSP) to develop a more potent DNA vaccine for therapy of HPV16-induced tumors. Their expression was characterized in mammalian cells and immunogenicity was tested by DNA vaccination with a gene gun using two different cell lines for induction of tumors: (1) MHC class I positive TC-1 cells [1] and (2) their clone TC-1/A9 with downregulated MHC class I expression [2]. The induced antitumor responses were enhanced in comparison with E7 and E7GGG alone. HSP70.1 fusion genes were also repeatedly more immunogenic than Sig/E7GGG/LAMP-1 [2] but the differences were not significant in any experiment. Comparison of E7GGGHSP with the fusion of E7GGG with *E. coli* $\beta$-glucuronidase (E7GGG.GUS) [40] showed superior efficacy of E7GGG.GUS. Furthermore, we demonstrated enhanced immunogenicity of double dose DNA vaccines based on E7GGGHSP and E7GGG.GUS fusion genes. Unfortunately, there was no evidence of improvement of the antitumor responses induced by combination of E7GGGHSP with another fusion gene, Sig/E7GGG/LAMP-1 or E7GGG.GUS.

The E7 protein is a very labile protein with a half-life of about 1 h [46]. The production of E7HSP and E7GGGHSP fusion proteins was confirmed by immunoblotting showing substantially elevated steady-state levels of these fusion proteins and also previously prepared Sig/E7GGG/LAMP-1 and E7GGG.GUS fusion proteins in comparison with E7 and E7GGG. Similar effects have been reported for the fusion of E7 with the herpes simplex virus ferry protein VP22 [41], staphylococcal nuclease Nuc [47], Myc tag [49] and HPV16 E6 [48]. However, the role of the described increase in steady-state levels of E7 fusion proteins in enhancement of immune responses is unclear as, interestingly, higher efficacy of the DNA vaccine was also demonstrated after E7 destabilization by fusion with ubiquitin [50] or mutagenesis in zinc-binding motifs [23]. While increased amount of the produced E7 antigen might enhance cross-presentation by APC, accelerated E7 degradation in *in vivo* transfected APC might augment direct presentation.

The E7HSP and E7GGGHSP proteins were localized in the cytoplasm of transfected cells which corresponded with location of HSP70.1, whereas the E7 and E7GGG proteins have been preferentially demonstrated in the nucleus, being less frequent in the cytoplasm [3]. The E7 oncoprotein can bind to the pRB tumor suppressor protein present in the nucleus thus contributing to malignant transformation of HPV16-infected cells. For safety reasons, we have introduced three point mutations into the pRB-binding site of the E7 protein [3]. However, oncogenic potential of E7 is not restricted to the pRb-binding site. Via interactions of various domains, E7 binds to a number of cellular proteins a portion of which is

localized in the nucleus [21]. Therefore, reduced nuclear location of the E7 fusion proteins might enhance the safety of corresponding DNA vaccines. Moreover, conformation changes of the E7 protein caused by fusion with a helper protein might inhibit E7 oncogenicity.

The mutated E7GGG gene has been shown more immunogenic than the wild-type E7 gene. However, no increase in immune responses has been observed for Sig/E7GGG/LAMP-1 in comparison with Sig/E7/LAMP-1 [2]. We did not detect any relevant difference in the immunogenicity of E7HSP and E7GGGHSP in this study supporting our hypothesis [2] that rather than the modified epitope, inactivation of the pRb-binding site is responsible for augmented immunogenicity of E7GGG.

The fusion of a gene coding for an antigen with another gene is a successful strategy for the enhancement of DNA vaccine potency [43]. Various mechanisms have been described to be responsible for this effect such as modification of protein production, stability [40,41] and targeting, i.e. cellular localization and/or antigen processing and presentation [36,44,45], and utilization of immunostimulatory sequences or helper epitopes of the fusion partner. Two mechanisms have been proposed to explain the immunostimulatory effect of HSPs: (1) delivery of antigens into APC via endocytosis mediated by HSP-specific receptors and (2) induction of maturation of APC [27]. In this study, we demonstrated that the DNA vaccine containing E7 linked to mouse HSP70.1 elicited significantly better protection against the growth of TC-1-induced tumors than the DNA vaccine containing a combination of E7 with HSP70.1. The same effect has been observed for the linkage of E7 to *Mycobacterium tuberculosis* HSP70 (TBHSP) [25] indicating that the fusion of a HSP gene to an antigen gene is necessary for the observed enhancement of DNA vaccine potency. Moreover, our results suggest that the immunostimulatory effects of HSP fusion genes are not due to the bacterial origin of the HSP moiety. This has also been confirmed for protein vaccines as the vaccination with the murine HSP70-ovalbumin fusion protein elicited CTL responses equivalent to those generated by the TBHSP-ovalbumin fusion protein [16].

The fusion gene linking E7 to TBHSP has been compared with some other E7-expressing fusion genes, showing induction of higher number of E7-specific $CD8^+$ T lymphocytes in comparison with the Sig/E7/LAMP-1 gene [44]. Here, we found that the antitumor effects generated by the fusion genes of E7 or E7GGG with mouse HSP70.1 were slightly higher than those of Sig/E7GGG/LAMP-1 and the counts of E7-specific IFN-$\gamma$-producing $CD8^+$ T cells induced by vaccination with either of these constructs were comparable. As fusion of E7 to TBHSP generates potent $CD8^+$ T-cell responses that are $CD4^+$ T-cell-independent, and whilst Sig/E7/LAMP-1 targets the E7 antigen to MHC class II presentation pathways and increases activation of $CD4^+$ lymphocytes, it has been proposed that administration of these two DNA vaccines in combination might induce further augmentation of antitumor effects [31]. Considering this hypothesis, we tested combinations of Sig/E7GGG/LAMP-1 with E7HSP or E7GGGHSP. As the constructs were investigated for their possible synergistic effect, we prepared bullets containing plasmids mixed at a 1 : 1 ratio thus reducing the amount of each from the two constructs received by immunized mice to 50%. We compared the vaccination with combinations of constructs and with single genes for their ability to induce antitumor effects against TC-1 and TC-1/A9 cells and to stimulate E7-specific $CD8^+$ T-cell responses and production of anti-E7 antibodies. In all of these attributes, any combination of LAMP-1 and HSP70.1 fusion genes did not exceed the effects induced with single genes. In the meantime, we developed the E7GGG.GUS fusion gene and showed significant protection against TC-1/A9 cells induced by this DNA vaccine [40]. Therefore, we included E7GGG.GUS in the next immunization experiments.

An analogous combination of E7GGGHSP with E7GGG.GUS did not induce better protection against the growth of TC-1/A9-induced tumors than E7GGG.GUS alone. We also tried to combine E7GGGHSP with E7GGG.GUS in a different manner, delivering each gene separately in one gene gun shot into opposite sides of the abdomen and thus eliciting immune responses by each gene in a different lymph node. However, neither type of combined immunization led to an enhancement of antitumor responses.

Vaccination with the Sig/E7GGG/LAMP-1 gene has not protected mice against the development of TC-1/A9-induced tumors in our previous work [2]. In this study, we found that the vaccination regimen with 1 μg of pBSC/E7GGGHSP, that protected all mice against development of TC-1-induced tumors, did not inhibit the growth of TC-1/A9-induced tumors. As Cheng *et al*. [34] reported that the DNA vaccination with 2 μg of E7 linked to TBHSP generated a significant antitumor effect against the TC-1-derived P3 (A15) clone with downregulated MHC class I expression, we decided to increase the dose of the plasmid. However, vaccination with the double dose of pBSC/E7GGGHSP still provided only mild protection against TC-1/A9 cells. The low efficacy of our DNA vaccine when compared with that of Cheng *et al*. [34] could be explained by the use of different cell line, HSP and/or immunization regimen.

We achieved a substantial increase in protection against the development of TC-1/A9-induced tumors by doubling the dose of the E7GGG.GUS gene. However, increasing the dose of the wild-type E7-containing DNA vaccine delivered by a gene gun has not improved protection against the TC-1-induced tumors [42]. Moreover, other studies have found that DNA vaccines containing the gene coding for the HIV env protein did not generate a dose-dependent effect on a number of stimulated HIV gp120-specific $CD8^+$ T cells [51,52]. However, a dose-dependent enhancement of influenza nucleoprotein-specific CTL precursor frequency by DNA vaccination has been demonstrated [53]. The described discrepancy in the effect of dosage on DNA vaccine efficacy may be related to the type of antigen, route of DNA administration,

vaccination regimen and/or disease models used in these studies.

Both humoral and cellular immune responses are affected by the type of vaccine and the route of its delivery. Enhanced humoral immune responses have been described after vaccination with TBHSP-antigen fusion protein vaccines [14] but no increase in antigen-specific antibodies has been detected after gene gun administration of such a DNA vaccine [25,30]. We did not detect E7-specific antibodies after DNA vaccination with E7, E7GGG, E7HSP or E7GGGHSP in concordance with results of other studies showing no E7-specific antibody responses after DNA vaccination with E7 [54] or E7 linked to TBHSP [30]. We did not detect E7-specific antibodies after vaccination with E7GGG.GUS either. Immunization with Sig/E7GGG/LAMP-1 induced E7-specific antibodies as reported for Sig/E7/LAMP-1 by others [54]. However, combinations with HSP fusion genes inhibited antibody production. The reduced dose of pBSC/E7GGGLAMP may have been responsible for the effect, but we cannot exclude possible interference of the immunity induced with the HSP70.1 fusions.

In summary, we compared immunogenicity of three fusion genes of the mutant HPV16 E7 gene. E7GGG linked with *E. coli* $\beta$-glucuronidase induced the best antitumor responses that were further enhanced by doubling the plasmid dose. Combined immunization with two fusion constructs delivered by a gene gun either in mix or separately did not exceed the effect of single genes.

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# DNA vaccines based on chimeric potyvirus-like particles carrying HPV16 E7 peptide (aa 44-60)

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**Abstract.** Vaccine strategies for the treatment of human papillomavirus-induced cervical cancer are based mainly on the human papillomavirus 16 E7 (HPV16 E7) oncoprotein. The immunogenicity of the E7 gene has been enhanced by its fusion to many different genes. Here, we linked a short sequence coding for the E7 peptide (aa 44-60) containing immunodominant epitopes for B and T cells to the 3' end of the gene coding for the whole coat protein (CP) of the potyvirus, potato virus A (PVA), and its deleted form (CPdel) with a short C-terminal deletion of 5 amino acids (LGVKG). CP-E7 and CPdel-E7 fusion proteins, just like CP alone, spontaneously assembled into virus-like particles in both procaryotic and eucaryotic cells. The CP-E7 and CPdel-E7 fusion genes induced slightly stronger E7-specific cytotoxic T-lymphocyte responses than the whole E7 gene, although they were still lower than those elicited by the previously constructed fusion gene, Sig/E7GGG/LAMP-1. The E7- and CP-specific antibody responses were not detected in mice vaccinated with CP-E7 and CPdel-E7 fusion genes. The CP-E7 and CPdel-E7 fusion genes protected mice against the development of tumors induced by TC-1 cells producing the E7 antigen and were also effective in the therapeutic setting, i.e. when the vaccination was performed after tumor cell administration. Their antitumor effect was comparable to those of the whole E7 gene and Sig/E7GGG/LAMP-1 fusion gene. There was no relevant difference between immune responses elicited by CP-E7 and CPdel-E7 DNA vaccination.

## Introduction

Human papillomaviruses (HPVs) are the causative agent of cervical cancer. HPV16 is the most prevalent type of HPV associated with cervical cancer, detected in about 50 percent of cervical cancer. Therefore, it is the most important target for development of both prophylactic and therapeutic anti-HPV vaccines. Preventive immunization is based on the structural L1 and L2 proteins of HPV and should result in the induction of neutralizing antibodies. The L1 monomers assemble spontaneously into virus-like particles and enter the mammalian cells through specific receptors (1). Vaccination with L1 virus-like particles has been shown to be very effective, generating 100% protection against persistent HPV infection (2). Therapeutic vaccination against HPV is aimed at eliciting cellular immune responses to viral E7 and/or E6 oncoproteins that are the only viral proteins constitutively expressed in cells of cervical cancer. The therapeutic vaccines against HPV-associated cancer have appeared promising, but there is still a need for improvement (3). Different strategies have been utilized, including the fusion of the HPV16 E7 protein or the E7-derived peptides to proteins forming virus-like particles (4-6).

VLPs (virus-like particles) are known to induce not only potent antibody responses but also strong cell-mediated responses (6,7). The nature of the induced immune responses against L1 VLPs has been shown to vary with different delivery systems. Monkeys immunized intramuscularly with plasmid DNA or a replicon incompetent adenoviral vector expressing HPV16 L1 developed strong Th1/Tc1 responses, potent humoral responses and only weak neutralizing antibodies, while immunization with HPV16 L1 VLPs led to a potent humoral response with high levels of neutralizing antibodies and a strong L1-specific Th2 response (8). VLPs alone have not proved very efficient at inducing cytotoxic T-lymphocyte (CTL) responses but have become a very powerful vaccine when applied together with adjuvants activating antigen-presenting cells (7). The delivery of DNA vaccines by a gene gun is considered to have such an adjuvant effect. Mild damage caused to the skin by the gold particles may act as maturation and stimulation signals for ) Langerhans cells and dermal dendritic cells (9).

PVA is a filamentous RNA virus which belongs to the genus Potyvirus (family Potyviridae), the largest group of plant viruses. Potyvirus particles are flexible rods, 680-900 nm long and 11-15 nm wide, consisting of more than 2000 copies of a coat protein subunit and one single-stranded RNA genome (10). The potyvirus CP is a multifunctional protein

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which plays a role in virus transmission by aphids (11), virus movement in plants (12) and virion formation. Three regions can be distinguished in the potyviral CP protein. The central core region involves 214-217 amino acids (aa) and is considered to be responsible for particle integrity. Both the N-terminal (29-94 aa) and C-terminal (18-20 aa) regions are surface exposed and are not required for virion assembly (13,14). The expression of potyvirus CP (Johnsongrass mosaic virus, JGMV) in bacteria (*Escherichia coli*), yeast (*Saccharomyces cerevisiae*), insect cells or mammalian cell culture has been demonstrated to result in the formation of potyvirus-like particles (PVLPs) (14,15). It has been shown that self-assembly of the particles is not impaired, even by the insertion of a foreign 26 kD protein into the JGMV CP (14,15). Such chimeric PVLPs have been highly immunogenic, even in the absence of any adjuvant (14,16).

The aims of this study were to investigate the ability of DNA vaccines, coding for the PVA CP protein associated with HPV16 E7 peptide (aa 44-60), to elicit E7-specific immune responses and to compare their efficacy with those of the E7 gene and the previously prepared Sig/E7GGG/LAMP-1 fusion gene (17). Furthermore, we assessed the influence of C-terminal deletion and fusion to the E7 peptide (aa 44-60) on the CP's ability to assemble into VLPs in procaryotic and eucaryotic cells.

## Materials and methods

*Animals*. Female C57BL/6 mice, aged 6-8 weeks (H-$2^b$; Charles River, Germany), were used in the immunization experiments. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals.

*Cell lines*. The efficacy of DNA vaccines was evaluated using TC-1 tumor cells (18) (kindly provided by T.C. Wu, Johns Hopkins University, Baltimore, MD), prepared by transformation of primary C57BL/6 mouse lung cells with HPV16 E6/E7 oncogenes and activated *H-ras*. To verify the expression of the E7 and CP proteins from constructed plasmids, mouse NIH 3T3 fibroblasts and human embryonic kidney 293T cells (kindly provided by J.A. Kleinschmidt, DKFZ, Heidelberg, Germany) were transfected and production of proteins was determined in cell lysates. All cells were grown in Dulbecco's Modified Eagle's medium (PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (PAA Laboratories), 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin.

*Plasmids*. The constructions of pBSC, pBSC/E7 (19), pBSC/E7GGGLAMP (17) and pMPM4Ω/PVA-CP (20) were described previously. Two fusion genes coding for the HPV16 E7-derived peptide (aa 44-60) fused to the C-terminus of full-length PVA CP (CP-E7) or PVA CP shortened by 5 aa (CPdel-E7) were prepared by two sequential PCR reactions modifying the 3' end of the PVA CP gene by primers with long overhangs coding for the E7 epitope. For the first PCR reaction, the upstream primer, 5'-AAAA<u>CCATGG</u>AAGCCG GAACTCTTGATGC-3', with the start codon ATG containing *Nco*I restriction site (underlined) on its 5' end was used. The downstream primers coding for the junction of CP and E7 were 5'-TGTTGTAGTGGGCGCGGTCCGGCTCGGCCTGAAC CCCCTTCACGCCTAGAAGGT-3' for the CP-E7 gene and 5'-GTTGTAGTGGGCGCGGTCCGGCTCGGCCTGAACA AGGTGATGCATGTTGCGATTAAC-3' for the CPdel-E7 gene. In the second PCR reaction, we used the same primers for construction of both the CP-E7 and the CPdel-E7 fusion genes, the upstream primer that had been used in the first PCR reaction and the downstream primer coding for the C-terminus of E7 peptide, 5'-AAA<u>AGATCT</u>ACTTGCAGCAG AAGGTCACGATGTTGTAGTGGGCGCGGTCCGGCTC-3', which contained the *Bgl*II restriction site (underlined). The PCR products were cloned into the *Nco*I and *Bgl*II restriction sites of pUC57T/A and pMPM4Ω.

PVA CP, PVA CP-E7 and PVA CPdel-E7 genes were cut from pMPM4Ω plasmids by *Nco*I and *Sal*I restriction enzymes and cloned to the corresponding sites in the pUC131 plasmid (kindly provided by J.A. Kleinschmidt). Finally, these three genes were excised from pUC131 by *Xho*I and *Sal*I restriction enzymes and cloned to the *Xho*I site in the pBSC plasmid. The accuracy of generated pBSC/CP, pBSC/CP-E7 and pBSC/CPdel-E7 was confirmed by DNA sequencing.

The plasmids were propagated in *Escherichia coli* XL1-blue or DH5α strains cultured in Luria-Bertani broth with 100 $\mu$g/ml ampicillin added, and purified with the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany).

*In-gel digestion of proteins for detection of E7 peptide by MALDI*. For in-gel digestion with trypsin, a method adapted from Shevchenko *et al* (21) was used. The gel was washed with water (two times for 10 min) and then the band of interest was excised and cut into 1-mm cubes. The gel particles were washed three times with 0.1 M $NH_4HCO_3$/acetonitrile 1:1 (v/v) for 15 min with a volume roughly equal to 3-fold the gel volume. All remaining liquid was removed and the gel pieces were covered with 100% acetonitrile. Acetonitrile was removed after 5 min and the gel was rehydrated with 0.1 M $NH_4HCO_3$ and an equal volume of acetonitrile was added. After 15 min, all liquid was removed and the gel particles were vacuum dried. Then reduction with dithiothreitol (DTT) followed by alkylation with iodoacetamide (IAA) was performed and the samples were freeze dried. The gel pieces were covered with trypsin solution from bovine pancreas (10 $\mu$g/ml) in 50 mM $NH_4HCO_3$ and incubated at 37˚C overnight. The peptides were extracted from the gel by addition of a volume of 25 mM $NH_4HCO_3$, followed by the same volume of acetonitrile, and sonicated for 10 min. The supernatant was recovered, 100 $\mu$l of 30% acetonitrile with 0.1% trifluoro acetic acid (TFA) was added to the gel particles and sonicated again for 15 min. This step was repeated with 50% acetonitrile containing 0.1% TFA. The extracts were pooled and vacuum dried. The samples were purified on $ZipTip_{C18}$ (Millipore) prior to MALDI-TOF mass spectrometry.

*Matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry*. Analyte (tryptic digest) (1 $\mu$l) was mixed with 3 $\mu$l of matrix solution prepared as follows: 10 mg of DHB (2,5-dihydroxybenzoic acid, Sigma) were dissolved in 1 ml of 30% acetonitrile/0.1% TFA (1:2, v/v). The mixture was spotted on a MALDI target plate. The peptide mixture for external calibration was purchased from

Bruker, Germany. A mass spectrometer BIFLEX IV (Bruker) with reflector was used for analyses. Spectra of the peptide mixtures were recorded in the reflector mode at a laser wavelength of 337 nm. Peptide mass maps were searched against theoretically derived maps of investigated proteins.

*Detection of CP proteins by immunoblotting*. 293T cells were grown on 6-cm dishes and transfected with 15 $\mu$g plasmids by modified calcium-phosphate precipitation (22). After two days, the cells were collected and spun at 1200 rpm for 5 min at 4˚C. The cell pellets were washed with 1 ml ice-cold PBS (23) and resuspended in 100 $\mu$l of lysis buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 2 mM EDTA, 100 mM Tris-HCl, pH 8) (24). Cell lysates were then passed five times through a 21-G needle and centrifuged at 13000 rpm for 3 min at 4˚C. The last two steps (i.e. passing through a needle and centrifugation) were repeated. The cell lysates were mixed 1:1 with 0.02% bromphenol blue solution and denaturated by incubation at 98˚C for 3 min. Proteins were further separated by 10% SDS-PAGE, electroblotted onto a polyvinylidene difluoride (PVDF) membrane and incubated with mouse anti-CP monoclonal antibody, clone PVA 634 (25) and secondary peroxidase-labelled anti-mouse IgG antibodies (Amersham Biosciences, Little Chalfont, UK). The membranes were stained using the ECL Plus kit (Amersham Biosciences).

*Detection of CP proteins by immunofluorescence*. NIH 3T3 cells were grown on slides in 24-well plates and transfected with 4 $\mu$g of plasmids by modified calcium-phosphate precipitation (22). Two days after transfection, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 containing 5 $\mu$g/ml DAPI for 3 min. The CP protein was stained with mouse anti-CP monoclonal antibody, clone PVA 634 (25), and secondary anti-mouse IgG antibodies labeled with FITC (Sigma).

*Electron microscopy of potyvirus-like particles produced in procaryotic cells*. Purified recombinant PVA-CP proteins were prepared from *E. coli* cells transformed with pMPM4Ω-derived plasmids by ultracentrifugation on a CsCl or sucrose gradient (20). The samples were applied directly to the carbonated microscopic grids and negatively stained with uranyl acetate (26).

*Electron microscopy of potyvirus-like particles in eucaryotic cells*. 293T cells grown on slides in 24-well plates were transfected with 4 $\mu$g plasmids by modified calcium-phosphate precipitation (22). Two days after transfection, the slides were flat embedded in LR white resin. In brief, cells were rinsed with Sörensen buffer (SB; 0.1M Na/K phosphate buffer, pH 7.3), fixed with 3% formaldehyde and 0.1% glutaraldehyde in SB, dehydrated through an ethanol series of increasing concentration, embedded in LR white resin and polymerized at 4˚C for two days. Sections (80-nm-thick) were contrasted with uranyl acetate and observed with a FEI Morgagni electron microscope operating at 80 kV.

*Preparation of gene gun cartridges*. Plasmid DNA was coated onto 1-$\mu$m gold particles (Bio-Rad, Hercules, CA) as described previously (19). Each cartridge contained 1 $\mu$g of DNA coated onto 0.5 mg of gold particles.

*Stimulation of splenocytes for in vitro assays*. For *in vitro* assays, mice (3 per group) were vaccinated using a gene gun with 1 $\mu$g of plasmid DNA into the shaven abdomen, at a discharge pressure of 400 psi, and boosted with the same dose two weeks later. Splenocytes from vaccinated mice were isolated two weeks after the second vaccination and restimulated for five or six days with 0.001 $\mu$g/ml E7-specific H-2D$^b$ CTL epitope, RAHYNIVTF (aa 49-57) (27), or with 10 $\mu$g/ml E7-derived peptide, QAEPDRAHYNIVTFCCKCD (aa 44-62), carrying H-2D$^b$ CTL epitope, B cell epitope and T helper cell epitope (28). Control splenocytes were incubated without peptides.

*ELISPOT assay*. The ELISPOT assay described by Miyahira *et al* (29) and Murali-Krishna *et al* (30) was modified to detect HPV16 E7-specific T cells. The 96-well filtration plates (Millipore Corp., Bedford, MA) were coated with 10 $\mu$g/ml rat anti-mouse IFN-γ or IL-4 antibody (BD Biosciences Pharmingen, San Diego, CA) in 50 $\mu$l of PBS. After overnight incubation at 4˚C, the wells were washed and then blocked with culture medium containing 10% fetal calf serum. Different concentrations of either non-restimulated or restimulated splenocytes from each vaccinated group of mice, starting from 1x10$^6$/well, were added to the wells. Cells were incubated at 37˚C for 24 h either with or without 0.001 $\mu$g/ml $E7_{49-57}$ peptide or 10 $\mu$g/ml $E7_{44-62}$ peptide. The plates were washed and incubated with 5 $\mu$g/ml biotinylated anti-IFN-γ or anti-IL-4 antibody (BD Pharmingen) in 50 $\mu$l of PBS at 4˚C overnight. After washing, the avidin-horseradish peroxidase conjugate (BD Pharmingen) was added and the plates were incubated for 2 h at room temperature. After washing, spots were developed by adding 50 $\mu$l of 0.5 mg/ml aminoethylcarbazole solution (Fermentas Inc., Hanover, MD) and 0.03% $H_2O_2$ and incubated at room temperature for 1 h. The spots were counted using a dissecting microscope.

*Tetramer staining*. E7-specific CD8$^+$ CTLs were detected by the tetramer-staining assay in lymphocyte bulk cultures, restimulated *in vitro* for 6 days with HPV16 $E7_{49-57}$ peptide, using the R-phycoerythrin labeled H-2D$^b$/$E7_{49-57}$ tetramer reagent, as described previously (31). The stained cells were analyzed on a FACScan instrument, using Cell Quest software (Becton-Dickinson).

*Detection of anti-E7 specific antibodies by GST capture ELISA*. The enzyme-linked immunosorbent assay (ELISA) was performed as described previously (32,33). Ninety-six-well plates (Dynatech, Chantilly, VA) were coated overnight at 4˚C with 200 ng/well of glutathione casein in 50 mM carbonate buffer, pH 9.6. Thereafter, the wells were incubated for 1 h at 37˚C with 100 $\mu$l of blocking buffer (0.2% casein in PBS with 0.05% Tween-20) and washed 3 times in washing buffer (PBS with 0.05% Tween-20). The cleared lysate from *E. coli* expressing the glutathion-S-transferase (GST)-E7-tag protein diluted in blocking buffer to 25 $\mu$g/100 $\mu$l was added to each well for 1 h at 37˚C. Unbound material was washed away 5 times in washing buffer. Mouse sera assayed for anti-E7 antibodies were diluted 1:50 in blocking buffer containing 0.25 $\mu$g/$\mu$l total lysate proteins from the GST-tag-transformed *E. coli* (to block reactivities of the sera with contaminating

*E. coli* proteins) and incubated for 1 h at 37˚C. After washing the plates 5 times, bound mouse antibodies were detected by sheep anti-mouse IgG antibodies conjugated to horseradish peroxidase (Amersham Biosciences) diluted 1:2000 in blocking buffer for 1 h at 37˚C. The plates were washed 5 times and stained with 100 $\mu$l of 10 $\mu$g/ml tetramethylbenzidine (Sigma) and 0.003% $H_2O_2$ for 5-10 min. The reaction was stopped by adding 50 $\mu$l of 1 M sulfuric acid and the absorbance was measured at 450 nm.

*Detection of anti-CP specific antibodies by DAS-ELISA*. For the detection of PVA CP, the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was employed using buffers described by Clark and Adams (34) as reported by Filigarova *et al* (35). The PVA CP expressed in *E. coli* and also in tobacco leaves infected with PVA isolate Lichte Industrie (kindly provided by Dr Dedic, Potato Research Institute, Havlickuv Brod, Czech Republic) was used as an antigen.

*Tumor protection experiment*. Mice (eight per group) were twice vaccinated using a gene gun with 1 $\mu$g of plasmids at a two-week interval. Two weeks after the second vaccination, mice were subcutaneously challenged into the back with $3x10^4$ TC-1 cells suspended in 0.15 ml PBS and then monitored twice a week for tumor growth.

*Tumor therapeutic experiment*. Mice (eight per group) were subcutaneously inoculated into the back with $3x10^4$ TC-1 tumor cells suspended in 0.15 ml PBS. Three and ten days later, mice were given 1 $\mu$g of plasmids using a gene gun. The mice were monitored twice a week for tumor growth.

*Statistical analysis*. Tumor formation after DNA immunization was analyzed by the log-rank test using Prism 3 software (GraphPad Software Inc., San Diego, CA, USA). A difference between groups was considered significant if $P<0.05$.

**CP protein**
**1 MEAGTLDAGETPAQKSEDRK**
**21 KEGEGNSSKAVAVKDKDVDL**
**41 GTAGTHSVPRLKSMTSKLTL**
**61 PMLKGKSVVNLDHLLSYKPK**
**81 QVDLSNARATHEQFQNWYDG**
**101 VMASYELEKSSMEIILNGFM**
**121 VWCIENGTSPDINGVWTMMD**
**141 DEEQVSYPLKPMLDHAKPSL**
**161 RQIMRHFSALAEAYIEMRSR**
**181 EKPYMPRYGLQCNLRDQSLA**
**201 RYAFDFYEITATTPVRAKEA**
**221 HLQMKAAALKNSNTNMFGLD**
**241 GNVTTSEEDTERHTATDVNR**
**261 NMHHLLGVKGV**

**The C terminus (aa 261-288) of the CP-E7 protein**
**261 NMHHLLGVKGV QAEPDRAHYNIVTFCCK**

**The C terminus (aa 261-283) of the CPdel-E7 protein**
**261 NMHHLV QAEPDRAHYNIVTFCCK**

Figure 1. Amino acid sequence of CP protein and C-termini of CP-derived fusion proteins. The E7-derived peptide is underlined.

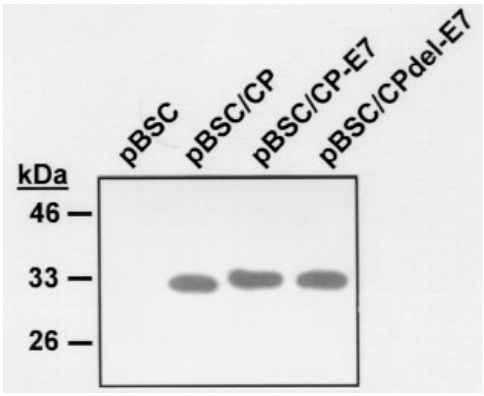


Figure 2. Immunoblotting detection of the CP antigen in 293T cells transfected with pBSC-derived plasmids. Proteins separated by 10% SDS-PAGE were transferred onto a PVDF membrane, detected by the CP-specific antibody (PVA 634) and visualized by ECL. The experiment was repeated with similar results.

## Results

*Generation of pBSC/CP, pBSC/CP-E7 and pBSC/CPdel-E7 DNA vaccines and detection of protein expression by immunoblotting*. By modifications of the 3' end of the CP gene, we generated two fusion genes linking the sequence coding for the HPV16 E7-derived peptide (aa 44-60) to the whole CP gene (CP-E7) or to the CP gene with a short C-terminal deletion (CPdel-E7) as described in Materials and methods (Fig. 1). The shortened form of CP was used because we were afraid that the fusion of the E7 peptide to the whole CP could disturb the formation of VLPs. The fusion genes and the CP gene alone were cloned into the mammalian expression plasmid, pBSC, downstream of its immediate early cytomegalovirus promoter. The expression of the CP antigen was detected in transfected 293T cells by immunoblotting. The levels of expression of CP, CP-E7 and CPdel-E7 proteins were comparable (Fig. 2). We observed small differences in the mobility of proteins depending on the size. The presence of the E7-derived peptide in the CP-E7 and CPdel-E7 proteins was further confirmed by MALDI mass spectrometry revealing the presence of 1.298 kD peptide corresponding to a C-terminal fragment of E7 peptide (AHYNIVTFCCK) generated after trypsin digestion of CP-E7 and CPdel-E7 proteins (data not shown).

*Detection of cellular location of CP proteins by immunofluorescence and visualization of PVA VLPs produced in procaryotic and eucaryotic cells by electron microsopy*. Transfection of NIH 3T3 cells and subsequent immunofluorescent staining were used to determine the cellular localization of the CP protein and CP-fusion proteins. The cells transfected with pBSC/CP, pBSC/CP-E7 or pBSC/CPdel-E7 showed cytoplasmic localization of the CP antigen (Fig. 3). In some cells, CP protein formed spindle-shaped aggregates, while aggregates of CP-E7 and CPdel-E7 proteins showed spherical morphology. In non-transfected cells no specific staining was detected (data not shown).

Electron microscopy of purified recombinant CP, CP-E7 and CPdel-E7 proteins obtained by centrifugation in a CsCl density gradient or on a sucrose cushion revealed the formation of VLPs in *E. coli* cells transformed with pMPM4Ω-derived plasmids (Fig. 4). This was the evidence that CP monomers

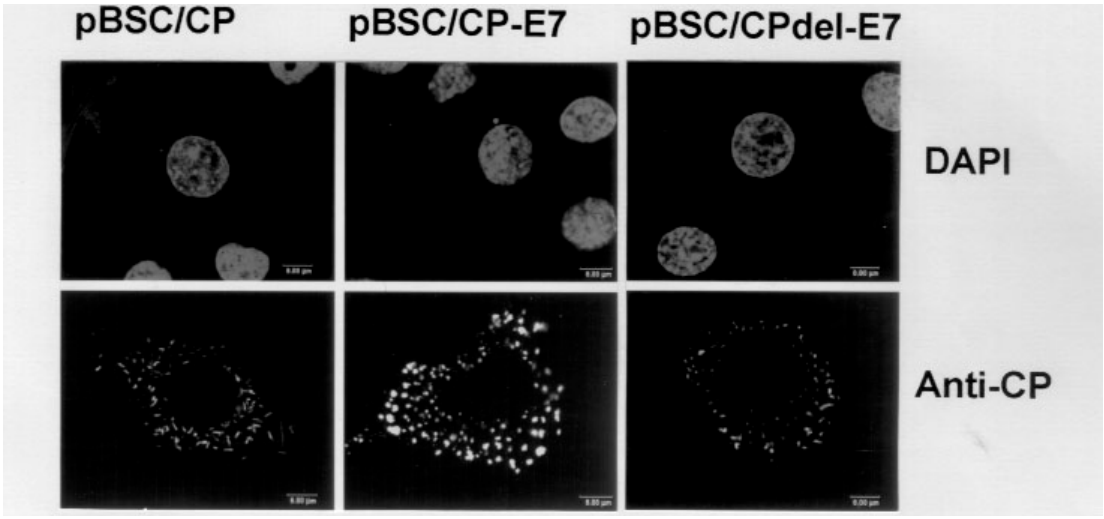


Figure 3. Immunofluorescence detection of the CP antigen in NIH 3T3 cells transfected with pBSC-derived plasmids. The paraformaldehyde-fixed cells were stained with anti-CP monoclonal antibody (PVA 634) and DAPI 2 days after transfection and examined by a confocal microscope. The scale bars represent 8 $\mu$m.

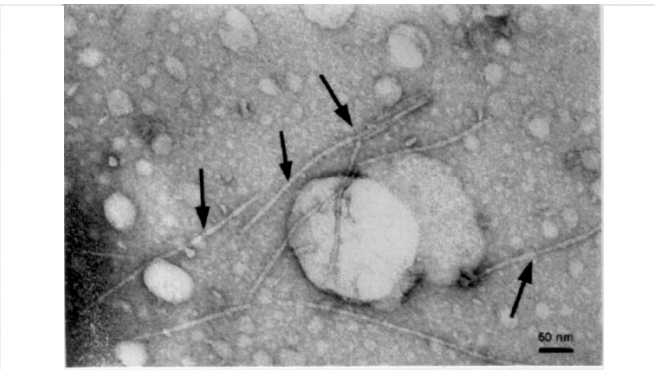


Figure 4. Transmission electron micrograph of potyvirus-like particles produced in procaryotic cells. PVA CP VLPs were purified from *E. coli* by centrifugation in CsCl density gradient. The arrows indicate VLPs. The scale bar represents 50 nm.

of PVA, when expressed in a heterologous host (*E. coli*), self-assembled to form VLPs. The morphology of CP-E7 and CPdel-E7 VLPs did not differ from that of CP VLPs, shown in Fig. 4.

Electron microscopy of 293T cells transfected with pBSC/CP, pBSC/CP-E7 or pBSC/CPdel-E7 revealed the formation of aggregates of VLPs (Fig. 5). While non-modified CP VLPs were aggregated into parallel straight bundles, aggregates of modified CP-E7 VLPs kept globular formation as the individual threads were curved. The CPdel-E7 VLPs also stuck to each other; the aggregates were similar in morphology to both CP and CP-E7 VLPs.

*Vaccination with pBSC/CP-E7 or pBSC/CPdel-E7 DNA vaccines enhanced an E7-specific CD8$^+$ T cell-mediated immune response*. We performed tetramer assays to detect the $E7_{49\text{-}57}$ peptide-loaded H-2D$^b$ tetramer positive CD8$^+$ T cells and ELISPOT assays to assess numbers of E7-specific IFN-γ- or IL-4-secreting cells in splenocytes from mice vaccinated with the set of DNA vaccines that were further investigated for their abilities to induce anti-E7 antibodies, to protect mice

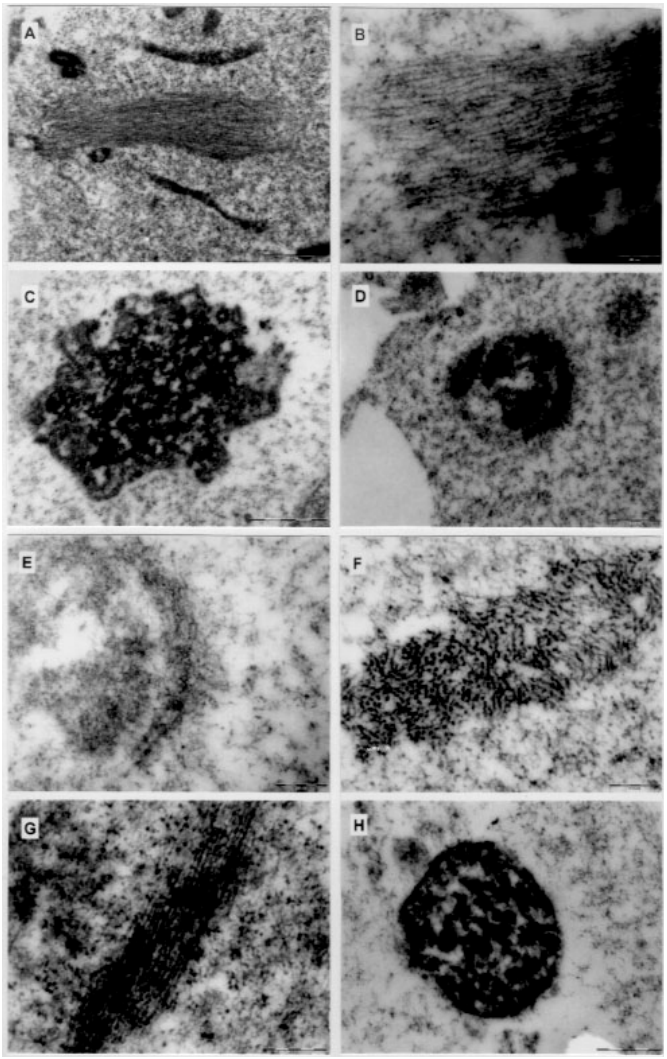


Figure 5. Transmission electron micrographs of potyvirus-like particles in mammalian cells. The 293T cells were transfected with pBSC-derived plasmids and examined by electron microscopy 2 days later. Aggregates of VLPs of PVA CP (A, B), CP-E7 (C-E) and CPdel-E7 (F-H) were found in cells. The scale bars represent 1 $\mu$m (A,C), 500 nm (D, H) or 200 nm (B, E-G).

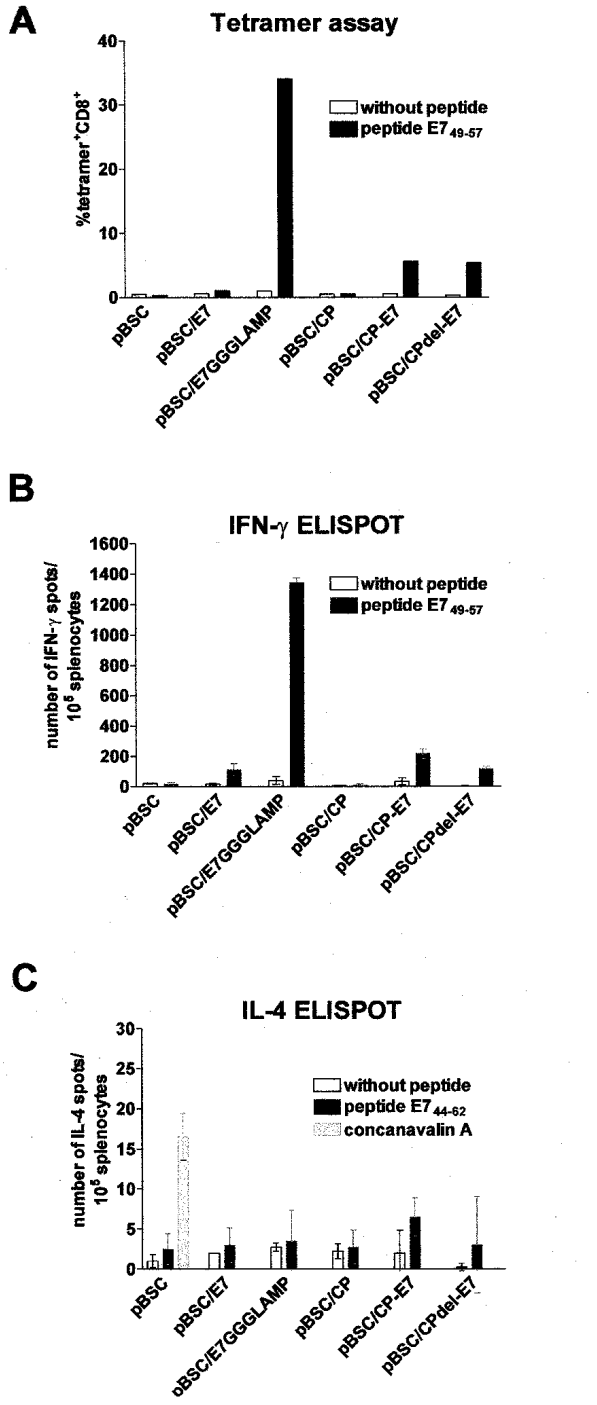


Figure 6. Detection of immune responses by tetramer staining and ELISPOT assays. Mice (n=3) were immunized two times with 1 $\mu$g of plasmids at a 2-week interval. Splenocytes were isolated two weeks after the second vaccination and incubated five days for ELISPOT assay or six days for tetramer staining with or without E7-derived peptides. $E7_{49-57}$ peptide-loaded H-2D$^b$ tetramer-positive CD8$^+$ cells were assessed by tetramer assay (A). IFN-γ-producing E7-specific CD8$^+$ lymphocytes were detected after the stimulation of splenocytes with $E7_{49-57}$ peptide (B). IL-4-secreting cells were detected in splenocytes incubated with $E7_{44-62}$ peptide (C). Splenocytes stimulated with concanavalin A were used as a positive control in the ELISPOT assay detecting IL-4 positive cells.

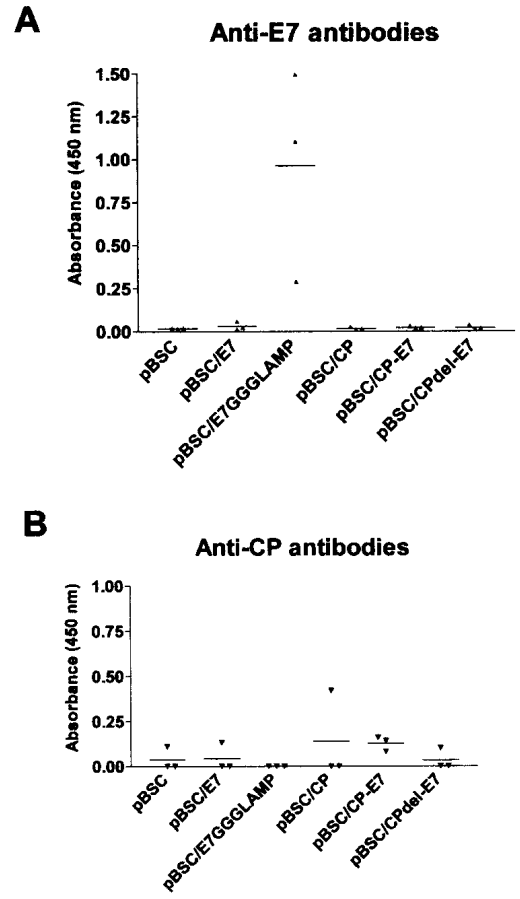


Figure 7. Detection of humoral immune responses. Mice (n=3) were immunized two times with 1 $\mu$g of plasmids. E7-specific (A) and CP-specific (B) antibodies were determined by ELISA two weeks after the last immunization in sera diluted 1:50 (A) or 1:100 (B).

against the growth of TC-1-induced tumors and cure TC-1-induced tumors.

For the tetramer assay, splenocytes were restimulated by 6-day-incubation with the $E7_{49-57}$ peptide. About 5% of restimulated splenocytes from mice vaccinated with pBSC/CP-E7 or pBSC/CPdel-E7 were both CD8 and tetramer positive, the corresponding figures for pBSC/E7-vaccinated and pBSC/E7GGGLAMP-vaccinated mice were 0.5 and 33%, respectively. No E7-specific responses were detected in mice immunized with either pBSC or pBSC/CP. The results of a representative experiment are shown in Fig. 6A.

Furthermore, we investigated the ability of the tested DNA vaccines to induce E7-specific IFN-γ-secreting splenocytes by ELISPOT assay after 5-day restimulation with the $E7_{49-57}$ peptide (Fig. 6B). About 200 and 100 E7-specific CD8$^+$ T cells were detected per 105 splenocytes isolated from the pBSC/CP-E7-vaccinated and pBSC/CPdel-E7-vaccinated mice, respectively. These counts were comparable to that of E7-specific CD8$^+$ T cells detected in pBSC/E7-vaccinated mice (about 100/10$^5$), but were approximately ten times lower than that in pBSC/E7GGGLAMP-vaccinated mice (about 1300/105). No E7-specific splenocytes were revealed in mice vaccinated with either pBSC or pBSC/CP.

We did not detect any E7-specific IL-4-secreting splenocytes by ELISPOT assay. Numbers of IL-4-secreting splenocytes did not exceed 12 per 10$^5$ regardless of whether or not they had been restimulated with the $E7_{44-62}$ peptide and, thus, neither E7-specific responses nor relevant differences among the tested groups could be observed (Fig. 6C). As a positive control, splenocytes stimulated with concanavalin A were used.

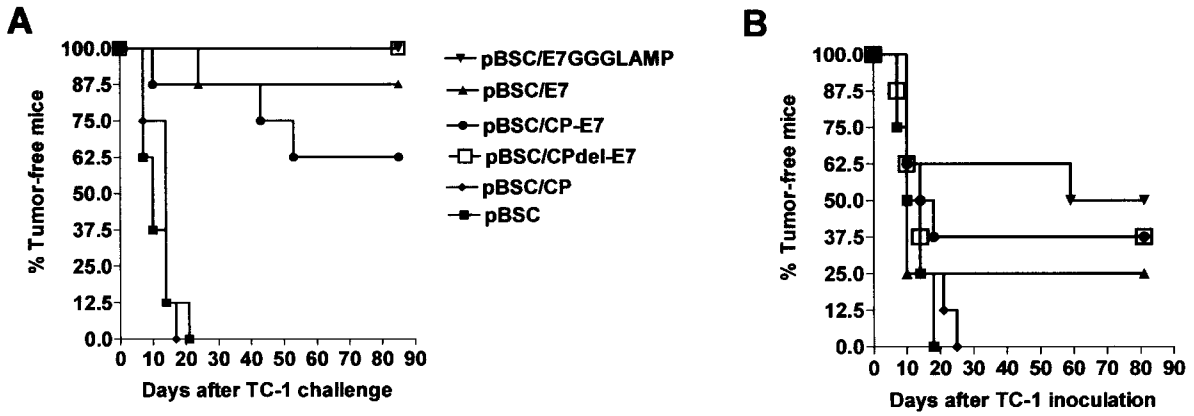


Figure 8. DNA immunization against TC-1 tumor cells. Mice (n=8) were either immunized two times at a two-week interval and challenged with $3x10^4$ mouse TC-1 tumor cells two weeks after the last vaccination (A) or inoculated with $3x10^4$ mouse TC-1 tumor cells and immunized on days 3 and 10 (B). Both experiments were repeated with similar results.

*Vaccination with the pBSC/CP-E7 or pBSC/CPdel-E7 DNA vaccines did not induce production of either anti-E7 or anti-CP antibodies*. ELISA was used to quantitate anti-E7 and anti-CP antibodies in sera of vaccinated mice (Fig. 7). Blood samples for ELISPOT assay were obtained simultaneously with splenocytes from the same mice. No anti-E7 or anti-CP antibodies were detected in sera of mice vaccinated with pBSC or pBSC-derived plasmids containing CP-E7, CPdel-E7 or E7 genes. The pBSC/E7GGGLAMP plasmid induced the production of anti-E7 antibodies in all three mice. One out of three mice immunized with the pBSC/CP plasmid was positive for anti-CP antibodies.

*Vaccination with the pBSC/CP-E7 or pBSC/CPdel-E7 DNA vaccines protected mice against the growth of E7-expressing TC-1 cells*. To determine whether vaccination with the CP-E7 or CPdel-E7 fusion genes protects mice against E7-expressing tumors, animals (8 per group) were immunized twice with plasmids by the gene gun and then challenged with TC-1 cells (Fig. 8A). All mice that received control plasmids pBSC or pBSC/CP developed tumors within 21 days. In contrast, all mice immunized with pBSC/CPdel-E7 and 5 out of 8 (62.5%) mice immunized with pBSC/CP-E7 remained tumor-free for 85 days after TC-1 challenge ($P<0.01$). Thus, both CP-E7 and CPdel-E7 generated protection against the formation of TC-1-induced tumors, comparably to E7 (87.5%) or Sig/E7GGG/LAMP-1 (100%).

The potential of DNA vaccines was also assessed by therapeutic immunization that was initiated three days after TC-1 inoculation (Fig. 8B). While all mice in control groups vaccinated with pBSC or pBSC/CP plasmids developed tumors within 25 days, 37.5% (3/8) of mice immunized with the pBSC/CP-E7 or pBSC/CPdel-E7 plasmids remained tumor-free for 80 days after inoculation of tumor cells. Both CP-E7 and CPdel-E7 cured mice with a similar efficacy to E7 (25%) or Sig/E7GGG/LAMP-1 (50%).

## Discussion

In this study, we linked the sequence coding for the HPV16 E7 peptide (aa 44-60) to the PVA CP gene, which could be helpful for the production of the E7 peptide in plants. Before the preparation of plant vaccines, we investigated the immunogenicity of fusion genes by DNA vaccination. We supposed that a DNA vaccine based on the virus-like particles expressing immunogenic epitopes could elicit a strong CTL response. Firstly, we confirmed the expression of the fusion proteins by immunoblotting and MALDI and demonstrated the formation of VLPs in procaryotic and eucaryotic cells by transmission electron microscopy. We investigated the immunogenicity of the products of the fusion genes by DNA vaccination with a gene gun on the mouse model using TC-1 cells for induction of tumors. The fusion genes significantly protected mice against the growth of TC-1-induced tumors and cured a portion of animals with pre-inoculated TC-1 cells. The antitumor effects of these fusion genes were comparable to those of the full-length E7 gene or Sig/E7GGG/LAMP-1 fusion gene. However, the other E7-fusion genes containing full-length E7 have been shown to induce more potent antitumor effects (36,37).

The chimeric CP monomers of JGMV potyvirus containing a decapeptide replacing 16 out of the 18 aa of the C-terminal surface-exposed region have been reported to retain the ability to assemble into hybrid VLPs in procaryotic as well as eucaryotic cells (14,15). In another study, the chimeric JGMV CP carrying the 98-amino-acid sequence replacing 14 amino acids at the C terminus produced in *E. coli* formed chimeric VLPs (16). Similarly, we found that the PVA CP monomers either alone or carrying the E7 peptide (aa 44-60) on their C-termini or modified C-termini with a short deletion (5 aa) could assemble into VLPs in both procaryotic and eucaryotic cells.

The model of three-dimensional structure of intact PVA particles has revealed that the N- and C- terminal regions are exposed on the surface of VLPs (38) just like in another potyvirus - JGMV (14). The JGMV VLPs carrying peptides of up to 27 aa have been shown to generate formations of parallel-laying VLPs in cells (15,16). In this study, we observed that the 'threads' of PVA CP VLPs aggregated into parallel bundles, while PVA CP-E7 VLPs formed spherical aggregates. The formation of both types of aggregates was observed with CPdel-E7 VLPs. These data suggested that the expression of the E7 epitope on the surface of PVA VLPs disturbed the laying of one VLP to another, resulting in the formation of morphologically different aggregates.

The induction of protective immunity against tumor development and low levels of antibody responses has been shown in the A31 lymphoma model for the DNA vaccine delivered by injection, comprising the fusion gene coding for the coat protein of potexvirus, linked to the modified idiotype, scFv (4). We found that the DNA vaccine coding for PVA CP, fused to the immunodominant E7 epitope and delivered by a gene gun, induced protection against the development of tumors expressing E7, but neither anti-E7 nor anti-CP antibodies were detected. The difference may result from a variation in the routes of vaccine delivery and/or the nature of both the E7-derived antigen and the CP.

In summary, we showed the potential of fusion genes linking the sequence coding for the HPV16 E7 immunodominant epitope to the PVA CP gene to induce E7-specific cellular immune responses in the mouse model.

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# Combined immunization with DNA and transduced tumor cells expressing mouse GM-CSF or IL-2

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**Abstract.** A combination of different types of vaccines usually induces enhanced immune responses in comparison to immunization with single vaccines. The highest efficacy of a heterologous prime-boost strategy is mostly achieved after priming with a DNA vaccine and boosting with a recombinant virus or a protein vaccine. The aim of this study was to determine whether the combination of a DNA and cellular vaccine elicits stronger antitumor immune responses than vaccines used alone and to find out whether the efficacy of this combined immunization depends on the sequence in which the vaccines were applied. We utilized experimental vaccines that proved to be partially effective in protection against mouse tumor cells representing models of human papillomavirus-induced malignancies. The fusion gene Sig/E7GGG/LAMP-1, inoculated via a gene gun, was used for DNA immunization. As cellular vaccines, HPV16 E6/E7 and H-ras transformed B9 or 181 cells transduced with the gene coding for GM-CSF or IL-2, respectively, were applied. In both preventive and therapeutic immunizations, inoculation first with the DNA vaccine followed by application of a cellular vaccine induced the best protection from tumor growth. These results were confirmed by detection of immune reactions with *in vitro* tests. We failed to enhance immune reactions by utilization of an equivalent mix of B9 and 181 cells, but the addition of the second DNA-vaccine dose applied simultaneously with a cellular-vaccine boost moderately increased antitumor response. Our findings suggest the benefit of the heterologous prime-boost strategy based on combination of a DNA vaccine with a cellular vaccine and importance of sequence in which the vaccines are administered.

## Introduction

Infection with human papillomaviruses (HPV) is the most important risk factor for the development of cervical cancer. As the HPV oncoproteins E6 and E7 are constitutively expressed in all tumor cells, they are considered suitable targets for immunotherapy of HPV-induced malignancies.

Some approaches in developing antitumor vaccines are based on the premise that tumor cells do not normally stimulate an effective tumor-specific immune response because they do not efficiently present tumor antigens to the relevant lymphocytes. To overcome such inadequate antigen presentation and achieve a more sustained and effective systemic antitumor response, tumor cells can be genetically modified to express immunostimulatory cytokine gene(s) and/or a suicide gene. Production of GM-CSF (1) or IL-2 (2) by transduced tumor cells was particularly efficient in stimulation of antitumor responses. In many animal models, killing tumor cells by herpes simplex virus thymidine kinase (HSV-TK) and its substrate ganciclovir (GCV) induced specific immune responses (3-6). Combinations of IL-2 or GM-CSF with HSV-TK/GCV treatment resulted in enhanced antitumor efficacy when compared with either therapy. Brokstedt *et al* (7) demonstrated that the potent antitumor response induced by the combination treatment was mediated by tumor-specific cytotoxic $CD8^+$ T lymphocytes and was not dependent on NK cells.

In our previous experiments, B9 and 181 cells expressing either mouse GM-CSF or IL-2 and the HSV-TK gene were prepared from mouse oncogenic MK16/1/IIIABC cells (MK16) transformed with HPV16 E6/E7 oncogenes and activated H-ras. Both cell lines were non-oncogenic in syngeneic mice. While two doses of subcutaneously-injected B9 cells prevented tumor development in almost all mice challenged with MK16 cells, the effect of 181 cells was insignificant. GCV treatment did not enhance antitumor response in this model (8).

A number of groups have shown that an enhanced level of immunity can be generated by using a heterologous prime-boost strategy in which animals are primed and boosted with different types of vaccines (9). The sequence in which the vaccines are applied is of great relevance to immunization efficacy. The strongest immunity is usually induced by priming with DNA and boosting with a recombinant virus or a protein vaccine (10-15).

Stimulation of $CD4^+$ T helper (Th) cells regulating effector immune cells is crucial for the induction of strong and long-lasting antitumor response (16). To achieve optimal tumor-specific immunity, the activation of both Th1 and Th2 response is required (17). Wu *et al* (18) constructed the fusion

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*Key words:* heterologous prime-boost, human papillomavirus, DNA vaccine, cellular vaccine

protein Sig/E7/LAMP-1 consisting of the HPV16 E7 oncoprotein and two sorting signals of lysosome-associated membrane protein 1 (LAMP-1). The signals target the fusion protein into the endosomal and lysosomal compartments, which results in enhanced presentation of the E7 epitopes by MHC class II molecules and increased activation of $CD4^+$ T cells (18). The enhancement of Sig/E7/LAMP-1 immunogenicity in comparison with that of wild-type E7 was demonstrated both after immunization with recombinant vaccinia viruses (18,19) and after inoculation of naked DNA via a gene gun (20).

To increase the safety of the fusion protein, we prepared the Sig/E7GGG/LAMP-1 construct in which the E7 part was mutated in the Rb-binding site. DNA immunization by a gene gun demonstrated that mutagenesis did not change the immunogenicity of the fusion gene (21).

In this study, we attempted to enhance the potency of vaccination against HPV-induced tumors by using a heterologous prime-boost strategy in which a DNA vaccine containing the Sig/E7GGG/LAMP-1 fusion gene was combined with a cellular vaccine producing IL-2 and/or GM-CSF. Furthermore, the influence of the order in which the vaccines are given on immunization efficacy was determined.

## Materials and methods

*Plasmid pBSC/E7GGGLAMP*. The plasmid pBSC/E7GGGLAMP (22) was used for DNA immunization. It carries the Sig/E7GGG/LAMP-1 gene containing three substitutions of $D_{21}$, $C_{24}$ and $E_{26}$ for glycine in the Rb-binding domain of the HPV16 E7 oncoprotein. The plasmid was propagated in *Escherichia coli* XL1-blue strain cultured in LB Broth with 100 μg/ml ampicillin added, and purified with the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany).

*Preparation of cartridges for the gene gun*. Plasmid DNA was coated onto 1-μm gold particles (Bio-Rad, Hercules, CA) as described previously (13). Each cartridge contained 1 μg of DNA coated onto 0.5 mg of gold particles.

*Cell lines*. Tumorigenic cell line MK16/1/IIIABC (MK16) was derived from C57BL/6 mouse kidney cell culture by its transfection with the HPV16 E6/E7 oncogenes and activated H-*ras* oncogene (23). Tumors induced after subcutaneous inoculation of MK16 cells metastasize spontaneously to lymph nodes and lungs. MK16 cells do not express surface MHC class I molecules *in vitro*, but their expression is inducible with interferon (IFN)-γ (24). Moreover, MHC class I molecules are also up-regulated in tumors induced by MK16 cells (25). MK16 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Linz, Austria), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete DMEM medium).

B9 and 181 cells were derived from 123IA cells, a thymidine kinase-deficient subline of MK16 cells, by transduction with the suicide gene HSV-TK and either the gene coding for mouse GM-CSF or IL-2 (8). These cells were non-oncogenic in syngeneic mice. B9 and 181 cell lines were grown in complete DMEM supplemented with HAT.

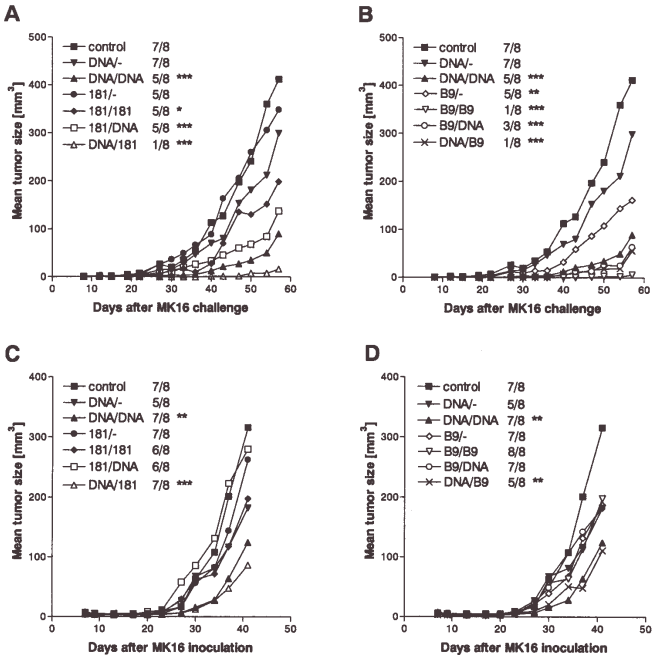


Figure 1. Tumor growth after combined immunization. In the preventive immunization experiment (A and B), mice were twice vaccinated at a 2-week interval and after another 2 weeks were s.c. challenged with $5x10^5$ MK16 cells into the back. In the therapeutic immunization experiment (C and D), mice were first inoculated with $5x10^5$ MK16 cells and then immunized on days 3 and 10. Priming vaccine/boosting vaccine, no. of mice with tumor/no. of mice in group, and statistical comparison with a control non-immunized group are indicated. $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

RMA cells are a mutagenized subline derived from the Rauscher leukemia virus-induced lymphoma RBL-5 of C57BL/6 origin (26). 2F11 cells were prepared from RMA cells by transfection with the HPV16 E7 oncogene (27). RMA and 2F11 cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol (complete RPMI-1640 medium).

*Animals*. Female 6-8-week-old C57BL/6 mice (H-$2^b$, Charles River Laboratories, Germany), were used in the immunization experiments. Animals were maintained under standard conditions, and UKCCCR guidelines for the care and treatment of animals with experimental neoplasia were followed.

*Immunization of animals*. Mice were immunized with the pBSC/E7GGGLAMP plasmid and/or the B9 and/or 181 cell lines. Plasmid DNA (1 μg) was administered by a gene gun (Bio-Rad, Hercules, CA) at a discharge pressure of 400 psi into the shaven skin of the abdomen. Cells were trypsinized and washed 3 times with PBS. A dose of $2.5x10^6$ cells in 0.2 ml PBS was injected subcutaneously (s.c.) into the flank.

*Tumor-protection experiments*. In the preventive immunization experiment, mice (8 per group) were twice vaccinated at a 2-week interval and after another 2 weeks were challenged s.c. with $5x10^5$ MK16 cells into the back. In the therapeutic immunization experiments, mice (8 per group) were first

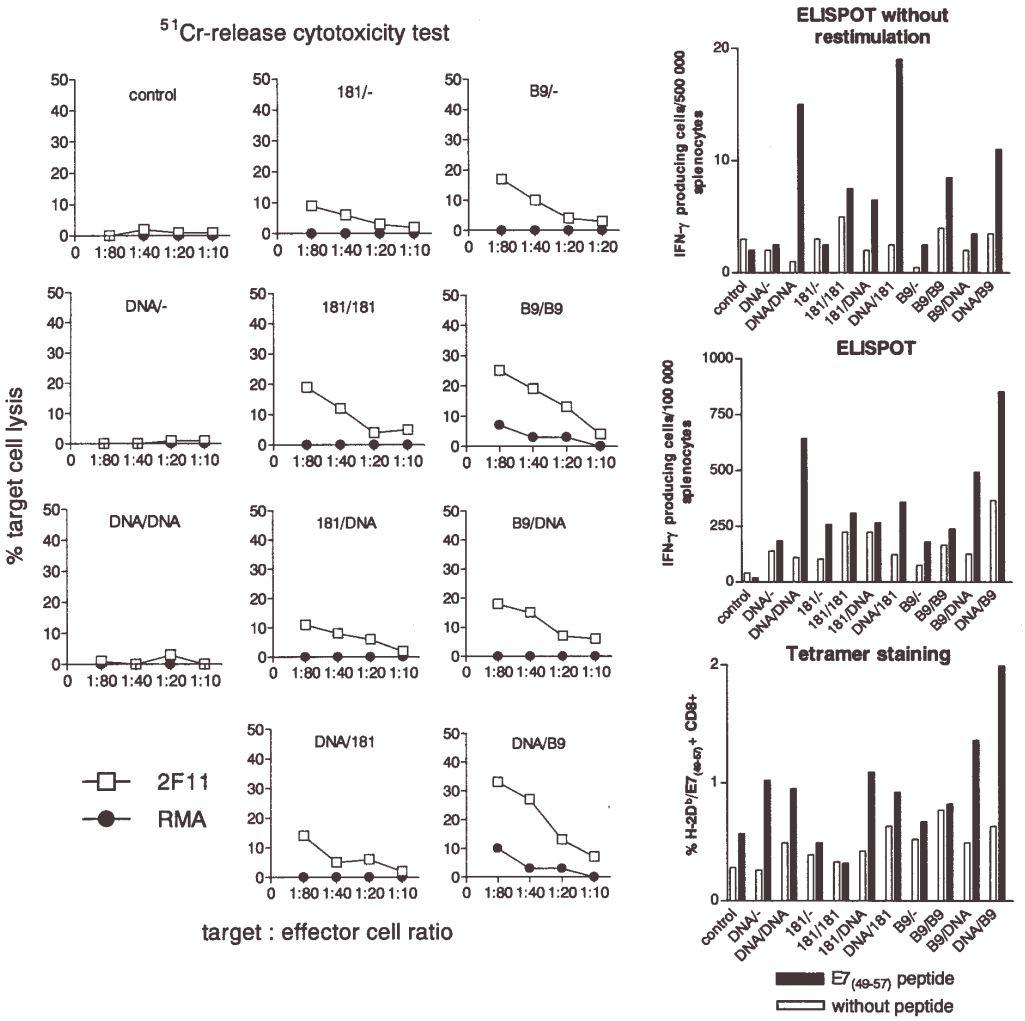


Figure 2. *In vitro* detection of immune responses after preventive immunization. Mice (3 per group) were vaccinated twice at a 2-week interval and after another 2 weeks were sacrificed and their splenocytes were pooled. Lymphocyte bulk cultures were restimulated with the E7-specific peptide (aa 49-57), and immunological assays were performed. Control lymphocytes were cultured without the peptide.

inoculated with $5x10^5$ MK16 cells and then immunized 3 and 10 days later. Tumor growth was monitored twice a week.

*Isolation of splenocytes*. Mice (3 per group) were twice immunized with plasmid DNA at a two-week interval. Two weeks after the last immunization animals were sacrificed and their splenocytes were pooled. Alternatively, mice inoculated with $5x10^5$ MK16 cells were immunized on days 3 and 10, and their splenocytes were isolated and pooled 10 days after the last immunization.

*Chromium-release cytotoxicity test*. Splenocytes ($2x10^6$/ml) were cultured for 5 days in 1 ml of complete RPMI medium supplemented with 6 ng of the RAHYNIVTF peptide derived from the HPV16 E7 oncoprotein (aa 49-57). Cells were then washed and their cytotoxicity was measured by $^{51}Cr$ release from target cells. The target cells ($2x10^6$ 2F11 or RMA cells) were labelled with 100 μCi $^{51}Cr$ for 90 min and washed 3 times with complete RPMI medium. Then, $1x10^3$ labelled cells/well (in triplicate) were incubated with effector cells using effector/target cell ratios 80, 40, 20 and 10. The percentage of specific lysis was calculated as follows: $100 \times [(cpm_{experimental}-cpm_{spontaneous})/(cpm_{maximal}-cpm_{spontaneous})]$.

Maximum release was determined by lysis of target cells in 2% Triton X-100. Spontaneous release was determined by incubating target cells in the absence of effector cells.

*ELISPOT assay*. Splenocytes were cultured in a 24-well plate at a concentration of $5x10^6$ cells/ml in complete RPMI medium. For restimulation, 6.25 ng/ml of the E7 peptide (aa 49-57) was added for 6 days. Alternatively, freshly-isolated splenocytes with 6.25 ng/ml of the peptide added were used. Detection of IFN-γ producing cells was performed as described previously (28).

*Tetramer staining*. Splenocytes were restimulated with the E7 peptide for 6 days as described in ELISPOT assay. E7-specific $CD8^+$ cells were stained with a mixture of $H\text{-}2D^b/E7_{(49\text{-}57)}$-PE tetramers and anti-mouse CD8a-FITC antibody as described previously (29). Samples were analyzed on a FACScan, using Cell Quest software (Becton-Dickinson).

*Statistical analysis*. Tumor growth was evaluated by two-way analysis of variance (ANOVA). Numbers of lung metastases were compared by two-tailed t-test. A difference between groups was considered statistically significant

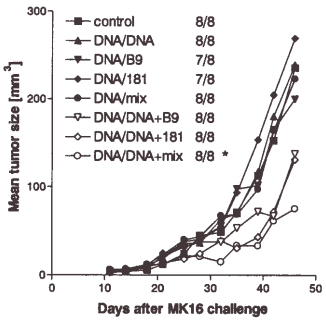


Figure 3. Tumor growth after enhanced therapeutic immunization. Mice were first inoculated with $5x10^5$ MK16 cells and then immunized on days 3 and 10. Priming vaccine/boosting vaccine(s), no. of mice with tumor/no. of mice in group, and statistical comparison with a control non-immunized group are indicated.

when $p<0.05$. Calculations were performed using GraphPad Prism version 3 (GraphPad Software, San Diego, CA).

## Results

*Superior efficacy of a DNA-vaccine prime combined with a cellular-vaccine boost*. To enhance the antitumor effect of immunization against HPV-induced malignancies, we applied a heterologous prime-boost strategy using a previously developed DNA vaccine and two cellular vaccines. For DNA immunization, the fusion gene Sig/E7GGG/LAMP-1 was inoculated via a gene gun. Live B9 and 181 cells derived from the mouse MK16 tumor cell line by transduction with GM-CSF or IL-2 genes, respectively, were utilized as cellular vaccines. C57BL/6 mice were immunized with combinations of the DNA and cellular vaccines. For comparison, immunization with 1 or 2 doses of single vaccines was also performed. In the first experiment, protection against challenge with MK16 cells was determined (Fig. 1A). After immunization with single vaccines, the best antitumor response was elicited with B9 cells (in the group immunized with 2 doses, only 1 animal developed a tumor). In view of the high immunization efficacy of B9 cells, we were not able to prove the enhancement of immune responses after combination with the DNA vaccine. However, combinations of the DNA vaccine with 181 cells clearly showed that the prime with DNA and the boost with cells was the most efficient immunization procedure utilizing 181 cells (1 mouse out of 8 developed a tumor).

In the next experiment, we tested the efficacy of a combined vaccination in a therapeutic setting. The immunization was initiated 3 days after inoculation of MK16 cells (Fig. 1B). In this scheme, a weak antitumor effect was induced. Tumors developed in most animals of all groups. Significant reduction of tumor growth was recorded only after immunization with DNA/181, DNA/B9 or DNA/DNA.

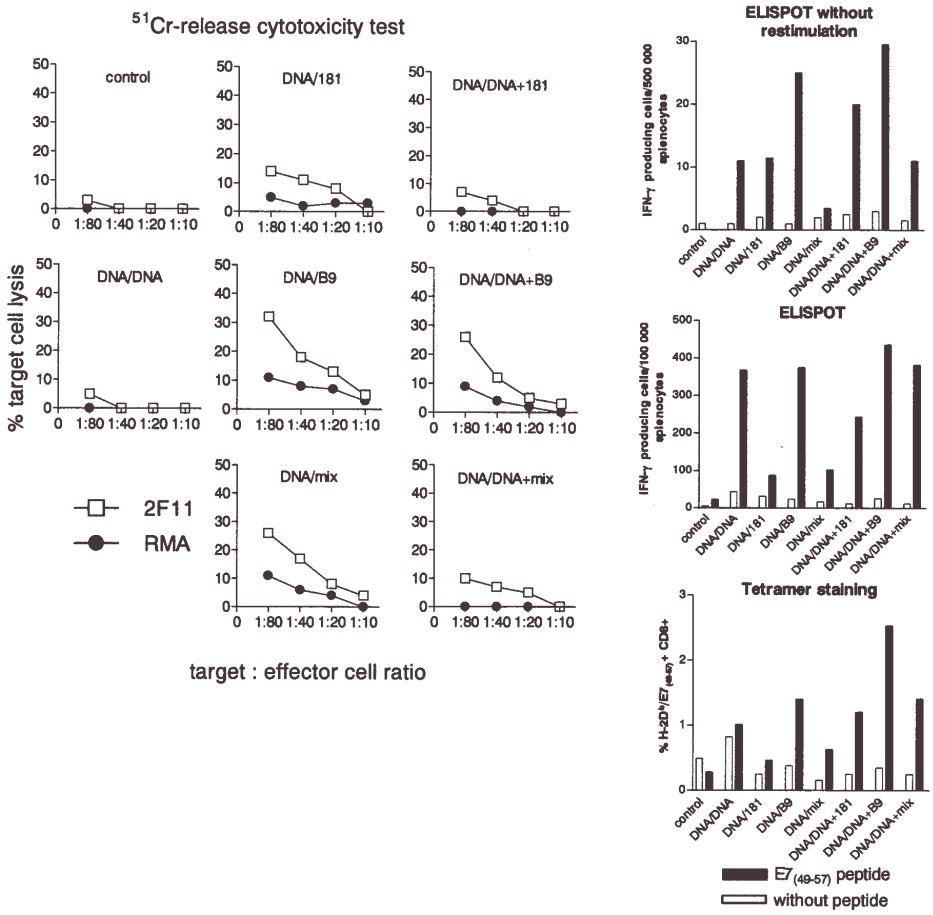


Figure 4. *In vitro* detection of immune responses after enhanced therapeutic immunization. Mice (3 per group) were first inoculated with $5x10^5$ MK16 cells and then immunized on days 3 and 10 to be sacrificed 10 days later; their splenocytes were pooled. Lymphocyte bulk cultures were restimulated with the E7-specific peptide (aa 49-57) and immunological assays were performed. Control lymphocytes were cultured without the peptide.

Table I. Metastases to lungs.
A, Metastases were determined after preventive immunization (Fig. 1A) in mice with primary tumors of about 15 mm in the longest diameter.

| | No. of mice | | | | |
|---|---|---|---|---|---|
| | With primary tumor | With macro-metastases | With micro-metastases | Without metastases | Mean no. of macro-metastases |
| Control | 7 | 6 | 1 | 0 | 13.3±2.6 |
| DNA/- | 7 | 6 | 0 | 1 | 15.3±3.9 |
| DNA/DNA | 5 | 4 | 0 | 1 | 10.8±4.2 |
| 181/- | 5 | 4 | 1 | 0 | 6.0±2.3 |
| 181/181 | 5 | 3 | 1 | 1 | 10.2±5.0 |
| 181/DNA | 5 | 4 | 0 | 1 | 14.6±5.9 |
| DNA/181 | 1 | 1 | 0 | 0 | 11.0 |
| B9/- | 5 | 3 | 1 | 1 | 9.5±5.5 |
| B9/B9 | 1 | 1 | 0 | 0 | 3.0 |
| B9/DNA | 3 | 3 | 0 | 0 | 14.3±8.4 |
| DNA/B9 | 1 | 1 | 0 | 0 | 14.0 |

B, Metastases were determined after therapeutic immunization (Fig. 1B) in mice with primary tumors of about 15 mm in the longest diameter.

| | No. of mice | | | | |
|---|---|---|---|---|---|
| | With primary tumor | With macro-metastases | With micro-metastases | Without metastases | Mean no. of macro-metastases |
| Control | 7 | 7 | 0 | 0 | 9.0±3.2 |
| DNA/- | 5 | 5 | 0 | 0 | 14.2±5.3 |
| DNA/DNA | 7 | 7 | 0 | 0 | 6.6±3.5 |
| 181/- | 7 | 6 | 1 | 0 | 8.1±3.1 |
| 181/181 | 7 | 6 | 1 | 0 | 4.3±1.0 |
| 181/DNA | 8 | 7 | 1 | 0 | 7.1±2.9 |
| DNA/181 | 8 | 7 | 0 | 1 | 8.1±2.4 |
| B9/- | 7 | 7 | 0 | 0 | 5.0±2.1 |
| B9/B9 | 8 | 8 | 0 | 0 | 8.0±1.7 |
| B9/DNA | 7 | 7 | 0 | 0 | 9.9±2.5 |
| DNA/B9 | 8 | 8 | 0 | 0 | 6.2±2.6 |

Immunoreactions elicited in the preventive immunization scheme were determined by several *in vitro* assays (Fig. 2). With the exception of the ELISPOT assay without re-stimulation, superior efficacy of B9 cells in comparison with 181 cells was confirmed, especially if combined with the DNA prime. Surprisingly, while ELISPOT assays and tetramer staining detected relatively high efficacy of 2 doses of the DNA vaccine alone, no positive reactivity was demonstrated in these animals in the $^{51}$Cr-release test.

*Attempts to enhance therapeutic immunization with combined vaccines*. In view of the low effect of therapeutic immunization, we tried to increase the efficacy of the most promising immunization procedure, DNA prime/cell boost, by two ways: (i) utilizing a mixed cellular vaccine containing equal numbers of B9 and 181 cells and (ii) adding the DNA vaccine to the second (boost) immunization (Fig. 3). However, with the exception of two mice, tumors developed in all animals. Significant inhibition of tumor growth was induced only in the group primed with DNA and boosted with DNA plus the mixture of both cells. DNA vaccine inoculated in the second immunization in addition to B9 and 181 cells also reduced tumor growth, but this effect was insignificant. *In vitro* tests of immunoreactivity (ELISPOT assays and tetramer staining) performed with splenocytes isolated from animals subjected to therapeutic immunization (including MK16 inoculation) confirmed the beneficial effect of the DNA vaccine applied as a boost together with transduced cells, but did not demonstrate the contribution of the cellular mix (Fig. 4).

*Inhibition of lung metastases*. As MK16 cells metastasize spontaneously to lungs, we assessed the ability of the vaccines to inhibit the formation of lung metastases in mice inoculated with MK16 cells. We sacrificed mice when the primary tumor reached 15 mm in diameter. In comparison with control mice, neither preventive nor therapeutic vaccination has any effect on the formation of metastases. Results of the preventive and first therapeutic immunization experiments are given in Table I. Similarly, no protection from metastases was recorded in the second therapeutic experiment (data not shown). Differences in numbers of superficial macrometastases were also insignificant (data not shown).

## Discussion

In our previous studies, we prepared and tested two types of experimental vaccines against tumors induced by HPV: (i) We modified the Sig/E7/LAMP-1 fusion gene developed by Wu *et al* (18) and used the resultant Sig/E7GGG/LAMP-1 gene for DNA immunization by a gene gun. We achieved relatively good protection from tumors induced by the original TC-1 cells, but a substantially lower effect was recorded when using TC-1 clones with down-regulated surface expression of MHC class I molecules for challenge (22). (ii) We transduced the 123IA subline of MK16 cells with the HSV-TK suicide gene and gene coding for GM-CSF (B9 cells) or IL-2 (181 cells). Production of both cytokines abolished oncogenicity of 123IA cells. However, while the preventive immunization with B9 cells almost completely inhibited tumor formation from MK16 cells, the effect induced with 181 cells was low and not enhanced by treatment with GCV (8).

In an attempt to increase the efficacy of vaccination against tumor cells producing the E7 oncoprotein, we tested a heterologous prime-boost strategy in this study. Immunization with transduced tumor cell lines B9 and 181 was combined with application of a DNA vaccine containing the Sig/E7GGG/LAMP-1 gene. In accordance with the published data (10-15) we showed the benefit of priming with a DNA vaccine and boosting with a vaccine of another type, a cellular vaccine in our case. Using this approach, we enhanced the efficacy of 181 cells in preventive immunization against MK16 cells. In therapeutic immunization, the effect of

combined vaccination was still evident, but antitumor response was weak. Therefore, various improvements to the immunization procedure were tested. A double dose of B9 or 181 cells (data not shown) or an equivalent mix of both cell lines failed to elicit a higher immune response in combination with the DNA vaccine. Addition of the second dose of the DNA vaccine administered simultaneously with the cellular-vaccine boost induced enhanced immune responses both in the tumor-protection experiment and *in vitro* immunological tests, but the effect was low.

In the study comparing the influence of GM-CSF and IL-2 secretion by tumor cells in a preventive and therapeutic immunization model, Castleden *et al* (30) have shown that while preventive immunization with cells producing GM-CSF generated long-term systemic protection, cells producing IL-2 were more efficient in therapeutic immunization against established tumors. Similar results were observed with the B9 and 181 cells in a previous study (31). The results obtained after preventive immunization in the present study seem to be in accordance with those data. B9 cells alone induced better protection from tumor growth than 181 cells. Owing to the high efficacy of the B9 vaccine, it was not possible to get the same effect after priming with DNA in the tumor-protection experiment, but in *in vitro* tests (ELISPOT assays and tetramer staining) after restimulation of splenocytes, both B9 cells alone and combined with DNA priming generated stronger immunity in comparison with 181 cells. In the ELISPOT assay without restimulation, inverse effects were observed; immunizations utilizing 181 cells resulted in more efficient activation of E7-specific T lymphocytes. This difference may reflect the difference in the types of detected cells. We suppose that after restimulation, memory T cells were mostly detected, while without restimulation, effector T cells were evaluated.

As tumors induced after s.c. inoculation of MK16 cells metastasize spontaneously to lungs and lymph nodes, we also determined the influence of immunization on the formation of metastases. We removed lungs from mice, when the primary s.c. induced tumors achieved approximately the same size, and counted the superficial lung metastases. Our results demonstrate that neither IL-2 nor GM-CSF inhibited metastatic growth. They are in accordance with those of experiments comparing secreted and membrane-bound GM-CSF in a mouse B16F10 melanoma model (32), but in discrepancy with the findings of some experiments using 181 cells in an MK16 tumor model (2). However, in latter experiments, 181 cells were inoculated peritumorally and lungs for metastasis evaluation were removed on the same day after such treatment without respect to the size of primary tumors. As correlation between the size of s.c. MK16-induced tumors and the number of lung metastases in non-immunized mice was reported (24), the reduction in the size of primary tumors could be closely associated with an antimetastatic effect of IL-2 (2).

In conclusion, we showed enhancement of immune responses against tumor cells producing HPV16 E7 oncoprotein by a heterologous prime-boost strategy. We confirmed the benefit of priming with a DNA vaccine and boosting with a more complex vaccine, e.g. a cellular vaccine as used in this study.

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**RESEARCH ARTICLE**

# Enhancement of immunogenicity of HPV16 E7 oncogene by fusion with *E. coli* β-glucuronidase

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## Abstract

**Background** Human papillomavirus type 16 (HPV16) E7 is an unstable oncoprotein with low immunogenicity. In previous work, we prepared the E7GGG gene containing point mutations resulting in substitution of three amino acids in the pRb-binding site of the HPV16 E7 protein.

**Methods and results** To increase E7GGG immunogenicity we constructed fusion genes of *E. coli* β-glucuronidase (GUS) with one or three copies of E7GGG. Furthermore, a similar construct was prepared with partial E7GGG (E7GGGp, 41 amino acids from the N-terminus). The expression of the fusion genes was examined in human 293T cells. Quantification of GUS activity and the amount of E7 antigen showed substantially reduced GUS activity of fusion proteins with complete E7GGG that was mainly caused by decrease of their steady-state level in comparison with GUS or E7GGGpGUS. Still, the steady-state level of E7GGG.GUS was about 20-fold higher than that of the E7GGG protein. The immunogenicity of the fusion genes with complete E7GGG was tested by DNA immunisation of C57BL/6 mice with a gene gun. TC-1 cells and their clone TC-1/A9 with down-regulated MHC class I expression were subcutaneously (s.c.) inoculated to induce tumour formation. All mice were protected against challenge with TC-1 cells and most animals remained tumour-free in therapeutic-immunisation experiments with these cells, in contrast to immunisation with unfused E7GGG and the fusion with the lysosome-associated membrane protein 1 (Sig/E7GGG/LAMP-1). Significant protection was also recorded against TC-1/A9 cells. Both tetramer staining and ELISPOT assay showed substantially higher activation of E7-specific $CD8^+$ lymphocytes in comparison with E7GGG and Sig/E7GGG/LAMP-1. Deletion of 231 bp in the GUS gene eliminated enzymatic activity, but did not influence the immunogenicity of the E7GGG.GUS gene.

**Conclusions** The findings demonstrate the superior immunisation efficacy of the fusion genes of E7GGG with GUS when compared with E7GGG and Sig/E7GGG/LAMP-1. The E7GGG.GUS-based DNA vaccine might also be efficient against human tumour cells with reduced MHC class I expression.


**Keywords** human papillomaviruses; E7 oncogene; GUS; DNA vaccine; fusion genes

## Introduction

Administration of plasmid DNA is a simple vaccination approach that induces both humoral and cellular immune responses. Due to a number of advantages, DNA vaccines hold promise for future extensive use. They were demonstrated to activate effective immunity against both infectious agents and tumour cells. However, the

immune responses induced are usually weak when compared with other types of vaccines. Several strategies have been developed to increase the efficacy of DNA vaccines. These include combination with genes coding for immunostimulatory factors, stimulation with adjuvants, construction of modified genes, and boosting with heterologous vaccine [1,2].

The E7 oncoprotein of human papillomavirus type 16 (HPV16) is frequently used as a model antigen for studies of anti-tumour immunity. In cooperation with the viral E6 oncoprotein, E7 is necessary for both the oncogenic transformation of cells and maintenance of the transformed state [3]. For this reason it represents a suitable target for vaccination. HPVs have been identified as a key aetiological agent of cervical carcinoma and their association with some other malignancies has been suggested [4]. Cervical carcinoma is the second most common malignancy in women worldwide with a mortality rate of about 60%. Therefore, anti-HPV vaccine development is the focus of current papillomavirus research. As the E7 oncogene is only a weak immunogen, several fusion and/or modified genes have been constructed and their enhanced efficacy has been demonstrated [5–8]. The Sig/E7/LAMP-1 gene has been prepared by fusion with the sorting signals of lysosome-associated membrane protein 1 (LAMP-1) [9]. To increase its safety, we have introduced three point mutations into the pRb-binding site of E7 (Sig/E7GGG/LAMP-1 gene) without reduction of immunogenicity in mice [10].

The gene coding for *E. coli* $\beta$-glucuronidase (GUS) is frequently used as a reporter gene in experiments with plant cells. The enzyme is very stable in these cells. Therefore, epitopes from animal viruses have been fused with GUS and transgenic plants expressing these constructs have been prepared [11,12]. Detection of GUS enzymatic activity has enabled easy selection of plants with the highest level of transgene expression (up to 3% of total soluble proteins). High immunogenicity of animal-virus epitopes has been demonstrated after immunisation of animals with plant extracts containing fusion products [11,12].

In this study, we constructed E7GGG-GUS fusion genes and characterised their expression in mammalian cells. Immunogenicity of these fusion genes was compared with the Sig/E7GGG/LAMP-1 gene in preventive and therapeutic DNA immunisation experiments. Increased efficacy was demonstrated against both TC-1 cells expressing the HPV16 E7 protein [13] and their derivative, TC-1/A9 cells, with down-regulated production of MHC class I molecules [10].

# Materials and methods

## Plasmids

MonoGUS plasmid [14] with short polylinker EcoRI-SmaI(1)-BamHI-SmaI(2) followed by the GUS gene was modified using procedures for the preparation of recombinant DNA plasmids described by Sambrook *et al*. [15]. The cauliflower mosaic virus transcription termination signal present in MonoGUS was excised with SacI. The 132-bp PvuII fragment E7GGGp was excised from the plasmid pBSC/E7GGG [6] and cloned into the SmaI(1) site of MonoGUS after partial digestion with SmaI. The resulting fusion gene E7GGGpGUS used in this study contains an open-reading frame (ORF) comprised of 41 codons from the N-terminus of E7GGG, 9 linker codons and 603 codons of GUS. Alternatively, the whole E7GGG gene was amplified from the pBSC/E7GGG using primers E7-1: 5′-CCAGGATCCATCATGCATGGAGATACACC-3′ (forward) and E7-2: 5′-CAGCCATGGTGGATCCTGGTTTCTGAGAACAG-3′ (reverse), digested with BamHI (underlined sequences in primers) and cloned into the unique BamHI site of MonoGUS. *In frame* fusions E7GGG.GUS with 8 linker codons between E7GGG and GUS and E7GGG(3×)GUS containing three copies of E7GGG and an additional 3 linker codons between E7GGG genes were selected. Finally, all these fusion genes and the GUS gene alone (Figure 1a) were excised with EcoRI and cloned into the mammalian expression plasmid pBSC [6].

To eliminate enzymatic activity of GUS, plasmids pBSC/GUS and pBSC/E7GGG.GUS were digested with EcoRV and religated. Then the clones with the deletion of 231 bp in GUS were isolated (Figure 1b).

Plasmids were purified with the Qiagen Plasmid Maxi kit (Qiagen, Hilden, Germany).

## Cell lines

TC-1 cells prepared by transformation of C57BL/6 primary mouse lung cells with the HPV16 E6/E7 oncogenes and activated *H-ras* [13] were kindly provided by T.-C. Wu

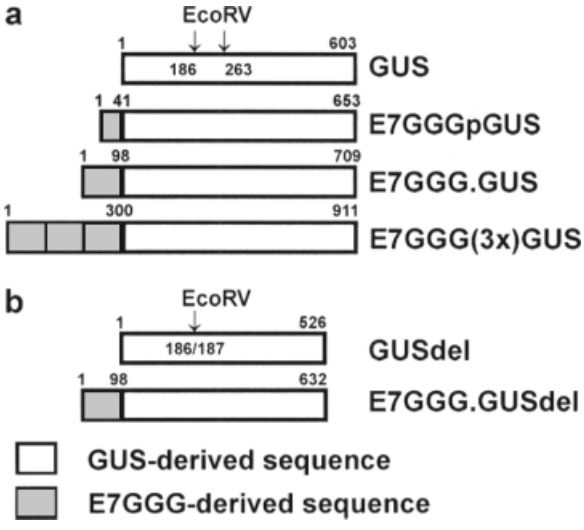


**Figure 1. Schematic representation of genes cloned into the mammalian expression plasmid pBSC. The genes contain either the complete GUS open reading frame (ORF; a) or the GUS ORF deleted between EcoRV sites as indicated (b). The deletion resulted in removal of 77 amino acids from the corresponding products**

(Johns Hopkins University, Baltimore, MD, USA). TC-1/A9 cells with down-regulated MHC class I expression were derived from TC-1 cells that formed a tumour in the mouse immunised with the mutated HPV16 E7 gene [10]. 293T cells (kindly provided by J. Kleinschmidt, DKFZ, Heidelberg, Germany) were derived from human embryonic kidney cells by transformation with adenovirus 5 DNA [16] and subsequently by transduction with simian virus 40 (SV40) large T antigen [17]. NIH 3T3 fibroblasts were established from a mouse embryo culture [18]. All cells were grown in high glucose Dulbecco's modified Eagle's medium (D-MEM; Life Technologies, Paisley, Scotland, UK) supplemented with 10% foetal calf serum (FCS; PAA Laboratories, Linz, Austria), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

## Animals

Six- to eight-week-old female C57BL/6 mice (H-$2^b$) (Charles River, Germany) were used in the immunisation experiments. Animals were maintained under standard conditions and the UKCCCR guidelines for the care and treatment of animals with experimental neoplasia were observed.

## Analysis of GUS activity

293T cells ($0.7 \times 10^6$) were seeded into 6-cm plates and transfected the next day by modified calcium phosphate precipitation in HEPES-buffered saline solution [19] with 6 μg of plasmids. Cells were incubated for 2 days, collected, and lysed on ice in 300 μl of GUS buffer (50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 10 mM $\beta$-mercaptoethanol, 0.1% Triton X-100). After two cycles of freezing and thawing, precipitate was removed by centrifugation. GUS activity in 10 μl of supernatant was assayed with the 4-methylumbelliferyl-$\beta$-D-glucuronide (MUG) substrate [20]. Protein concentration was measured according to Bradford [21].

## Immunoblotting staining

Lysates in GUS buffer were analysed by 10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Little Chalfont, UK). The membrane was blocked with 10% non-fat milk in PBS and incubated with mouse monoclonal anti-E7 antibody (clone 8C9; Zymed, San Francisco, CA, USA) or rabbit polyclonal anti-GUS antibodies (Molecular Probes, Eugene, OR, USA) and, subsequently, with horseradish-peroxidase-conjugated secondary antibodies of appropriate specificity (Amersham Biosciences). Blots were stained using the ECL Plus system (Amersham Biosciences) or by incubation with 1 mg/ml diaminobenzidine (DAB) and 0.03% $H_2O_2$.

## Immunofluorescence staining

NIH 3T3 cells were grown on 24-well slides and transfected with 0.5 μg plasmids using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany). Two days after transfection cells were fixed with 4% paraformaldehyde for 10 min and permeabilised with 0.2% Triton X-100 in PBS for 3 min. E7 antigen was stained with E7-specific mouse monoclonal antibody (Zymed) followed by FITC-conjugated secondary anti-mouse IgG antibody (Sigma, St. Louis, MO, USA). The slides were examined using a Nikon Eclipse 600 epifluorescence microscope.

## Northern blot hybridisation

293T cells ($2 \times 10^6$) were seeded into 10-cm plates and transfected the next day by modified calcium phosphate precipitation in HEPES-buffered saline solution [19] with 15 μg of plasmids. After 2 days total RNA was isolated using the RNeasy Mini kit (Qiagen). RNA (5 μg) was separated on a 1% agarose/formaldehyde gel and blotted onto a Hybond $N^+$ membrane (Amersham Biosciences). The membrane was hybridised with the digoxigenin-labelled GUS or $\beta$-actin probe in DIG Easy Hyb solution (Roche Diagnostics) and the bound probe was visualised by incubation with CSPD-ready-to-use substrate (Roche Diagnostics). The digoxigenin-labelled probes were prepared by polymerase chain reaction (PCR) with digoxigenin-dUTP (Roche Diagnostics). In PCR, we used primers for GUS (forward, 5′-TCGATGCGGTCACTCATTAC-3′; reverse, 5′-CCACGGTGATATCGTCCAC-3′) and $\beta$-actin (forward, 5′-CCAGAGCAAGAGAGGTATCC-3′; reverse, 5′-GAGTCCATCACAATGCCTGT-3′) as described previously [10].

## Preparation of cartridges for gene gun

Plasmid DNA was coated onto 1-μm gold particles (Bio-Rad, Hercules, CA, USA) as described previously [6]. Each cartridge contained 0.5 mg gold particles coated with 1 μg DNA.

## Immunisation experiments

Mice were immunised with plasmids using the gene gun at a discharge pressure of 400 psi into the shaven skin of the abdomen. Each immunisation consisted of one shot delivering 1 μg of plasmid DNA. In immunisation/challenge experiments, mice (5 or 8 per group) were vaccinated with two doses administered at a 2-week interval, and 2 weeks after the last vaccination the animals were challenged s.c. into the back with

$3 \times 10^4$ TC-1 or TC-1/A9 cells suspended in 150 µl PBS. In therapeutic immunisation experiments, mice (8 per group) were first inoculated with $3 \times 10^4$ TC-1 cells and then immunised 3 or 4 and 10 days later. Tumour cells were administered under anaesthesia with etomidatum (0.5 mg i.p./mouse; Janssen Pharmaceutica, Beerse, Belgium). Tumour growth was monitored twice a week.

## Tetramer staining

Mice were immunised by a gene gun with two doses of plasmids given at a 2-week interval. Two weeks after the second dose, tetramer staining was performed as described previously [22]. In brief, lymphocyte bulk cultures were prepared from splenocytes of three immunised animals and restimulated with the HPV16 $E7_{(49-57)}$ peptide (RAHYNIVTF) for 6 days. After incubation with anti-mouse CD16/CD32 antibody (Fc-block; BD Biosciences Pharmingen, San Diego, CA, USA), lymphocytes were stained with a mixture of H-$2D^b/E7_{(49-57)}$-PE tetramers and anti-mouse CD8a-FITC antibody (BD Biosciences Pharmingen). The stained cells were analysed on a FACScan instrument using CellQuest software (Becton Dickinson).

## ELISPOT assay

Ninety-six-well filtration plates (MAHA 45; Millipore, Bedford, MA, USA) were coated with 5 µg/ml of rat anti-mouse IFN-$\gamma$ monoclonal antibody (clone R4-6A2; BD Biosciences Pharmingen) and blocked with RPMI-1640 supplemented with 10% FCS. Lymphocytes ($5 \times 10^5$, prepared as described for tetramer staining) were added to the wells and cultivated at 37 °C in 5% $CO_2$ for 20 h in the presence or absence of the $E7_{(49-57)}$ peptide (25 ng/ml). Wells were washed and incubated overnight at 4 °C with 2 µg/ml of biotinylated rat anti-mouse IFN-$\gamma$ monoclonal antibody (clone XMG 1.2; BD Biosciences Pharmingen). Plates were then washed and incubated for 3 h at 37 °C with avidin-horseradish-peroxidase conjugate (BD Biosciences Pharmingen). Spots were stained with 3-amino-9-ethylcarbazole.

## Statistical analysis

Tumour formation in immunisation experiments was analysed by log-rank test. Groups of mice with regression of some tumours were compared in contingency tables by two-tailed Fisher's exact test. Results were considered significantly different if $P < 0.05$. Calculations were performed using GraphPad Prism version 3.0 (GraphPad Software, Inc., San Diego, CA, USA).

# Results

## Construction of fusion genes and GUS activity of their products in transfected 293T cells

To increase the immunogenicity of the E7 protein we fused the mutated HPV16 E7 gene (E7GGG) or the portion corresponding to 41 amino acids from the N-terminus (partial E7GGG, E7GGGp) with the 5′-end of the gene coding for GUS. Ligation resulted in fusion genes with one copy of E7GGG or E7GGGp or with three copies of E7GGG. The fusion genes (and also the GUS gene alone) were inserted into the mammalian expression plasmid pBSC (Figure 1a). Expression of cloned genes was detected after transfection of human 293T cells by analysis of enzymatic activity of GUS (Figure 2). High GUS activity was recorded for GUS alone and for the fusion protein containing E7GGGp (E7GGGpGUS). Fusions with complete E7GGG had markedly reduced GUS activity – in comparison with GUS, only about 5 and 2% of GUS activity was detected for E7GGG.GUS and E7GGG(3×)GUS, respectively.

## Detection of fusion proteins in transfected 293T cells

Lysates of transfected 293T cells used for determination of GUS activity of fusion proteins were analysed by SDS-PAGE and immunoblotting. After staining of the gel with Coomassie brilliant blue, strong bands corresponding in size to GUS and E7GGGpGUS were detected in respective

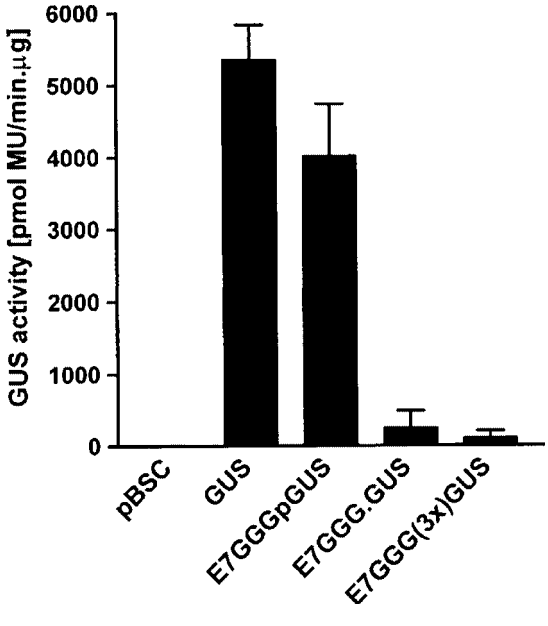


**Figure 2. Detection of GUS activity in 293T cells transfected with pBSC-derived plasmids. Enzymatic activity in cellular extracts was tested by reaction with 4-methylumbelliferyl-β-D-glucuronide (MUG) substrate. The results are shown as produced amount of 4-methylumbelliferone (MU) per min per µg of soluble proteins. Bars represent means of three independent experiments (error bars–standard deviations)**

samples of 293T lysates (Figure 3a). Immunoblotting analysis with GUS- and E7-specific antibodies verified that the bands were specific for these proteins (see below). Comparison of diluted samples with serial dilution of bovine serum albumin (BSA) revealed that GUS and E7GGGpGUS comprised about 6 and 8% of total extracted proteins, respectively (Figure 3b).

Although the production of E7GGG.GUS and E7GGG (3×)GUS was not visible after staining with Coomassie brilliant blue, these products were detected by immunoblotting with an E7-specific monoclonal antibody (Figure 4a). This approach also revealed that, after transfection of the pBSC/E7GGG(3×)GUS plasmid, E7GGG(2×)GUS and E7GGG.GUS proteins were probably also produced in 293T cells, but at substantially lower level than E7GGG(3×)GUS (Figure 4a).

Because of large differences in the amount of GUS fusion proteins that were produced, we performed serial dilutions of samples to determine their relative level of production. From these experiments we concluded that the steady-state level of the E7GGGpGUS protein was about 10–20-fold and 30–40-fold higher than that of the E7GGG.GUS and E7GGG(3×)GUS proteins, respectively (Figure 4b). Furthermore, we found that the steady-state level of the E7GGG.GUS protein was 20–30-fold higher than that of the E7GGG protein (Figure 4c).

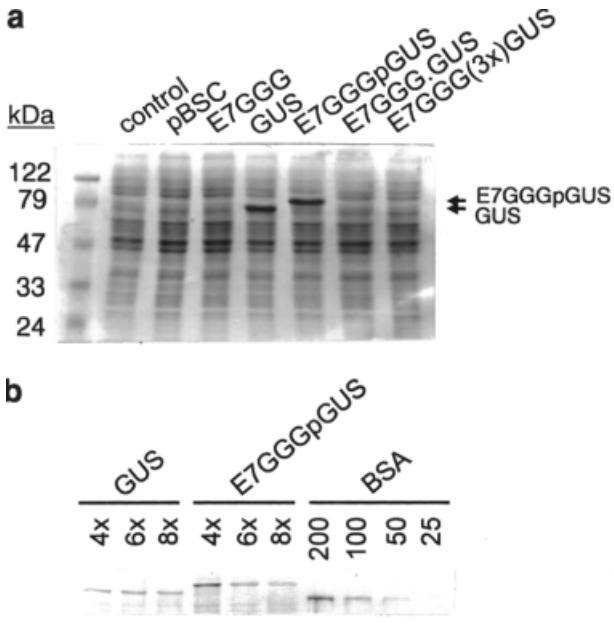


**Figure 3. SDS-PAGE gels stained with Coomassie brilliant blue. (a) Proteins (10 μg/lane) from extracts of 293T cells transfected with pBSC constructs were separated in 10% gel. (b) Quantification of GUS and E7GGG.GUS proteins in transfected 293T cells. Dilutions of cellular extracts are indicated. Amount of total soluble proteins in undiluted extracts corresponds to 10 μg/lane. For comparison, dilution of BSA was used. Amounts of BSA in ng per lane are indicated. The experiment was repeated with similar results**

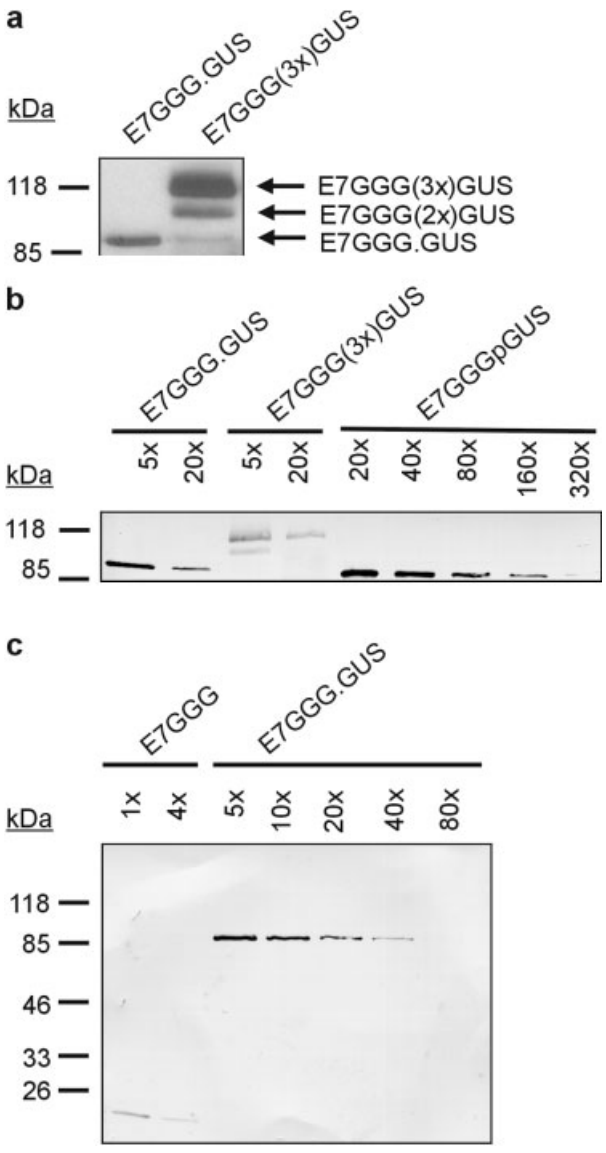


**Figure 4. Immunoblotting detection of E7 antigen in 293T cells transfected with pBSC-derived plasmids. Proteins separated in 7% (a), 10% (b) or 12% gel (c) were transferred onto a polyvinylidene difluoride (PVDF) membrane, detected with HPV16 E7-specific monoclonal antibody, and visualised with enhanced chemiluminiscence (ECL; a) or diaminobenzidine (DAB; b, c). Dilutions of cellular extracts are indicated. Amount of total soluble proteins in undiluted extracts corresponds to 20 μg/lane. The experiment was repeated with similar results**

## Tumour formation after immunisation with fusion genes

Immunogenicity of GUS fusion genes was tested in C57BL/6 mice by DNA immunisation with a gene gun. Oncogenic TC-1 cells expressing E7 and E6 oncoproteins and MHC class I molecules were s.c. administered to induce tumour formation. In preventive vaccination experiments, animals were twice immunised with plasmids and TC-1 cells were inoculated 14 days later. All mice vaccinated with the E7GGG.GUS or E7GGG(3×)GUS genes were protected against tumour formation. In control groups immunised with E7GGG

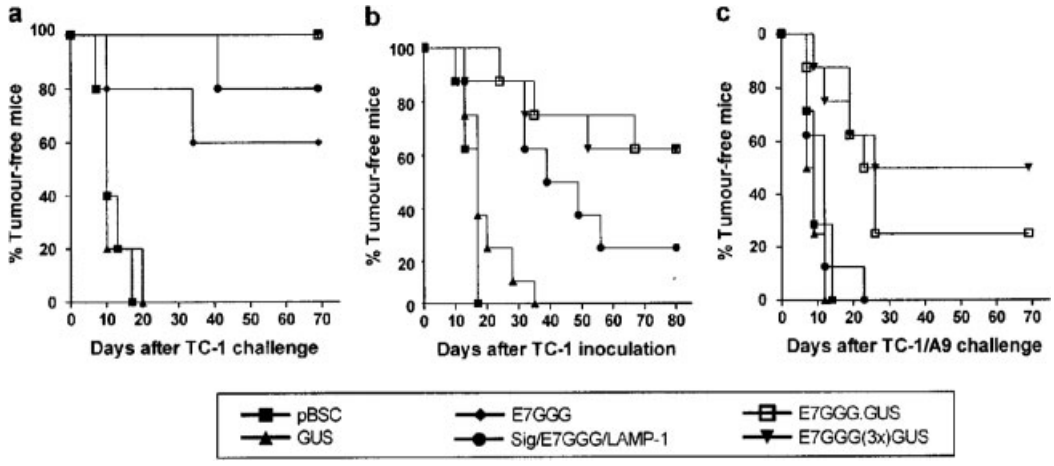


**Figure 5. Tumour formation after immunisation with pBSC-derived plasmids. Preventive (a, c) or therapeutic immunisation (b; on days 4 and 10) by gene gun was performed as described in Materials and methods. TC-1 (a, b) or TC-1/A9 cells (c) were s.c. administered to induce tumour formation. Five (a) or 8 mice (b, c) per group were used in experiments**

or Sig/E7GGG/LAMP-1 genes some animals developed a tumour (Figure 5a).

In therapeutic vaccination experiments, mice were immunised 4 and 10 days after TC-1 inoculation. After both E7GGG.GUS and E7GGG(3×)GUS gene administration, tumour formation was observed in 3 out of 8 mice ($P < 0.01$ compared with pBSC; Figure 5b). However, these tumours developed later and their growth was reduced in comparison with tumours in negative control groups treated with plasmids pBSC or pBSC/GUS. Furthermore, the tumour incidence in mice immunised with the control Sig/E7GGG/LAMP-1 gene was two-fold higher (6/8; $P < 0.05$).

In our previous work, we developed the TC-1/A9 subline with down-regulated production of MHC class I molecules and demonstrated the negligible effect of vaccination with the Sig/E7GGG/LAMP-1 gene against these cells [10]. However, after preventive immunisation with both the E7GGG.GUS and E7GGG(3×)GUS genes, the formation and growth of TC-1/A9 tumours were significantly reduced in comparison with both pBSC and Sig/E7GGG/LAMP-1 ($P < 0.05$; Figure 5c).

## Detection of E7-specific CD8$^+$ cells

Induction of immune responses after immunisation with E7GGG-GUS fusion genes was also tested by *in vitro* tetramer staining of activated CD8$^+$ cells specific for the main H-2D$^b$ epitope of the E7 antigen. For comparison, the E7GGG and Sig/E7GGG/LAMP-1 genes were administered. Results of this tetramer staining (Figure 6) corresponded to *in vivo* immunisation experiments. Stimulation with the E7-derived synthetic peptide RAHYNIVTF resulted in strong activation of CD8$^+$ cells in splenocytes isolated from E7GGG.GUS- and E7GGG(3×)GUS-immunised animals. The number of stained T lymphocytes was about 10- and 3-fold higher than after immunisation with the E7GGG and Sig/E7GGG/LAMP-1 genes, respectively.

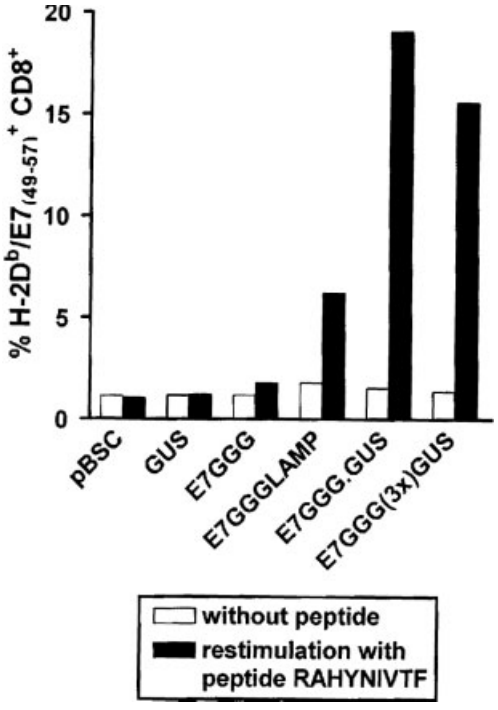


**Figure 6. Detection of E7-specific CD8$^+$ T lymphocytes by tetramer staining. Lymphocyte bulk cultures were prepared 2 weeks after the second dose of plasmids administered by a gene gun, restimulated with the E7$_{(49-57)}$ peptide for 6 days, and stained with a mixture of H-2D$^b$/E7$_{(49-57)}$-PE tetramers and anti-mouse CD8a-FITC antibody. Control lymphocytes were cultivated without the peptide**

## Elimination of GUS activity in E7GGG.GUS

To study the contribution of GUS activity to enhanced immunogenicity of the E7GGG.GUS gene, we deleted 77 codons from the GUS portion of the fusion gene (E7GGG.GUSdel) and from the GUS gene alone (GUSdel; Figure 1b). Assays for GUS activity proved complete elimination of enzymatic reactivity (Figure 7).

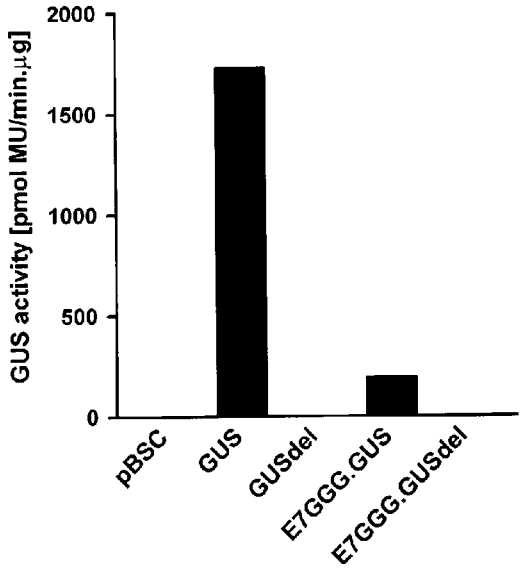


**Figure 7. Detection of GUS activity in 293T cells transfected with deleted constructs. Non-deleted genes were used for comparison. Enzymatic activity in cellular extracts was tested as in Figure 2. The experiment was repeated with similar results**

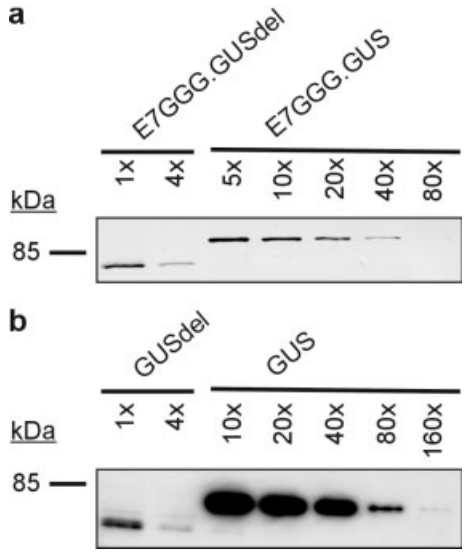


**Figure 8. Immunoblotting detection of E7 or GUS antigen in 293T cells transfected with GUSdel constructs. Non-deleted genes were used for comparison. Proteins separated in 10% gel were transferred onto a PVDF membrane, detected with E7 (a) or GUS (b) specific antibodies, and stained with DAB (a) or visualised by ECL (b). Dilutions of cellular extracts are indicated. Amount of total soluble proteins in undiluted extracts corresponds to 20 μg/lane. The experiment was repeated with similar results**

Immunoblotting analysis verified the production of deleted proteins, but at substantially reduced level (Figure 8). Quantification of proteins with an E7-specific antibody showed about a 10-fold lower steady-state level of E7GGG.GUSdel in comparison with E7GGG.GUS (Figure 8a). Similarly, after staining with GUS-specific antibodies, the amount of detected GUSdel was about 60-fold lower than that of GUS (Figure 8b).

Immunogenicity of the E7GGG.GUSdel gene was compared with the E7GGG.GUS gene by therapeutic immunisation against TC-1 cells (Figure 9). Similar efficacy (5/8 tumour-free mice; $P < 0.05$) was shown for both genes. In this experiment we recorded regression of tumours 2–3 mm in diameter. In the control group immunised with the Sig/E7GGG/LAMP-1 gene only one mouse did not develop a tumour ($P > 0.05$).

Activation of E7-specific $CD8^+$ cells was compared *in vitro* by ELISPOT detection of IFN-$\gamma$-producing cells. Results of this assay (Figure 10) corresponded to the *in vivo* tumour-protection experiment. After stimulation with the peptide RAHYNIVTF, production of IFN-$\gamma$ by splenocytes isolated from E7GGG.GUS- and E7GGG.GUSdel-immunised mice was similar. The number of IFN-$\gamma$-producing cells was about 2.5-fold higher than after immunisation with the Sig/E7GGG/LAMP-1 gene.

## RNA analysis of transfected 293T cells

Immunoblotting analyses demonstrated large differences in the production of detected proteins. To evaluate the contribution of mRNA expression, we performed Northern blot hybridisation with a GUS-specific probe (Figure 11a). Detection of $\beta$-actin transcripts was used as an internal control (Figure 11b). The results showed only slightly reduced amounts of E7GGG.GUS and E7GGG(3×)GUS transcripts in comparison with GUS and E7GGGpGUS transcripts. Furthermore, the expression of mRNAs from

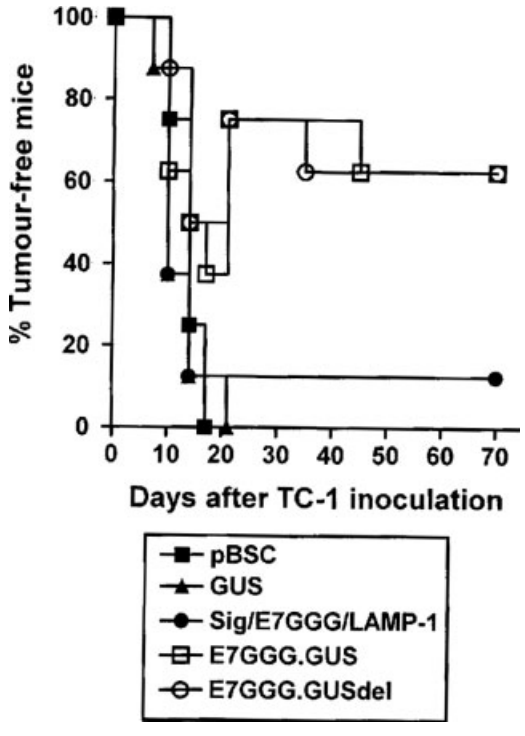


**Figure 9. Tumour formation after therapeutic immunisation with pBSC/E7GGG.GUSdel plasmid. pBSC/E7GGG.GUS was used for comparison. Mice (8 per group) were s.c. inoculated with $3 \times 10^4$ TC-1 cells and immunised twice with a gene gun on days 3 and 10**

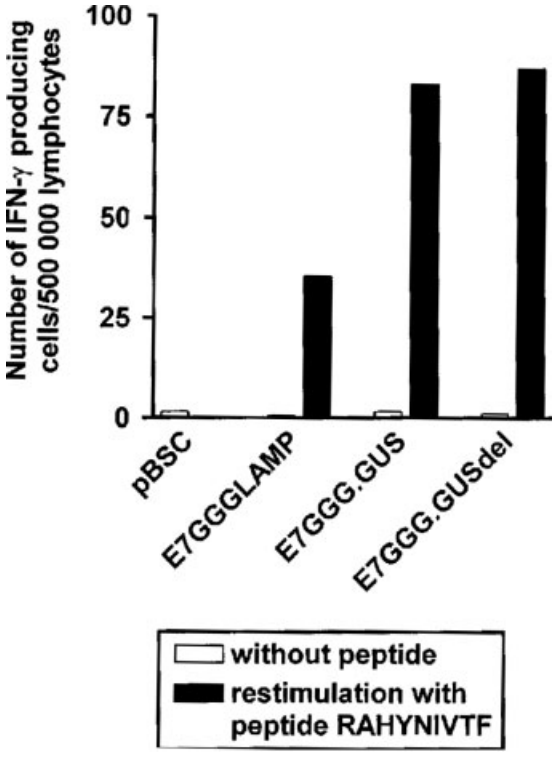


**Figure 10. Detection of E7-specific $CD8^+$ T lymphocytes by ELISPOT assay. Lymphocyte bulk cultures were prepared 2 weeks after the second dose of plasmids administered by a gene gun. IFN-$\gamma$-producing cells were detected 1 day after stimulation with the $E7_{(49-57)}$ peptide. Control lymphocytes were cultivated without the peptide**

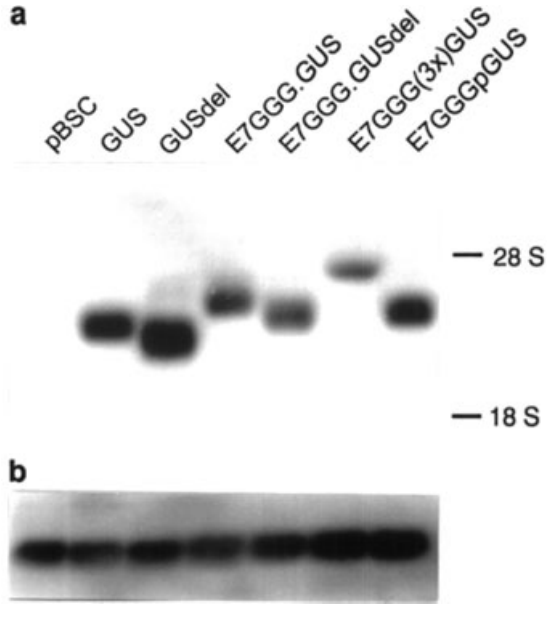


**Figure 11. Northern blot analysis of RNA extracted from 293T cells transfected with pBSC-derived plasmids. The membrane was hybridised with digoxigenin-labelled GUS (a) or $\beta$-actin probe (b)**

genes with deletion in GUS was not decreased. In fact, a moderately increased steady-state level of GUSdel mRNA was recorded.

## Subcellular localisation of proteins in transfected NIH 3T3 cells

The localisation of proteins in transfected NIH 3T3 cells was determined by immunofluorescence staining with anti-E7 monoclonal antibody (Figure 12). The E7GGG protein was distributed predominantly in the nucleus. Depending on the efficiency of transfection of individual cells, the cytoplasm also stained with various intensities. Fusion of E7GGG or E7GGGp with GUS resulted in cytoplasmic localisation of the E7 antigen that was not altered after deletion in the GUS portion of E7GGG.GUS.

# Discussion

We constructed fusion genes of the mutated E7 oncogene (E7GGG) with GUS to increase the stability of the E7 antigen, which could be helpful for the production of the E7 antigen in plants. Simultaneously with the expression of the fusion genes in plant cells and preparation of transgenic plants (data to be published), we characterised their expression in mammalian cells and tested their immunogenicity by DNA vaccination with a gene gun in this study. We demonstrated enhanced immunogenicity in comparison with both E7GGG and Sig/E7GGG/LAMP-1.

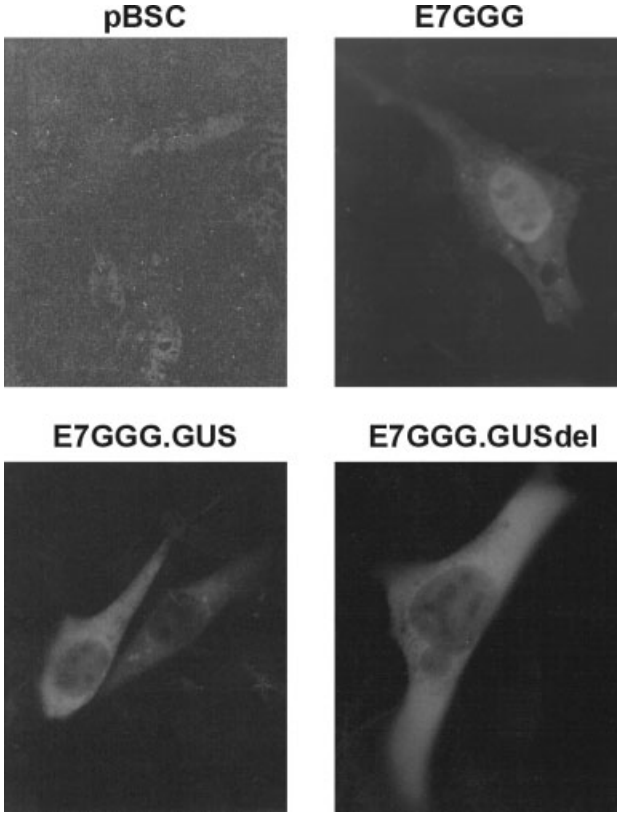


**Figure 12. Immunofluorescence detection of E7 antigen in NIH 3T3 cells transfected with pBSC-derived plasmids (original magnification ×1000). The paraformaldehyde-fixed cells were stained with anti-E7 monoclonal antibody 2 days after transfection**

We also compared GUS fusions with one or three copies of E7GGG. However, we did not observe significantly higher efficacy for the fusion gene with three copies of E7GGG.

Similar to expression in plant cells, the GUS protein was very stable after transfection of human 293T cells. It comprised about 6% of extracted proteins. However, fusion with complete E7GGG resulted in marked reduction of both GUS activity and the steady-state level of fusion proteins. These effects were even more evident for the construct with three copies of E7GGG. Quantification of protein production showed that the decrease in steady-state levels of fusion proteins was the main cause of reduced GUS activity. However, we cannot exclude that the decrease of enzymatic activity also resulted from conformational changes induced by fusion, because the reduction of GUS activity was slightly higher than the reduction of protein steady-state level. Moreover, GUS activity of fusion with partial E7GGG (E7GGGpGUS) was also only slightly reduced, although its steady-state level was comparable to GUS alone.

The mechanism responsible for the large difference in the steady-state level of GUS fusion proteins with complete and partial E7GGG is not quite clear from the present study. Potentially, the portion of the E7GGG gene absent in E7GGGp could contain sequence(s) decreasing mRNA expression and/or stability. Indeed, we detected slightly reduced amounts of E7GGG.GUS and E7GGG(3×)GUS transcripts, but this decrease was not high enough to explain the decrease of protein content. Moreover, experiments in which E7 codons were modified suggest that such regulatory sequences are not present in the E7 gene [23].

HPV16 E7 is a very unstable protein with a half-life of about 1 h [24]. We have shown similar production of E7 and E7GGG proteins after transfection of 293T cells [6]. The E7 C-terminus can probably influence the protein stability as mutagenesis in this region changed (accelerated) E7 degradation [5]. Therefore, we can hypothesise that fusion of E7GGG with GUS might destabilise the GUS protein and that elimination of the E7GGG C-terminus from the fusion protein might restore its stability. Alternatively, the part of the E7GGG gene absent in partial E7GGG could contain codons that are rate-limiting for E7GGG translation.

Deletion of 77 amino acids in GUS decreased the steady-state levels of both the GUS and E7GGG.GUS proteins. As transcription of deleted genes was not down-regulated, the most probable explanation for the reduction of amounts of deleted proteins is the decrease of their stability.

Immunoblotting analysis of E7GGG(3×) GUS production with an E7-specific monoclonal antibody showed that besides the dominant band representing the E7GGG(3×)GUS protein, two minor bands corresponding in size to E7GGG(2×)GUS and E7GGG.GUS were detected. As addition of protease inhibitor cocktail into the lysis buffer did not alter the band intensity (data not shown) we suppose that the two bands do not represent degradation products of E7GGG(3×)GUS, but rather the E7GGG(2×)GUS and E7GGG.GUS proteins. During HPV16 infection the E7 protein is translated from the E6/E7 bicistronic mRNA. Leaky scanning, which is the predominant mechanism responsible for its translation [25], also might be involved in the production of the E7GGG(2×)GUS and E7GGG.GUS proteins from the E7GGG(3×)GUS gene.

We examined immunogenicity of the GUS fusion genes in comparison with the fusion gene Sig/E7GGG/LAMP-1 that previously was shown to prevent tumour formation by TC-1 cells, but which only reduced the growth of TC-1 tumours in therapeutic immunisation experiments and failed to protect animals against TC-1/A9 cells with down-regulated production of MHC class I molecules [10]. As reduced MHC class I expression is one of the most important mechanisms responsible for tumour-cell escape from the host immune system, and this reduction occurs in most cervical carcinoma patients, successful immunisation against such cells could offer great benefits. The GUS fusion genes exhibited enhanced efficacy against both established TC-1 tumours and TC-1/A9 cells administered after vaccination. Both the tetramer staining and ELISPOT assay demonstrated increased activation of E7-specific $CD8^+$ lymphocytes that probably play the main role in elimination of TC-1 cells. Which immune cells are efficient against TC-1/A9 cells is currently unknown. As up-regulation of MHC class I molecules on TC-1/A9 cells was demonstrated *in vivo* [10], the contribution of $CD8^+$ CTL is also possible in TC-1/A9 elimination.

Fusion of a gene coding for a protein immunogen with a helper gene is an efficient method of increasing protein immunogenicity. Various mechanisms resulting in enhanced immunity induced by vaccination with fusion genes have been described including modifications of cellular localisation and/or antigen processing and presentation, utilisation of helper epitopes in the fusion partner, and modification of protein production and stability [26]. For the E7GGG.GUS fusion, we showed that enzymatic activity did not likely contribute to enhancement of E7GGG immunogenicity as its elimination did not influence immunogenicity of the fusion gene. More likely, subcellular localisation of the E7GGG.GUS protein and/or helper epitopes included in GUS might be responsible for the effect. The increased steady-state level of the E7GGG.GUS protein (in comparison with E7GGG) probably did not play a crucial role, as the steady-state level of E7GGG.GUSdel was about 10-fold decreased without reduction of its immunogenicity.

In conclusion, we enhanced the immunogenicity of the E7GGG gene by its fusion with GUS. The effect was evident in DNA immunisation experiments against both TC-1 cells and, to a lesser extent, TC-1/A9 cells with down-regulated MHC class I expression. Tumour-escape mechanisms probably represent the main obstacle for successful anti-tumour immunisation. In fact, we have found that some TC-1 sublines isolated from immunised mice which retained MHC class I expression were quite resistant to preventive immunisation with

the E7GGG.GUS gene (unpublished data). We hope that further enhancement of anti-tumour immunity resulting in killing of such immunoresistant cells can be achieved by combination with immunostimulatory gene(s) and/or by application of heterologous boost.

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