Charles University in Prague

Gene Immunotherapy of Cancer: DNA Vaccines against HPV 16

Mgr. Ingrid Poláková

PhD. Thesis

Supervisor: RNDr. Michal Šmahel, PhD.

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I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning.

LIST OF ABBREVIATIONS

Aa = amino acid

Ab = antibody

Ag = antigen

APC = antigen presenting cell

APM = antigen-processing machinery

CC = cervical cancer

cFLIP = cellular FLICE-inhibitory protein

CIN = cervical intraepithelial neoplasia

CMV = human cytomegalovirus

CRT = calreticulin

CTL = cytotoxic T lymphocyte

CTLA-4 = cytotoxic T-lymphocyte antigen-4

DAC = 5-aza-2'-deoxycytidine

DC = dendritic cell

DNA = deoxyribonucleic acid

DNMT = DNA methyltransferase

EP = electroporation

ER = endoplasmic reticulum

FA = Freund adjuvant

FasL = Fas ligand

FrC = Fragment C of tetanus toxin

GM-CSF = granulocyte-macrophage colony-stimulating factor

GUS = *E.coli* β-glucuronidase

HIV = Human Immunodeficiency Virus

HPV =human papillomavirus

HR = high-risk

HSIL = high grade squamous intraepithelial lesions

Hsp = heat shock protein

ICS = intracellular cytokine staining

IFN = interferon

i.d. = intradermal

IL = interleukin

IRF = interferon regulatory factor

i.m. = intramuscular

KLH = keyhole limpet hemocyanin

LAK cells = lymphokine activated killer (LAK) cells

LAMP-1 = lysosome-associated membrane protein-1

LC = Langerhans cells

LCR = long control region

LN = lymph nodes

LR = low-risk

LSIL = low grade squamous intraepithelial lesions

MHC CIITA = Class II, major histocompability complex, transactivator

MIP-1α = macrophage inflammatory protein 1α

MSC = myeloid suppressor cell

NCR = non-coding region

NMR = nuclear magnetic resonance

ORF = open reading frame

pDNA = plasmid DNA

PI3K = phosphatdylinositol 3'-kinase

PMED = Particle Mediated Epidermal Delivery

pRb = retinoblastoma protein

PV = papillomavirus

s.c. = subcutaneous

SIL = squamous intraepithelial lesions

SCT = single chain trimer

SS = signal sequence

TAA = tumour-associated antigen

TAM = tumour-associated macrophages

TBK 1 = TANK-binding kinase-1

TCR = T cell receptor

TGF = tumour growth factor

TIL = tumour-infiltrating lymphocytes

TLR = Toll-like receptor

T_{reg} = regulatory T cells

TSG = tumour suppressor gene

VLP = virus-like particle

wt = wild-type

CONTENTS

1. PREFACE

Cervical carcinoma represents the second most frequent cancer in women. Today it is well established that cervical tumours are mostly associated with human papillomavirus (HPV) infection. More then 95 % of cervical cancer biopsies contain high-risk HPV genomes (zur Hausen 2009). Infections with certain genotypes of HPV can lead to other anogenital and head and neck cancers or can cause benign warts. However, 91% of HPV-related cancer deaths, on the global scale, are due to cervical cancer (Cutts *et al.*, 2007). Nowadays, two prophylactic vaccines, protecting against HPV 16 and HPV 18, are licensed. Nevertheless, development of therapeutic vaccines is desirable to eliminate current HPV infections and to treat progressing tumours.

Immunotherapy has become a common approach in cancer treatment due to advances in understanding cellular and molecular mechanisms of the immune system. One of the several strategies of immunotherapy is the induction of antigen-specific immune responses. As a source of antigens may serve vaccines based on vectors, dendritic cells, peptides and DNAs. The discovery of DNA immunisation in the early 1990s (Wolff *et al.*, 1990; Wang *et al.*, 1993; Ulmer *et al.*, 1993) brought new options into immunotherapy of cancer. The novel vaccines based on DNA carry the genetic material that encodes an antigen, rather than the antigen itself. Moreover, the administration of the DNA vaccines leads to the induction of both humoral and cellular immune responses (Coban *et al.*, 2008). The immunogenicity of DNA vaccines is well established in animal models. Several DNA plasmid products are licensed for veterinary application. Unfortunately, the immunogenicity of DNA vaccines in large animals and particularly in humans is significantly lower. New strategies developed to improve the immunogenicity of DNA vaccines show that DNA immunization can indeed induce antigen-specific immune responses in humans. Clinical studies with DNA vaccines against HBV, influenza virus, malaria, HIV-1 and various cancers were reported (Lu *et al.*, 2008). DNA vaccines have emerged as an attractive form also for therapeutic treatment of HPV-associated lesions. The ideal targets of therapeutic HPV vaccines are the viral E7 and E6 oncoproteins that are essential in cellular transformation and constitutively expressed in malignant cells. Currently, several ongoing studies (also clinical trials) are focused on strategies enhancing the efficacy and safety of DNA vaccines against HPV.

Objectives of the PhD. Thesis:

- The enhancement of immunogenicity of DNA vaccines against human papillomavirus type16 E7 and E6 oncoproteins: construction of plasmid DNA carrying *Escherichia coli* β-glucuronidase-fused E7 or E6 genes

- Modifications of the viral E6 oncogene in order to increase the production of the full-length E6 protein and to decrease its oncogenicity

- Observation of immune responses induced after administration of the prepared plasmid DNA constructs

- Evaluation of the efficacy of the E7- and E6-derived peptide vaccines delivered with a tattoo device

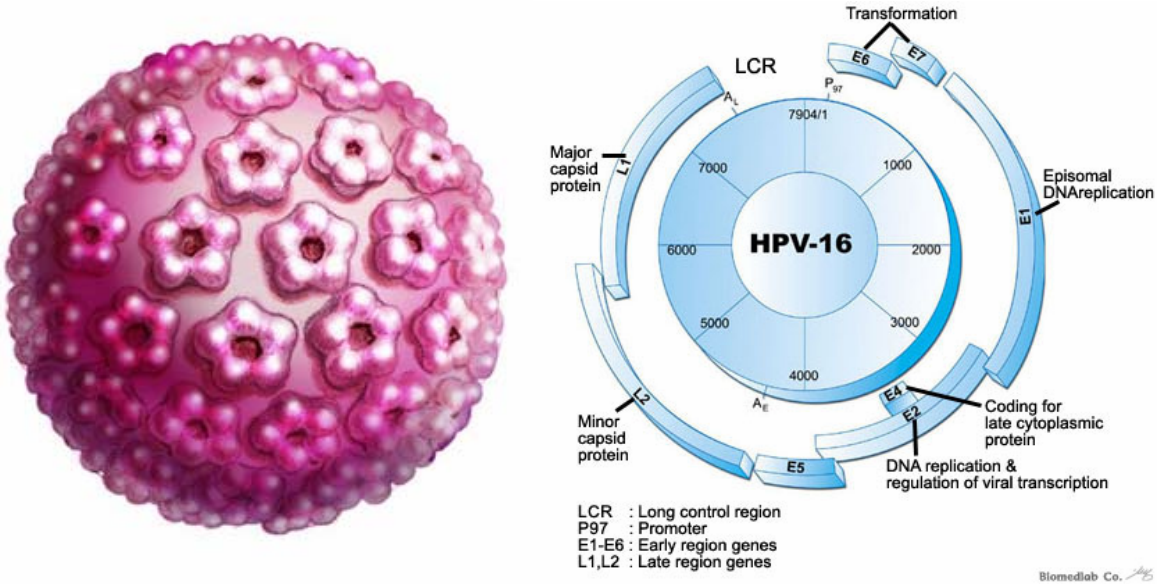
2. INTRODUCTION

2.1 Human papillomavirus

2.1.1 Genome structure and replication

Papillomaviruses (PVs) are a group of small non-enveloped icosahedral DNA tumour viruses with a virion size of ~55 nm in diameter and 72 capsomers (Fig.1). They form a distinct taxonomic family, the *Papillomaviridae.* All papillomaviruses contain a double-stranded, circular DNA genome approximately 8 kb in size that is generally divided into three major regions: early, late and a long control region (LCR or non-coding region [NCR]). The early region encodes six common open reading frames (ORFs; E1, E2, E4, E5, E6 and E7) that translate to individual proteins. Two other ORFs, E3 and E8, were also assigned to this region initially, but only the E8 (in bovine PV-1) has been proven to encode a protein. The late region lies downstream of the early region and contains L1 and L2 ORFs for translation of major L1 and minor L2 capsid proteins. The LCR region does not encode any protein, but bears the origin of replication and multiple transcription factor binding sites.

Figure 1. Model of papillomavirus capsid and genome of HPV 16



Papillomaviruses replicate and assemble exclusively in the nucleus of keratinocytes. Viruses infect the basal layers of a squamous epithelium and are in the phase of latent infection whereby the viral genome is maintained episomally at a low copy number without production of virions. The viral gene expression leads only to the expression of six non-structural viral regulatory proteins from the early region in the undifferentiated or intermediately differentiated keratinocytes. The function of these proteins is summarised in Table 1. In the upper layer, as the filial cells move towards the surface and undergo differentiation, two structural viral capsid proteins are expressed from the late region in terminally differentiated keratinocytes. Finally, the virions are released from the uppermost layer to search for new host cells (Fig. 2).

Papillomavirus DNA is frequently found to be integrated into host chromosomes in cervical cells. The integration sites are not distributed to hotspot areas and there is no evidence of insertional mutagenesis. Viral integration occurs downstream of the early genes E6 and E7, often in the E1 and E2 region; this disruption results in a loss of negative-feedback control on E6 and E7 oncogene expression by the viral regulatory E2 protein (Zheng & Baker, 2006; Woodman *et al.*, 2007; Yugawa & Kiyono, 2009).

Table 1. Papillomavirus proteins and their function

Protein	Functions
Early	
E1	Viral DNA replication, ATP dependent DNA helicase, DNA-dependent ATPase
E2	Viral DNA replication, regulation of transcription of viral genes
E3	Not known
E4 (late)	Disruption of cytokeratin filament network, virus maturation
E5	Transforming activity, downregulation of MHC class I expression
E6	Viral oncoprotein inducing cell immortalisation and transformation, binding to p53 protein
E7	Viral oncoprotein inducing cell immortalisation and transformation, binding to pRb protein
E8	Not known
Late	
L1	Major capsid protein: can form virus like particles
L2	Minor capsid protein: possible DNA packaging protein

2.1.2 Pathogenesis and epidemiology

Papillomaviruses infects various animals from birds to mammals, including humans. They are highly host-specific and are not transmissible between species. PVs are classified into genotypes based on the sequence of L1 ORF with more than 10% of difference. Up to the last year more than 100 different genotypes just of human PVs (HPVs) have been identified and sequenced. They are also strictly tissue-specific: they infect only epithelial cells of the skin and mucosa.

Although PV infections usually result in benign lesions, HPV infection may progress to the development of malignant lesions. According to their ability to induce malignancy, HPVs are classified as non-oncogenic low-risk (LR) or oncogenic high-risk (HR) types (Table 2). The human genital tract may be infected by about 40 HPV genotypes.

Table 2. Human papillomavirus genotypes

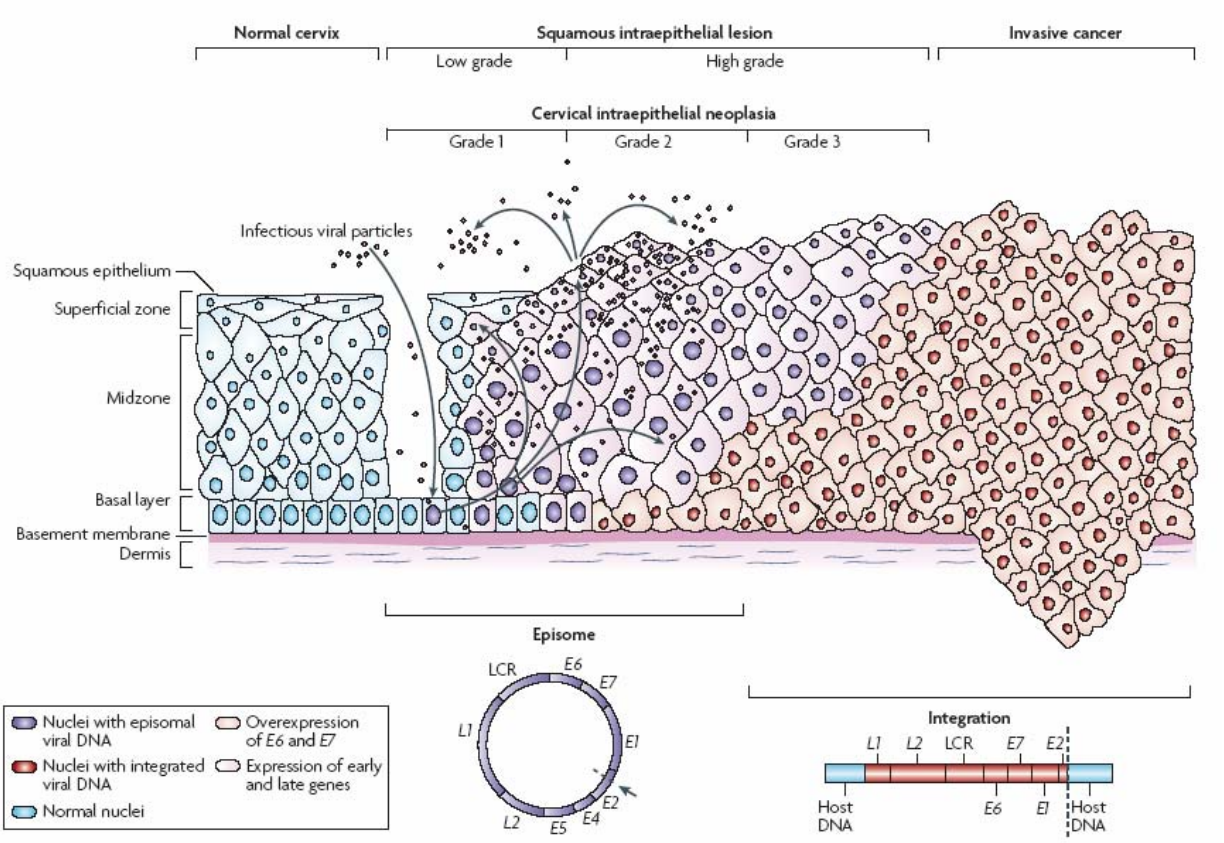
	Genotypes
High-risk HPVs	16,18,31,33,35,39,45,51,52,56,58,59,68,73,82
Probably high-risk HPVs	26,53,66
Low-risk HPVs	6,11,40,42,43,44,54,61,70,72,81,cand89

Genital HPVs are primarily transmitted by genital skin-to-skin contact, usually, but not necessarily, during sexual intercourse. High-risk HPV genotypes can lead to cervical cancer (CC) and are associated with other mucosal anogenital and head and neck cancers. Infections with LR HPVs can cause benign or low-grade cervical tissue changes and genital warts (*condylomata accuminata*), which are growths on the cervix, vagina, vulva and anus in women and the penis, scrotum or anus in men.

Cervical cancer is the second most common cancer among women worldwide affecting approximately 500 000 women each year with mortality rate about one-third of these cases. There are over one thousand new cases of cervical carcinoma only in the Czech Republic. Most women are infected shortly after beginning their first sexual relationship. In young women the infection is mostly asymptomatic, with only mild changes in the epithelium, and transient. In serious cases, the ongoing virus replication may induce abnormal growth of squamous cells called squamous intraepithelial lesions (SIL) of low (LSIL) or high (HSIL) grade (Fig.2). Cervical intraepithelial neoplasia (CIN) is a term for abnormal cells in the cervix; grades from 1 to 3 describe the proportion of thickness of the cervical epithelium.

The final stage of the pathological process is CC. The most common types of HPV detected in LSIL are the HPV 16 (26%), 31 (12%) and 51 (11%) (Cutts *et al.*, 2007; Vonka & Hamsikova, 2007; Woodman *et al.*, 2007; Tachezy & Rob, 2007).

Figure. 2 HPV-mediated progression to cancer



Woodman *et al.*, Nat Rev Cancer. 2007; 7:11-22.

2.1.3 Carcinogenesis

More than enough evidence exists about the connection between HPV infection and cervical cancer: the presence of the viral DNA in the infected cells, serological findings and the most important of all, the experimental confirmation of the transformation ability of the viral E6 and E7 oncoproteins.

2.1.3.1 Oncoproteins of HPVs

E5 protein

The HPV 16 E5 protein is a small hydrophobic membrane protein located downstream of the E2 ORF. Although this gene is not well conserved at the DNA level among HPVs or animal viruses, the expressed proteins are always hydrophobic and membrane-bound (Tsai & Chen, 2003). The E5 presents as a dimer and is distributed predominantly in the endoplasmic reticulum, the Golgi and the cytoplasmic membrane (Oetke *et al.*, 2000).

The E5 protein is under-expressed (because of the fragment deletion of the E5 ORF) in cervical carcinoma cells, which suggests that E5 may play a critical role in the genesis of CC but not in the persistence or progression and maintaining the malignant phenotype (Yang *et al.*, 2003). There are well recognised cellular targets for the E5 protein due to E5 may contribute to cell transformation: interaction with a subunit of vacuolar ATPase induces enhanced epidermal growth factor receptor signalling and so cell proliferation (Genther Williams *et al.*, 2005). Moreover, E5 causes the retention of MHC class I in the Golgi apparatus and restrain its transport to the cell surface (Kim & Yang, 2006), enhances the MAP kinase activation (Crusius *et al.*, 1997), stimulates the nuclear oncogenes, such as c-jun and c-fos (Jin *et al.*, 2001), and down-regulates the expression of the p21 tumour suppressor gene (Tsao *et al.*, 1996).

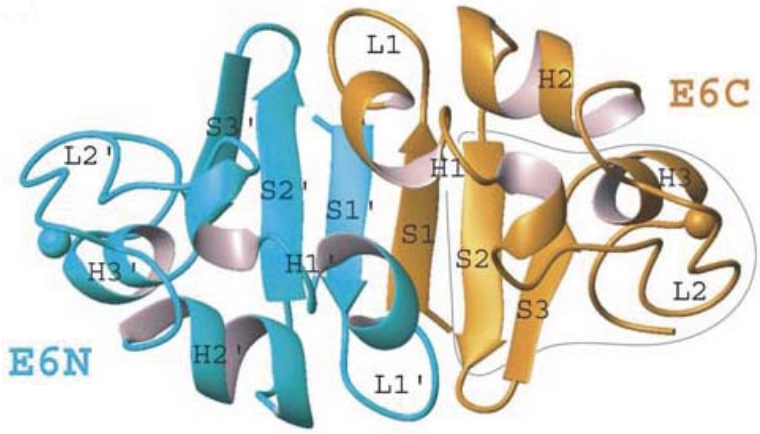
E6 protein

The E6 protein is nuclear and cytoplasmic protein of about 18 kDa. Most PVs have tandem ATGs of which the second one (151 aa form) usually, but not always, is the start. In HPV 16, a protein of 158 residues can be generated (Androphy *et al.*, 1987; Barbosa & Wettstein, 1988; Neary & DiMaio, 1989). The protein contains two CX_2C-X_{29}-CX_2C zinc-fingers joined by an interdomain linker of 36 amino acids and flanked by short amino (N) and carboxy (C) terminal domains (Howie *et al.*, 2009). Recently, the solution structure of the C-terminal half of HPV 16 E6 was solved by nuclear magnetic resonance (NMR) and a model for the whole protein was proposed (Fig. 3) (Nomine *et al.*, 2006).

Analysis of the genome of HR HPVs (but not LR HPVs) reported spliced isoforms of the E6 gene that lead to the expression of truncated E6 proteins denoted as E6*I and E6*II. The studies detected the E6*I transcript as the most abundant one in HPV 16 transformed cells, CC cell lines and clinical samples (Smotkin *et al.*, 1989; Cornelissen *et al.*, 1990; Griep

et al., 1993). E6* binds to the interface of the N- and C-terminus of the full-length E6 protein and thus inactivates its function (Nomine *et al.*, 2006).

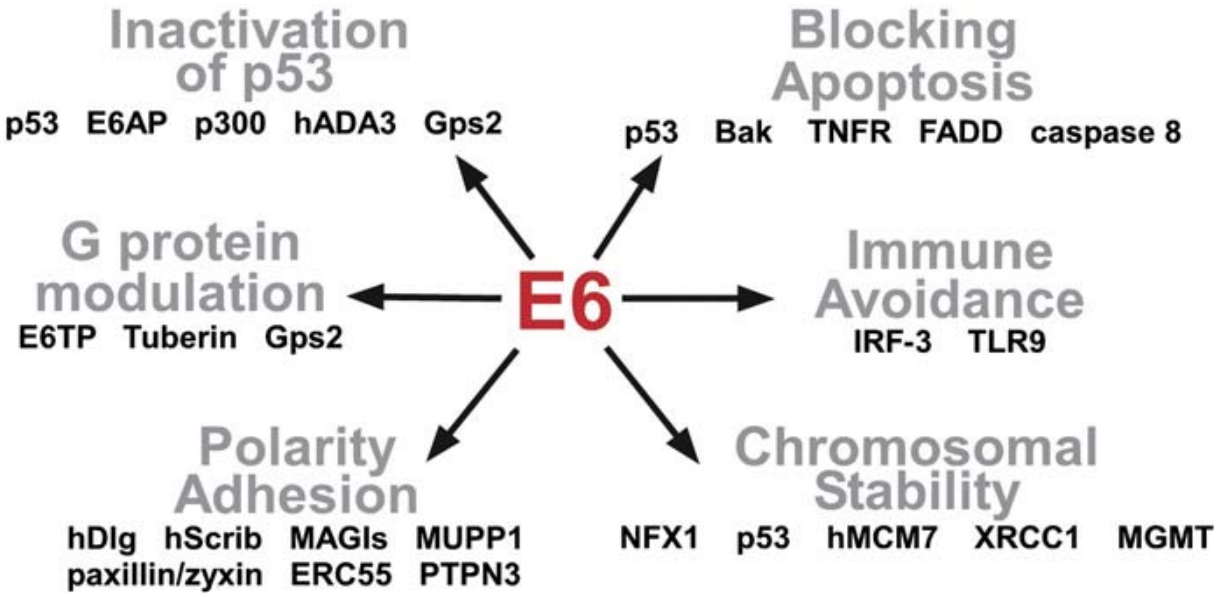
Figure 3. Proposed model of the E6 protein; pseudodimeric arrangement of E6N and E6C



Taken and modified from Nominé *et al.* Mol Cell. 2006; 21:665-78.

E6 interacts with a number of different proteins that mediate the apoptotic pathway, regulate transcription and mediate chromosomal stability, differentiation, cell-cell adhesion etc. The cellular proteins affected by the E6 protein are represented in Fig. 4.

Figure 4. Binding partners of E6



Howie *et al.* Virology. 2009; 384:324-34.

There are two main binding motifs for the E6 protein, the well characterized LXXLL motif and the specific domain on cellular proteins known as PDZ. The LXXLL motif was firstly described on the E6 associated protein (E6AP) that forms a complex with both E6 and target proteins leading to ubiquitination of the target protein and subsequent proteasome mediated degradation (Huibregtse *et al.*, 1991; Scheffner *et al.*, 1993; Be *et al.*, 2001). Other proteins binding to E6 by way of this motif are E6BP (ERC55), IRF3, paxillin and tuberin (Tong & Howley, 1997; Elston *et al.*, 1998; Ronco *et al.*, 1998). Binding to the LXXLL motif is highly conserved in PVs and E6AP binds to both HR and LR HPVs (Chen *et al.*, 1998). Only HR HPV E6 proteins (Kiyono *et al.*, 1997) have the ability to bind to the PDZ domain containing proteins such as hDLg1 and 4 (Lee *et al.*, 1997), hScrib (Nakagawa & Huibregtse, 2000) and MAGI 1, 2 and 3 (Glaunsinger *et al.*, 2000; Thomas *et al.*, 2002). The p53, Bak p300/CBP, Gps2, FADD proteins and procaspase 8 have been reported to bind various E6 proteins, but they lack both LXXLL and PDZ domains (Howie *et al.*, 2009).

One of the first identified and best characterised interacting partner of the E6 protein is the p53 tumour suppressor. Once activated, p53 induce DNA repair, cell cycle arrest and/or apoptosis, based upon the extent of damage (reviewed by Murray-Zmijewski *et al.*, 2008). During the carcinogenesis, to overcome this obstacle, the E6 protein causes the degradation of p53. The principle mechanism is through the ubiquitin-proteasome pathway (Scheffner *et al.*, 1990). Interestingly, both HR and LR HPV E6 proteins have been shown to be able to bind the p53 C-terminus but only the HR E6 proteins are capable of binding to the core region of p53 that is required for its degradation (Li & Coffino, 1996). There are some other mechanisms described how the HPV can block the function of the p53 independently on the protein degradation: (i) The interaction of E6 with p53 can inhibit the binding of p53 to its site-specific sequences, what correlates with the affinity that each E6 has for p53. This association may also cause conformational change in the p53 protein (Lechner & Laimins, 1994; Thomas *et al.*, 1995). (ii) Binding of the E6 to p53 may lead to sequestration of the p53 in the cytoplasm (Mantovani & Banks, 2001). (iii) Interaction of HR E6 with p300 inhibits the p53 acetylation and decreases its ability to bind to the DNA (Zimmermann *et al.*, 2000).

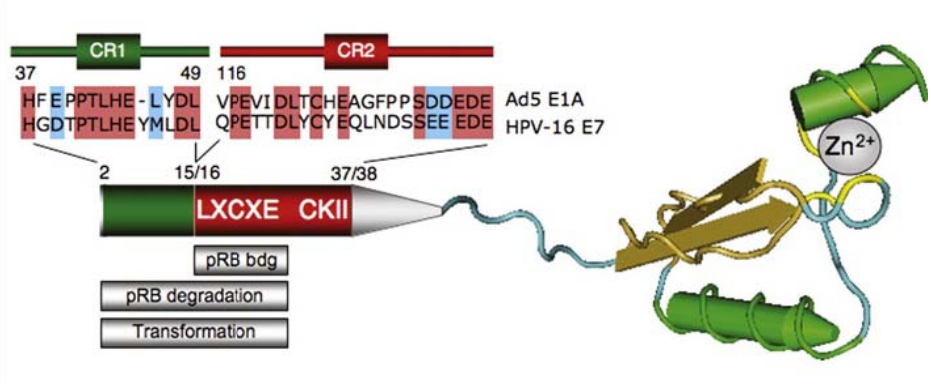
E7 protein

The E7 proteins are small, acidic proteins composed of about 100 amino acids (Fig 5). The N-terminus of E7 contains two conserved regions, CR1 and CR2, with sequence similarity to adenovirus E1A and polyomavirus SV40 T antigen (Phelps *et al.*, 1988; Vousden & Jat, 1989). The C-terminal part of E7 contains a zinc-binding domain composed of two

Cys-X-X-Cys motifs that function as a dimerization domain (Barbosa *et al.*, 1989; Clemens *et al.*, 1995).

Although the predicted molecular weight of HPV E7 is ~11 kDa, in polyacrylamide gels migrates with a molecular size of 18 to 20 kDa (Smotkin & Wettstein, 1986), which is mediated by the CR1 domain or the high content of acidic residues (Munger *et al.*, 1991; Armstrong & Roman, 1993). HPV 16 E7 is located mainly in the cytoplasm but also exists in the nucleus, and its half-life is about one hour (Smotkin & Wettstein, 1987; Greenfield *et al.*, 1991).

Figure 5. Schematic structure of HPV E7 oncoprotein



Taken and modified from McLaughlin-Drubin *et al.* Virology. 2009; 384:335-44.

The biological activities of the E7 protein are linked to its ability to associate with and disrupt the normal activities of cellular regulatory complexes (see Table 3).

HPV E7 proteins associate with pRb and the related pocket proteins that regulate G1/S entry and modulate the E2F transcription factors (Munger *et al.*, 1989). The pRB/E2F complex controls cellular processes such as cellular differentiation, apoptosis and genomic instability (Dyson, 1998). High-risk HPV E7 binds the E2F-bound pRB, destabilises it through proteasomal degradation and thus activates E2F-mediated transcription and uncontrolled S-phase entry (Jones *et al.*, 1997). This pRb-binding ability of the E7 protein leads to several alterations in cellular processes (see Table 3) and the outcome is to retain the differentiating keratinocytes in a DNA synthesis competent state.

Table 3. Cellular protein targets of the E7 protein (summarised from Zwerschke & Jansen-Durr, 2000; McLaughlin-Drubin & Munger, 2009; Yugawa & Kiyono, 2009)

E7 target	Cellular function	E7 domain	Result of E7 binding
pRb/E2F complex	transcriptional repressor, G1 arrest	CR2, C-terminus	proteasomal degradation of pRb, uncontrolled G1 exit
p107, p130	transcriptional repressors, G1 arrest	CR2	disruption of E2F-p107 and E2F-p130 complexes, derepression of E2F
cyclin A, E	subunits of cdk2	CR2	increased levels of cyclin A,E; cell cycle dysregulation
$p27^{KIP1}$,$p21^{CIP1}$	cdk inhibitors	C-terminus	inactivation of $p27^{KIP1}$,$p21^{CIP1}$, activation of cdk2
p600	? chromosome segregation, synaptic transmission, MAP	CR1	inactivation of p600, deregulation of anoikis (a form of apoptosis)
pyruvate kinase	glycolytic control enzyme	C-terminus	weakly active pyruvate kinase, increased glycolytic processes
S4 ATPase subunit	subunit of 26S proteasome	C-terminus	increased ATPase activity
AP-1	transcriptional activator	C-terminus	increased AP-1-dependent transcription
TBP	transcriptional activator	Ser31/32 of N-terminus	? modulation of transcription , interaction with TAF110
HATs	transcriptional co-activators	C-terminus	inactivation of HATs
HDACs	transcriptional co-repressors	C-terminus	increased E2F2-mediated transcription
IGFBP-3	regulation of cell survival	C-terminus	proteasomal degradation of IGFBP-3
IRF-1	transcriptional activator, IFN signaling	CR1, CR2	inhibition of activity

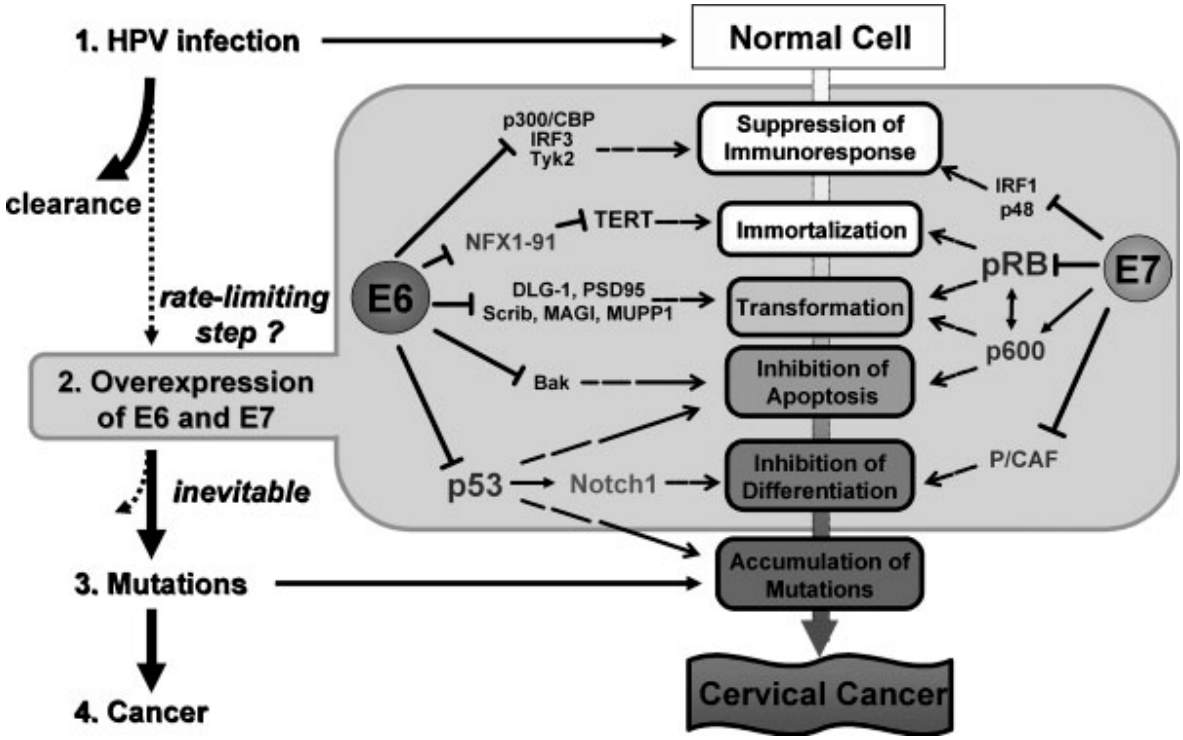
KIP1 – kinase activating protein 1, cdk – cyclin-dpendent kinase, p600 – pRb associated factor, AP-1 – activating protein 1, TBP – TATA binding protein, HATs – histone acetyl transferases, HDACs – class I histone deacetylases, IGFBP-3 – insulin-like growth factor binding protein, IRF-1 – interferon regulatory factor 1

2.1.3.2 Mechanisms in HPV-mediated carcinogenesis

HPVs have been characterised as causative agents for CC. Their DNA is found to be frequently integrated into the chromosomes in cancer cells. As the E6 and E7 viral oncoproteins are constantly expressed in high levels in the CC cells, these proteins play an important role in carcinogenesis and maintenance of transformed phenotype. However, several studies confirmed that the expression of E6 and E7 itself is not sufficient for cancer

development, but it seems to be involved in every stage of multi-step carcinogenesis (Fig. 6) (reviewed in Yugawa & Kiyono, 2009).

Figure 6. Multi-step carcinogenesis for HPV-induced cervical cancer



Yugawa *et al.* Rev Med Virol. 2009; 19:97-113.

Cooperation of E6 and E7 does not merely immortalise normal human epithelial cells but confers tumourigenic properties to transformed cells. One of the supposable epigenetic changes that can also contribute to carcinogenesis, is the aberrant methylation of CpG islands in tumour suppressor genes (TSGs) by DNA methyltransferases (DNMTs), which are over-expressed in several malignancies (Esteller, 2006). There is no evidence for HPV-induced methylation of TSGs so far, but HPV 16 E7 has been shown to bind DNMT1 and stimulate its enzymatic activity (Burgers *et al.*, 2007). Moreover, the transcription of the DNMT1 is under the control of E2F transcription factor that can be stimulated (as mentioned before) by the E7 oncoprotein. There are several other genes, which are commonly found methylated in increased levels in women with invasive disease and are reviewed in (Woodman *et al.*, 2007).

2.1.4 Immunology

Papillomaviruses are very successful infectious agents; the infection they induce is generally long lasting with a risk of progression to malignancy and the host immune response to the infection is weak. There are several reasons why HPVs are poor natural immunogenes (summarised by Frazer, 2004; Stanley, 2009):

- HPVs are dsDNA viruses, with no dsRNA intermediate to induce innate immune responses.
- Infection is strictly intra-epithelial: the virus is not lytic, there is no antigen (Ag) release and also no inflammation to alert the innate immune system.
- Infection has no viraemic phase and therefore less systemic Ag presentation occurs leading to weak humoral immune response.
- Viral non-structural proteins are expressed at low levels compared with more immunogenic proteins from other viruses.
- Additional mechanisms are provided for evading the induction of immune response: interaction of E6 with IRF3 and E7 with IRF1 etc.

Cell mediated immune response is the most important effector mechanism for the control and clearance of viral infections. Both in the regressing genital warts and in the cervical tissues, HPV specific cytotoxic T lymphocytes (CTLs) against early non-structural proteins can be detected. $CD4^+$ and $CD8^+$ are involved in these responses (Nakagawa *et al.*, 1999). HPV 16 E2 and E6 specific $CD4^+$ T cells are measurable in patients with regressing cervical disease and are not seen in persistent infection, what suggests that CTL response to E6 is important for viral clearance (Nakagawa *et al.*, 2000; van Poelgeest *et al.*, 2006).

The cell-mediated immune response is accompanied or closely followed by seroconversion – generation of serum neutralizing antibodies against the major L1 viral capsid protein. Antibodies to the minor L2 capsid protein are not detectable in natural infections with HPVs (Dillner, 1999). Antibody concentrations are low (absence of a viraemia) (Kirnbauer *et al.*, 1994) and 20-50% of women with HPV DNA do not have detectable specific anti-HPV antibodies, which may happen due to the fact that current serological assays are relatively insensitive. Anti-HPV L1 antibodies persist for many years; however, it is still unclear whether these low levels of antibodies protect sufficiently against reinfection with the same HPV type.

2.1.5 Vaccines against HPVs

The well-characterised features of HPV infections provide unique opportunities for development of vaccines aimed against these oncogenic viruses. Two different types of HPV vaccines can be designed, the prophylactic (preventive) vaccines for prevention of HPV infection and the therapeutic vaccine inducing regression of lesions evoked by the virus infection.

2.1.5.1 Prophylactic vaccines

Traditionally, prophylactic vaccines are aimed against virus-specific neutralising antibodies; however, the modest production of anti-HPV L1 antibodies complicated the development of HPV vaccines. The problem was solved after the recognition that the L1 capsid protein, if produced in large amounts in recombinant systems, is able to self-assemble to so-called virus-like particles (VLPs) (Hagensee *et al.*, 1993; Vonka & Hamsikova, 2007; Stanley, 2007).

Two HPV prophylactic vaccines have been developed: Cervarix™, a bivalent HPV 16/18 VLP vaccine, and Gardasil™ also known as Silgard, a quadrivalent HPV 16/18/6/11 vaccine (Table 4). The vaccines are subunit vaccines consisting of L1 VLPs and do not contain any live biological product or DNA, so they are non-infectious (reviewed in Cutts *et al.*, 2007; Stanley, 2009). Vaccines are injected intramuscularly in a three-dose immunisation scheme. The inoculated antigens access the local lymph nodes and thus circumvent the immune avoidance strategy of viral intra-epithelial life cycle. These antigens are highly immunogenic with ability to activate both innate and adaptive immune responses (Harro *et al.*, 2001). VLPs induce high concentrations of neutralizing antibodies to L1 (Harper *et al.*, 2004; Villa *et al.*, 2006) and there is also evidence that HPV L1 VLP vaccines generate not only type specific but also cross-neutralizing antibodies (Smith *et al.*, 2007).

Both vaccines show high efficacy in the Phase II and Phase III randomised control trials achieving over a 5 year period 100% protection against high-grade cervical lesions in 15-26 years old women naïve for HPV 16 and 18 at trial entry (Paavonen *et al.*, 2007; Ault, 2007). The vaccination had only weak effect on lesion development in women who had evidence of past HPV infection and women with persistent HPV infection were not protected at all (Hildesheim *et al.*, 2007). The optimal time for immunisation with VLP vaccines is

before puberty. Studies with the quadrivalent vaccine show that antibody levels are higher in 9-15 year-old girls than in 16-23-year-old women (Block *et al.*, 2006).

Table 4. Characteristics of HPV L1 VLP vaccines (modified from Cutts *et al.*, 2007; Stanley, 2007)

Manufacturer and trade name	Quadrivalent vaccine	Bivalent vaccine
	Merck /Gardasil, Silgard/	**GlaxoSmithKline /Cervarix/**
L1 VLP antigens	HPV 6, 11, 16, 18	HPV 16, 18
Expression system	Yeast (*S. cerevisiae*)	Baculovirus
Adjuvant	Proprietary aluminium hydroxyphosphate sulphate (225μg)	ASO_4 (500 μg aluminium hydroxide + 50 μg 3-deacylated monophosphoryl lipid A)
Injection volume	0.5 ml i.m.	0.5 ml i.m.
Immunisation schedule	0, 2 and 6 months	0, 1 and 6 months
Adolescent safety and immunogenicity bridging trials	Children 9-15 years Women 15-26 years	Females 10-14 years Males 10-18 years

Future generations of preventive vaccines should possess increased number of HPV types to maximize the protection against HPV malignancies. A polyvalent L1 vaccine containing VLPs for nine HPV types is in Phase II clinical trial at present (http://clinicaltrials.gov/ct2show/NCT00943722). Also an L2-based vaccine can be employed, which is less immunogenic than the L1 vaccine, but this may be overcome by using strong adjuvants such as Toll-like receptor agonists (Alphs *et al.*, 2008).

2.1.5.2 Therapeutic vaccines

The existing preventive HPV vaccines targeting L1, as mentioned before, have no therapeutic effect and are unable to eliminate pre-existing HPV infection. Therefore, women already infected with oncogenic HPVs are at risk of developing cancer. It is estimated that it would take ~ 20 years from the mass preventive vaccination to affect the cervical cancer rates.

In order to treat currently infected patients, it is important to develop therapeutic vaccines against HPV (reviewed in Hung *et al.*, 2008; Cid-Arregui, 2009).

Recently developed therapeutic vaccines are aimed to induce cellular immune responses against HPV early antigens. The ideal targets are the E6 and E7 proteins, which unlike capsid proteins are constitutively expressed in HPV precancerous lesions and tumours and are important for induction and maintenance of cellular transformation.

Therapeutic HPV vaccine approaches include live-vector-based, peptide- and protein-based, nucleic-acid-based and cell-based vaccines, each with advantages and disadvantages. These vaccines control HPV infection through cell mediated immunity, mainly through $CD8^+$ T cells, which requires the collaboration of $CD4^+$ helper T cells to get them completely effective (Cid-Arregui, 2009).

The success of therapeutic vaccines may be decreased by the tumour microenvironment. For instance, T regulatory cells release immunosuppressive cytokines that can paralyse T cell functions (Lin *et al.*, 2010a). Therefore, depletion of T regulatory cells from the tumour microenvironment significantly enhances the potency of therapeutic HPV DNA vaccines (Chuang *et al.*, 2009).

The Table 5 summarises the current therapeutic HPV vaccines in clinical trials.

Table 5. Therapeutic HVP vaccine clinical trials (summarised from Hung *et al.*, 2008; Cid-Arregui, 2009; Lin *et al.*, 2010b)

Type of vaccine	Vaccine construct + adjuvant	Antigen(s)	Target subtype(s)	Phase of study/subjects	Sponsor	Reference(s)
Live vector based (bacterial, viral vectors)	Attenuated recombinant *Listeria monocytogenes* encoding antigen (Lovaxin C)	E7 protein fused to listeriolysin O	HPV 16	Phase I, CIN III	Advaxis	(Radulovic *et al.*, 2009)
	Recombinant Vaccinia virus (TA-HPV)	E6/E7 fusion protein	HPV 16, HPV 18	Phase I/II, CIN III	Xenova	(Kaufmann *et al.*, 2002)
	Attenuated recombinant Modified Vaccinia virus Ankara (MVA)	E2 protein	HPV 16, HPV 18	Phase II, CIN III	IMSS	(Garcia-Hernandez *et al.*, 2006)
Peptide based	Set of overlapping long peptides + Montanide ISA-51	E6 protein (9x) + E7 protein (4x) 25-35aa long	HPV 16	Phase I, CIN III	NA	(Kenter *et al.*, 2008)
	Lipopeptide	lipidated E7 (86-93aa)	HPV 16	Phase I	NCI	(Steller *et al.*, 1998)
Protein based	Fusion protein (SGN-00101/HSPE7)	HSP/E7 fusion protein	HPV 16	Phase II, CIN III	Nventa/ StressGen	(Roman *et al.*, 2007)
	Fusion protein (TA-CIN)	L2/E6/E7 fusion protein	HPV 16	Phase I	Xenova	(de Jong *et al.*, 2002)
	Fusion protein + ISCOMATRIX®	E6/E7 fusion protein	HPV 16	Phase I, CIN I-III	CSL Limited	(Frazer *et al.*, 2004)
Dendritic cell based	Autologous dendritic cells (DCs)	DCs pulsed with recombinant HPV 16 E7 or HPV 18 E7	HPV 16, HPV 18	Clinical pilot study, CIN II-III	NA	(Santin *et al.*, 2006; Ferrara *et al.*, 2003)
DNA based	*see under DNA vaccines against HPV*					
Prime-boost	Prime with: TA-CIN Boost with: TA-HPV	L2/E6/E7 fusion protein + E6/E7 fusion protein	HPV 16 + HPV 16, 18	Phase II, AGIN III	Xenova	(Fiander *et al.*, 2006)

aa – amino acid, AGIN – Anogenital intraepithelial neoplasia, CIN – cervical intraepithelial neoplasia, HSP – Heat shock protein, IMSS – Instituto Mexicano del Seguro Social, NCI – National Cancer Institute

2.2 Gene immunotherapy of cancer

2.2.1 Immunotherapy of cancer in general

Immunotherapy, beyond the conventional methods, chemotherapy, radiotherapy and surgery, turned out to be an effective strategy in the fight against malignancies. The purpose of tumour immunotherapy is to stimulate or restore the ability of the immune system, which is commonly weakened during tumour development, to fight. Generally, the goal is to provide either active or passive immunity against cancer. Table 6 gives a brief overview of several methods of anti-tumour immunotherapy.

Passive humoral or cellular immunotherapy consists in administration of components of the immune system to patients: anti-tumour antibodies or specific effector cells that are isolated from patients, activated *ex vivo* and introduced to the bloodstream to affect directly the tumour. However, the application of monoclonal antibodies has some limitations. They do not provide long-lasting effect and therefore repeated delivery is needed. Moreover, these antibodies are potentially immunogenic, which may be a problem for repeated administration (King *et al.*, 2008). The main limitation of autologous effector cells is their preparation – not all cells grow well enough in culture to generate the quantity of cells that is required to produce a useful anti-tumour effect.

Active immunotherapy may be specific or non-specific depending on the properties of the induced immune response. Active **specific** immunotherapy makes efforts to activate effector mechanisms, generally the cytotoxic T cells, which are specific against tumour-associated antigens (TAAs). In comparison with passive immunotherapy, active immunotherapy with vaccines has the potential to induce besides tumour-specific effectors also memory T cells (Disis *et al.*, 2009). Several strategies can be used to induce the cellular immunity and to stimulate the host response; they usually involve administration of peptides, proteins, DNA, DCs pulsed with antigens or tumour cells, which serve as a source of antigens. The purpose of the **non-specific** immunotherapy is to induce the global immune system by application of recombinant cytokines or parts of microorganisms. Clinical trials indicate the effectiveness of recombinant interferons and certain bacterial adjuvants (Table 6), usually simultaneously with chemotherapy or radiotherapy.

Table 6. Anti-tumour immunotherapy

Type of immunotherapy	Treatment principle
Passive immunotherapy	
Humoral	**Monoclonal anti-tumour antibodies (mAbs)** - conjugated with toxins/radioisotopes - linkage to second Ab reacting with CTL
Cellular	**Lymphokine activated killer (LAK) cells** - patient's T cells exposed to IL-2 *ex vivo* and returned to the bloodstream
	Tumour-infiltrating lymphocytes (TILs) - isolated from tumour tissue, exposed to IL-2 and injected to the patient's bloodstream
	TCR gene transfer into T cells - patients treated with autologous peripheral blood T cells transduced with the α and β chains of TCR by viral vectors
Active immunotherapy	
Specific	**Peptide-based vaccines** - short peptide segments from defined TAAs
	DNA-based vaccines - plasmid DNA encoding a specific Ag
	Tumour-cell-based vaccines - *ex vivo* treated autologous tumour cells or allogeneic tumour cell lines (irradiated, treated with neuraminidase, genetically modified *etc.*)
	Vector- based vaccines - recombinant bacterial vaccines or recombinant viruses expressing tumour Ags, immunostimulatory cytokines *etc.*
	Dendritic-cell-based vaccines - generated *in vitro* or *ex vivo* and introduced to patients - DCs pulsed with tumour lysates or peptide tumour epitopes, fused with irradiated tumour cells, transfected with nucleic acids encoding TAAs *etc.*
Non-specific	**Bacterial adjuvants** - BCG and its derivatives, killed suspensions of *Corynebacterium parvum*
	IFN-α,β,γ

Ab – antibody, Ag – antigen, BCG – bacille Calmette- Guérin, CTL – cytotoxic T lymphocytes, DC – dendritic cell, IFN – interferon, IL – interleukin, TAA – tumour-associated antigen, TCR – T cell receptor

As most types of immunotherapy, both active and passive, are designed to be targeted against specific antigens of cancer cells, this therapy may lead to one serious disadvantage: the tumour cells can mutate and thus avoid immune responses. Moreover, the same tumour may produce a slightly modified antigen in different patients. Therefore, the immunotherapy directed against a certain antigen might become ineffective.

A term **gene immunotherapy** of cancer was established for immunotherapies that apply methods of gene therapy trying to use the genetic pattern of tumour cells to fight with cancer. The strategies of gene therapy, such as gene modification of tumour or non-tumour cells and utilisation of therapeutic anti-cancer vaccines, induce anti-tumour immune responses or use the mechanisms of immune system to eliminate tumour cells.

2.2.2 Mechanisms of tumour escape from anti-tumour immunity

In early stages of tumour development, effective anti-tumour immune response occurs that persists even during the tumour growth. The concept of tumour immunoediting gives an explanation for the role of the immune system in tumour development. Three phases, the elimination phase, the phase of equilibrium and the escape phase, form this concept. During the phase of elimination, also called tumour immune surveillance, the immune system eliminates the detected tumour cells. This elimination can be complete, when all tumour cells are cleared, or incomplete. The phase of equilibrium represents a period of cancer persistence. During this phase, tumours accumulate changes that help them to escape from or to suppress the immune responses. The balance between the activation and suppression of immune responses determines the fate of the tumour. If the immune system fails to contain the tumour growth, the tumours progress and the tumour development leads to the escape phase (Khong & Restifo, 2002; Swann & Smyth, 2007).

The ability of tumours to evade the host immune system may affect the immunotherapy. Therefore, several factors, described in the following two chapters, have to be considered when designing therapeutic vaccines.

2.2.2.1 Tumour microenvironment and cellular mechanisms of tumour escape

The tumour microenvironment is a unique environment comprised primarily of tumour cells, immune cells, fibroblasts and the extra-cellular matrix. Inflammatory cells found in tumours contribute to the progression of tumours and their escape from the host immune system. Modification of the function of infiltrating cells by the tumour cells leads to creating a microenvironment suitable for tumour growth. The immune cells in tumours are represented generally with T lymphocytes, dendritic cells and macrophages and occasionally with B cells and natural killer (NK) cells (reviewed in Whiteside, 2008).

Lymphocytes in the tumours, also called tumour-infiltrating lymphocytes (TILs), are the major component of the tumour microenvironment. These cells are commonly $CD4^+$ and $CD8^+$ T cells and many of them are specific for TAAs but mostly ineffective in avoiding tumour growth (Whiteside, 2006). A subset of T cells with $CD4^+$ $CD25^{high}$ $Foxp3^+$ characteristics is a population of regulatory T cells (T_{reg}) with suppressing abilities affecting the proliferation of effective TAA-specific T cells thus contributing actively to tumour development. T_{reg} depletion or inhibition of their function belongs to the main strategies of cancer immunotherapy (Colombo & Piconese, 2007).

Tumour-associated macrophages (TAMs) play a key role in tumour growth, dissemination and angiogenesis and, through releasing of inhibitory molecules, they suppress lymphocyte functions (al-Sarireh & Eremin, 2000; Martinez *et al.*, 2008). Furthermore, immature myeloid precursors, also known as myeloid suppressor cells (MSCs), are accumulated in the tumour site and peripheral blood of cancer patients. These $CD14^+$ $CD11b^+$ HLA-$DR^{neg/low}$ myeloid precursors influence the immune system by suppressing the development of specific T cell responses. They inhibit T cell immunity through TGF-β release or arginase I production (Serafini *et al.*, 2006; Filipazzi *et al.*, 2007).

When talking about tumour microenvironment, it is necessary to mention vesicular structures called microvesicles or exosomes, which are released by tumour cells. As the content of these recently re-discovered organelles involves tumour antigens, they were supposed to be potential anti-tumour vaccines. Unfortunately, it turned out that tumour microvesicles have negative effect on anti-cancer immune responses. They also retain molecules, which promote the tumour progression *in vivo* (reviewed in Iero *et al.*, 2008).

2.2.2.2 Molecular mechanisms leading to tumour escape

Tumour cells attempt to escape mostly from immune *recognition*. A number of molecular mechanisms enabling the tumour to become invisible for the immune system were described. Here is a brief summary of the well-known escape strategies:

MHC class I loss or down-regulation:

Several MHC class I phenotypes and changes in MHC class I expression were described in animal or human tumours. These alterations are mostly results of mutations and defects in the antigen-processing machinery (APM), which lead to down-regulation of MHC I molecules. The total loss of MHC I expression and the loss of MHC class I haplotype or allelic loss may occur through mutations in genes for the MHC I. Such abnormalities affect the MHC I-dependent antigen presentation and the tumour cells escape from recognition by $CD8^+$ T cells (reviewed in Ahmad *et al.*, 2004). Moreover, epigenetic modifications were reported to be an important factor in regulation of the APM. DNA methylation or histone deacetylation of genes encoding components of APM may lead to reduced MHC class I molecule expression on the tumour cell surface (Manning *et al.*, 2008).

Tumour antigen loss, down-regulation or mutation:

Modifications in TAA expression are another reason for the escape of tumour cells from the immune system, even in the presence of TAA-specific CTLs. Mutations in the immunodominant epitope of the antigen (antigenic drift) disable the MHC-peptide interaction and the binding to TCR (Ahmad *et al.*, 2004). The suboptimal expression level of the antigen is associated with the *in vivo* immunoselection of antigen-loss variants of the tumour (Lozupone *et al.*, 2003).

Alterations in signalling pathways:

Fas/Fas ligand (FasL) signal pathway plays a key role in tumour immune escape as the activation of this pathway leads to apoptosis of the immune cells. Suppression of the FasL expression in tumour cells, further blocking the TNF apoptotic signal pathway of the immune cells, can increase the survival of the cells of the immune system (Zhang & Xu, 2007; Xu & Zhang, 2008). Furthermore, the Fas/FasL complex generally activates the caspase-8 pathway that finally leads to cell death. Therefore, in many tumours, cellular FLICE-inhibitory protein (cFLIP), a caspase-8 inhibitor, is expressed (Medema *et al.*, 1999). Several human cancers escape from apoptosis by activation of the survival signal (antiapoptotic factors of the Bcl-2 family) with phosphatidylinositol 3'-kinase (PI3K) and protein kinase B (Osaki *et al.*, 2004).

Alteration in signal transduction molecules:

The CD3 ζ chain is a part of the TCR complex and is concerned in inducing the activation signal in T lymphocytes. The total loss of the CD3 ζ chain or its reduced expression is associated with elevated production of immunosuppressive cytokines and thus contributes to immune evasion (Ahmad *et al.*, 2004).

Lack of co-stimulation:

Cancers may progress even in the presence of TAAs due to the lack of expression of co-stimulatory molecules on a tumour cell surface. The insufficient co-stimulation induces anergy in the T cells (Abken *et al.*, 2002). Viral vectors co-expressing IL-12 and B7.1 could be used in the immunotherapy of cancer, which reverses the expression of co-stimulatory molecules and thus increases the immunogenicity of tumour cells (Wen *et al.*, 2001).

2.2.3 Tumour-escape mechanisms used by HPVs

Various reasons were described why the immune system fails to recognise the replicating HPV. As the virus is non-lytic, has no blood-borne phase and the early proteins are expressed at low levels, the production of the viral antigens is limited and insufficient to induce pro-inflammatory signals to activate the host immune system. Other reasons could be the different gene-codon usage and the ability of viral proteins to mimic the host proteins to take advantage of the host's self-tolerance (reviewed in Kanodia *et al.*, 2007).

The alteration of antigen presentation in HPV infected tumour cells represents an essential role in immune evasion. Several evidence exists suggesting that the immunogenic peptides from the E6 and E7 proteins are not efficiently processed by tumour cells, and a down-regulation of MHC class I molecules and TAPs was recorded (Bauer *et al.*, 2000; Evans *et al.*, 2001). The regulation of transcription of genes involved in antigen presentation is one of the many functions of the E7 protein, which leads to reduced protein presentation and the virus easily escapes from CTL attack. Moreover, the HPV 16 E5 protein affects the stability of the peptide-MHC class I complex by alkalinisation of the Golgi apparatus and endosomes (Ashrafi *et al.*, 2005).

HPVs, like many other viruses, also disrupt the IFN type I pathway, which has anti-viral and immunostimulatory activities. The E6 protein binds to IRF-3 to inhibit its trans-

activation function and to block the IFN-β gene transcription (Ronco *et al.*, 1998). The E7 protein also prevents the transcription of IFN-β due to IRF-1 binding (Park *et al.*, 2000).

Furthermore, HPVs up-regulate the PI3K pathway in Langerhans cells (LC) at the site of primary infection and thus inhibit LC from inducing immune response (Fausch *et al.*, 2005). The E6 protein contributes to inhibition of apoptosis of the infected cells – this protein prevents cells from p53-dependent cell death by binding to p53 (Howie *et al.*, 2009).

2.3 DNA vaccines

DNA vaccines are a relatively new vaccination strategy but the beginnings of their development date back to the early 1990s. A number of animal model studies indicated the success of vaccination with DNA preparations (Wolff *et al.*, 1990; Williams *et al.*, 1991) and with time DNA vaccines emerged as an attractive form of immunotherapy.

A DNA vaccine is composed of a plasmid DNA encoding the antigen of interest under the control of a mammalian promoter, traditionally the human cytomegalovirus (CMV) immediate-early or CMV-Chicken-β actin promoter, and can be easily produced in bacteria. Commonly utilized selectable markers are the antibiotic resistance markers. The expression is generally higher if an intron is present downstream of the promoter. However, a number of prokaryotic sequences, negatively affecting the gene expression in eukaryotic cells, have been identified. Therefore, it is important to evaluate all changes made in the composition and orientation of elements within the prokaryotic region of the plasmid (reviewed in Williams *et al.*, 2009). Moreover, there are several components build into the plasmid DNA that can affect the immunogenicity of the vaccines (see Chapter *Enhancement of DNA vaccine potency*).

2.3.1 Immune responses and vaccine delivery

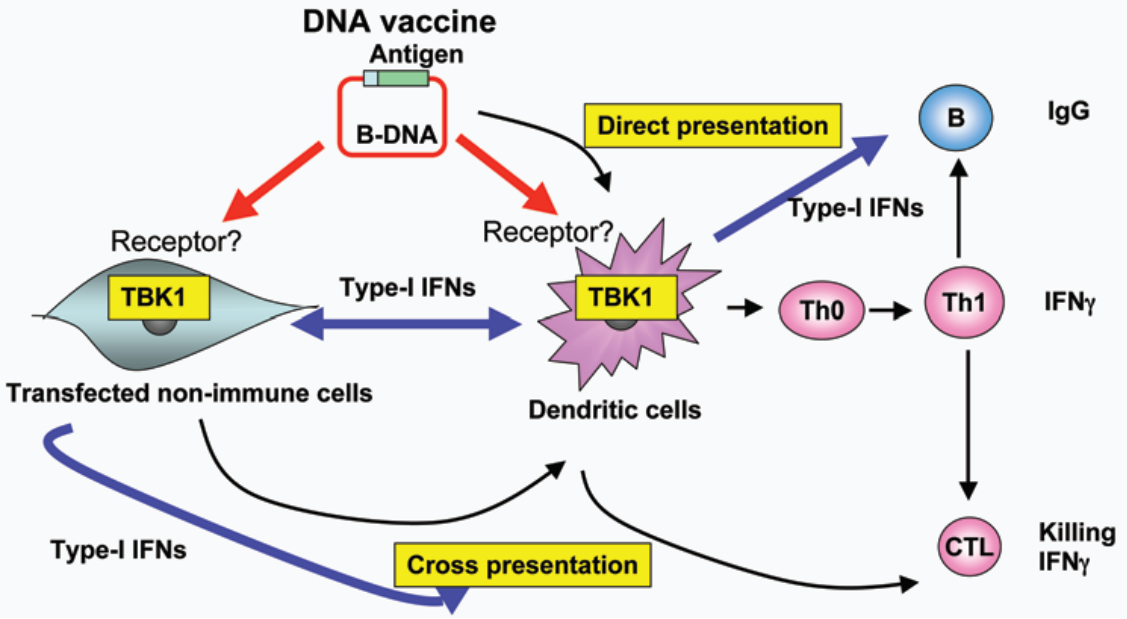
Once the plasmid DNA is administered *in vivo*, the encoded antigen is expressed in the host cells and presented by antigen presenting cells (APCs). This occurs mainly in the lymph nodes and leads to the induction of both the cellular and humoral immune responses that is a unique feature of the DNA vaccines (Coban *et al.*, 2008).

APCs, generally dendritic cells (DCs), play the key role in the activation of the innate immunity. They process and present the endogenously expressed antigens to class I and class II MHC molecules that leads to the priming of naïve $CD8^+$ and $CD4^+$ T cells. The activated $CD8^+$ cytotoxic T cells then kill tumour cells by inducing apoptosis in the target cells and the induction of the $CD4^+$ T cells can help to augment the $CD8^+$ T cell immune response (Lin *et al.*, 2010b). As the DNA vaccines are ordinarily injected into muscle or skin, the antigen is mainly produced in myocytes or keratinocytes, which are not professional APCs. The antigen must be transferred to DCs and this indirect process of presentation is termed cross-presentation. DCs may acquire the antigen from exogenous source into the MHC class I pathway also from dead or dying cells by phagocytosis (reviewed in Rice *et al.*, 2008;

Abdulhaqq & Weiner, 2008). The transfected myocytes and keratinocytes can contribute to immune activation through production of cytokines and chemokines and through the up regulation of the MHC class I expression (Larregina & Falo, Jr., 2000; Shirota *et al.*, 2007).

However, there are still many unclarities about the precise cellular and molecular mechanisms by which the DNA vaccine works *in vivo*. For instance: it has been thought that the CpG motifs, sequences of an unmethylated C followed by G, are the built-in immunostimulatory elements of the DNA vaccines. The addition of many CpG motifs into the plasmid DNA enhances the immunogenicity of the vaccines (Coban *et al.*, 2005). The Toll-like receptor 9 (TLR9) is the mediator for the induction of the protective immune response (Krieg, 2006). Nowadays, it is known that DNA vaccines can stimulate the innate immune system independently of TLRs. The double stranded structure of the DNA vaccine is essential for the activation of type I IFN-mediated immune response. The key signalling molecule in this process is the TANK-binding kinase-1 (TBK1, Fig. 7). This way of activation affects both the direct and the cross-presentation of an antigen (reviewed in Coban *et al.*, 2008).

Figure 7. Priming of the immunity after DNA vaccination



Coban *et al.* Hum Vaccin. 2008; 4:453-6.

Different administration methods may influence the immune response by the rate of transfection efficacy or by affecting the way of antigen presentation thus the immunogenicity of the DNA vaccines could be different depending on the way of delivery. Moreover, the high immune response relies not on the amount of administered DNA but on the ability of the

DNA to enter efficiently the targeted cells. There are four currently leading delivery methods for DNA vaccine administration and each of them has its advantages and disadvantages.

Intramuscular (i.m.) needle injection

The DNA vaccine designed for i.m. injection contains the plasmid DNA dissolved mostly in saline or other solutions (Lu *et al.*, 2008). The optimum dose for the vaccination is approximately 50-100 μg for mice. The predominant cells transfected after the inoculation of the vaccine are myocytes. A very recent observation characterised the distribution and presentation of the plasmid-encoded antigen *in vivo* as following: The injected DNA vaccine promptly enters the peripheral blood and lymphatics from the site of injection and reaches the lymphoid tissue as free DNA within 24 h. DNA in lymph nodes (LNs) is taken up by peripheral cells that then migrate deeper to the LN. pDNA and/or the expressed antigen is then transferred to DCs ($CD11c^+$) for presentation to naïve T cells. Simultaneously, DNA from the blood reaches the bone marrow and spleen where it is taken up by DC precursors. After three days, antigen-specific $CD4^+$ T cells are detectable in these tissues. In summary, the immune responses are induced by DNA vaccines within days and become systemic very rapidly (Rush *et al.*, 2010).

Intradermal (i.d.) delivery via gene gun

The commercial name used for the gene gun technology is Particle Mediated Epidermal Delivery (PMED) that presents a needle free device (Fig. 8). The plasmid DNA is coated onto gold particles and delivered via the gene gun under pressure into the epidermal layer of a skin. Thus the DNA penetrates directly into the cytoplasm (Tang *et al.*, 1992) of keratinocytes and DCs (Langerhans cells) present in the epidermis. Generally, only a small amount (1-2 μg for mice) of the plasmid DNA is delivered and the antigen expression persists 4-14 days in the site of administration. Transfected DCs migrate to regional LNs within 12-24 h and present antigen to naïve $CD8^+$ T cells (Porgador *et al.*, 1998).

Intradermal (i.d.) delivery via tattooing

Tattooing represents a method of solid vibrating needle (Fig.8) that repeatedly punctures the skin and wounds the epidermis and dermis. This procedure causes dermal haemorrhage and necrosis and induces cutaneous inflammation followed by healing (Gopee *et al.*, 2005). 50-100 μg of plasmid DNA dissolved in saline is usually delivered to the skin at

the dorsum of animals and is able to induce cellular and humoral antigen-specific responses (Baxby, 2002). Tattooing involves much larger area of the skin than other intradermal administrations, what potentially leads to more transfected cells (Bins *et al.*, 2005). However, the gene expression after tattoo delivery peaks after 6 h and vanishes within 4 days (Corder *et al.*, 1996). Moreover, studies with an *ex vivo* human skin model showed extremely low transfection efficiency of this technique that indicates a necessity to develop strategies for enhancing the *in vivo* transfection efficacy of tattoo-delivered DNA vaccines (van den Berg *et al.*, 2009).

Figure 8. Helios gene gun from Bio-Rad and tattoo machine from Bortech Tattoogrosshandel

Electroporation-mediated i.m. delivery

Electroporation (EP) is a method in which multiple electric pulses (20-30 ms) of low voltage (50-200 V/cm) are applied to the vaccination site to improve transfection efficacy in the tissues where the DNA vaccine was delivered before by conventional needle injection. The transfection efficacy is enhanced by electric pulse by two potential ways: either the electric pulse creates pores in a cell membrane, which facilitates the entry of naked DNA into a cell, or the tissue damage induces inflammation and recruits DCs and lymphocytes to the site of injection (reviewed in Abdulhaqq & Weiner, 2008). The doses of DNA applied in this vaccination method are comparable with those used by i.m. injection. EP elicits high cellular and humoral immune responses in various animals and also in humans. However, a special device is needed for human application. It is important to be proved that EP does not generate high risk of DNA integration into the host-cell genome (Lu *et al.*, 2008).

Several DNA delivery studies were published that compare the different DNA vaccine immunisation methods (Trimble *et al.*, 2003; Wang *et al.*, 2008). Based on these results, only

the gene gun-mediated approach is able to elicit protective levels of immune responses in humans. EP-mediated administration is also highly effective in inducing antigen-specific immune responses in animal models and in prime-boost combination protocols is successful in humans, too (Chiarella *et al.*, 2010). Indeed, both the gene-gun immunisation and the administration with EP are several times more immunogenic than the simple i.m. needle injection. A more recent study performed a comparison of EP and gene-gun delivery methods in ability to generate antigen-specific $CD8^+$ T cell responses and anti-tumour immune responses against the HPV E7 protein. The DNA vaccine administered with EP induced the higher number of E7-specific cytotoxic cells when compared to gen-gun delivery (Best *et al.*, 2009). Comparison of the efficacy of DNA vaccines delivered by a tattoo device or needle injection revealed that the humoral and cellular immune responses induced by tattooing are significantly higher than those after i.m. administration of DNA (Pokorna *et al.*, 2008). To the best of my knowledge, there is no published data about the comparison of gene gun or EP-mediated delivery of a DNA vaccine with tattooing.

2.3.2 Advantages and disadvantages

DNA vaccines have several advantages when compared to other forms of vaccines. First of all, they are relatively safe, safer than live attenuated vaccines or inactivated viral vaccines, which may cause infection *in vivo*. Moreover, DNA vaccines are unable to revert to viral forms, unlike live vector-based vaccines, and can be administered repeatedly to the same individual (Abdulhaqq & Weiner, 2008; Lin *et al.*, 2010b). Studies with DNA vaccines have shown that DNA plasmids themselves are not immunogenic and they do not generate anti-DNA antibodies, even after multiple administrations (Smith, 2000). Additionally, DNA vaccines are stable and relatively simple to design and prepare at high purity. As they are not really temperature sensitive, their storage and transportation is inexpensive thus DNA vaccines are highly suitable for mass production and distribution.

Potential integration of the plasmid DNA into the host cell genome may represent a risk in term of clinical application of DNA vaccines. Integration of the foreign DNA into the site of proto-oncogenes or tumour-suppressor genes could lead to tumour progression. However, there is still no evidence about connection between tumour development and the integration of the plasmid after DNA vaccination (Nichols *et al.*, 1995; Ramirez *et al.*, 2008).

Other important limitation of DNA vaccines is their low immunogenicity, particularly in large animals and humans, due to the inability to spread from transfected cells into surrounding cells *in vivo*.

2.3.3 Enhancement of DNA vaccine potency

As mentioned above, the suboptimal immunogenicity of DNA vaccines requires solutions. Nowadays, numerous strategies exist to enhance the DNA vaccine efficacy. Here is the summary of the main improvements:

1. *Modification of plasmid sequences*

Induction of a strong immune response after plasmid DNA immunisation depends on the high constitutive expression levels of the encoded antigen. Therefore, utilising strong regulatory elements, the promoter/enhancer, intron and polyadenylation signal, is a key parameter in plasmid DNA vaccine vector design. Unmethylated CpG motifs also contribute to immune system stimulation (Williams *et al.*, 2009). Additionally, differences between codon usage in a heterologous gene and host organisms may affect antigen expression. Therefore, codon adjustment of the plasmid expressing the antigen is considerable to maximise translational efficiency and consequently the immune response. For instance, the replacement of wild-type codons in the HIV-1 gp120 DNA vaccine increased the expression of the gp120 compared to the wild-type gene and significantly enhanced the $CD8^+$ T cell response. Such codon optimisation of the antigen increased the immunogenicity of DNA vaccines also against tetanus or malaria (Garmory *et al.*, 2003).

2. *Improved delivery methods*

The different routes of administration may determine or affect the induced immune responses. Various vaccine deliveries are described precisely in the Chapter *Immune responses and vaccine delivery*.

3. *Utilisation of adjuvants*

Nowadays, novel types of adjuvants, the molecular adjuvants, are used beyond the conventional ones (e.g. Freund adjuvant) including cytokines, chemokines and costimulatory molecules. Co-injection of DNA encoding cytokines IL-2 and IFN-γ stimulates the T_H1 immune response and thus the activation of $CD8^+$ T cells (Chow *et al.*, 1998). Co-administration of plasmids encoding GM-CSF (granulocyte-macrophage colony-stimulating

factor) and MIP-1α (macrophage inflammatory protein 1α) with a DNA vaccine recruits macrophages and DCs to the site of inoculation that leads to activation of $CD4^+$ and $CD8^+$ T cells (McKay *et al.*, 2004). However, an optimal combination of adjuvants with DNA vaccines is needed to be further proposed to significantly enhance the DNA vaccine potency (Ohlschlager *et al.*, 2009).

The co-delivery of "helper antigens" with weak antigens of interest may be an alternative strategy. Helper antigens, such as keyhole limpet hemocyanin (KLH), tetanus toxin or β-galactosidase, are foreign antigens that activate strong T cell immune responses. The gene encoding the helper antigen may be administered separately from the gene for the target antigen on a second plasmid but to the same site of immunisation, or the two plasmids may be co-coated onto the same gold particles when delivered by a gene-gun device (Leitner *et al.*, 2009).

4. *Modification of antigen*

Construction of DNA fusion-gene vaccines is another strategy to activate effective immune response. Co-expression of helper antigens by generating fusion proteins with the target antigen enhances immune responses, for example heat shock proteins (Hsp; Chen *et al.*, 2000; Qazi *et al.*, 2005) or *E.coli* β-glucuronidase (GUS; Smahel *et al.*, 2004) fused to target antigen induce high $CD8^+$ T cell response. Moreover, the linkage to Fragment C (FrC) of tetanus toxin results in tumour growth suppression (Stevenson *et al.*, 2004).

Several modifications of the plasmid-expressed antigens are focused on their targeting to antigen presentation pathways. Attachment of a signal sequence permits the antigen to entry the endoplasmic reticulum (ER) and subsequently, the antigen is presented on the cell surface through MHC class I molecules to $CD8^+$ T cells (Leifert *et al.*, 2004). Furthermore, antigens can be directed to the lysosome/endosome by linkage to lysosome-associated membrane protein-1 (LAMP-1) and thus induce the $CD4^+$ T cell responses via MHC class II molecules (Wu *et al.*, 1995).

The fusion of the antigen with VP22, a viral translocatory protein from HSV-1 or the Marek's disease virus, helps the spread of the antigen from cells where they are abundantly expressed into neighbouring APCs (Manoj *et al.*, 2004). Linking the antigen to cytotoxic T-lymphocyte antigen-4 (CTLA-4) targets the antigen directly to APCs through the recognition with the B7 costimulatory molecule (Boyle *et al.*, 1998).

2.3.4 DNA vaccines against HPV

DNA vaccines against HPV represent therapeutic vaccines focused on two, E6 and E7, viral antigens. The administration of the E6 and E7 genes may lead to cell transformation as the produced E6 and E7 proteins are oncogenic. Thus a modification is needed to turn E6 and E7 to proteins incapable of such transformation.

Limitation of HPV E6 and E7 DNA vaccines is definitely their low immunogenicity. Therefore, several strategies have been developed to overcome this obstacle (for review see Lin *et al.*, 2010a; Lin *et al.*, 2010b):

- Increasing the number of HPV antigen-expressing or HPV antigen-loaded DCs – including different routes of administration or utilisation of microencapsulated vaccines, increasing intercellular spreading of HPV antigens to DCs etc.
- Improving HPV-antigen expression, processing and presentation – codon optimisation, directing the antigen to MHC presentation pathways etc.
- Enhancing DC and T cell interaction – prolonging DC survival and increasing cytokine expression, priming helper T cells etc.

The following paragraphs detail some of these methods of enhancing the immunogenicity of therapeutic HPV DNA vaccines:

An effective strategy to enhance antigen expression is the employment of demethylation agents. It has been shown that DNA methylation, particularly the methylation of CpG motifs in the plasmid of DNA vaccines, silenced gene expression (Hirasawa *et al.*, 2006). Thus, application of a nucleoside analogue 5-aza-2'-deoxycytidine (DAC), which inhibits DNA methyltransferases (DNMTs), could prevent DNA methylation. The combination of the DNA vaccine encoding calreticulin linked to HPV 16 E7 (CRT/E7) with DAC treatment led to the up-regulation of CRT/E7 expression and enhanced E7-specific $CD8^+$ T cell response (Lu *et al.*, 2009).

CIITA (Class II, major histocompability complex, transactivator) is a regulator of the expression of MHC I and MHC II molecules on the DC surface. Therefore, the co-delivery of CIITA with HPV DNA vaccines leads to enhanced antigen presentation through both MHC pathways and subsequently to stronger $CD4^+$ and $CD8^+$ T cell responses (Kim *et al.*, 2008).

A technology called single chain trimer (SCT) represents a DNA vaccine encoding the antigenic peptide fused to β2-microglobulin and MHC I heavy chain. The fusion protein (antigenic peptide/MHC class I molecule) is expressed on the DC surface as MHC I

molecules already loaded with the antigenic peptide. Mice immunised with the HPV E6 SCT vaccine were completely protected from E6-producing TC-1 tumour cells and an increased E6-specific $CD8^+$ T cell response was detected (Huang *et al.*, 2005).

To disable DCs or T cells from undergoing apoptosis after their mutual interaction, the pro-apoptotic proteins and the pro-apoptotic signalling protein of the DCs are essential to be blocked. The co-administration of the anti-HPV 16 E7 DNA vaccine with siRNA silencing the expression of the pro-apoptotic Bak and Bax proteins or with DNA encoding shRNA blocking the pro-apoptotic signalling protein, the Fas ligand, resulted in significantly enhanced E7-specific $CD8^+$ T cell response and strong anti-tumour effect in vaccinated mice (Kim *et al.*, 2005; Huang *et al.*, 2008).

Several DNA vaccines have already been tested on humans and the following table summarises the finished or ongoing clinical trials with therapeutic HPV DNA vaccines (Table 7).

Table 7. Clinical trials with therapeutic HPV DNA vaccines (modified from Lin *et al.*, 2010b)

Vaccine construct + adjuvant	Antigen(s)	Target subtype(s)	Route of administration	Phase of study/subjects	Sponsor	Reference(s)
DNA (ZYC101)	E7 epitope (83-95aa)	HPV 16	i.m. injection with microencapsulation of the DNA vaccine	Phase I, anal HSIL Phase I, CIN II-III	MGI Pharma	(Klencke *et al.*, 2002; Sheets *et al.*, 2003)
DNA (ZYC101a)	E6, E7	HPV 16, HPV 18	i.m. injection with microencapsulation of the DNA vaccine	Phase II, CIN II-III	MGI Pharma	(Garcia *et al.*, 2004)
DNA (pNGVL4a-sig/E7(detox)/HSP70)	E7	HPV 16	i.m. injection	Phase I, CIN II-III	NCI	(Trimble *et al.*, 2009)
Prime: DNA (pNGVL4a-Sig/E7(detox)/HSP70) Boost: rVV (TA-HPV) ± imiquimod	E7 E6, E7	HPV 16 HPV 16, HPV 18	i.m. injection (DNA vaccine and rVV) topical (imiquimod)	Phase I, CIN III	NCI	(Lin *et al.*, 2010b)
DNA (pNGVL4a-CRT/E7(detox))	E7	HPV 16	i.d. injection via gene gun	plans for Phase I, CIN II-III	NCI	(Lin *et al.*, 2010b)
DNA (VGX-3100)	E6, E7	HPV 16, HPV 18	i.m. injection with electroporation	Phase I, CIN II-III	VGX Pharmaceuticals	(Lin *et al.*, 2010b)

aa – amino acid, CIN – cervical intraepithelial neoplasia, CRT – calreticulin, HSIL – high-grade squamous intraepithelial lesions, HSP – heat shock protein, i.d. – intradermal, i.m. – intramuscular, NCI – National Cancer Institute, rVV (TA-HPV) – recombinant vaccinia virus

3. PAPERS

3.1 Paper I:

Mutation in the immunodominant epitope of the HPV 16 E7 oncoprotein as a mechanism of tumor escape

Michal Smahel, Pavla Tejklova, Jana Smahelova, Ingrid Polakova,
Jana Mackova

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Contribution of the author of this PhD. thesis:

I participated on the processing of splenocytes for the ELISPOT assay and tetramer staining and carried out the ELISPOT assays.

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ORIGINAL ARTICLE

Mutation in the immunodominant epitope of the HPV16 E7 oncoprotein as a mechanism of tumor escape

Michal Smahel · Pavla Tejklova · Jana Smahelova · Ingrid Polakova · Jana Mackova

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Abstract Infection with high-risk types of human papillomavirus (HPV) can cause the development of malignant tumors. To study mechanisms responsible for immune escape of tumor cells infected with HPV16, we previously used mouse oncogenic TC-1 cells producing HPV16 E6 and E7 oncoproteins to derive TC-1 clones resistant to immunization against E7. We have found immunoresistance of the clones to correlate with the point mutation in the E7 oncogene, which resulted in the N53S substitution in the immunodominant epitope RAHYNIVTF (aa 49–57). Here, we have shown that this mutation reduced stabilization of H-2D^b molecules on RMA-S cells and eliminated immunogenicity of E7. The resistance of TC-1 clones was E7-specific as immunization against E6 inhibited tumor growth. Transduction of the TC-1/F9 clone carrying the mutated epitope with the wild-type E7 gene restored susceptibility to immunization against E7. Our results suggest that mutagenesis of tumor antigens can lead to the escape of malignant cells and should be considered in the development and evaluation of cancer immunotherapy.

Keywords Tumor escape · Epitope · Human papillomavirus · E7 oncogene

Introduction

Evasion of tumor cells from the host immune responses is probably the main obstacle to the successful utilization of anti-tumor vaccines in clinical practice. Various mechanisms contributing to this phenomenon were revealed [27]. As CD8$^+$ cytotoxic T lymphocytes (CTLs) play the crucial role in the elimination of tumor cells, impaired presentation of tumor antigen epitopes by MHC class I molecules is the most frequent reason for tumor escape. It can be caused by down-regulation of MHC class I expression [12], defects in antigen-processing machinery (APM) [34], and/or elimination or reduction of one or more tumor antigenic epitopes [1].

Reduction or loss of expression of a tumor antigen is a well-characterized event after specific immunotherapy of human melanomas [18, 31]. Such down-regulation only involves melanoma antigens dispensable for proliferation of transformed cells and progression of malignant tumors. However, some tumor antigens are oncoproteins whose production is vital for cellular transformation and its maintenance. As their down-regulation is unlikely to happen, other mechanisms of tumor escape may evolve. Bai et al. [2], have shown that mutations in a tumor-antigenic epitope can have the same effect as the elimination of the whole antigen in the escape of tumor cells to lysis by CTLs.

High-risk types of human papillomavirus (HPV) can induce the development of malignant tumors, of which cervical carcinoma (CC) is the most serious disease. HPV16 is the most prevalent HPV type found in about 50–60% of CC patients [40]. It encodes three proteins with oncogenic properties: E5, E6, and E7. While E5 is usually absent in late stages of tumor progression, E6 and E7 are the only viral proteins constitutively produced in all tumor cells and are indispensable for both the oncogenic transformation of cells and maintenance of the transformed state [24]. The level of E6 and E7 expression correlates with their oncogenic potential. Both oncoproteins have been shown to be a suitable target of immune responses induced in animal

M. Smahel (✉) · P. Tejklova · J. Smahelova · I. Polakova · J. Mackova
Department of Experimental Virology, Institute of Hematology and Blood Transfusion, U Nemocnice 1, 128 20 Prague 2, Czech Republic
e-mail: smahel@uhkt.cz

models by therapeutic vaccines [6, 7] and E6- and E7-specific cell-mediated immunity has also been observed in HPV-infected people [33].

From a tumor developed from mouse oncogenic TC-1 cells producing E6 and E7 oncoproteins, we have recently derived clones resistant to DNA immunization against E7. Furthermore, we have shown that immunoresistance of the clones correlated with the N53S mutation in the immunodominant H-2D^b-binding E7 epitope RAHYNIVTF [38]. In this study, the N53S mutation has been shown to eliminate immunogenicity of E7 and to be responsible for evasion of TC-1 clones carrying the mutated E7 from E7-specific immune responses induced by vaccination.

Materials and methods

Plasmids

The point mutation of the HPV16 E7 oncogene recently found in four immunoresistant TC-1 clones and resulting in the N53S substitution in the immunodominant E7 epitope [38] was introduced into the E7 gene and the fusion gene E7GGG.GUS. These genes were first isolated from plasmids pBSC/E7 [37] and pBSC/E7GGG.GUS [35], respectively, after digestion with *Eco*RI and cloned into the *Eco*RI site of pALTER-MAX (Promega, Madison, WI). Mutagenesis was then performed using the Altered Sites II Mammalian Mutagenesis System (Promega) and oligonucleotide GGTTACAATACTGTAATGGGC (the altered nucleotide is underlined). The resultant genes E7S and E7GGGS.GUS were cloned back between the *Eco*RI sites of pBSC. The fusion gene E7GGG.GUS consists of the mutated HPV16 E7 gene (E7GGG) and the gene encoding *E. coli* (β-glucuronidase (GUS). The E7GGG gene contains three point mutations resulting in substitutions D21G, C24G, and E26G in the Rb-binding site [37].

For transduction of TC-1/F9 cells, plasmids pPUR/E7.FLAG and pPUR/E7S.FLAG were constructed as follows: the E7 and E7S genes were amplified from plasmids pBSC/E7 and pALTER/E7S, respectively, using primers 5′-TCAGTACAGCTGTAATCATGCATG-3′ (forward) and 5′-TGACTCGAGTTACTTGTCGTCATCGTCTTTGTAGTCGACTGGTTTCTGAGAACAGAT-3′ (reverse). The reverse primer contains the sequence encoding the FLAG epitope (underlined). The PCR products were cleaved with *Pvu*II and *Xho*I and the resulting two fragments were cloned into pPUR/E7, from which the E7 gene was removed with *Pvu*II and *Xho*I. The pPUR/E7 plasmid was previously prepared by ligation of pPUR (Clontech, Palo Alto, CA) linearized with *Pvu*II and a fragment containing the enhancer/promoter, intron, E7 gene, and polyadenylation signal that was prepared from pBSC/E7 by digestion with *Kpn*I (partial) and *Xba*I and treatment with T4 DNA polymerase.

The pcDNA3-CRT/E6 plasmid (a generous gift from T.-C. Wu, Johns Hopkins University, Baltimore, MD) carries the HPV16 E6 oncogene fused with the gene encoding calreticulin [28].

Plasmids were propagated in *E. coli* XL1-blue strain and purified with the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany).

Peptides

The peptides RAHYNIVTF and RAHYSIVTF (>90% pure) were custom synthesized by Clonestar Biotech (Brno, Czech Republic).

Mice

Six- to eight-week-old female C57BL/6 mice (H-2^b) (Charles River, Germany) were used in immunization experiments. The animals were maintained under standard conditions at the National Institute of Public Health, Prague.

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* [20] were kindly provided by T.-C. Wu. TC-1/F9 and TC-1/C6 clones were derived from TC-1 cells that had formed a tumor in the mouse immunized with a DNA vaccine against the HPV16 E7 antigen. The clones are resistant to immunization with the E7GGG.GUS gene [38]. Furhermore, 293T cells derived from human embryonic kidney cells [8] were kindly provided by J. Kleinschmidt, DKFZ, Heilderberg, Germany. NIH 3T3 fibroblasts established from mouse embryo culture [13] were obtained from the German National Resource Centre for Biological Material. RMA-S cells (kindly provided by T. Schumacher, The Netherlands Cancer Institute, Amsterdam) are transporter-associated with antigen processing 2 (TAP-2)-defective lymphoma T cells [14]. TC-1, TC-1/F9, TC-1/C6, and 293T cells were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (FCS; PAA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. NIH 3T3 cells were cultured in high-glucose DMEM supplemented with 10% calf serum (PAA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. RMA-S cells were grown in RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10^{-5} M 2-mercaptoethanol.

H-2D^b stabilization assay

Several 6-cm dishes were seeded with 4×10^6 RMA-S cells/dish and incubated at 26°C for 16 h to increase surface expression of MHC class I molecules [22]. The cells were then incubated with 10-μM synthetic peptides for 20 min at 26°C, washed twice with PBS and incubated at 37°C. At different times, 0.5×10^6 cells were removed and surface H-2D^b molecules were stained with FITC-labeled specific antibody (clone CTDb; Serotech, Oxford, England) and analyzed by flow cytometry.

Detection of the E7 antigen by immunoblotting

To verify the expression of the modified E7 genes cloned into pBSC, 293T cells grown in 6-cm dishes were transfected with plasmids by calcium-phosphate precipitation [5]. Two days after transfection, the cells were washed twice with ice-cold PBS and resuspended in GUS buffer (50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% Triton X-100). After two cycles of freezing and thawing, the precipitate was removed by centrifugation and 10 μg of proteins were further analyzed by SDS-PAGE. To determine production of the E7 antigen in TC-1/F9 cells transduced with the E7.FLAG or E7S.FLAG genes, the cells were resuspended in lysis buffer (4% sodium dodecyl sulphate, 20% glycerol, 10% mercaptoethanol, 2 mM EDTA, 100 mM Tris, pH 8) and proteins corresponding to 2×10^5 cells were analyzed by SDS-PAGE. Separated proteins were electroblotted onto a PVDF membrane (Amersham Biosciences, Little Chalfont, England), incubated with monoclonal anti-E7 antibody (clone 8C9; Zymed, San Francisco, CA) and, subsequently, with secondary peroxidase-labeled antibodies. Blots were stained using the ECL Plus system (Amersham Biosciences).

Analysis of GUS activity

Enzymatic activity of the fusion proteins E7GGG.GUS and E7GGGS.GUS was determined after transfection of 293T cells with the corresponding plasmids as described previously [35]. In brief, the cells were transfected by modified calcium-phosphate precipitation in HEPES-buffered saline solution, incubated for 2 days, and lysed in GUS buffer. GUS activity was assayed with the 4-methylumbelliferyl-β-D-glucuronide (MUG) substrate. The protein concentration was measured according to Bradford [4].

Transduction of TC-1/F9 cells

TC-1/F9 cells were seeded at 2×10^5 cells/dish into 4-cm dishes and transfected the following day with 4 μg pPUR-derived plasmids using the METAFECTENE transfection reagent (Biontex, Munich, Germany). Three days after transfection, the cells were selected with 8 μg/ml puromycin and individual colonies were isolated thereafter using cloning cylinders.

Immunization/challenge experiments

Plasmid DNA was coated onto 1-μm gold particles (Bio-Rad, Hercules, CA) as described previously [37]. Each cartridge contained 0.5 mg gold particles coated with 1 μg DNA. Mice (5 per group) were immunized with the plasmids by a gene gun at a discharge pressure of 400 psi into the shaven skin of the abdomen and challenged s.c. into the back with 3×10^4 cells suspended in 150 μl of PBS under intraperitoneal anesthesia with etomidate (0.5 mg/mouse, Janssen Pharmaceutica, Beerse, Belgium). Tumor growth was monitored twice a week.

Tetramer staining

Mice were immunized 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 [26]. In brief, lymphocyte bulk cultures were prepared from splenocytes of three immunized 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), 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 analyzed on a FACScan instrument using CellQuest software (Becton Dickinson).

ELISPOT assay

Lymphocytes producing IFN-γ were detected by the ELISPOT assay simultaneously with tetramer staining as described previously [35]. Spots were evaluated by the Eli.Scan ELISPOT Scanner (A.EL.VIS, Hannover, Germany).

Sequence analysis of the E7 gene

Cell lines were established from TC-1-induced tumors as described previously [36]. The clones were isolated from the cell lines by limiting dilution. The E7 gene in the clones was sequenced using primers described by He et al. [11].

Statistical analysis

Tumor formation in immunization experiments was analyzed by log-rank test or using contingency tables by

two-tailed Fisher's exact test. Results were considered significantly different if $P < 0.05$. Calculations were performed with GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA).

Results

The N53S mutation destabilizes binding of the HPV16 $E7_{49–57}$ peptide to H-2D^b molecules

In our previous study, we have shown correlation between immunoresistance of some clones derived from a TC-1-induced tumor formed in a mouse immunized against the HPV16 E7 oncoprotein and the N53S substitution in the immunodominant E7 epitope RAHYNIVTF (aa 49–57) [38]. Computational analysis [29, 30] predicted decreased binding of the mutated peptide to H-2D^b molecules. To verify this prediction, we tested stabilization of H-2D^b molecules on RMA-S cells (Fig. 1). We pulsed the cells with synthetic peptides at 26°C and followed the kinetics of surface H-2D^b expression at 37°C. While the RAHYNIVTF peptide substantially prolonged the presence of H-2D^b molecules on RMA-S cells, H-2D^b loss after incubation with the RAHYSIVTF peptide carrying the N53S mutation was similar to that observed for RMA-S cells with empty H-2D^b (no peptide added). This result implies the involvement of the N53S substitution in immunoresistance of TC-1 clones.

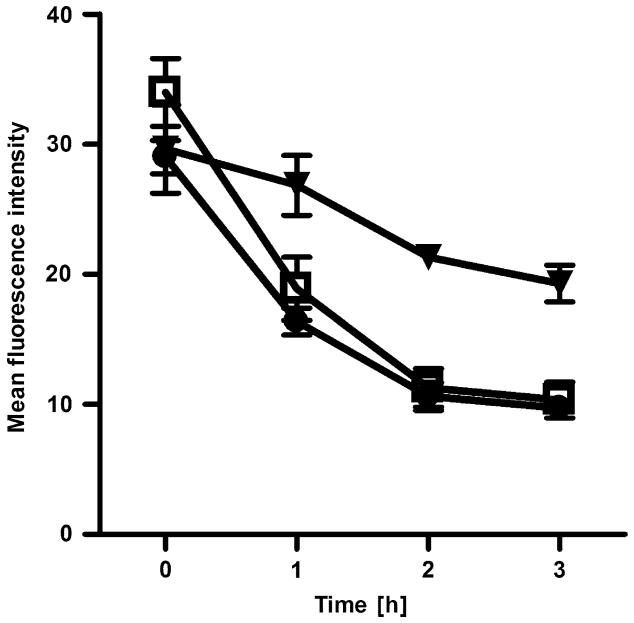

Fig. 1 Kinetics of peptide-induced H-2D^b stabilization. RMA-S cells were incubated at 26°C for 16 h and pulsed for 20 min with the peptide RAHYNIVTF (*filled inverted triangle*) or RAHYSIVTF (*open square*). The cells incubated in medium without any peptide were used as a negative control (*filled circle*). After washing twice with PBS, the cells were incubated at 37°C and surface H-2D^b molecules were detected by flow cytometry at 1-h interval. The data represent means of three independent experiments; *bars* ±SD

The N53S mutation eliminates immunogenicity of the HPV16 $E7_{49–57}$ epitope

To test the influence of the N53S mutation on immunogenicity of the E7 protein, we introduced the corresponding nucleotide alteration into the fusion gene E7GGG.GUS and demonstrated the expression of the mutated E7GGGS.GUS gene by immunoblotting analysis of transfected 293T cells (Fig. 2a). We also quantitatively compared the production of the E7GGGS.GUS and E7GGG.GUS proteins by detection of GUS activity, which showed that both proteins were produced with the same efficiency (Fig. 2a). Then, we immunized C57BL/6 mice with pBSC-derived plasmids by a gene gun and determined E7-specific immune reactions. Both ELISPOT analysis (Fig. 2b) and tetramer staining (Fig. 2c) of splenocytes restimulated with the RAHYNIVTF peptide demonstrated loss of immunogenicity of the E7GGGS.GUS gene. When using the RAHYSIVTF peptide for restimulation in the ELISPOT assay, we did not observe any specific lymphocyte activation in mice vaccinated with either E7GGG.GUS or E7GGGS.GUS. Furthermore, we tested anti-tumor immunity in an immunization/challenge experiment using the E7-expressing TC-1 cell line for tumor induction. While the E7GGG.GUS vaccine protected 4 of 5 mice and a tumor in the non-protected mouse appeared on day 74 after TC-1 administration ($P < 0.01$), all mice immunized with E7GGGS.GUS developed a tumor within 15 days (Fig. 2d). In summary, these data demonstrate the elimination of the immunodominant $E7_{49–57}$ epitope by the N53S mutation.

TC-1 clones with the N53S mutation are sensitive to immunization against the E6 oncoprotein

Besides the E7 oncoprotein, TC-1 cells produce the E6 oncoprotein of HPV16. Therefore, we tested whether resistance of TC-1/F9 and TC-1/C6 clones to E7-specific immunity was associated with resistance to immunization against E6. We vaccinated mice with the pcDNA3-CRT/E6 plasmid and challenged them with tumor cells. Vaccination against E6 prevented the growth of tumors from TC-1/F9 and TC-1/C6 cells (Fig. 3). This suggests that immunoresistance of TC-1/F9 and TC-1/C6 clones is E7-specific.

Transduction with the wild-type E7 restores immunosensitivity of TC-1/F9 cells

To show directly that the N53S mutation was responsible for immunoresistance of the four TC-1 clones, we tried to

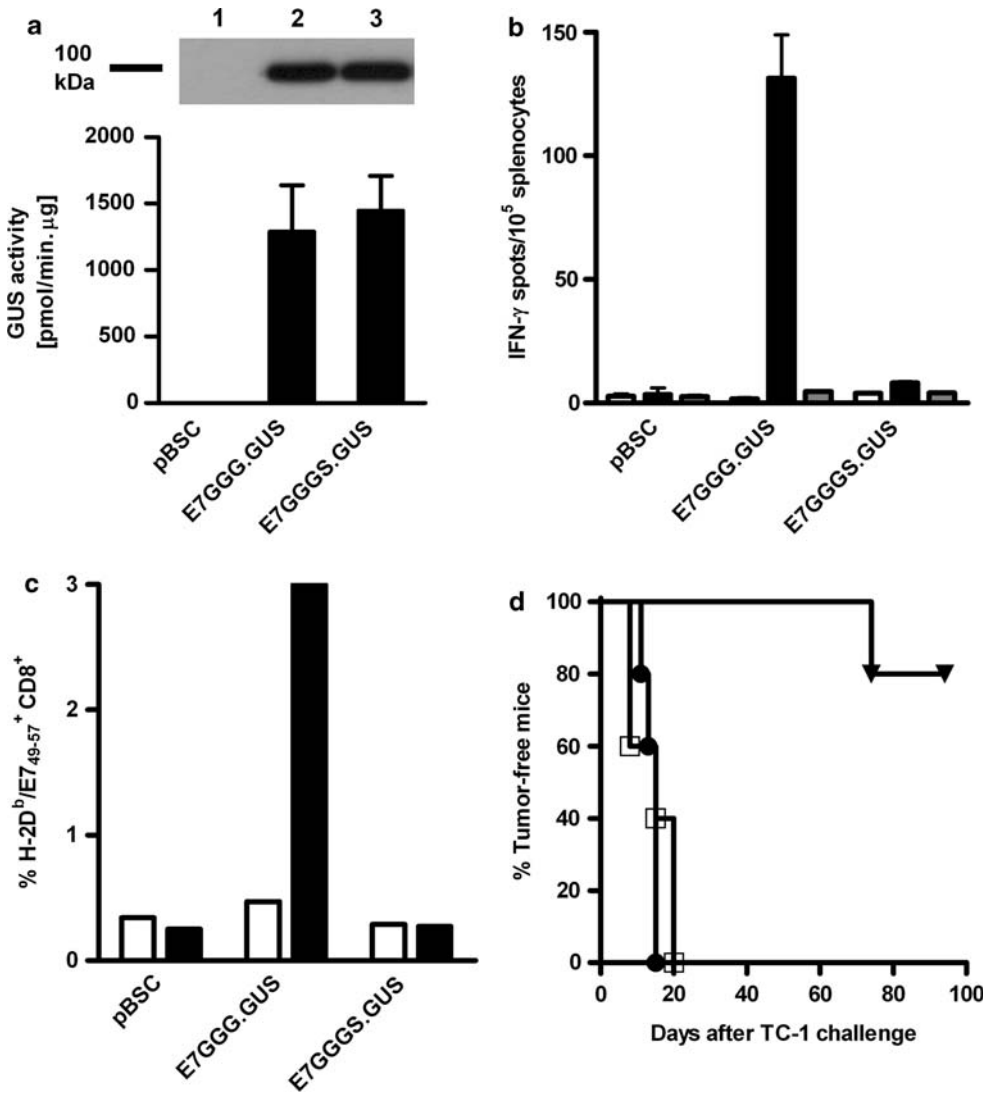

Fig. 2 Immunogenicity of the E7-derived GUS fusion protein carrying the N53S mutation. **a** 293T cells were transfected with plasmids, lysed 2 days thereafter, and tested for the production of fusion proteins: *upper part*, immunoblotting staining with a mouse monoclonal anti-E7 antibody. *Lane 1* pBSC, *lane 2* pBSC/E7GGG.GUS, *lane 3* pBSC/E7GGGS.GUS. *lower part*, analysis of GUS activity by incubation with the MUG substrate. *Columns* mean of four independent experiments; *bars* SD. **b**, **c** Mice ($n = 3$) were twice immunized by a gene gun and 2 weeks after the second immunization lymphocyte bulk cultures were prepared from splenocytes, restimulated with the peptide RAHYNIVTF (*black columns*) or RAHYSIVTF (*gray columns*) for 6 days, and analyzed by IFN-γ ELISPOT assay (**b**) or stained with a mixture of H-2D^b/E7$_{49-57}$-PE tetramers and anti-mouse CD8a-FITC antibody (**c**). Control lymphocytes were cultivated without the peptide (*white columns*). *Columns* mean of duplicate samples; *bars* ±SD. The experiment was repeated with similar results. **d** Mice ($n = 5$) were twice immunized at a 2-week interval by a gene gun with 1 µg of plasmids pBSC (*open square*) pBSC/E7GGG.GUS (*filled inverted triangle*) or pBSC/E7GGGS.GUS (*filled circle*) and challenged s.c. with 3×10^4 TC-1 tumor cells 2 weeks later. The experiment was repeated with similar results

restore immunosensitivity of TC-1/F9 cells by transduction with the wild-type E7 gene. We first added the sequence encoding the FLAG epitope to the E7 and E7S genes, which enabled us to distinguish the products of the endogenous E7S gene and the transferred genes. Then, we transfected TC-1/F9 cells with FLAG-marked genes, selected and isolated the transduced clones and found clones TC-1/F9/C1 and TC-1/F9/B5 producing E7-FLAG and E7S-FLAG, respectively, at a level similar to that of the E7 antigen in TC-1 cells (Fig. 4).

To detect immunosensitivity of TC-1/F9/C1 and TC-1/F9/B5 clones, we tested tumor protection in mice immunized with the E7GGG.GUS gene. While TC-1/F9/B5 cells (similar to parental TC-1/F9 cells) formed tumors in all immunized animals, we observed inhibition of the tumor growth in mice challenged with TC-1/F9/C1 cells (Table 1). However, this inhibition was not significant because of the reduced oncogenicity of TC-1/F9/C1 cells that was evident from the decrease of tumor formation in pBSC- and E7GGGS.GUS-treated mice. Therefore, we

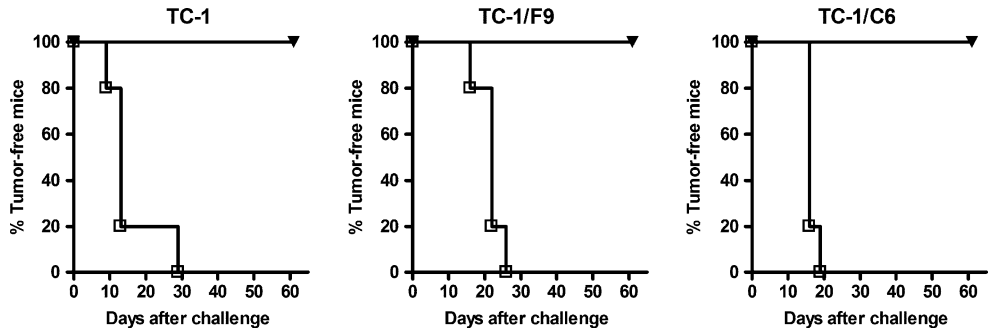

Fig. 3 Immunosensitivity of TC-1/F9 and TC-1/C6 cells to vaccination against the E6 oncoprotein. Mice ($n = 5$) were three times immunized at a 1-week interval by a gene gun with 2 μg of pBSC (*open square*) or pcDNA3-CRT/E6 plasmid (*filled inverted triangle*) and challenged s.c. with 3×10^4 tumor cells 1 week after the last immunization. TC-1 cells were used as a positive control

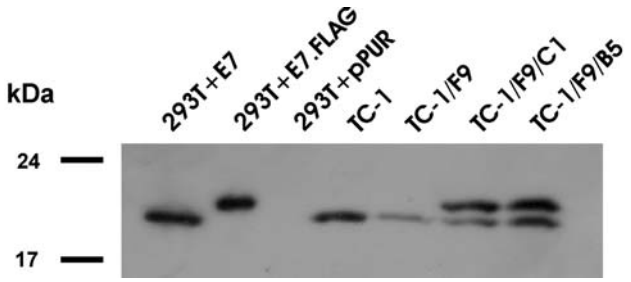

Fig. 4 Immunoblotting analysis of the E7 antigen in transduced TC-1/F9 cells. TC-1/F9 cells were transfected with pPUR/E7.FLAG and pPUR/E7S.FLAG plasmids and TC-1/F9/C1 and TC-1/F9/B5 clones were selected, respectively. Proteins isolated from 2×10^5 TC-1-derived cells were separated by electrophoresis in 12% polyacrylamide gel and transferred onto a PVDF membrane. The E7 antigen was detected with specific monoclonal antibody. 293T cells transfected with pPUR, pPUR/E7, or pPUR/E7.FLAG were used as controls. Production of proteins with the FLAG tag was also proved by staining with anti-FLAG antibody (data not shown)

derived clones from TC-1/F9/C1 cells and examined immunosensitivity of four clones (A7, H10, G4, E12) with the highest production of the wild-type E7 antigen. We found enhanced susceptibility to immunization against E7 in all four clones, but again oncogenicity of the clones was decreased in comparison with TC-1/F9 cells (Table 1). Nevertheless, after summarizing the results from both experiments, we have shown significant inhibition of tumor formation from TC-1/F9/C1 cell line or its clones after immunization with the E7GGG.GUS gene ($P < 0.01$). Thus, these data provide evidence that the N53S mutation caused immunoresistance of TC-1/F9 cells.

Tumor escape by the N53S mutation is a rare event in TC-1 cell lines

We tried to derive TC-1 clones with the mutated $E7_{49–57}$ epitope from other tumors formed in mice immunized against E7 with a DNA vaccine. Therefore, we isolated cell lines from ten tumors that had developed in several immunization experiments, with most animals protected from TC-1 cells. We preferred tumors that had started to grow after a relatively long period of dormancy (approximately 40–70 days). In most cases, TC-1 cells formed small nodules (less than 1 mm in diameter) shortly after inoculation that did not enlarge for several weeks. From the isolated cell lines, clones were derived in vitro and the whole E7 gene was sequenced from cellular DNA. However, no mutation in the E7 gene was found in any of 52 analyzed clones.

Discussion

High-risk types of HPV can induce the development of human malignancies, but most infections with these HPVs are asymptomatic and transient. Furthermore, untreated mild precancerous lesions usually regress spontaneously [25]. HPV-infected cells are probably eliminated by cell-mediated immunity elicited against early viral proteins. In our effort to search for mechanisms of evasion of HPV-infected cells, we previously found the N53S mutation in the HPV16 E7 oncoprotein of some clones derived from a tumor induced by TC-1 cells in a mouse immunized against E7. The presence of this mutation correlated with immunoresistance of the clones [38]. In the current study, we have demonstrated that the N53S mutation was actually responsible for immunoresistance of the clones. We first experimentally verified the computational analysis that had predicted decreased binding of the mutated $E7_{49–57}$ epitope to H-$2D^b$ molecules. Then we showed that the N53S mutation eliminated the immunodominant H-$2D^b$ E7 epitope and we also proved that immunoresistance of TC-1 clones was E7-specific. Finally, we restored TC-1/F9 clone susceptibility to immunization against E7 by transduction with the wild-type E7 gene.

As immunogenicity of peptides has been shown to correlate with their ability to stabilize surface MHC class I expression [21], the experiments with the RAHYSIVTF peptide and RMA-S cells suggested reduced immunogenicity of

Table 1 Immunosensitivity of TC-1/F9 cells transduced with E7S or wild-type E7

Experiment no.	Tumor cells	pBSC	pBSC/E7GGGS.GUS	pBSC/E7GGG.GUS
1	TC-1	5/5[a]	5/5	1/5
	TC-1/F9	5/5	5/5	5/5
	TC-1/F9/B5	5/5	5/5	5/5
	TC-1/F9/C1	3/5	3/5	1/5
2	TC-1/F9/C1	3/5	4/5	1/5
	TC-1/F9/C1/A7	2/5	2/5	1/5
	TC-1/F9/C1/H10	5/5	3/5	1/5
	TC-1/F9/C1/G4	3/5	3/5	1/5
	TC-1/F9/C1/E12	4/5	4/5	2/5
1 + 2	TC-1/F9/C1 + subclones	20/30	19/30	7/30[b]

Mice ($n = 5$) were twice immunized at a 2-week interval by a gene gun with 1 μg of pBSC, pBSC/E7GGGS.GUS or pBSC/E7GGG.GUS plasmid and challenged s.c. with 3×10^4 tumor cells 2 weeks after the second immunization. Tumor growth was monitored for 60 days

[a] No. of mice with a tumor/no. of mice per group

[b] $P < 0.01$, compared with both pBSC- and pBSC/E7GGGS.GUS-immunized mice

the mutated $E7_{49–57}$ epitope. To test the influence of the N53S mutation on immunogenicity of the E7 protein, we introduced the corresponding nucleotide alteration into the fusion gene E7GGG.GUS and compared the original and mutated genes for immunogenicity. We used the fusion gene instead of E7 alone because E7 is poorly immunogenic [37] and the enhanced immunogenicity of the fusion with GUS provides a more sensitive system for the evaluation of the N53S effect. The mutated fusion gene E7GGGS.GUS did not induce CTLs specific for the $E7_{49–57}$ epitope and did not protect mice from TC-1-induced tumors. Moreover, our results demonstrate that the N53S mutation did not generate a new H-2^b epitope, because (1) activated CTLs were not found after restimulation of splenocytes from E7GGGS.GUS-immunized mice with the mutated RAHYSIVTF peptide and (2) the E7GGGS.GUS gene did not protect animals against TC-1/F9 and TC-1/F9/B5 cells producing the E7S protein.

For C57BL/6 mice, the RAHYNIVTF epitope is the only immunodominant epitope identified in the HPV16 E7 oncoprotein [10, 32]. The synthetic peptide DLYCYEQL derived from E7 (aa 21–28) was found to bind efficiently to H2-K^b molecules but controversial data about its processing and presentation in tumor cells have been reported [3, 32]. As the potential subdominant $E7_{21–28}$ epitope had been mutated in the E7GGG gene [37], it could not be implicated in the protection against TC-1 cell lines after immunization with either the E7GGG.GUS or E7GGGS.GUS construct. To test possible involvement of the $E7_{21–28}$ epitope in the induction of anti-tumor immunity after elimination of the $E7_{49–57}$ epitope in our model, we utilized the E7S.GUS gene (containing only the N53S mutation in E7). As immunization with E7S.GUS did not elicit any protection against TC-1 cells (data not shown), our results support the conclusion [32] that the $E7_{21–28}$ peptide is not an H-2^b epitope. Therefore, the escape of TC-1-induced tumors after immunization against E7 in inbred C57BL/6 mice is enabled by elimination of a single T-cell epitope. Nevertheless, this model might resemble the conditions in at least a part of an outbred population as the number of E7 CTL epitopes is probably limited by the small size of the E7 protein [16].

Our attempt to show that transduction of TC-1/F9 cells with the wild-type E7 oncogene could restore the susceptibility of these cells to immunization against E7, was complicated by surprisingly reduced oncogenicity of transduced TC-1/F9/C1 cells. However, the tumor-cell population is heterogeneous in various properties including oncogenicity. Moreover, we observed a similar phenomenon for TC-1 cells previously showing that all clones derived from one TC-1-induced tumor had lower oncogenicity than TC-1 cells or parental cells isolated directly from the tumor. Their oncogenicity did not correlate with E7 expression [38]. However, we have shown variability in expression of cytokines influencing immune cells, namely MCP-1, osteopontin, and midkine, in TC-1 cells and their clones derived from tumors [38]. Therefore, oncogenicity of TC-1 clones might be influenced by immunomodulatory cytokines produced by these cells.

To assess the frequency of the escape mutation in the E7 oncogene in our experimental system, we derived 10 cell lines from TC-1-induced tumors formed in mice immunized against E7, isolated 52 clones from these cell lines, and determined the sequence of the E7 gene amplified from the clones. However, we did not find any mutation in E7. Therefore, we analyzed the expression of MHC class I molecules on eight cell lines derived from tumors and found its substantial reduction on all tested cell lines (data not shown). We also demonstrated the MHC class I

down-regulation on TC-1 cells after immunization against E7 in our previous study, in which we showed that both H-2D^b and H-2K^b molecules were simultaneously reduced [36]. These results imply that down-regulation of MHC class I expression is the principal mechanism of escape of TC-1 cells from the host immunity. Similarly, reduced production of MHC class I molecules has been found in most cases of CC [15, 17]. Nevertheless, mutations in HPV16 oncoproteins can be implicated in the evasion of infected cells from immune surveillance and in the pathogenesis of cervical lesions as the R10G and L83V substitutions in the E6 protein that alter the HLA class I-binding epitopes pose an increased risk of HPV persistence and progression of premalignant lesions [9, 23, 39].

Mutations in oncoproteins that would enable escape of transformed cells and subsequent progression of tumors should not markedly inhibit carcinogenic activity of these oncoproteins, otherwise malignancy of cells would be compromised. Indeed, the E6 variants carrying the R10G or L83V substitutions keep most of their activities important for malignant transformation of cells [19]. We did not find any substantial alteration to the E7S oncogenic potential tested in NIH 3T3 transformation assay either (V. Lucansky, unpublished results).

Antigenic drift, a well-known mechanism of viral evasion of host immunity, was reported for the first time as a mechanism of tumor escape after adoptive transfer of purified transgenic T cells into immunodeficient mice [2]. In our study, we have shown generation of a tumor variant with a mutated tumor antigenic epitope after anti-tumor immunization of normal mice, which further indicates the potential impacts of this phenomenon on outcomes of cancer immunotherapy, especially when specific immunotherapy is targeted at a limited set of epitopes.

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3.2 Paper II:

Enhancement of T cell-mediated and humoral immunity of β-glucuronidase-based DNA vaccines against HPV 16 E7 oncoprotein

Michal Smahel, Ingrid Poláková, Dana Pokorná, Viera Ludvíková, Martina Dusková, Josef Vlasák

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Contribution of the author of this PhD. thesis:

I participated on the processing of splenocytes and carried out the ELISPOT assays.

Enhancement of T cell-mediated and humoral immunity of ß-glucuronidase-based DNA vaccines against HPV16 E7 oncoprotein

MICHAL SMAHEL[1], INGRID POLÁKOVÁ[1], DANA POKORNÁ[1], VIERA LUDVÍKOVÁ[1], MARTINA DUSKOVÁ[1] and JOSEF VLASÁK[2]

[1]Department of Experimental Virology, Institute of Hematology and Blood Transfusion, Prague;
[2]Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, Ceské Budejovice, Czech Republic

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Abstract. Therapeutic DNA vaccines against oncogenic infection with human papillomavirus type 16 (HPV16) are mostly targeted against viral oncoproteins E7 and E6. To adapt the E7 oncoprotein for DNA immunization, we have previously reduced its oncogenicity by modification of the Rb-binding site and enhanced immunogenicity of the modified E7GGG gene by the fusion with the 5'-terminus of the gene encoding *E. coli* ß-glucuronidase (GUS). In this study, we attempted to improve immunogenicity of the GUS-based anti-E7 vaccines by increasing the steady-state level of fusion proteins. We fused deletion mutants of E7GGG and codon-optimized E7GGG with the 5'-terminus of GUS and unaltered E7GGG with the 3'-terminus of GUS. Furthermore, we mutated the initiation codon of the GUS gene in the E7GGG.GUS construct, as GUS alone was produced from this fusion gene. We found that only the fusion of E7GGG with the 3'-terminus of GUS (GUS.E7GGG) and deletion mutants of E7GGG with the 5'-terminus of GUS increased the steady-state level of fusion proteins in transfected human 293T cells. Analysis of immune reactions induced in mice by vaccination via a gene gun showed that the increased steady-state level of fusion proteins resulted in augmented production of E7-specific antibodies, but did not enhance cell-mediated anti-tumor immunity. Finally, we joined the signal sequence of the adenoviral E3 protein with GUS.E7GGG. This modification led to the predominant localization of the fusion protein in the endoplasmic reticulum and enhancement of $CD8^+$ T-cell response, while antibody production was reduced. In conclusion, we found modifications of the E7GGG.GUS fusion gene that augmented either humoral or cell-mediated immune responses.

Correspondence to: Dr Michal Smahel, Department of Experimental Virology, Institute of Hematology and Blood Transfusion, U Nemocnice 1, 128 20 Prague 2, Czech Republic
E-mail: smahel@uhkt.cz

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Introduction

Vaccination with plasmid DNA is a rapidly developing method for induction of immune responses. It was proved to induce both humoral and cell-mediated immunity, but the level of the immune responses was often unsatisfactory in comparison with traditional vaccination strategies. The efficacy of DNA vaccines can be enhanced by modifications of a gene coding for an antigen. These modifications include changes resulting in the increase of protein production or the alteration of protein stability and/or cellular localization (1).

The E7 oncoprotein of human papillomavirus type 16 (HPV16) is a suitable target for development of therapeutic DNA vaccines against malignant diseases associated with HPV16 infection (2). As it is only a weak immunogen, numerous modifications of the E7 oncogene were constructed and their immunogenicity was examined on mouse tumor models. Some of them have already been tested in clinical trials (3). Enhanced immunity against E7 was demostrated for instance after codon optimization of the E7 sequence (4), addition of intracellular localization signals (5), and fusion with genes encoding chaperones (6), bacterial toxins (7) or viral proteins capable of intercellular spreading (8).

As oncogenicity is a characteristic of the HPV16 E7 gene inconsistent with its use for DNA immunization of humans, we have reduced its tumorigenic potential by the preparation of the modified E7GGG gene encoding the protein with substitution of three amino acids in the pRb-binding site (9). To enhance immunogenicity of E7GGG, we fused it with sorting signals of lysosome-associated membrane protein 1 (LAMP-1) (10), *E. coli* ß-glucuronidase (GUS) (11), and mouse heat shock protein 70 (Hsp70) (12). As the fusion with GUS (E7GGG.GUS) yielded the highest anti-tumor efficacy, we performed further modifications to enhance its immunogenicity. In this study, we demonstrated that stabilization of the fusion protein increases the generation of E7-specific antibodies, but only targeting into the endoplasmic reticulum (ER) enhances cell-mediated immunity and anti-tumor effect of the DNA vaccine delivered by a gene gun.

Materials and methods

Plasmids. The construction of plasmids pBSC/GUS, pBSC/E7GGG.GUS, and pBSC/E7GGGpGUS (designated pBSC/E7GGG41.GUS in this report) was described previously (11). To perform some of the modifications specified below, the genes GUS and E7GGG.GUS were cloned into the *Eco*RI site of pALTER-MAX (Promega, Madison, WI). The modified genes were then cloned back into the *Eco*RI site of pBSC.

The genes E7GGG60.GUS and E7GGG75.GUS were prepared from the GUS gene by addition of a sequence encoding 60 or 75 amino acids from the N-terminus of the E7GGG protein, respectively. These sequences were amplified from the plasmid pBSC/E7GGG (9) using the forward primer E7-1 (11) and the reverse primer E7-60 (5'-CCAGGATCCC TTGCAACAAAAGGTTACAAT-3') or E7-75 (5'-CCAGGA TCCGTCTACGTGTGTGCTTTGTA-3'), respectively. The PCR products were digested with *Bam*HI and cloned into the *Bam*HI site upstream of the GUS gene.

The termination codon of the GUS gene was eliminated and the *Hin*dIII site was created using the Altered Sites II Mammalian Mutagenesis System (Promega) and the primer 5'-CAGGAGAGTTGTTGAAGCTTGTTTGCCTCCCTG-3' (substituted nucleotides are underlined). Into the *Hin*dIII site, the E7GGG gene was inserted after amplification with the primers 5'-CACAAGCTTTGATGCATGGAGATACACC TAC-3' (forward) and 5'-CACAAGCTTTTATGGTTT CTGAGAACAGAT-3' (reverse) which resulted in the GUS.E7GGG gene. To prepare the SS.GUS.E7GGG gene containing the signal sequence from the adenoviral E3 gene, annealed oligonucleotides 5'-GATCCGCCGCCATGAGGT ACATGATTTTAGGCTTGCTCGCCCTTGCGGCAGTCT GCAGCGCTGCCG-3' and 5'-GATCCGGCAGCGCTGCAG ACTGCCGCAAGGGCGAGCAAGCCTAAAATCATGTA CCTCATGGCGGCG-3' were cloned into the *Bam*HI site upstream of the GUS.E7GGG gene.

The initiation codon of the GUS gene was eliminated and the *Xho*I site was created in the resultant EGUS gene using the Altered Sites II Mammalian Mutagenesis System and the primer 5'-TTCTACAGGACGTAACTCGAGGGACT GACCACC-3' (substituted nucleotides are underlined). This modification was also performed in the E7GGG.GUS gene (E7GGG.EGUS).

The E7GGG gene optimized for expression in human cells (hE7GGG; the GenBank accession no. EU443245) was designed and synthesized by GeneArt (Regensburg, Germany). The sequence was amplified using the primers 5'-TTCCGGA TCCATCATGCATGG-3' (forward) and 5'-CATGCATGGG ATTAGGATCC-3' (reverse) and cloned into the *Bam*HI site upstream of the GUS gene (hE7GGG.GUS).

The modified constructs were verified by sequencing. pBSC-derived plasmids were propagated in *E. coli*, XL1-blue strain, and purified with the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany).

Mice. Six- to eight-week-old female C57BL/6 mice (H-2^b) (Charles River, Germany) were used in immunization experiments. Animals were maintained under standard conditions at the Center for Experimental Biomodels, Charles University, Prague.

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. TC-1/A9 cells with down-regulated MHC class I expression were derived from TC-1 cells that formed a tumor in the mouse immunized against the HPV16 E7 antigen (10). 293T cells derived from human embryonic kidney cells (14) were kindly provided by J. Kleinschmidt, DKFZ, Heidelberg, Germany. NIH 3T3 fibroblasts established from mouse embryo culture (15) were obtained from the German National Resource Centre for Biological Material. TC-1, TC-1/A9, and 293T cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (FCS; PAA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. NIH 3T3 cells were cultured in high glucose DMEM supplemented with 10% calf serum (PAA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Analysis of GUS activity. The enzymatic activity of GUS was determined after transfection of 293T cells with corresponding plasmids as described previously (11). In brief, cells were transfected by modified calcium-phosphate precipitation in HEPES-buffered saline solution (16), incubated for 2 days, and lysed in the GUS buffer (50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 10 mM ß-mercaptoethanol, 0.1% Triton X-100). GUS activity was assayed with the 4-methylumbelliferyl-ß-D-glucuronide (MUG) substrate. Protein concentration was measured according to Bradford (17).

Immunoblotting staining. 293T cells seeded in 6-cm dishes were transfected with 6 μg of plasmids by calcium-phosphate precipitation in HEPES-buffered saline solution. Two days after transfection, the cells were washed twice with ice-cold PBS and lysed in the GUS buffer or Laemmli buffer. Proteins separated by SDS-PAGE were electroblotted onto a PVDF membrane (Amersham Biosciences, Little Chalfont, UK), incubated with polyclonal anti-GUS antibodies (Molecular Probes, Eugene, OR) and, subsequently, with secondary peroxidase-labeled antibodies (Amersham Biosciences). Blots were stained using the ECL Plus system (Amersham Biosciences).

Pulse-chase analysis of protein stability. 293T cells grown in 15-cm dishes were transfected with 30 μg of plasmids by calcium-phosphate precipitation in HEPES-buffered saline solution. One day after transfection, $2x10^6$ cells were seeded in 6-cm dishes. The next day, the cells were starved in methionine/cysteine-free DMEM supplemented with 5% dialyzed FCS (PAA) for 1 h and incubated with ^{35}S-labeled methionine/cysteine (100 μCi/ml in DMEM with 5% dialyzed FCS; GE Healthcare). After washing with PBS, the cells were cultured in DMEM supplemented with 1 mM methionine and 0.5 mM cysteine for indicated time intervals and lysed in the RIPA buffer with a protease inhibitor cocktail (Sigma, Saint Louis, MO) added. The E7-containing fusion proteins were immunoprecipitated using anti-E7 monoclonal antibody (clone TVG710Y; Santa Cruz Biotechnology, Santa Cruz, CA) and protein G PLUS-Agarose (Santa Cruz Biotechnology) and separated by SDS-PAGE. The dried gels were exposed to

the Fuji BAS-MS imaging plate and luminescence was measured by the Fuji FLA 2000 phosphorimager (Fujifilm, Stamford, CT) and analyzed with ScanPack 3.0 software (Biometra, Goettingen, Germany).

Immunofluorescence staining. NIH 3T3 cells were grown on coverslips in 24-well plates and transfected with 0.5 μg plasmids using the Metafectene transfection reagent (Biontex, Martinsried/Planegg, Germany). Two days after transfection, cells were fixed in methanol for 10 min at -20˚C. The E7 antigen was stained with E7-specific mouse monoclonal antibody (clone 8C9; Zymed, San Francisco, CA) followed by Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies (Molecular Probes). Simultaneously, the ER or the Golgi apparatus (GA) were stained with rabbit anti-calreticulin or anti-GM130 antibodies (both Santa Cruz Biotechnology), respectively, and secondary Alexa Fluor 546-conjugated goat anti-rabbit IgG antibodies (Molecular Probes). The slides were examined by a Leica TCS SP2 AOBS confocal microscope, images were processed by Leica Confocal Software (Leica Microsystems, Germany).

Preparation of gene gun cartridges. Plasmid DNA was coated onto 1-μm gold particles (Bio-Rad, Hercules, CA) as described previously (9). Each cartridge contained 0.5 mg of gold particles coated with 1 μg DNA.

Immunization experiments. Mice were immunized with plasmids by a gene gun (Bio-Rad) at a discharge pressure of 400 psi into the shaven skin of the abdomen. Each immunization consisted of one shot delivering 1 μg of plasmid DNA. In immunization/challenge experiments, mice 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 $3x10^4$ TC-1/A9 cells suspended in 150 μl PBS. In therapeutic immunization experiments, mice were first inoculated with $3x10^4$ TC-1 cells and immunized 3 and 10 days later. Tumor cells were administered under anesthesia with intraperitoneal etomidate (0.5 mg/mouse; Janssen Pharmaceutica, Beerse, Belgium). Tumor growth was monitored twice a week. Tumor size was calculated from three perpendicular measurements using the formula:

$$\frac{\pi}{6} \text{ (a x b x c)}$$

ELISPOT assay. Mice were immunized by a gene gun with two doses of plasmids given at a two-week interval. Two weeks after the second dose, an ELISPOT assay was performed as described previously (11). Cells producing interferon γ (IFN-γ) were detected in lymphocyte bulk cultures prepared from splenocytes of three immunized animals and cultivated with the HPV16 $E7_{49-57}$ peptide (RAHYNIVTF) for 20 h. Spots were evaluated by the Eli.Scan ELISPOT Scanner (A.EL.VIS, Hannover, Germany).

Intracellular cytokine staining (ICS). Lymphocytes were prepared and cultivated with the E749-57 peptide as for an ELISPOT assay. Twelve hours before staining, GolgiStop (BD Biosciences Pharmingen, San Diego, CA) was added to the culture medium according to the manufacturer's instructions. Surface CD8 molecules were stained with PE-conjugated rat anti-mouse CD8a monoclonal antibody (BD Biosciences Pharmingen). Then, cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences Pharmingen) and intracellular IFN-γ was stained with FITC-conjugated rat anti-mouse IFN-γ monoclonal antibody (BD Biosciences Pharmingen). The stained cells were measured on a Coulter Epics XL flow cytometer (Coulter, Miami, FL) and analyzed by FlowJo 7.1.2. software (TreeStar, Ashland, OR).

ELISA. Sera were collected from mice immunized for the ELISPOT assay and ICS. E7-specific antibodies were detected in sera diluted 1:50 by ELISA as described previously (12).

Statistical analysis. Tumor formation in the immunization experiments was analyzed by a log-rank test. Tumor growth was evaluated by two-way analysis of variance. Results were considered significantly different at $P<0.05$. Calculations were performed using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA).

Results

Generation of deletion mutants of E7GGG.GUS and 3'-terminus fusion GUS.E7GGG and characterization of their steady-state level production. At least in some conditions, cross-presentation of antigens seems to be the principal mechanism of induction of immune responses after DNA immunization with a gene gun (18). Therefore, we tried to enhance the efficacy of the E7GGG.GUS vaccine by modifications of the fusion gene that could increase the steady-state level of resultant proteins. In our previous study, we showed that while fusion of the full-length E7GGG gene with GUS decreases the steady-state level of the GUS antigen about 10-fold, expression of the fusion gene E7GGG41.GUS containing 41 amino acids from the N-terminus of E7GGG is comparable with that of GUS alone (11). As E7GGG41.GUS does not contain the $H\text{-}2D^b$ RAHYNIVTF epitope (aa 49-57), we constructed other two deletion mutants, E7GGG60.GUS and E7GGG75.GUS, comprising 60 and 75 amino acids from the E7GGG N-terminus, respectively (Fig. 1).

Stability and/or immunogenicity of fusion proteins can be influenced by position of fusion partners. This effect has also been demonstrated for the HPV16 E7 oncogene fused with the mouse Hsp70 gene (19). To verify the position effect of the E7GGG fusion with GUS, we joined E7GGG with the 3'-terminus of GUS, thus generating the GUS.E7GGG gene (Fig. 1).

We have demonstrated previously that fusion of E7GGG with GUS does not markedly affect the enzymatic activity of GUS (11). Therefore, we measured the GUS activity to quantitatively compare the production of fusion proteins after transfection of 293T cells with pBSC-derived plasmids (Fig. 2A). We found that the steady-state level of the 5'-terminus fusion proteins decreased with increasing the portion of E7GGG added to GUS. The main difference (corresponding to about 60% of the enzymatic activity of GUS alone) was found between the production of the proteins E7GGG60.GUS

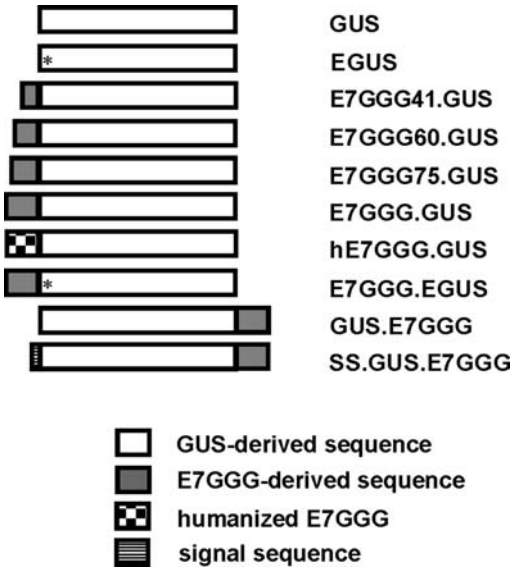

Figure 1. Schematic representation of genes cloned into the mammalian expression plasmid pBSC. An asterisk (*) indicates the mutation in the GUS initiation codon.

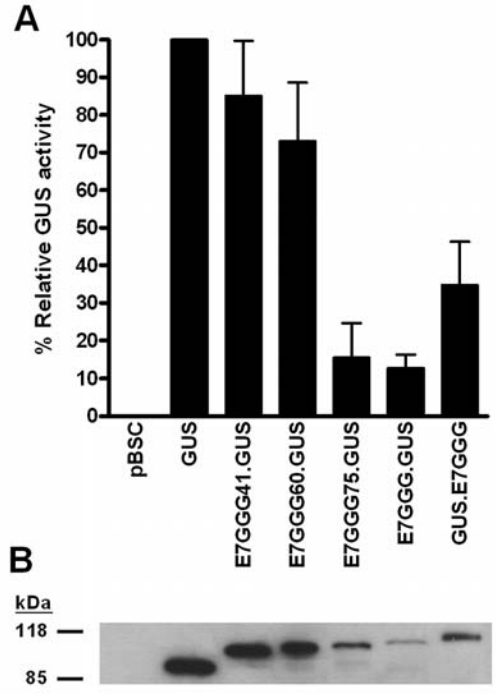

Figure 2. Expression of deletion mutants and C-terminus fusion. 293T cells were transfected with pBSC-derived plasmids, lysed in GUS buffer two days thereafter, and tested for the production of proteins: A, Analysis of GUS activity by incubation with the MUG substrate - columns, mean of three independent experiments; bars ± SD. B, Immunoblotting staining with anti-GUS antibodies. Two μg of proteins were separated in 7% SDS-PAGE gel.

and E7GGG75.GUS. The GUS activity of GUS.E7GGG was about twice higher than that of E7GGG.GUS. These quantitative data were confirmed by immunoblotting analysis of cell lysates (Fig. 2B).

Immunogenicity of deletion mutants and 3'-terminus fusion. We examined immunogenicity of GUS fusion genes after DNA immunization of C57BL/6 mice by a gene gun. In our experimental setting, induction of E7-specific antibodies by E7GGG.GUS was negligible. All modified genes elicited higher production of antibodies against E7. The level of antibodies correlated with the steady-state level of protein production in transfected 293T cells (Fig. 3A).

Cell-mediated immunity against E7 was tested by an ELISPOT assay detecting splenocytes that produced IFN-γ after restimulation with the H-2D^b $E7_{49-57}$ epitope. The number of IFN-γ-producing cells after immunization with E7GGG.GUS was at least twice higher than after immunization with the modified fusion genes (Fig. 3B). Furthermore, we examined the anti-tumor effect of the modified genes. For induction of s.c. tumors, we used TC-1 cells producing the E7 oncoprotein and their derivative with the reduced surface expression of MHC class I molecules, TC-1/A9 cells. In preventive immunization against TC-1/A9 cells, all mice developed a tumor with the exception of two animals immunized with the E7GGG.GUS gene (Fig. 3C), which corresponds with the highest efficiency of this gene in the ELISPOT assay. In therapeutic immunization against TC-1 cells, both the E7GGG.GUS gene and the modified genes induced elimination of tumor cells in about half of mice (Fig. 3D). In summary, the increase in the steady-state level of the GUS fusion proteins enhanced the production of E7-specific antibodies, but did not improve the cytotoxic T-lymphocyte (CTL) response.

Subsequent modifications of E7GGG fusions with GUS: optimization of E7GGG, elimination of the GUS initiation codon and addition of a signal sequence. Codon optimization can substantially increase the production of a protein. Such modification of the HPV16 E7 gene resulted in enhanced immune responses after DNA immunization (4,20). This effect was achieved even after fusion of the codon optimized E7 gene with the unmodified lysosome-associated membrane protein 1 (LAMP-1) (21). Therefore, we joined the E7GGG gene optimized for expression in human cells (hE7GGG) with GUS, thus generating the hE7GGG.GUS gene. However, despite the fact that the hE7GGG gene produced about a 6-fold higher amount of the E7GGG protein in comparison with the E7GGG gene (data not shown), production of the E7GGG.GUS protein from the hE7GGG.GUS and E7GGG.GUS genes was comparable (Fig. 4A).

After detection of the E7GGG.GUS protein with anti-GUS polyclonal antibody, we found a minor band corresponding in size with the GUS protein. We supposed that the translation of the E7GGG.GUS gene could occasionally start from the GUS initiation codon. To test this hypothesis and possibly increase the production of the E7GGG.GUS fusion protein, we mutated the GUS initiation codon, which resulted in the substitution of methionine for glutamic acid (E7GGG.EGUS). Similarly, we introduced this mutation into the GUS gene (EGUS). By using the GUS-specific antibodies, immunoblotting analysis of lysates from the transfected 293T cells showed that the mutation eliminated the production of the protein corresponding in size with the GUS protein from both of the mutated genes (Fig. 4B). However, the steady-state level of the mutated E7GGG.EGUS protein was a bit lower than that of the E7GGG.GUS protein. Moreover, the GUS

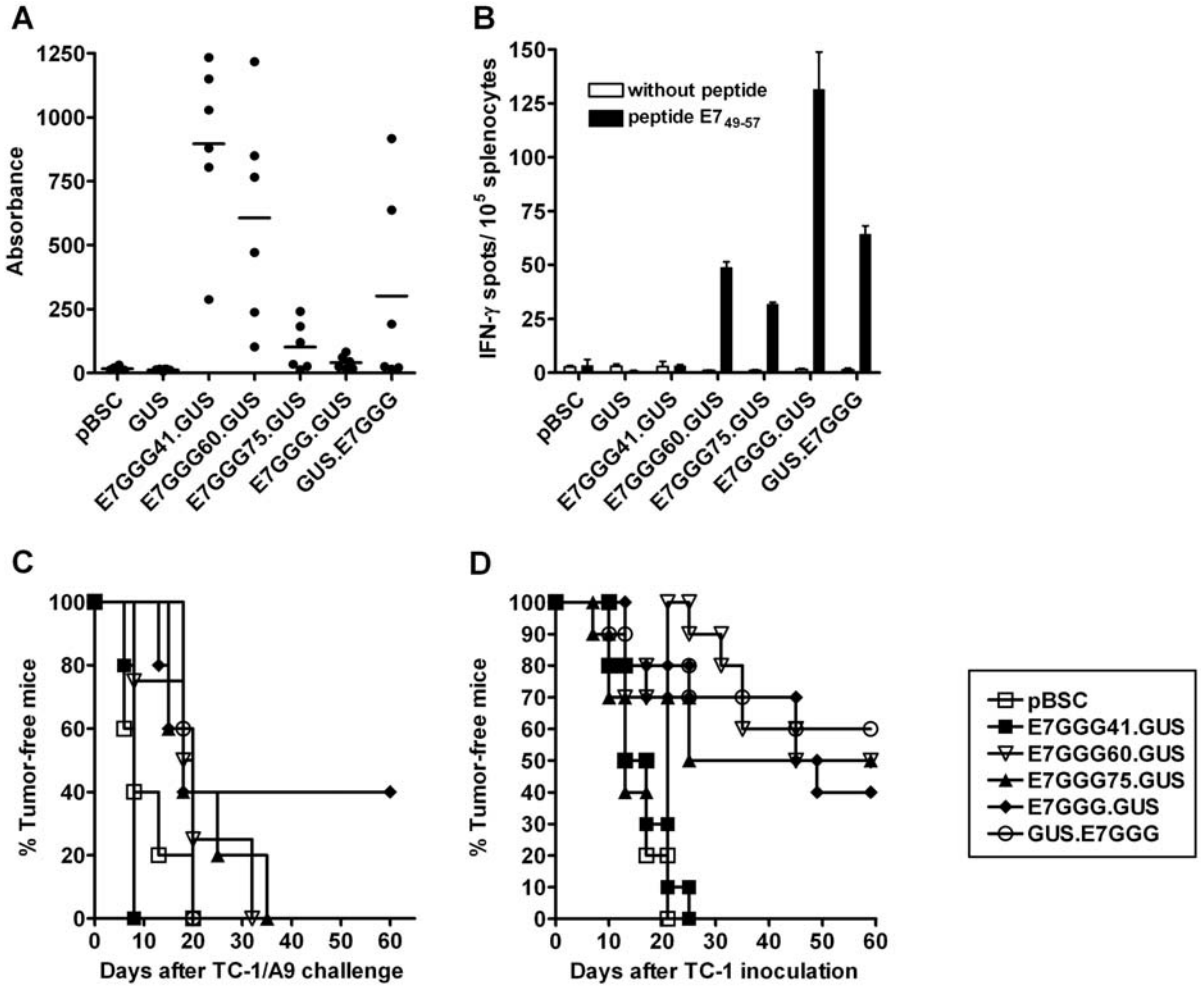

Figure 3. Immunogenicity of deletion mutants and C-terminus fusion. A and B, Mice (n=3) were twice immunized at a 2-week interval by a gene gun with 1 μg of plasmids and two weeks after the second immunization, blood was collected and lymphocyte bulk cultures were prepared from splenocytes. A, E7-specific antibodies were examined in 1:50 diluted sera by ELISA. B, Lymphocytes producing IFN-γ were detected after incubation with the peptide RAHYNIVTF by an ELISPOT assay. Control lymphocytes were cultivated without the peptide. Columns, mean of duplicate samples; bars ± SD. C, Mice (n=5) were twice immunized at a 2-week interval by a gene gun and challenged s.c. with $3x10^4$ TC-1/A9 tumor cells two weeks later. D, Mice (n=10) were inoculated s.c. with $3x10^4$ TC-1 cells and immunized by a gene gun 3 and 10 days later.

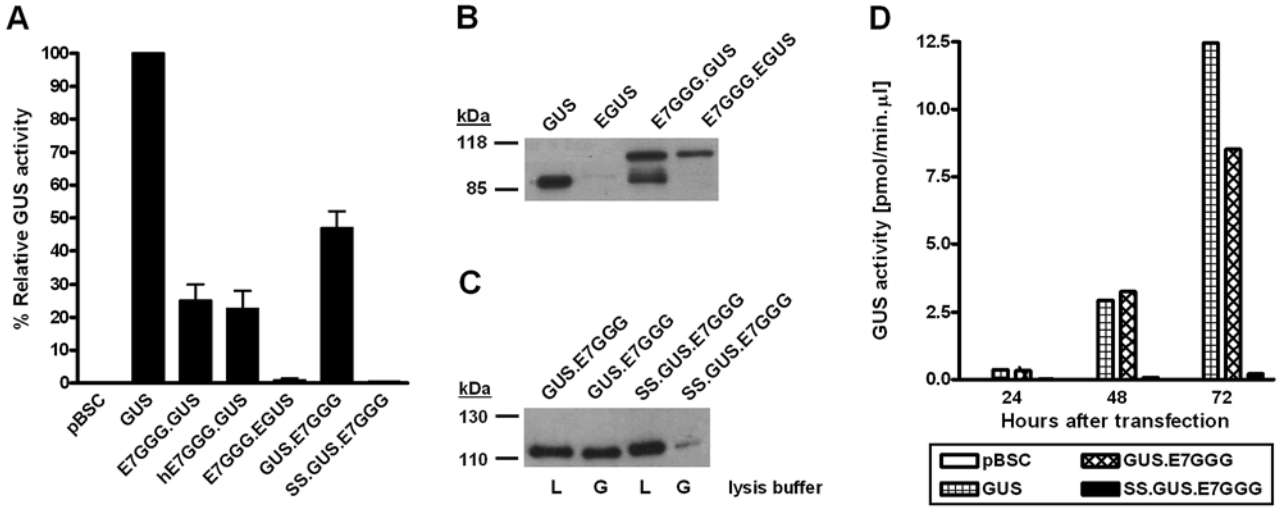

Figure 4. Expression of the modified genes hE7GGG.GUS, E7GGG.EGUS, and SS.GUS.E7GGG. The production of the fusion proteins was examined after transfection of 293T cells with pBSC-derived plasmids. A and B, Two days after transfection, cells were lysed in the GUS buffer and GUS was detected in lysates by analysis of GUS activity with the MUG substrate (A, columns, mean of three independent experiments; bars ± SD) or by immunoblotting staining with anti-GUS antibodies [B, 4 μg (GUS) or 13 μg of proteins (EGUS, E7GGG.GUS, E7GGG.EGUS) were separated in 7% SDS-PAGE gel]. C, Two days after transfection, cells were lysed in the GUS (G) or Laemmli buffer (L) and proteins in 2 μl of lysates were separated in 7% SDS-PAGE gel, transferred onto a PVDF membrane and stained with anti-GUS antibodies. D, Samples of media were collected 24, 48, and 72 h after transfection and examined for GUS activity.

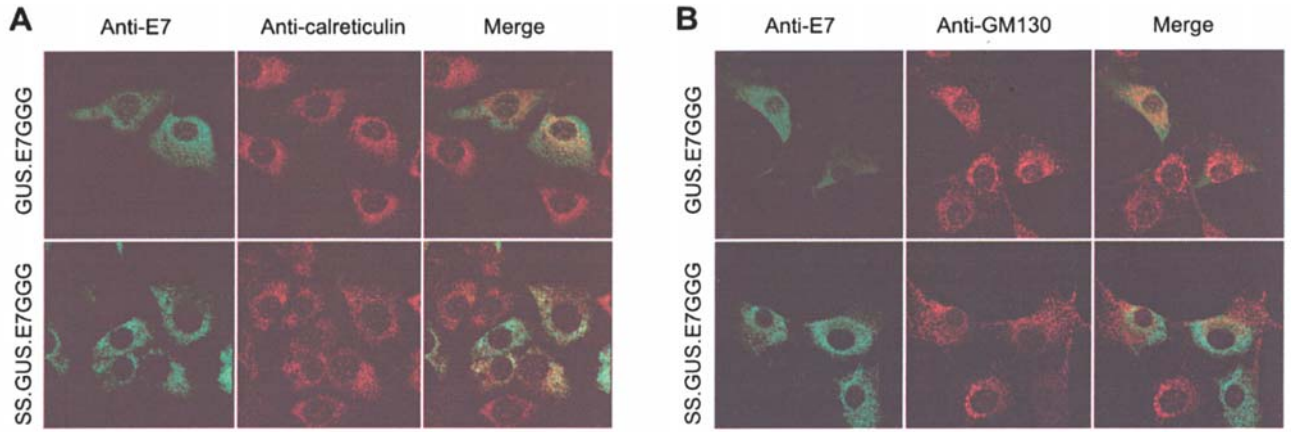

Figure 5. Intracellular localization of SS.GUS.E7GGG. NIH 3T3 cells were transfected with plasmids pBSC/GUS.E7GGG or pBSC/SS.GUS.E7GGG and fixed with methanol 2 days thereafter. The E7 antigen was stained using anti-E7 monoclonal antibody (green). Simultaneously, the ER (A) or GA (B) were stained using polyclonal anti-calreticulin or anti-GM130 antibodies, respectively (red). The slides were examined by a confocal microscope.

activity of E7GGG.EGUS was almost completely eliminated (Fig. 4A). These data suggest that the enzymatic activity of the mutated protein was reduced by the modification of its conformation caused by the substitution of methionine for glutamic acid.

The efficacy of DNA vaccines can be enhanced by the alteration of antigen cellular localization. As addition of a signal sequence targeting a protein into the ER has been reported to increase immunogenicity of some cytoplasmic or nuclear proteins including the E7 protein (5,22), we joined the adenoviral E3 signal sequence with GUS.E7GGG (SS.GUS.E7GGG). This modification resulted in almost complete elimination of the GUS activity in lysates of the transfected 293T cells (Fig. 4A). Immunoblotting analysis showed that while after lysis with the Laemmli buffer the level of the detected SS.GUS.E7GGG protein was comparable with that of the GUS.E7GGG protein, it was markedly reduced in the GUS lysis buffer (Fig. 4C), which suggested localization of the SS.GUS.E7GGG protein into subcellular compartments. Furthermore, we tested possible secretion of the SS.GUS.E7GGG protein by determination of the GUS activity in media of the transfected cells. However, the enzymatic activity of SS.GUS.E7GGG was about 40-fold lower when compared with GUS.E7GGG (Fig. 4D). The higher amount of GUS.E7GGG in the medium was confirmed by immunoblotting analysis (data not shown), which excluded the possibility that a post-translational modification caused the lower GUS activity of the SS.GUS.E7GGG protein. Furthermore, colocalization analysis demonstrated that while the E7-specific antibody diffusely stained the cytoplasm of the NIH 3T3 cells transfected with the GUS.E7GGG gene, the SS.GUS.E7GGG protein showed perinuclear staining and colocalization with calreticulin, a chaperone resident in the ER, but not with GM130, a marker of the GA (Fig. 5).

Immunogenicity of hE7GGG.GUS, E7GGG.EGUS, and SS.GUS.E7GGG. Similarly to E7GGG.GUS, the modified genes hE7GGG.GUS, E7GGG.EGUS, and SS.GUS.E7GGG elicited a negligible production of E7-specific antibodies (data not shown). Examination of cell-mediated immunity by intracellular staining of IFN-γ in $CD8^+$ T lymphocytes showed that only immunization with SS.GUS.E7GGG

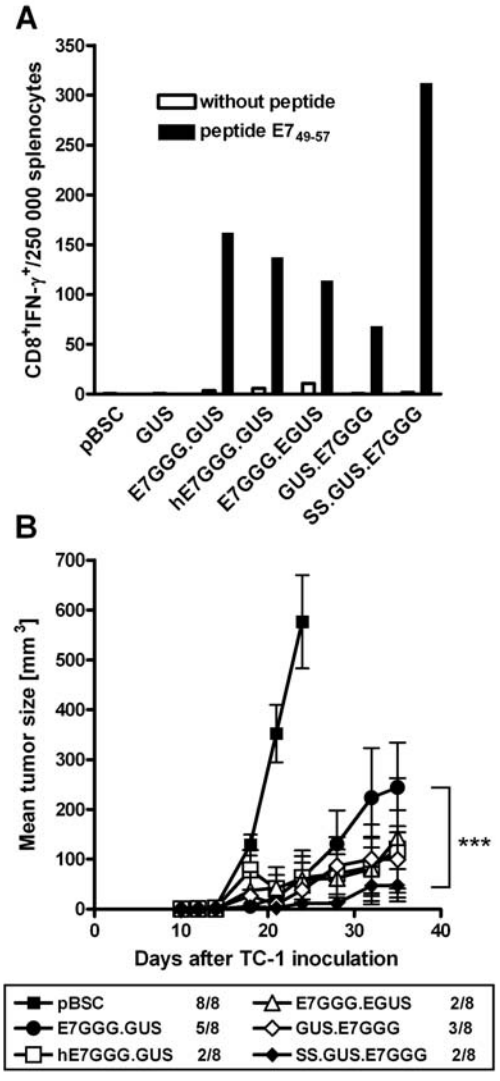

Figure 6. Immunogenicity of the genes hE7GGG.GUS, E7GGG.EGUS, and SS.GUS.E7GGG. A, Mice (n=3) were twice immunized at a 2-week interval by a gene gun with 1 μg of plasmids and two weeks after the second immunization, lymphocyte bulk cultures were prepared from splenocytes. $CD8^+$ lymphocytes producing IFN-γ were detected after incubation with the peptide RAHYNIVTF by ICS. Control lymphocytes were cultivated without the peptide. B, Mice (n=8) were inoculated s.c. with $3x10^4$ TC-1 cells and immunized by a gene gun 3 and 10 days later. No. of mice with a tumor/no. of mice in group is indicated.

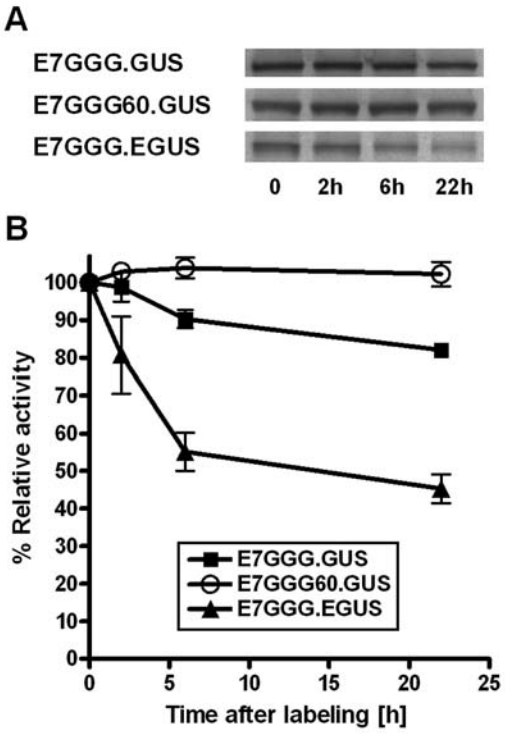

Figure 7. Stability of proteins E7GGG.GUS, E7GGG60.GUS, and E7GGG.EGUS. 293T cells were transfected with pBSC-derived plasmids, starved in a methionine/cysteine-free medium for 1 h, and incubated with ^{35}S-labeled methionine/cysteine for 1 h. After cultivation in the medium containing methionine/cysteine for indicated time-points, the cells were lysed and the E7-containing fusion proteins were immunoprecipitated using anti-E7 monoclonal antibody and analyzed by SDS-PAGE and phosphor-imaging (A). Protein quantification was performed using ScanPack 3 software. The data represent means of two independent experiments; bars ± SD (B).

enhanced this immune response in comparison with the original E7GGG.GUS gene (Fig. 6A). Accordingly, after therapeutic vaccination against TC-1 cells, the growth of tumors in mice immunized with SS.GUS.E7GGG was significantly slower ($P<0.001$) compared with E7GGG.GUS-immunized animals (Fig. 6B). In summary, localization of the fusion protein of E7GGG with GUS into the ER increased the CTL response, but did not affect the induction of antibodies against E7.

Fusion protein stability. Immunogenicity of DNA vaccines can be substantially influenced by the stability of the produced immunogens (23,24). As modifications of the E7GGG.GUS gene resulted in considerable differences in the steady-state level of the produced fusion proteins possibly caused by the alteration of protein stability, we compared the stability of the cytoplasmic proteins E7GGG.GUS, E7GGG60.GUS, and E7GGG.EGUS by pulse-chase labeling followed by the immunoprecipitation with anti-E7 monoclonal antibody. We found that the stability of these proteins corresponded with their steady-state level in transfected 293T cells (Fig. 7). While the amount of the labeled E7GGG60.GUS protein did not recognizably decrease after 22-h chase, about a half of E7GGG.EGUS and 20% of E7GGG.GUS was degraded. Our data suggest that the stability of fusion proteins corresponded with the induction of E7-specific antibodies but we did not find any relation between the protein stability and cell-mediated immune responses.

Discussion

The rate of antigen synthesis is an important factor determining the efficacy of DNA vaccines. It can be affected by both the plasmid backbone and the sequence of the gene encoding the antigen (25). Moreover, the steady-state level of an antigen in transfected cells can be increased by the stabilization of the antigen, which might enhance its cross-presentation. As cross-priming was identified as the predominant mechanism inducing a cytotoxic T-lymphocyte (CTL) response after DNA immunization by a gene gun (18), we attempted to enhance the steady-state level of the HPV16 E7 antigen produced after DNA vaccination. We showed previously that the fusion of the E7 protein modified in the pRb-binding site (E7GGG) with the N-terminus of GUS (E7GGG.GUS) increased the steady-state level of the E7 antigen about 20-fold and substantially enhanced the efficacy of gene-gun DNA immunization (11). To increase further the steady-state level of the E7 antigen, here we modified the E7GGG.GUS fusion gene by: i) the deletions of E7GGG portions encoding C-terminal amino acids, ii) the fusion of E7GGG with the 3' GUS terminus (GUS.E7GGG), iii) the codon optimization of E7GGG, and iv) the elimination of the GUS initiation codon. However, only deletion mutants and the GUS.E7GGG fusion led to increase in the E7 production. Moreover, none of the modifications improved the CTL response when compared with E7GGG.GUS, despite the fact that the augmented steady-state level of fusion proteins corresponded with the improvement of production of E7-specific antibodies, which suggested that a higher amount of the E7 antigen was released from transfected cells and thus E7 cross-priming might also be enhanced.

Codon optimization of E7GGG (hE7GGG) increased the E7GGG protein production about 6-fold, but we did not record any effect after the fusion of the codon-optimized E7GGG with GUS - both the production of the fusion protein and immune responses induced by gene-gun immunization were comparable with those of the E7GGG.GUS gene. We suppose that the translation of the non-optimized GUS part of the hE7GGG.GUS gene (that is about 6-fold longer than the hE7GGG part) suppressed the beneficial effect of E7GGG optimization.

The HPV16 E7 antigen is an unstable protein with a half-life of about 30-60 min (26,27). We have shown previously that the steady-state level of the E7 antigen can be substantially augmented by fusion of E7GGG with the N-terminus of GUS (11), but induction of E7-specific antibodies was still negligible in our experimental setting. Only after further increase of the E7 antigen level achieved by the removal of E7GGG C-terminal amino acids from the E7GGG.GUS protein (i.e. in constructs E7GGG41.GUS, E7GGG60.GUS, and E7GGG75.GUS) or by fusion of E7GGG with the C-terminus of GUS, we elicited production of anti-E7 antibodies that corresponded with the steady-state level of the E7 antigen and the stability of the fusion proteins in transfected cells as demonstrated for E7GGG60.GUS. Antibodies against

E7 do not probably contribute to the protection against HPV-associated tumors, but stabilization of an antigen by fusion with GUS could be utilized for the induction of antibodies against other infectious agents or preparation of poly- or monoclonal antibodies.

Stability of the E7 antigen also affects induction of cell-mediated immunity. For instance, the destabilization of the E7 antigen by the mutations in zinc-binding motifs (23) or the fusion with ubiquitin (28,29) significantly enhanced CTL responses. We prepared modifications of the E7GGG.GUS protein that are either more (E7GGG60.GUS) or less stable (E7GGG.EGUS) than the original protein. However, none of them induced a stronger CTL response. Our results are in accordance with the data reported by Golovina *et al* (30) who showed that while targeted degradation of an antigen substantially enhanced epitope production, misfolding of an antigen caused by a point mutation or deletion, did not impact on epitope production despite decreasing antigen stability.

Intracellular and extracellular targeting of antigens is another key factor influencing immunogenicity of DNA vaccines and modulating immune responses (1,25). Addition of a signal sequence targeting an antigen into the ER and possibly into a secretory pathway can enhance humoral and/or cell-mediated immune responses after DNA immunization. However, both cellular localization and the type of enhanced immune response after fusion with a signal sequence depend on the nature of the antigen (31,32). Addition of a signal sequence to the E7 oncoprotein probably resulted in E7 secretion and an improved E7-specific CTL response (5,22). Therefore, in the SS.GUS.E7GGG construct, we fused the signal sequence from the adenoviral E3 gene to the GUS.E7GGG gene. We showed that the protein produced from the SS.GUS.E7GGG gene accumulated in the ER and was not secreted from cells. In comparison with the E7GGG.GUS gene, SS.GUS.E7GGG did not enhance the production of E7-specific antibodies, but improved the anti-tumor effect mediated by $CD8^+$ T lymphocytes. We suppose that the retrograde transport of the fusion protein from the ER into cytosol followed by degradation with proteasomes (32) could be responsible for the enhanced immunogenicity of SS.GUS.E7GGG.

In conclusion, we observed that the increase in the steady-state level of the GUS-based fusion proteins containing HPV16 E7-derived sequences augmented the production of antibodies against E7, but did not enhance CTL responses. Localization of the fusion protein into the ER had an opposite effect. These results further evoke the issue of the contribution of cross-priming versus direct priming to the induction of anti-tumor immunity with GUS-based DNA vaccines.

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3.3 Paper III:

Vaccination with human papillomavirus type 16-derived peptides using a tattoo device

Dana Pokorná, Ingrid Poláková, Martina Kindlová, Martina Dušková,
Viera Ludvíková, Pavel Gabriel, Luďa Kutinová, Martin Müller, Michal Šmahel

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Contribution of the author of this PhD. thesis:

I participated on the processing of splenocytes for the ELISPOT assay and tetramer staining and carried out the ELISPOT assays. Occasionally, I helped with tattooing.

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Vaccination with human papillomavirus type 16-derived peptides using a tattoo device

Dana Pokorná[a,*], Ingrid Poláková[a], Martina Kindlová[a], Martina Dušková[a], Viera Ludvíková[a], Pavel Gabriel[a], Lud'a Kutinová[a], Martin Müller[b], Michal Šmahel[a]

[a] Institute of Hematology and Blood Transfusion, Department of Experimental Virology, U Nemocnice 1, 12820 Prague 2, Czech Republic
[b] German Cancer Research Center, Research Group of Tumorvirus-Specific Vaccination Strategies, F035 Im Neuenheimer Feld 242, 69120 Heidelberg, Germany

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ABSTRACT

Tattooing has been shown to be very efficient at inducing immunity by vaccination with DNA vaccines. In this study, we examined the usability of tattooing for delivery of peptide vaccines. We compared tattooing with subcutaneous (s.c.) needle injection using peptides derived from human papillomavirus type 16 (HPV16) proteins. We observed that higher peptide-specific immune responses were elicited after vaccination with the simple peptides ($E7_{44–62}$ and $E7_{49–57}$) and keyhole limpet hemocyanin-(KLH)-conjugated peptides ($E7_{49–57}$, $L2_{18–38}$ and $L2_{108–120}$) with a tattoo device compared to s.c. inoculation. The administration of the synthetic oligonucleotide containing immunostimulatory CpG motifs (ODN1826) enhanced the immune responses developed after s.c. injection of some peptides ($E7_{44–62}$, KLH-conjugated $L2_{18–38}$ and $L2_{108–120}$) to levels close to or even comparable to those after tattoo delivery of identical peptides with ODN1826. The highest efficacy of tattooing was observed in combination with ODN1826 for the vaccination with the less immunogenic $E6_{48–57}$ peptide and KLH-conjugated and non-conjugated $E7_{49–57}$ peptides which form the visible aggregates that could negatively influence the development of immune responses after s.c. injection but probably not after tattooing. In summary, we first evidenced that tattoo administration of peptide vaccines that might be useful in some cases efficiently induced both humoral and cell-mediated immune responses.

1. Introduction

Peptide-based vaccines are being developed for the treatment of infections [1], allergic and autoimmune diseases [2–4] and cancer [5,6]. The synthesis of peptides in clinical grade is relatively easy and peptides are free of any oncogenic potential, in contrast to DNA vaccines directed against oncoproteins [5]. Moreover, no major toxicity has been reported after peptide vaccination. To design peptide-based vaccines, the identification of specific antigens is initially required followed by immunogenic epitope(s) identification [5]. The major disadvantage of peptide-vaccine therapy is the restriction of one peptide to a single MHC molecule. However, this problem may be overcome by administration of combinations of various peptides, so-called peptide cocktail vaccines [6]. In some clinical trials, partial or complete tumor regression has been observed in approximately 10–30% of patients after therapeutic peptide vaccinations [7].

To enhance the immunogenicity of peptide-based vaccines, different strategies are used. The natural epitopes may be modified to improve the affinity to the MHC molecules, the stability of MHC complexes, or proteolytic stability and bioavailability [5]. Peptides are typically administered with an adjuvant such as incomplete Freund adjuvant (FA), aluminium salts and immunomodulatory molecules [6,8] or conjugated with helper proteins [5] or lipids [9]. A number of adjuvants have been described and may be used in animals, yet in many cases adjuvants induce untoward reactions that limit their broad applicability, e.g. they provoke high inflammation, irritation, ulceration, etc. Currently, only few adjuvants are approved for use in humans [10]. Therefore, delivery methods that allow efficient immunization without adjuvants are highly desirable.

For DNA vaccination, tattooing has been shown to induce higher cellular and humoral immune responses than intramuscular needle injection [11,12]. The tattoo procedure causes many minor mechanical injuries followed by hemorrhage, necrosis, inflammation, and regeneration of the skin and thus non-specifically stimulates the immune system [13]. Therefore, tattooing may partially substitute for the function of adjuvants [12]. To the best of our knowledge, tattooing has not yet been tested for administration of peptide vaccines. However, tattoo delivery of a modified amino acid, bleomycin, has been reported as a promising therapeutic modality in large keloids and hypertrophic scars [14].

* Corresponding author. Tel.: +420 221 977 302; fax: +420 221 977 392.
E-mail address: danap@uhkt.cz (D. Pokorná).

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In this study, we examined the implication of tattoo delivery on peptide vaccination. As model peptide antigens, we used human papillomavirus type 16 (HPV16)-derived peptides containing either a CTL epitope ($E7_{49-57}$ and $E6_{48-57}$), a B-cell epitope ($L2_{18-38}$ and $L2_{108-120}$) or combined CTL, T_{helper}- and B-cell epitopes ($E7_{44-62}$). Our results indicate that tattoo delivery is a more efficient method of peptide immunization than s.c. needle injection.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice aged 6–8 weeks (H-2^b; Charles River, Sulzfeld, 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 at the Center for Experimental Biomodels, Charles University, Prague.

2.2. Cell lines

The efficacy of HPV16-derived peptide vaccines was evaluated using TC-1 tumor cells [15] prepared by transformation of primary C57BL/6 mouse lung cells with HPV16 E6/E7 oncogenes and activated *H-ras* (kindly provided by T.-C. Wu, Johns Hopkins University, Baltimore, MD). Cells were grown in Dulbecco's Modified Eagle's Medium (D-MEM; PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (PAA Laboratories), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Plasmid

The construction of the plasmid pBSC/E7GGG.GUS has been described previously [16]. Briefly, the fusion gene E7GGG.GUS coding for the mutated (D21G, C24G and E26G) HPV16 E7 protein denoted E7GGG and *Escherichia coli* beta-glucuronidase was inserted downstream of the CMV promoter into the EcoRI site of the mammalian expression plasmid pBSC [17].

2.4. Synthetic peptides

The HPV16-derived peptides, $E7_{49-57}$ (RAHYNIVTF), $E7_{44-62}$ (QAEPDRAHYNIVTFCCKCD, acetylated on the first amino acid), $E6_{48-57}$ (EVYDFAFRDL), $L2_{18-38}$ (LYKTCKQAGTCPPDIIPKVEG) and $L2_{108-120}$ (LVEETSFIDAGAP), were used for immunization and detection of induced immune responses. In addition, the peptide $L2_{107-122}$ (SLVEETSFIDAGAPTS) was utilized in the ELISA detecting antibodies against the $L2_{108-120}$ peptide. The peptides were synthesized by the Fmoc solid-phase method (Clonestar Peptide Services, Brno, Czech Republic). Peptide purity was determined to be >90% by high-pressure liquid chromatography (HPLC) and peptide sequences were validated by mass spectrometry (MALDI-TOF). The $L2_{18-38}$, $L2_{108-120}$ and $E7_{49-57}$ peptides were conjugated with the carrier KLH protein by the glutaraldehyde method (Clonestar Peptide Services). Lyophilized peptides were dissolved in water ($E7_{49-57}$, $E7_{44-62}$ and $L2_{18-38}$) or a small amount of DMSO followed by addition of water ($E6_{48-57}$, $L2_{108-120}$ and $L2_{107-122}$). For immunizations, 10× concentrated PBS was added to the dissolved peptides to obtain the final dilutions of peptides in 1× PBS. The $E7_{49-57}$ peptide (both simple and KLH-conjugated) tended to aggregate in PBS.

2.5. Immunizations

Mice were immunized two or three times at 2-week interval. One immunization dose contained 50 or 100 μg of a peptide with or without 50 μg of CpG adjuvant (ODN1826: TCCATGACGTTCCTGACGTT; GENERI BIOTECH, Hradec Kralove, Czech Republic), 50 μg of a KLH-peptide conjugate or 1 μg of DNA. For needle delivery, peptides were s.c. injected into the dorsum of mice in a final volume of 200 μl PBS using a 29G needle (Chirana, Stara Tura, Slovak Republic) hold in horizontal orientation and inserted about 5 mm subcutaneously. The KLH-conjugated peptides in PBS were delivered either without FA or mixed 1:1 (v/v) with complete FA (Gibco) for the first s.c. needle injection and with incomplete FA (Sigma) for the following immunizations. For tattoo delivery, peptides were administered in 20 μl PBS on the shaved skin at the dorsum and then a commercial tattoo machine (Rotary 12000 AL, Bortech Tattoogrosshandel, Wuppertal, Germany) was used for delivery. The tattoo device was adjusted to allow exposure of the needle tip 1–2 mm beyond the barrel guide. This depth of tattooing into the mouse skin has been shown to result in the immediate location of tattooed inks mainly in the dermis and to a lower extent in the epidermis [13]. A skin surface area of approximately 2 cm × 1 cm was tattooed by 50 one-second treatments with a five-needle unit (5-linear tattoo needle, Bortech Tattoogrosshandel) oscillating at a voltage of 17.4 V set on the power supply (DC POWER SUPPLY, DF 1730 SB3A, Bortech Tattoogrosshandel) corresponding to a frequency of 145 Hz (145 punctures per second). Thus, every tattooed mouse received during one immunization a total number of 36 250 ($5 \times 50 \times 145 = 36\,250$) solid needle pricks. The tattoo procedure was well tolerated. The preparation of cartridges for DNA vaccination and gene gun immunization were performed as described previously [16]. Briefly, 1 μg of the pBSC/E7GGG.GUS plasmid was coated onto 0.5 mg of 1 μm gold particles and was delivered by the gene gun at a discharge pressure of 400 psi into the shaven skin of the abdomen as a single immunization.

2.6. Preparation of splenocytes for assays of E7-specific T-cell responses

For *in vitro* assays, pools of splenocytes from each vaccinated group (3 mice per group) were prepared 2 weeks after the last vaccination and used either fresh (*ex vivo*) or after restimulation with peptides for 5–7 days. Splenocytes were stimulated with the HPV16-derived $E6_{48-57}$ [18], $E7_{49-57}$ [20] or $E7_{44-62}$ [21] peptides carrying the H-2^b CTL epitope or cultured in medium without the peptides (negative control). All samples were cultivated in two parallel wells.

2.7. ELISPOT assay

The IFN-γ-ELISPOT assay was performed using both fresh splenocytes (1×10^6/well) and splenocytes after 6-day restimulation. The 96-well filtration plates (Millipore Corp., Bedford, MA) were coated with 10 μg/ml rat anti-mouse IFN-γ antibody (BD Biosciences Pharmingen, San Diego, CA) in 50 μl of PBS per well. 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-stimulated or stimulated splenocytes from each vaccinated group of mice, starting from 1×10^6/well, were added to the wells. Cells were incubated at 37 °C for 20 h either with or without different concentrations (from 0.001 to 10 μg/ml) of the $E7_{49-57}$, $E7_{44-62}$ or $E6_{48-57}$ peptide. The plates were washed and incubated with 5 μg/ml biotinylated anti-IFN-γ antibody (BD Biosciences Pharmingen) in 50 μl of PBS per well at 4 °C overnight. After washing, the avidin-horseradish peroxidase conjugate (BD Biosciences 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, Hanover, MD) and 0.03% H_2O_2 and incubated at room temperature for 1 h. Spots were

evaluated by the Eli.Scan ELISPOT Scanner (A.EL.VIS, Hannover, Germany).

2.8. Intracellular cytokine staining (ICS)

The intracellular cytokine staining of IFN-γ in $CD8^+$ cells was performed as described recently [22]. In brief, the splenocytes were incubated with the peptides for 20 h (*ex vivo* assay) or 6 days with one exchange of media (assay after restimulation). Twelve hours before staining, GolgiStop (BD Biosciences Pharmingen) was added to the culture medium according to the manufacturer's instructions. Surface CD8 molecules were stained with PE-conjugated rat anti-mouse CD8a monoclonal antibody (BD Biosciences Pharmingen). Then, cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences Pharmingen) and intracellular IFN-γ was stained with FITC-conjugated rat anti-mouse IFN-γ monoclonal antibody (BD Biosciences Pharmingen). The stained cells were measured on a Coulter Epics XL flow cytometer (Coulter, Miami, FL) and analyzed by FlowJo 7.1.2. software (TreeStar, Ashland, OR).

2.9. Tetramer staining

In the tetramer-staining assay, the $E7_{49–57}$-specific $CD8^+$ cells were detected in the splenocytes restimulated with the $E7_{49–57}$ peptide for 5 or 7 days as described previously [23]. After restimulation, splenocytes were centrifuged and resuspended in 50 μl RIA buffer (PBS supplemented with 1% BSA and 0.09% sodium azide) containing 5 μg/ml rat anti-mouse CD16/CD32 (Fc-block; BD Biosciences Pharmingen) and incubated on ice for 15 min. Washed cells were stained in 20 μl with 0.5 μl the PE-labeled H-$2D^b$/$E7_{49–57}$ tetramer reagent (PeliMer H-$2D^b$/E7, Sanquin, Amsterdam, Netherlands) for 20 min at room temperature. The 5 μl of RIA buffer with 50 μg/ml of FITC-conjugated rat anti-mouse CD8a monoclonal antibody (BD Pharmingen) was added and splenocytes were incubated for another 20 min at room temperature. After washing, the stained cells were measured on a Coulter Epics XL flow cytometer (Coulter) and analyzed by FlowJo 7.1.2. software (TreeStar).

2.10. Detection of peptide-specific antibodies by ELISA

Sera of immunized mice were collected either 10 days after each immunization or 14 days after the final immunization, and analyzed in enzyme-linked immunosorbent assay (ELISA). The 96-well plates (Maxisorp F96, Nunc) were coated overnight at 4 °C with 50 μl PBS containing 10 μg/ml of the $E7_{44–62}$, $L2_{18–38}$ or $L2_{107–122}$ peptides or KLH protein (Merck Biosciences, Nottingham, UK). Antibodies against the $L2_{108–120}$ peptide were detected using the $L2_{107–122}$ peptide because ELISAs with other coating substances like the simple or BSA-conjugated $L2_{108–120}$ peptide were less sensitive. Plates were washed three times in washing buffer (PBS with 0.3% Tween 20) and blocked with 100 μl 3% milk in washing buffer for 1 h at 37 °C. Mouse sera diluted in 50 μl of 1.5% milk in washing buffer were added to the plate either at a single dilution of 1:50 or at a dilution of 1:10 followed by twofold dilutions starting at 1:50 and ending at 1:104,857,600 and incubated for 1 h at 37 °C. Non-specific binding was determined using the dilution of 1:10 or 1:50 of the mouse sera on plates coated with PBS only. Then, the plates were washed and incubated with 50 μl/well of sheep anti-mouse IgG antibodies conjugated to horseradish peroxidase (HRP; Amersham Biosciences, Buckinghamshire, UK) diluted 1:2000 or rat anti-mouse IgG1 or IgG2a antibodies conjugated to HRP (BD Pharmingen) diluted 1:500 in 1.5% milk in washing buffer for 1 h at 37 °C. After final washings, the plates were stained with 100 μl of 10 μg/ml tetramethylbenzidine (Sigma) and 0.003% H_2O_2 in 0.1 M acetic acid (pH 6) for 20–30 min. The reaction was stopped by addition of 50 μl of 1 M sulfuric acid and the absorbance was measured at 450 nm. Titres were expressed as reciprocals of the final serum dilution giving an absorbance higher than 0.2. Sera with a titre less than 10 were considered negative and a value of 1 was assigned to them for computational purposes.

2.11. The HPV16-neutralization assay

The neutralization assay was performed as described previously [24]. Briefly, the 293TT cells were incubated with a mixture of HPV16 L1/L2 pseudovirions encapsidating a plasmid carrying the gene coding for secreted alkaline phosphatase (SEAP) and sera diluted 1:50 and 1:200 in DMEM (Sigma). Polyclonal neutralizing rabbit antisera specific for HPV16 L1 and L2 were used as positive controls. Detection of SEAP in cell culture supernatant was performed with the chemiluminescent SEAP Reporter Gene Assay (Roche). All sera were tested in duplicates.

2.12. Tumor protection experiment

Mice (5 per group) were vaccinated three times at 2-week interval. Two weeks after the last vaccination, mice were s.c. challenged into the back with 3×10^4 TC-1 cells suspended in 0.15 ml PBS. Tumor cells were administered under anaesthesia with etomidate (0.5 mg i.p./mouse; Janssen Pharmaceutica, Beerse, Belgium). Tumor growth was monitored twice a week. Tumor size was calculated from three perpendicular measurements using the formula $(a \times b \times c)\pi/6$.

2.13. Statistical analysis

Tumor formation was analyzed by the log-rank test. Tumor growth was evaluated by two-way analysis of variance. Data of endpoint ELISAs were analyzed by the Mann–Whitney test (Wilcoxon Rank sum test). A difference between groups was considered significant at $P < 0.05$. Calculations were performed using Prism software, version 4.0 (Graph-Pad Software, San Diego, CA).

3. Results

3.1. Tattoo delivery of the $E7_{49–57}$ peptide with CpG motifs elicited cellular immune responses comparable to those after gene-gun DNA vaccination with pBSC/E7GGG.GUS

To evaluate the efficacy of peptide immunization by tattooing, we compared the peptide vaccine based on the HPV16 E7 immunodominant CTL epitope and our most efficient DNA vaccine against the E7 oncoprotein [16]. We immunized mice three times with 100 or 50 μg of the $E7_{49–57}$ peptide in combination with 50 μg of CpG motifs by tattooing and with 1 μg of the pBSC/E7GGG.GUS plasmid administered by the gene gun. Then, we performed the ELISPOT assay to detect IFN-γ-secreting $CD8^+$ T cells in splenocytes from vaccinated mice. The results of a representative experiment, in which splenocytes were restimulated with the $E7_{49–57}$ peptide for 6 days, are shown in Fig. 1A. The immune response induced by tattooing was comparable with that after gene-gun DNA immunization.

The *in vitro* assays, i.e. ELISPOT (Fig. 1A), ICS and tetramer staining (data not shown), revealed that after vaccination with 50 μg of the $E7_{49–57}$ peptide combined with 50 μg of CpG motifs, the counts of E7-specific $CD8^+$ T lymphocytes reached 60–100% of those observed after vaccination with 100 μg of the $E7_{49–57}$ peptide with 50 μg of CpG motifs.

The potential of tattooing with the $E7_{49–57}$ peptide vaccine was also assessed by the preventive immunization against TC-1 cells (Fig. 1B). In contrast to naive mice that developed tumors within 19 days after inoculation of tumor cells, all tattooed mice remained

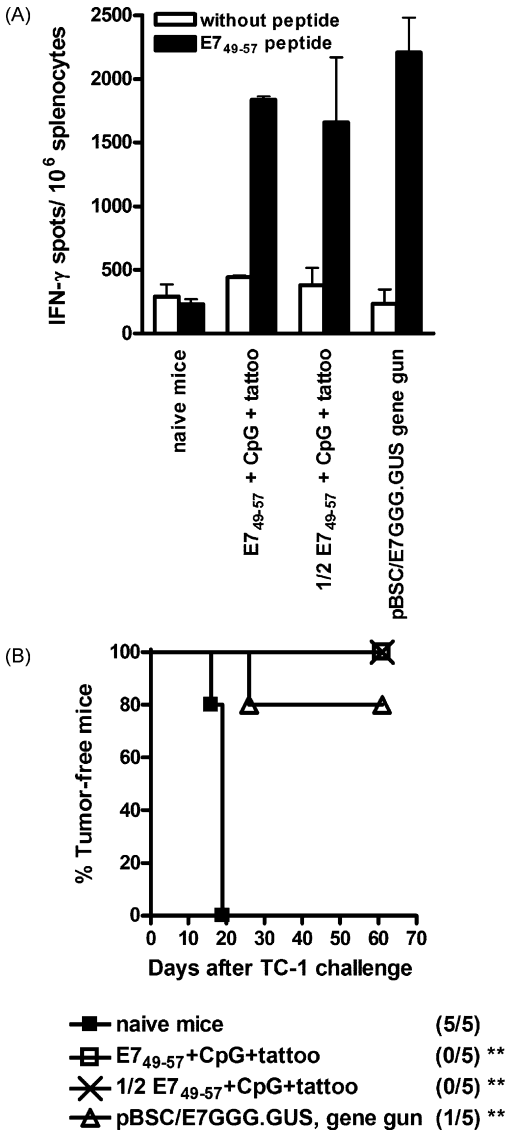

Fig. 1. Comparison of peptide and DNA immunization. Mice ($n = 8$) were immunized three times at 2-week interval with the $E7_{49–57}$ peptide (100 or 50 μg) supplemented with CpG motifs (50 μg) using a tattoo device or with the pBSC/E7GGG.GUS plasmid (1 μg) by a gene gun. The lower dose of the $E7_{49–57}$ peptide, i.e. 50 μg, is marked as 1/2. Fourteen days after the last vaccination: (A) Three mice were sacrificed, lymphocyte cultures were prepared from pools of splenocytes, and IFN-γ-producing cells were detected after 6-day restimulation with the $E7_{49–57}$ peptide by an ELISPOT assay. Control lymphocytes were cultivated in the absence of the peptide. Columns, mean of two samples; bars, S.D. (B) Five mice were challenged with 3×10^4 TC-1 tumor cells and the development of tumors was monitored twice a week. No. of mice with tumors/ no. of mice per group is indicated. Asterisks, statistical significance (**$P < 0.01$) in comparison with the group of naive mice.

tumor-free for the whole observation period of 61 days ($P < 0.01$). DNA immunization with pBSC/E7GGG.GUS protected 4 out of 5 mice ($P < 0.01$). To conclude, comparison with DNA vaccination confirmed high immunogenicity of peptide vaccines administered by tattooing.

3.2. Tattooing with the $E7_{49–57}$ and $E7_{44–62}$ peptides induced higher immune responses than s.c. injection

To further evaluate the usage of tattooing for peptide vaccination, we immunized mice with HPV16 E7-derived peptides by either a tattoo device or s.c. needle injection as described in Section 2. Furthermore, we determined whether CpG motifs (ODN1826) affect immune responses induced by tattooing with peptide vaccines.

Specific activation of $CD8^+$ lymphocytes isolated from spleens was tested by the staining of intracellular IFN-γ and tetramer staining. Intracellular IFN-γ was determined in an *ex vivo* assay after incubation with the $E7_{49–57}$ or $E7_{44–62}$ peptide. Stimulation of splenocytes by both peptides resulted in similar IFN-γ production. The results of a representative experiment are shown in Fig. 2A. About 1% of lymphocytes isolated from the spleens of mice tattoo vaccinated with the $E7_{49–57}$ peptide and CpG motifs were E7-specific $CD8^+$ T cells. This count was about 50-fold higher than that from mice vaccinated with the identical vaccine subcutaneously. Similarly, higher counts of E7-specific $CD8^+$ T cells in tattooed mice were also found after immunization with the $E7_{44–62}$ peptide and CpG motifs (about 0.4% *versus* 0.2% IFN-γ^+ $CD8^+$ T cells), the $E7_{49–57}$ peptide (0.04% *versus* 0.01%) and the $E7_{44–62}$ peptide (0.02% *versus* 0.01%).

For the tetramer assay, splenocytes were restimulated by a 7-day incubation with the $E7_{49–57}$ peptide (Fig. 2B). Higher rates of $CD8^+$ lymphocytes were tetramer positive in splenocytes from tattooed mice than from animals after s.c. administration of the $E7_{49–57}$ peptide with CpG motifs (about 9% *versus* 0.1%), the $E7_{44–62}$ peptide with CpG motifs (about 6% *versus* 4%), the $E7_{49–57}$ peptide alone (about 0.7% *versus* 0.4%) and the $E7_{44–62}$ peptide alone (about 0.9% *versus* 0.5%). The stimulation of $CD8^+$ T cells was so high in groups of mice tattooed with the $E7_{49–57}$ or the $E7_{44–62}$ peptide with CpG motifs that the numbers of tetramer$^+$ $CD8^+$ T cells were enhanced not only after incubation with the $E7_{49–57}$ peptide but also after cultivation in a medium without the peptide (about 1% or 0.3%, respectively, *versus* 0.02%, compared with the negative control naive mice). No E7-specific splenocytes were revealed in the control groups of naive mice and those treated with CpG motifs by tattooing.

Humoral responses induced by peptide immunization were determined by ELISA using the $E7_{44–62}$ peptide. All mice immunized with the $E7_{44–62}$ peptide (12/12) developed E7-specific antibodies, while all animals vaccinated with the $E7_{49–57}$ peptide (12/12) or CpG motifs alone (3/3) and naive controls (3/3) did not produce E7-specific antibodies (Fig. 2C). The end-point titration of sera from mice immunized with the $E7_{44–62}$ peptide revealed that the geometric mean titre of E7-specific IgG antibodies produced after tattoo vaccination with the peptide in combination with CpG motifs was about 60-fold higher than after subcutaneous immunization with the identical vaccine. Similarly, an about 10-fold higher geometric mean titre was found in mice tattooed with the peptide alone than in those immunized subcutaneously. The addition of CpG motifs enhanced the levels of E7-specific antibodies after immunization by both s.c. needle injection and tattooing. Furthermore, we determined subclasses of E7-specific IgG antibodies, IgG1 and IgG2a, produced in mice after the vaccination with the $E7_{44–62}$ peptide (Fig. 2D). In correlation with the previous detection of total IgG antibodies (Fig. 2C), the summation of IgG1 and IgG2a antibodies was higher in mice vaccinated with the peptide by tattoo and/or in combination with CpG motifs than that in mice immunized subcutaneously and/or in the absence of CpG motifs. The production of IgG1 antibodies was slightly decreased in the presence of CpG motifs. While IgG2a antibodies were not found in mice vaccinated with the $E7_{44–62}$ peptide in the absence of CpG motifs, all 3 mice and 2 out of 3 mice vaccinated with the peptide in combination with CpG motifs by tattooing or s.c. needle injection, respectively, produced IgG2a antibodies.

To determine whether tattooing with the $E7_{49–57}$ or $E7_{44–62}$ peptide is able to protect mice against E7-expressing tumors, animals were immunized three times and then challenged with TC-1 cells (Fig. 2E). The efficacy of this antitumor immunization corresponded with the activation of $CD8^+$ T cells found in the *in vitro*

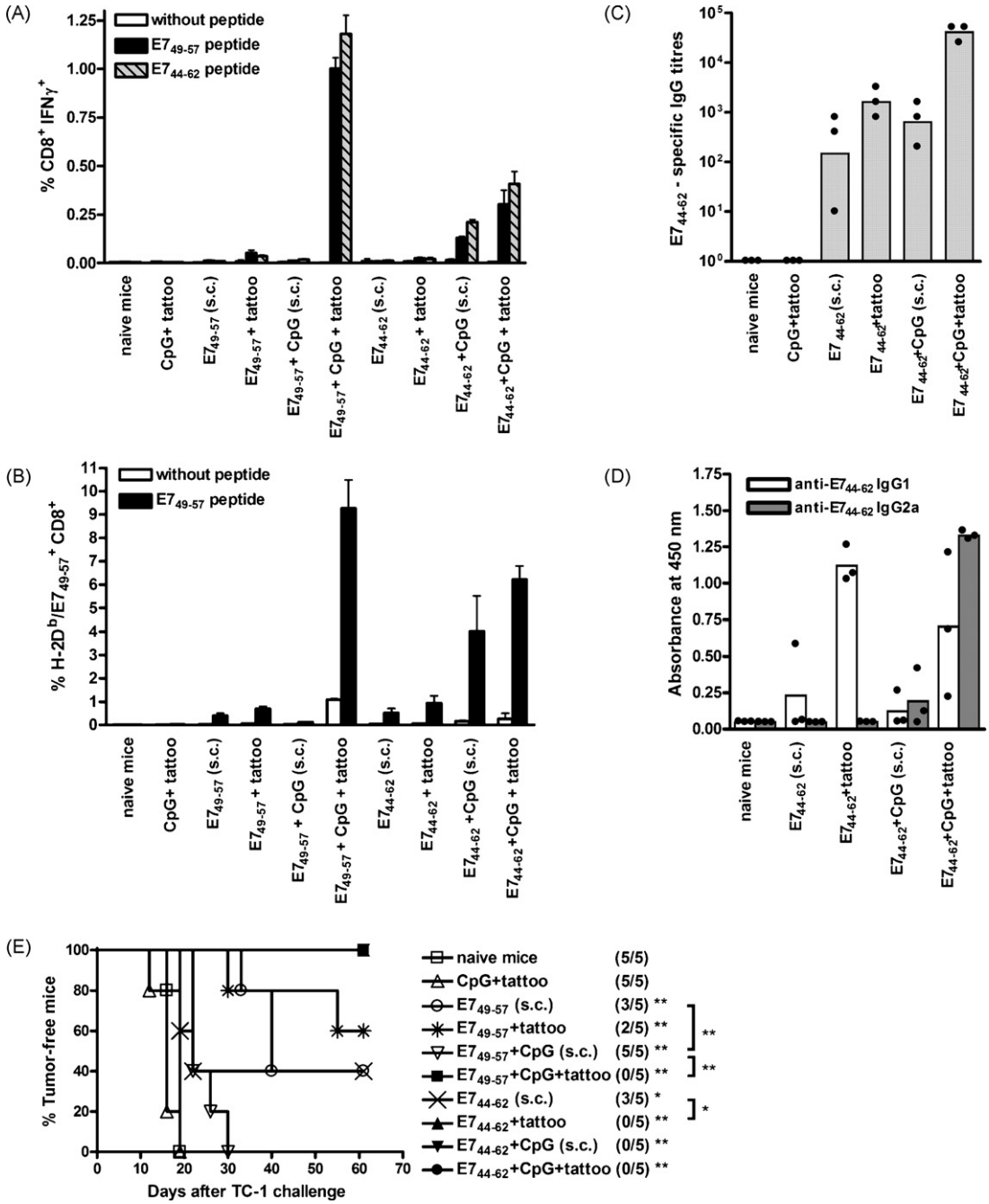

Fig. 2. Comparison of tattooing with s.c. needle injection using E7-derived peptides. Mice ($n = 8$) were immunized three times at 2-week interval with 100 μg of the $E7_{49–57}$ or $E7_{44–62}$ peptide with or without 50 μg CpG motifs by either tattooing or s.c. injection. Fourteen days after the last vaccination, three mice were sacrificed and their blood sera and splenocytes were used in *in vitro* assays (A–D), and five mice were inoculated with 3×10^4 TC-1 tumor cells. The development of tumors was monitored twice a week (E). Naive mice and mice tattooed with CpG motifs were used as controls. (A) *Ex vivo* intracellular staining of IFN-γ produced by $CD8^+$ cells in lymphocyte cultures prepared from pools of splenocytes and (B) tetramer staining of $CD8^+$ cells after 7-day restimulation of lymphocyte cultures prepared from pools of splenocytes. (A and B) Control lymphocytes were cultivated without the peptide. Columns, mean of two samples; bars, S.D. (C) E7-specific IgG antibodies in individual sera. Columns, geometric mean titres. (D) E7-specific IgG1 and IgG2a antibodies in individual sera. Columns, mean absorbances. (E) Formation of TC-1-induced tumors. No. of mice with tumors/no. of mice per group is indicated. Asterisks, statistical significance ($^*P < 0.05$, $^{**}P < 0.01$) in comparison with the group of naive mice; right square brackets followed by asterisks, statistical comparison of the indicated groups of mice.

assays. All naive mice and mice tattooed with CpG motifs developed tumors within 19 days. In contrast, all groups of mice immunized with the $E7_{49–57}$ or $E7_{44–62}$ peptides were significantly protected against the formation of TC-1-induced tumors ($P < 0.05$, compared to naive mice). Surprisingly, while vaccination with the $E7_{49–57}$ peptide alone given either subcutaneously or using the tattoo device protected 2 or 3 out of 5 mice, respectively (non-significant, tattoo *versus* s.c. delivery), and all mice tattoo vaccinated with the $E7_{49–57}$ peptide in combination with CpG motifs remained tumor-free for the whole observation period of 61 days, all mice s.c. immunized with the $E7_{49–57}$ peptide in combination with CpG motifs developed tumors within 30 days ($P < 0.01$, tattoo *versus* s.c. delivery). All animals immunized with the $E7_{44–62}$ peptide in combination with CpG motifs either by tattooing or subcutaneously or with the $E7_{44–62}$ peptide alone delivered by tattooing remained tumor-free, while 3 out of 5 mice immunized subcutaneously with the $E7_{44–62}$ peptide alone developed tumors ($P < 0.05$, tattoo *versus* s.c. delivery).

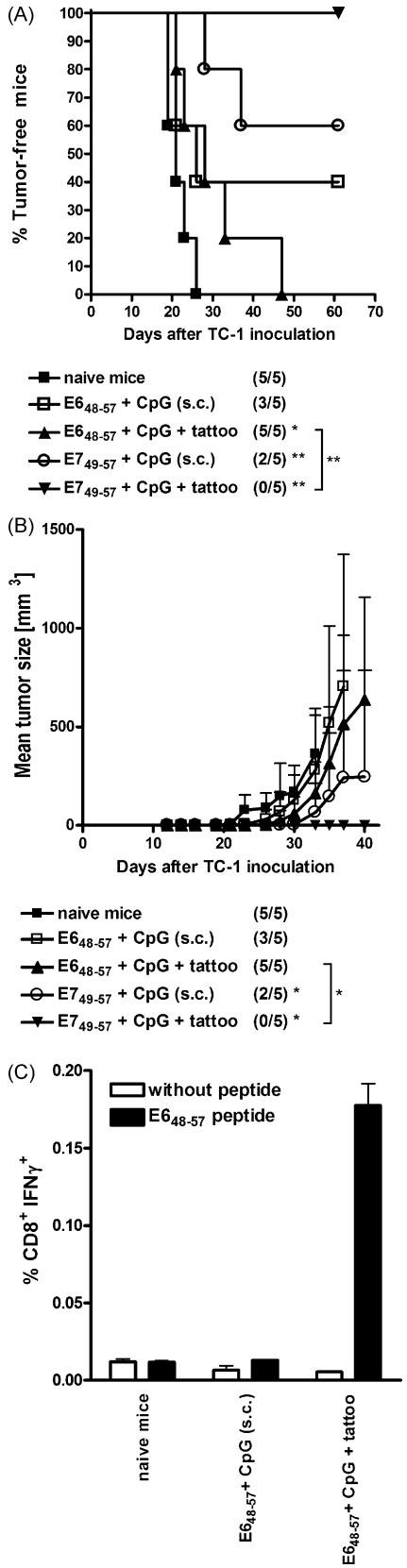

Fig. 3. Comparison of tattooing with s.c. needle injection using the E6-derived peptide. Mice ($n = 8$) were immunized three times at 2-week interval with the $E6_{48-57}$ or the $E7_{49-57}$ peptides (100 μg) in combination with CpG motifs (50 μg) either by tattooing or subcutaneous injection. Fourteen days after the last vaccination: (A and B) Mice ($n = 5$) received TC-1 tumor cells and the development (A) and growth of tumors (B) was monitored twice a week. No. of mice with tumors/no. of mice per group is indicated; bars, S.D.; asterisks, statistical significance (*$P < 0.05$, **$P < 0.01$) in comparison with the group of naive mice; right square brackets followed by asterisks, statistical comparison of indicated groups of mice. (C) Three mice were sacrificed, lymphocyte cultures were prepared from pools of splenocytes and intracellular IFN-γ-staining of $CD8^+$ cells was performed after *ex vivo* incubation with the $E6_{48-57}$ peptide. Columns, means of two samples; bars, S.D.

Altogether, our data indicate that in comparison with s.c. injection, tattoo delivery of peptide vaccines induced higher both cell-mediated and humoral immune responses. Addition of CpG motifs further enhanced the efficacy of vaccination by the tattoo device.

3.3. Tattoo delivery of the $E6_{48-57}$ peptide with CpG motifs induced higher CTL responses than s.c. injection

To examine whether tattooing is a more efficient method than subcutaneous needle injection for peptide vaccination carrying different CTL epitopes, we performed an immunization experiment with the $E6_{48-57}$ peptide, using the $E7_{49-57}$ peptide as a positive control.

Mice were immunized three times with 100 μg of peptides combined with 50 μg of CpG motifs and challenged with TC-1 cells (Fig. 3A and B). Naive mice developed tumors in 26 days after challenge, while all animals tattoo vaccinated with the $E7_{49-57}$ peptide in combination with CpG motifs were protected against tumor formation ($P < 0.01$, compared to naive mice) and 3 out of 5 mice immunized subcutaneously with the $E7_{49-57}$ peptide in combination with CpG motifs remained tumor-free for 61 days after challenge ($P < 0.01$, compared to naive mice). The data indicated that the conditions in the experiment shown in Fig. 3A and B were less stringent than those in the preceding experiment (Fig. 2E), allowing to notice the antitumor effect of less efficient vaccines. All mice tattoo immunized with the $E6_{48-57}$ peptide with CpG motifs developed tumors within 47 days; however, the tumors formed later and grew much more slowly than in mice (3/5) immunized s.c. with the identical vaccine during 61 days of screening (non-significant, tattoo *versus* s.c. delivery, Fig. 3A and B). The tattoo delivery of the $E6_{48-57}$ peptide together with the CpG motifs elicited significant protection against TC-1-tumor formation ($P < 0.05$, compared to naive mice), while the antitumor effects observed after s.c. injection of the identical vaccine were not significant in comparison with naive mice. Moreover, higher tumor formation and tumor growth were observed after injection of TC-1 cells in mice vaccinated with the $E6_{48-57}$ peptide in comparison with the $E7_{49-57}$ peptide ($P < 0.05$, comparison of tattoo delivery of peptides with CpG motifs; non-significant, comparison of s.c. delivery).

The intracellular IFN-γ staining of $CD8^+$ splenocytes revealed enhancement of E6-specific $CD8^+$ T cells in splenocytes isolated from mice tattooed with the $E6_{48-57}$ peptide with CpG motifs in comparison with those from naive animals or mice vaccinated with the identical vaccine subcutaneously (0.2% *versus* 0.01%, Fig. 3C). Similar results were obtained in the ELISPOT assay (data not shown). The immunization with the $E7_{49-57}$ peptide in combination with CpG motifs by tattooing induced E7-specific CTLs detected by ELISPOT and ICS (data not shown) in concordance with the previously obtained results (Fig. 2A and B).

3.4. Tattooing with the KLH-conjugated $E7_{49-57}$ peptide induced higher CTL responses than s.c. injection

To evaluate the effects of tattooing on the induction of specific cellular responses after vaccination with KLH-conjugated peptides, we immunized mice with the KLH-conjugated HPV16 $E7_{49-57}$ peptide using the non-conjugated $E7_{49-57}$ peptide as a positive control.

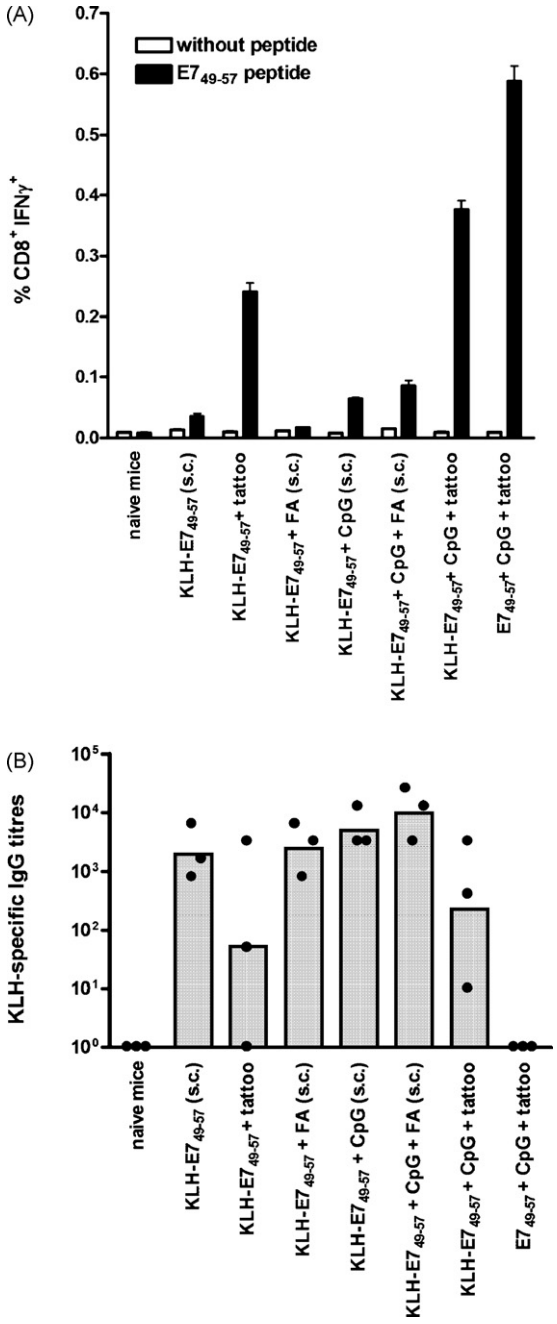

Fig. 4. Comparison of immune responses after vaccination with the KLH-conjugated $E7_{49–57}$ peptide. Mice ($n = 3$) were immunized three times at 2-week interval with 50 μg of the KLH-conjugated $E7_{49–57}$ peptide with or without 50 μg of CpG motifs by either tattooing or s.c. injection. Fourteen days after the last vaccination, mice were sacrificed and their blood sera and splenocytes were used in *in vitro* assays. Naive mice were used as a negative control, mice tattooed with 100 μg of the $E7_{49–57}$ peptide with 50 μg CpG motifs served as a positive control. (A) *Ex vivo* intracellular staining of IFN-γ produced by $CD8^+$ cells in lymphocyte cultures prepared from pools of splenocytes. Columns, mean of two samples; bars, S.D. (B) KLH-specific IgG antibodies in individual sera. Columns, geometric mean titres.

Specific activation of $CD8^+$ lymphocytes isolated from spleens was determined by the staining of intracellular IFN-γ in an *ex vivo* assay after incubation with the $E7_{49–57}$ peptide (Fig. 4A). About 0.4% of lymphocytes isolated from spleens of mice vaccinated with the KLH-conjugated $E7_{49–57}$ peptide and CpG motifs by tattooing were E7-specific $CD8^+$ T cells. This count was about 10-fold higher than that from mice vaccinated with the identical vaccine subcutaneously. Similarly, higher counts of E7-specific $CD8^+$ T cells in tattooed mice were also found after immunization with the KLH-conjugated $E7_{49–57}$ peptide without CpG motifs (about 0.25% *versus* 0.04% IFN-γ^+ $CD8^+$ T cells). The CpG motifs enhanced specific CTL responses induced by tattoo delivery of the KLH-conjugated $E7_{49–57}$ peptide but to the lower extent in comparison with tattoo delivery of the non-conjugated $E7_{49–57}$ peptide (Fig. 2A). The addition of FA did not substantially modify the detected counts of E7-specific CD8+ T cells after s.c. injections of the KLH-conjugated $E7_{49–57}$ peptide with or without CpG motifs (Fig. 4A). Similar results were obtained in the ELISPOT assay, staining of intracellular IFN-γ after restimulation and tetramer staining (data not shown).

KLH-specific antibodies induced by immunization with the KLH-conjugated $E7_{49–57}$ peptide were determined by ELISA using the KLH protein (Fig. 4B). Three immunizations with the KLH-conjugated $E7_{49–57}$ peptide induced KLH-specific antibodies in 17 out of 18 mice, while all mice vaccinated with the non-conjugated $E7_{49–57}$ peptide with CpG motifs (3/3) and naive mice (3/3) did not produce KLH-specific antibodies. The end-point titration of sera revealed that the geometric mean titre (GMT) of KLH-specific IgG antibodies produced in mice tattooed with the KLH-conjugated peptide in combination with CpG motifs was about 20-fold lower than that in mice immunized with the identical vaccine subcutaneously (about 250 *versus* 5000). Similarly, an about 40-fold lower GMT was found in mice tattooed with the KLH-conjugated peptide alone than in those immunized subcutaneously (about 50 *versus* 2000). The addition of CpG motifs enhanced the levels of KLH-specific antibodies after immunization by both s.c. needle injection and tattooing. Furthermore, the mixture with FA slightly enhanced the GMT of KLH-specific antibodies after s.c. delivery.

3.5. *The production of L2-specific antibodies after immunization with KLH-conjugated peptides $L2_{18–38}$ and $L2_{108–120}$ using a tattoo device was higher than that after s.c. injection*

To reveal the effects of tattooing on the induction of specific humoral responses after vaccination with the KLH-conjugated peptides, we immunized mice three times with the KLH-conjugated HPV16-derived $L2_{18–38}$ and $L2_{108–120}$ peptides either with or without CpG motifs using a tattoo device or s.c. injection.

The follow-up of the production of L2-specific antibodies after individual immunizations showed that the first vaccination with the KLH-conjugated peptides induced antibodies in 37 out of 60 mice and that the number of positively reacting mice increased to 58 out of 60 mice after the second vaccination. After three immunizations, mice (10/10) vaccinated with the KLH-conjugated $L2_{18–38}$ and $L2_{108–120}$ peptides subcutaneously without CpG motifs developed low levels of L2-specific antibodies (GMT of about 300), while mice (10/10) immunized with identical vaccines by tattoo produced high titres of L2-specific antibodies (GMT about 10 000, $P < 0.01$, tattoo *versus* s.c. delivery) and comparable differences in titres of L2-specific antibodies were also found in mice immunized with the KLH-conjugated $L2_{18–38}$ or $L2_{108–120}$ peptides in combination with CpG motifs ($P < 0.05$ and non-significant, respectively, tattoo *versus* s.c. delivery, Fig. 5A and B). Addition of CpG motifs enhanced the antibody production both after s.c. injection and tattoo delivery. Antibody production was also increased by FA for the s.c. delivered KLH-conjugated peptides. The effect of FA was higher than that of CpG motifs ($P < 0.05$ for the $L2_{18–38}$ peptide) and was not further augmented by the combination of FA with CpG motifs. The levels of L2-specific antibodies observed after tattoo delivery of the KLH-conjugated L2 peptides in combination with CpG motifs were comparable with the humoral responses after s.c. injection of the vaccines mixed with FA. However, none of these L2-specific antibodies showed any neutralization activity in the HPV16-neutralization assay (titre <1:200; data not shown). The

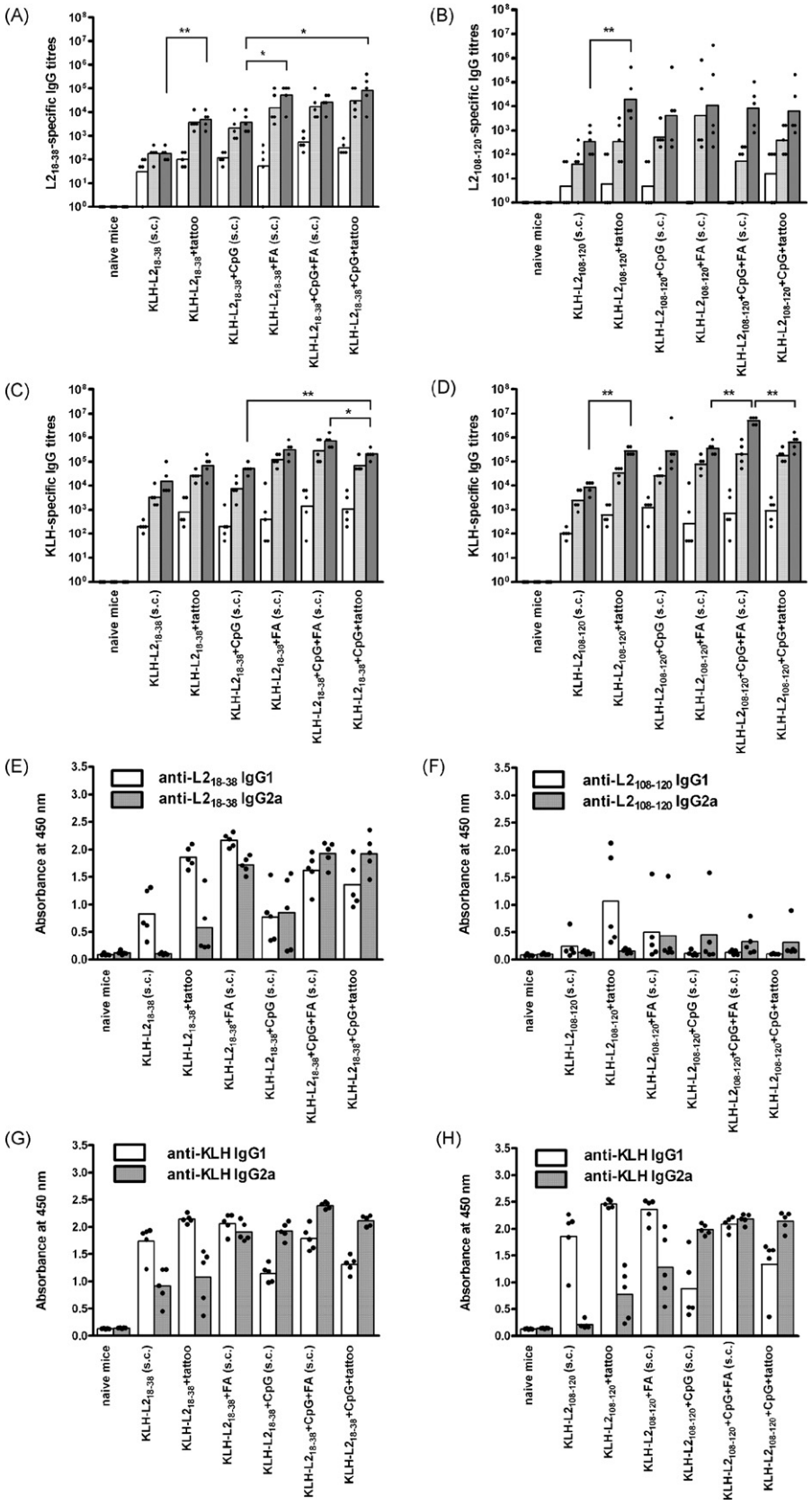

Fig. 5. Comparison of humoral responses after vaccination with the KLH-conjugated L2 peptides. Mice ($n = 5$) were immunized three times at 2-week interval with 50 μg of the KLH-conjugated $L2_{18–38}$ or $L2_{108–120}$ peptide with or without 50 μg of CpG motifs using a tattoo device or s.c. needle injection. Ten days after each immunization, sera from mice were collected and $L2_{18–38}$-specific (A), $L2_{108–120}$-specific (B) or KLH-specific (C and D) antibodies were detected in ELISA. Columns, geometric mean titres after the first (white), the second (light grey), and the third (dark grey) immunization dose; square brackets followed by asterisks, statistical comparison ($^{*}P < 0.05$, $^{**}P < 0.01$) of indicated groups. After the third vaccination, the $L2_{18–38}$-specific (E), $L2_{108–120}$-specific (F) or KLH-specific (G and H) IgG1 and IgG2a antibodies were assessed in individual sera. Columns, mean absorbances.

production of L2-specific antibodies was not found in the control group of naive mice (0/5).

Production of KLH-specific antibodies was found in all mice (60/60) immunized with KLH-conjugated peptides after the first immunization (GMT of about 500) and further increased after the second and the third immunizations (to a GMT of about 10 000 and 100 000, respectively; Fig. 5C and D). The levels of KLH-specific antibodies shown in Fig. 5C and D mostly corresponded to the production of L2-specific antibodies (Fig. 5A and B). Higher levels of KLH-specific antibodies were found in mice immunized with the KLH-conjugated $L2_{18–38}$ or $L2_{108–120}$ peptides by tattooing in comparison with subcutaneous immunization (non-significant and $P < 0.01$, respectively, tattoo *versus* s.c. delivery). Similarly, mice tattooed with the KLH-conjugated $L2_{18–38}$ or $L2_{108–120}$ peptides combined with CpG motifs developed higher titres of KLH-specific antibodies than those immunized subcutaneously ($P < 0.01$ and non-significant, respectively, Fig. 5C and D). There was no relevant difference in the production of KLH-specific antibodies between groups of mice immunized with the KLH-conjugated L2 peptides either in the mixture with FA by s.c. needle injection or in combination with CpG motifs by tattoo. However mice immunized with the KLH-conjugated $L2_{18–38}$ or $L2_{108–120}$ peptides combined with CpG motifs in mixture with FA developed higher titres of KLH-specific antibodies than mice immunized with identical peptides in combination with CpG motifs by tattoo ($P < 0.01$ and $P < 0.001$, respectively) or with FA by s.c. application ($P < 0.01$ for the $L2_{108–120}$ peptide). No KLH-specific antibodies were found in the control group of naive mice (0/5).

The analysis of the production of IgG1 and IgG2a antibodies revealed higher levels of both L2- and KLH-specific IgG1 antibodies in groups of mice immunized without CpG motifs, while the IgG2a antibodies were enhanced in groups of mice immunized with vaccines containing CpG motifs (Fig. 5E–H). We did not find any difference in the IgG1 to IgG2a ratio in association with peptide delivery method.

In summary, our results indicate that s.c. injection of the KLH-conjugated L2 peptides without any adjuvant induced lower levels of specific antibodies in comparison with tattoo delivery. However, the addition of adjuvanting CpG motifs substantially enhanced antibody production elicited after s.c. delivery to the extent comparable to that after tattooing.

4. Discussion

In this study we compared various methods of immunization with peptides and peptide-conjugates and observed that peptide vaccines delivered by tattooing consistently elicited higher specific both cellular and humoral immune responses than s.c. needle injection. Mostly, the co-administration of CpG motifs (ODN1826) markedly enhanced the immune responses.

The $E7_{49–57}$ epitope is considered to be the immunodominant H-2^b CTL epitope of the HPV16 E7 protein [25,19]. For vaccination purposes, the $E7_{49–57}$ peptide is usually administered with an adjuvant, e.g. FA [25–27]. We found that three s.c. immunizations with 100 μg of the $E7_{49–57}$ peptide or the longer $E7_{44–62}$ peptide in PBS solution in the absence of any adjuvant induced the E7-specific CTL response that was demonstrated in the tetramer assay (Fig. 2B) and also protected a portion of mice against the tumor formation after challenge with 3×10^4 TC-1 cells (Fig. 2E). Previously, two s.c. injections with 20 or 50 μg of the $E7_{49–57}$ peptide in PBS failed to induce CTL responses detectable in the chromium-release cytotoxicity test [28,29] and two doses of 20 μg of this peptide did not protect mice against development of tumors after injection of 5×10^5 TC-1 cells [28]. Moreover, two intraperitoneal injections of 100 μg of the $E7_{49–57}$ peptide were not able to protect mice against the challenge with 5×10^4 TC-1 cells and did not elicit the E7-specific CTL response detectable in the tetramer assay [30]. The higher dose and number of immunizations used for peptide vaccination and also lower dose of challenging TC-1-tumor cells administered in this work could explain the breaking through the detection limit.

Surprisingly, the addition of the CpG motifs to the s.c. injection of the $E7_{49–57}$ peptide substantially decreased induced CTL responses and protection against the challenge with TC-1 cells (Fig. 2A, B and E). We observed that addition of PBS to the water-dissolved $E7_{49–57}$ peptide caused mild precipitation that was further increased after the addition of CpG motifs, which could influence the presentation of the $E7_{49–57}$ peptide by antigen-presenting cells (APC) and result in the observed decrease in cellular immune responses [31]. Similarly, the KLH-conjugated $E7_{49–57}$ peptide was fully aggregated in PBS buffer in both the presence and absence of CpG motifs, which could contribute to poor immunogenicity induced after s.c. delivery of the KLH-conjugated $E7_{49–57}$ peptide (Fig. 4A). In contrast, the tattoo delivery of either the KLH-conjugated or non-conjugated $E7_{49–57}$ peptide in combination with CpG motifs induced a very high activation of E7-specific $CD8^+$ T cells (Figs. 2 and 4A). As the peptide aggregates can be solubilized by ultrasound [32], the tattoo procedure might also mechanically disrupt the aggregates of the peptide and the CpG motifs and thus result in their higher accessibility for APC uptake. Alternatively, different APCs presenting peptides after s.c. and tattoo delivery might have different ability to present a precipitated peptide. The experiment comparing the immunization effects of the aggregated and solubilized $E7_{49–57}$ peptide was not performed because we did not find convenient conditions (including sonication of the solution, utilization of a physiological solution, and decreasing a dose of the peptide and CpG motifs) for stable solubilization of peptide and CpG motifs at concentrations inducing immune responses above detection limits. Precipitation was not visible in any immunization solution of the $E7_{44–62}$ peptide, $E6_{48–57}$ peptide or KLH-conjugated $L2_{18–38}$ and $L2_{108–120}$ peptides.

The addition of the CpG motifs in the immunization with the $E7_{44–62}$ peptide and KLH-conjugated $E7_{49–57}$, $L2_{18–38}$ and $L2_{108–120}$ peptides enhanced humoral and/or cell-mediated immune responses both after the tattoo administration and s.c. injection (Figs. 2, 4 and 5). In both peptide delivery methods, the adjuvant also modulated the type of immune response as it shifted E7-, L2- and KLH-specific antibody production from IgG1 to IgG2a antibodies (Figs. 2D and 5D–F).

The CpG motifs alone have been shown to activate both CTL and NK cells and the treatment by intratumoral or s.c. injections of CpG motifs has reduced the growth of TC-1-induced tumors [33,34]. However, we did not observe any protection against TC-1 challenge or non-specific activation of the immune system in mice tattooed with CpG motifs in a preventive immunization setting (Fig. 2), which is in concordance with the published results showing that preventive administration of CpG motifs (ODN1826) has no obvious antitumor effects [35].

To evaluate the potency of tattooing for the delivery of peptide vaccines, we also compared this immunization strategy with highly efficient DNA vaccination against the E7 oncoprotein, the E7GGG.GUS fusion gene administered intradermally by the gene gun [16]. We found that three tattoo immunizations with 100 μg of the $E7_{49–57}$ peptide in mixture with 50 μg of CpG motifs stimulated immunity comparably to three immunizations with 1 μg of pBSC/E7GGG.GUS plasmid delivered by the gene gun (Fig. 1).

The H-2^b $E6_{50–57}$ minimal CTL epitope has been shown to bind MHC class I K^b molecules and also to be naturally presented by E6-expressing TC-1 cells [18], but two immunizations with 150 μg of this peptide and 20 μg of saponin Quil A into both hind foot pads were not able to prime mice *in vivo* [36,37]. In this work, we used the longer $E6_{48–57}$ peptide described previously to be optimal for

activation of the E6-specific $CD8^+$ T cells [18]. Three immunizations with 100 μg of the $E6_{48–57}$ peptide combined with the 50 μg of CpG motifs inhibited the growth of TC-1 tumor cells in mice, but only the tattoo delivery induced detectable levels of the E6-specific CTLs, while subcutaneous injection of the identical vaccine failed to prime the E6-specific CTLs (Fig. 3). For efficient induction of CTL responses in H-2^b mice by the E6 peptide, some improvements of the E6 vaccine have been achieved, e.g., by the D51I mutation of the E6 epitope leading to the higher stability of the peptide [37]. The tattoo delivery of peptides in combination with CpG motifs described in this work is another improvement.

The $E7_{44–62}$ peptide alone injected in PBS induced E7-specific antibodies (Fig. 2C) but both the $L2_{18–38}$ and the $L2_{108–120}$ peptides needed to be conjugated with KLH to elicit detectable humoral response (data not shown). This can be explained by the need for T-cell help in the induction of efficient antibody production. While the $E7_{44–62}$ peptide contains both the B-cell and Th-cell epitope [27,38], the $L2_{18–38}$ and $L2_{108–120}$ peptides lack a Th-cell epitope and so activation of Th lymphocytes is probably mediated only by the conjugated KLH protein [39–41]. The induction of the L2-specific antibodies after s.c. injection of the KLH-conjugated $L2_{108–120}$ peptide observed in this work is in concordance with the previous findings of Slupetzky et al. [40]. Antibodies against the $L2_{18–38}$ peptide have been previously elicited in rabbits [39]. Comparable levels of L2-specific antibodies were elicited by tattooing with the KLH-coupled L2 peptides and s.c. injection of the vaccines in the mixture with CpG motifs or FA (Fig. 5A and B), which indicates that adjuvanting effects of tattoo delivery could support humoral responses. Similarly, tattoo delivery of the $E7_{44–62}$ peptide with or without CpG motifs induced more E7-specific antibodies than s.c. injection of the identical vaccines (Fig. 2C), suggesting that multiple puncturing of the skin was advantageous for the induction of immune responses. Surprisingly, tattoo delivery of the KLH-conjugated $E7_{49–57}$ peptide induced lower titres of KLH-specific antibodies than s.c. injection (Fig. 4B). We suggest that the observed effect might be caused by aggregation of the KLH-conjugated $E7_{49–57}$ peptide which could have been less detrimental to the induction of humoral responses after s.c. delivery.

Tattooing with peptides in combination with CpG motifs might be a method of choice for the induction of peptide-specific immune responses. The utility of tattoo delivery is above all for vaccination with peptides of low immunogenicity or with a tendency to aggregate. Moreover, a major advantage of tattooing lies in the possible elimination of adjuvants that are necessary for vaccination by s.c. needle injection and may cause severe adverse effects. Furtheremore, peptide tattooing allows the replacement of the less safe DNA vaccines. Tattooing is well tolerated by animals and thus could be applied in laboratory conditions or veterinary practices. However, the usage of tattooing for vaccination of humans is less acceptable since it is associated with local damage of skin and a longer and painful vaccination procedure in comparison with single needle injection. Nevertheless, tattooing might be a method of choice for the treatment of severe diseases such as cancer.

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3.4 Paper IV:

DNA vaccine against human papillomavirus type 16: Modifications of the E6 oncogene

Ingrid Poláková, Dana Pokorná, Martina Dušková, Michal Šmahel

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Contribution of the author of this PhD. thesis:

I carried out almost all experimental work, from plasmid design, through *in vitro* and *in vivo* assays to immunisation experiments and evaluation of the results. The experimental work and the preparation of the manuscript were realized under the supervision and coordination of Michal Šmahel.

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DNA vaccine against human papillomavirus type 16: Modifications of the E6 oncogene

Ingrid Poláková*, Dana Pokorná, Martina Dušková, Michal Šmahel

Institute of Hematology and Blood Transfusion, Department of Experimental Virology, U Nemocnice 1, 12820 Prague 2, Czech Republic

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ABSTRACT

Since its discovery, DNA vaccination has become an effective strategy for the development of vaccines against cancer including cervical carcinoma (CC). The formation of CC is associated with human papillomavirus (HPV) infection. Viral E6 and E7 oncoproteins are suitable targets for therapeutic vaccination. To adapt the HPV16 E6 oncogene for DNA immunisation, we performed several modifications. First we fused the E6 gene with the 5′ or 3′-terminus of the *Escherichia coli* β-glucuronidase (GUS) gene and showed enhanced immunogenicity of the 3′ fusion (GUS.E6). Then, as the E6 oncogene contains two alternative introns that result in the production of truncated forms of the E6 protein, we abolished the 5′ splice site in the E6 gene. This modification completely eliminated the expression of the truncated E6 transcripts and thus increased the production of the full-length E6 protein. At the same time, it moderately reduced the immunogenicity of the modified non-fused (E6cc) or fused (GUS.E6cc) genes, probably as a consequence of the substitution in the immunodominant E6 epitope following the abolishment of the splice site. Furthermore, we reduced the oncogenicity of the E6 protein by two point mutations (E6GT) that, together, prevented E6-mediated p53 degradation. Finally, we constructed the GUS.E6GT gene characterized by enhanced safety and immunogenicity when compared with the wild-type E6 gene.

1. Introduction

Persistent infection with human papillomaviruses (HPV) is the main etiological factor in cervical cancer, the second most common cancer in women worldwide. Oncogenic high-risk (HR) HPV genotypes 16 and 18 are responsible for approximately 70% of all cervical cancers. Recently, two prophylactic vaccines based on virus-like particles (VLPs) produced by recombinant technology and protecting against infection with HPV16 and HPV18 have been licensed [1]. However, the development of therapeutic vaccines is still a topical problem, as preventive vaccination is of limited use and cannot cope with current HPV infection [2]. Since the viral oncoproteins E6 and E7 that are constitutively produced in all HPV-infected cells and that contribute to the transformation of epithelial skin or mucosal cells are also necessary for the maintenance of the transformed state [3], they became promising targets for the development of the therapeutic HPV vaccines.

DNA vaccines represent a potential form of antigen-specific immunotherapy of tumours because they can induce cytotoxic T-lymphocyte (CTL) response [4]. However, the low efficacy of DNA immunisation hampered its clinical use. Several strategies enhancing immunogenicity of the DNA vaccines have been developed including the modification of an antigen-encoding gene [5]. For clinical use of DNA vaccines, their safety must also be carefully considered. In our previous studies, we focused on the modification of the HPV16 E7 oncogene. To reduce its transformation potential, we altered it by point mutations resulting in the substitution of three amino acids in the pRb-binding site of the E7 protein [6]. Furthermore, to enhance its immunogenicity, we fused the modified E7GGG gene with sequences encoding sorting signals of lysosome-associated membrane protein 1 (LAMP-1), *Escherichia coli* β-glucuronidase (GUS) or mouse heat shock protein 70 (Hsp70) and demonstrated a superior antitumour effect of the E7GGG.GUS chimeric construct [7–9]. As the E7 oncoprotein is a relatively small protein (98 amino acids) with a limited number of epitopes [10] and immunity against the E6 oncoprotein (158 amino acids) is more readily induced in HPV16-infected people and is probably more important for the elimination of infected cells [11,12], E6 should also be included in the therapeutic HPV vaccines.

The HPV16 E6 oncoprotein is a multifunctional protein with several cellular targets. The first identified target, and apparently the most relevant, is the p53 tumour suppressor protein that can promote cell cycle arrest or apoptosis in infected cells. To overcome this obstacle, the E6 protein abrogates the functions of the p53 protein by inducing its degradation through the ubiquitin-proteasome pathway. The cellular E6AP ubiquitin ligase that binds both E6 and p53 plays a critical role in this process. Furthermore, the E6 oncoprotein can inhibit p53 activity independently of inducing

* Corresponding author. Tel.: +420 221 977 302; fax: +420 221 977 392.
E-mail address: ingrid.polakova@uhkt.cz (I. Poláková).

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its degradation [13]. Mutations that affect E6 binding with p53 or α-helix partners, including E6AP, have been shown to reduce E6 oncogenicity in *in vivo* assays [14–16].

In the HPV16 E6 open reading frame (ORF), one 5′ (donor) splice site and two 3′ splice (acceptor) sites have been identified. Alternative splicing results in the production of E6*I and E6*II transcripts from which truncated E6*I and E6*II proteins are transcribed. The E6*I transcript is the most abundant E6 mRNA in cervical cancer, premalignant lesions and cancer-derived cell lines [17–19].

In this study, we modified the E6 oncogene by fusion with GUS and by mutagenesis aiming at abolition of alternative splicing in the E6 gene or at reduction of the ability of the E6 protein to induce p53 degradation. The immunogenicity of the constructs was evaluated in mice after DNA immunisation by a gene gun.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (H-2^b; Charles River, Germany), 6–8-week-old, were used in the immunisation experiments. All animals were maintained under standard conditions and in accordance with the guidelines for the proper treatment of laboratory animals at the Center for Experimental Biomodels, Charles University, Prague.

2.2. Cell lines

Human embryonic kidney 293T cells transducted with simian virus 40 (SV40) large T antigen [20] were supplied by courtesy of J. A. Kleinschmidt, DKFZ, Heidelberg, Germany. TC-1 cells (kindly provided by T.-C. Wu, John Hopkins University, Baltimore, MD) were prepared by transformation of C57BL/6 mouse primary lung cells with the HPV16 E6/E7 oncogenes and human activated *H-ras* [21]. Saos-2 cells (obtained from DSMZ, Braunschweig, Germany) were established from primary human osteogenic sarcoma [22]. All cells were grown in Dulbecco's modified Eagle's medium (D-MEM; PAA Laboratories, Linz, Austria) supplemented with 10% foetal calf serum (FCS; PAA Laboratories), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

2.3. Plasmids

The construction of the plasmids pBSC and pBSC/E7GGG.GUS was described previously [6,8]. The plasmid pEA16E6 (kindly provided by F. Momburg, DKFZ, Heidelberg, Germany) contains the HPV16 E6 oncogene that was excised with *Eco*RI and *Bam*HI and cloned into pBK-CMV (Stratagene, La Jolla, CA) between the *Eco*RI and the *Bam*HI sites generating the pBK/E6 plasmid. The plasmid pBSC/E6 was constructed by excision of the E6 gene from pBK/E6 with *Sal*I and *Xho*I and its ligation into the *Xho*I restriction site of the pBSC plasmid. The plasmid pBSC/GUS [8] was modified by mutagenesis to generate the pBSC/GUS-STOP vector with the abolished termination codon of GUS and the *Hind*III site added [23].

For the generation of the pBSC/E6.GUS plasmid, the E6 gene was amplified from pBK/E6 using primers 5′-CTGACCCGGGGCCG-CCATGCACCAAAAGAGAACTG-3′ (forward) and 5′-CTAGCCCGGG-CCCAGCTGGGTTTCTCTACGT-3′ (reverse). The PCR product was digested with the *Xma*I restriction enzyme (underlined sequences in primers) and cloned into pBSC/E7GGG.GUS between the *Xma*I sites thus replacing E7GGG. To construct pBSC/GUS.E6 plasmid, the DNA fragment encoding E6 was amplified from pBSC/E6 using primers 5′-CACAAGCTTTGATGCACCAAAAGAGAACTGC-3′ (forward) and 5′-CACAAGCTTTTACAGCTGGGTTTCTCTACG-3′ (reverse), digested with *Hind*III (underlined sequences in primers) and ligated into the *Hind*III site of the pBSC/GUS-STOP plasmid.

The pBSC/EGUS plasmid with an eliminated initiation codon of the GUS gene was prepared previously [23]. The pBSC/E6.EGUS plasmid was constructed as follows: the E6 gene was excised from the pBSC/E6.GUS plasmid with the *Xma*I restriction enzyme and subsequently ligated into the *Xma*I site of pBSC/EGUS.

The E6 gene was mutated with the Altered Sites® II Mammalian Mutagenesis System (Promega, Madison, WI). The 5′ splicing site was abolished by two point mutations introduced with the oligonucleotide 5′-AAGTCATATAGCTCGCGTCGCAGTA-3′ (underlined nucleotides represent the mutations) resulting in the E6cc gene (Fig. 1). This mutation was also introduced into the E6.GUS fusion gene.

Substitutions C70G and/or I135T were created using the oligonucleotides 5′-CATTTATCACCTACAGCATA-3′ and 5′-CCGACCCCTTGTATTATGGA-3′, respectively. The resultant genes with one or both mutations were designated E6G, E6T and E6GT (Fig. 1).

The mutated genes were cloned between the *Eco*RI sites of the pBSC plasmid. The modified GUS.E6 fusion genes were prepared by the amplification of the mutated E6 genes from the pBSC/E6cc and pBSC/E6GT plasmids and their subsequent introduction into the *Hind*III site of pBSC/GUS-STOP.

The accuracy of all modified genes was confirmed by DNA sequencing.

The p53 expressing plasmid, pcDNA3.1wtp53, was kindly provided by M. Brazdova (Institute of Biophysics, Brno, Czech Republic). pTR-UF2 is a green fluorescent protein (GFP) expression plasmid [24].

The plasmids were propagated in *E. coli* XL1-blue strain cultured in Luria Broth Medium with 100 μg/ml of ampicillin added and purified with the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany).

2.4. Reverse transcription PCR

293T cells (7×10^5) were grown on 6-cm plates and transfected by modified calcium phosphate precipitation in HEPES-buffered saline solution [25] with 6 μg of plasmids. Total RNA was extracted from transfected cells after 2 days of incubation by the RNeasy Kit (Qiagen) following the manufacturer's instructions. Two μg of RNA were incubated with 2 U of RNase-Free DNase (Promega) in a 20-μl reaction volume containing 1× reaction buffer (Promega) at 37 °C for 30 min. DNase was stopped with 2 μl of DNase stop solution (10 min at 65 °C; Promega). Reverse transcription was performed with 11 μl of DNase-treated RNA in a 20-μl reaction volume containing 1.25 μM oligo(dT) primer, 0.5 mM of each dNTP, 1× reaction buffer (Promega) and 200 U of M-MLV reverse transcriptase (Promega). PCR amplification of E6 cDNA was performed with HotStarTaq DNA polymerase (Qiagen) and Q-solution (Qiagen) using E6-derived primers 5′-GCAAGCAACAGTTACTGCGA-3′ (forward) and 5′-GCTGGGTTTCTCTACGTGTT-3′ (reverse). The lengths of the expected products were 357, 174, and 57 bp. The reaction was performed in a PTC-200 thermal cycler (MJ Research, Watertown, MA): activation of the polymerase at 95 °C for 15 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min; incubation at 72 °C for 5 min. Amplification of β-actin served as an internal control [7]. The amplified products were separated in a 3% agarose gel, stained with ethidium bromide, photographed under UV light and analysed with ScanPack 3.0 software (Biometra, Göttingen, Germany) for quantification of the PCR products.

2.5. Immunoblotting staining of E6-containing proteins

293T cells were transfected with 6 μg of the appropriate plasmids and after 2 days, the cells were collected and lysed on

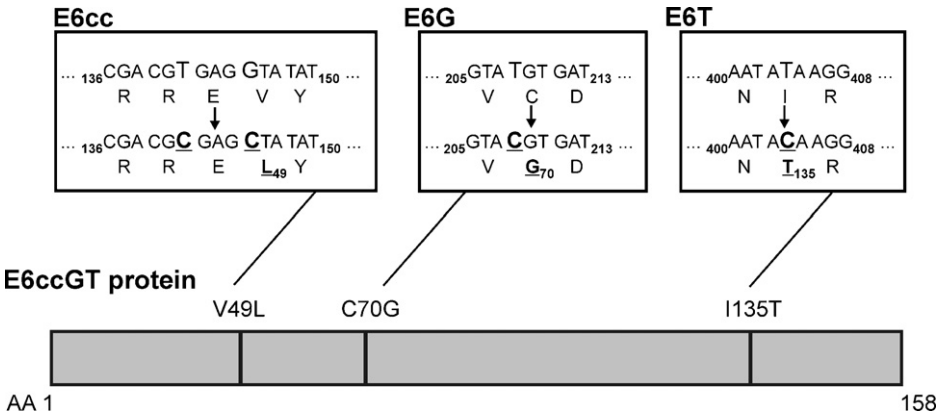

Fig. 1. Mutagenesis of the HPV16 E6 oncogene. The modified positions are highlighted.

ice in GUS buffer (50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 10 nM β-mercaptoethanol, 0.1% Triton X-100). The precipitate was removed by centrifugation. Proteins from cell lysates were separated by 7 or 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Little Chalfont, UK). The membrane was blocked with 10% non-fat milk in PBS and incubated with mouse monoclonal anti-E6 antibody (kindly provided by L. Gissmann, DKFZ, Heidelberg, Germany) or rabbit polyclonal anti-GUS antibodies (Molecular Probes, Eugene, OR) and subsequently with horseradish-peroxidase-conjugated secondary antibodies of the appropriate specificity (GE Healthcare). The blots were stained using the ECL Plus system (GE Healthcare). For verification of protein loading, the immunoblots were reprobed with mouse monoclonal anti-β-tubulin antibody (Sigma–Aldrich, Steinheim, Germany). Subsequently, the blots with protein bands were scanned and densitometry was performed using ScanPack 3.0 software.

2.6. In vivo p53 degradation assay

Saos-2 p53-null cells (7×10^5) were cotransfected with the GFP and wild-type (wt) p53 expression plasmids at a ratio of 1:6 and with the wt or mutated E6 protein expressing plasmids. The ratio of p53 to E6 plasmids was 20:1. The cells were harvested after 48 h and lysed in modified RIPA buffer (20 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1% NP-40 or IGEPAL, 1% deoxycholate and 0.1% SDS) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma, Saint Louis, MO). The protein concentration in the lysates was determined by Bradford assay and 15 μg of proteins were separated in a 10% gel by SDS-PAGE. Proteins were transferred onto a PVDF membrane and incubated with p53-specific monoclonal antibody DO-1 (kindly provided by B. Vojtesek, Masaryk Memorial Cancer Institute, Brno, Czech Republic) or with mouse anti-GFP mAb mixture (Roche, Basel, Switzerland). Proteins were visualised with the ECL Plus system utilising anti-mouse IgG horseradish-peroxidase-conjugated secondary antibodies.

2.7. Preparation of cartridges for the gene gun

Plasmid DNA was coated onto 1-μm gold particles (Bio-Rad, Hercules, CA) by the procedure recommended by the producer. Each cartridge contained 1 μg DNA coated onto 0.5 mg of gold particles.

2.8. Immunisation experiments

Mice were immunised with three 2 μg doses of plasmid DNA at 1-week intervals by a gene gun (g.g., Bio-Rad). Vaccines were delivered into the shaven skin of the abdomen at a discharge pressure of 400 psi. One week after the last vaccination, the animals (5 or 8 per group) were challenged s.c. into the back with 3×10^4 TC-1 tumour cells suspended in 150 μl of PBS and/or pools of lymphocytes were isolated from splenocytes (three mice per group) using Histopaque-1077 (Sigma) and analysed by an ELISPOT assay or intracellular cytokine staining. The tumour growth in the challenged animals was monitored twice a week and the tumour size was calculated from three perpendicular measurements using the formula $(\pi/6)(a \times b \times c)$.

2.9. ELISPOT assay

MultiScreen 96-well filtration plates (Millipore, Molsheim, France) were coated with 10 μg/ml of rat anti-mouse IFN-γ antibody (BD Biosciences Pharmingen, San Diego, CA) in 50 μl of PBS and incubated overnight at 4 °C. Plates were washed and blocked with an RPMI-1640 medium supplemented with 10% FCS. Lymphocytes were added to the plate (10^6/well) and incubated at 37 °C in 5% CO_2 for 20 h either with or without 3 μg/ml of the $E6_{48–57}$ peptide (EVYDFAFRDL) carrying an H-$2K^b$ cytotoxic T-lymphocyte (CTL) epitope [26]. The cells were removed by two washes with deionized H_2O and three washes with PBS–0.05% Tween 20, each washing taking 5 min. Then, 4 μg/ml of biotinylated rat anti-mouse IFN-γ antibody (BD Biosciences Pharmingen) in 50 μl PBS were added per well and cultivated at 4 °C overnight. The wells were washed three times with PBS–0.05% Tween 20 for 2 min and incubated for 1 h with 50 μl of 1:100 dilution of streptavidin-horseradish peroxidase (BD Biosciences Pharmingen) in PBS at 37 °C in 5% CO_2. After washing four times with PBS–0.05% Tween 20 for 2 min, followed by two washing steps with PBS alone, the spots were developed by adding 50 μl of an AEC chromogen and AEC substrate mixture (AEC Substrate Set, BD Biosciences Pharmingen) and incubation at room temperature for 5–15 min. They were counted by an Eli.Scan ELISPOT Scanner (A.EL.VIS, Hannover, Germany; software version 4.1).

2.10. Intracellular cytokine staining (ICS)

Lymphocytes were cultivated for 20 h with the $E6_{48–57}$ peptide as for an ELISPOT assay. The staining of IFN-γ in $CD8^+$ cells was performed as described previously [23]. The stained cells were measured on a Coulter Epics XL flow cytometer (Coulter, Miami, FL) and analysed by FlowJo 7.2.2 software (TreeStar, Ashland, OR).

2.11. Statistical analysis

Tumour growth after DNA immunisation was evaluated by two-way analysis of variance. A difference between groups was

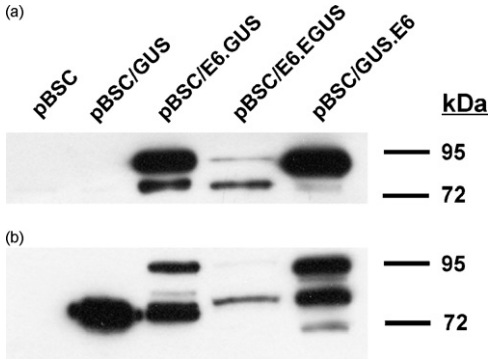

Fig. 2. Immunoblotting detection of GUS fusion proteins. Protein lysates from transfected 293T cells were analysed by 7% SDS-PAGE. Separated proteins were electroblotted onto a PVDF membrane and stained with anti-E6 monoclonal antibody (a) or rabbit anti-GUS antibody (b).

considered significant if $P < 0.05$. Calculations were performed using Prism software, version 5.0 (Graph-Pad Software, San Diego, CA).

3. Results

3.1. Fusion with the 3′ terminus of GUS enhanced the immunogenicity of anti-E6 DNA vaccines

In our previous studies, we found that the fusion of the modified HPV16 E7 gene (E7GGG) with the GUS gene markedly enhanced E7-specific immune reactions induced by DNA immunisation [8,23]. Therefore, we fused the E6 gene with the 5′ or 3′ terminus of GUS and thus prepared the E6.GUS and GUS.E6 constructs as described under Section 2. The expression of the fusion proteins was verified after transfection of 293T cells by immunoblotting analyses with E6- and GUS-specific antibodies (Fig. 2a and b). After the detection of the E6.GUS fusion protein with anti-GUS polyclonal antibody, we found a dominant band corresponding in size to the GUS protein. This band was eliminated after abolition of the GUS initiation codon by a mutation described recently [23]. However, as this mutation resulted in the substitution of methionine by glutamic acid, the stability of the mutated E6.EGUS protein, according to the previous results with E7GGG.EGUS [23], was supposedly substantially decreased and the upper band, corresponding to the full-length E6.EGUS, was visible only after longer exposition than that shown in Fig. 2b. Other unidentified bands found following the protein detection are supposed to be the products of expression from different initiation codons or the products of alternative splicing (see below) or degradation.

We examined the immunogenicity of the GUS fusion genes after immunisation of C57BL/6 mice with plasmid DNA by a gene gun. The E6-specific cell-mediated immunity was tested by an ICS assay (Fig. 3a) and an ELISPOT assay (data not shown) detecting IFN-γ-producing lymphocytes after incubation with the H-2K^b $E6_{48–57}$ epitope. The immunogenicity of the E6 fusion with the 5′ terminus of GUS (E6.GUS) was comparable with that of the E6 gene alone, but the fusion with the 3′ terminus (GUS.E6) induced 3–4 times higher immune response. Accordingly, after challenging the animals with TC-1 tumour cells, immunisation with GUS.E6 resulted in a significantly slower tumour growth ($P < 0.001$) compared with that in pBSC control group (Fig. 3b). The elimination of the GUS initiation codon in the E6.EGUS construct had a weak effect on the immunogenicity. In summary, the fusion of the E6 gene with the 3′ terminus of GUS led to an enhanced E6-specific immune response and a slower tumour growth.

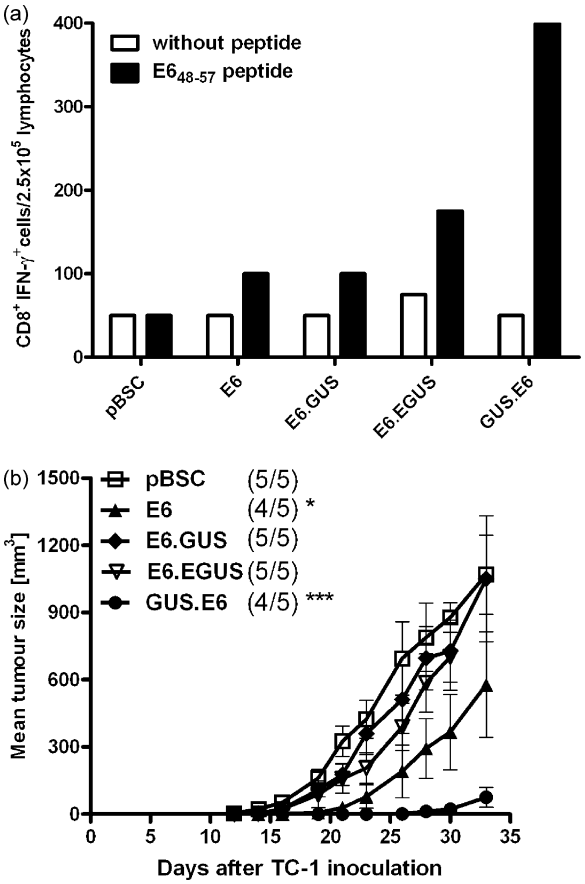

Fig. 3. Immunogenicity of GUS fusion genes. (a) Mice ($n = 3$) were immunised three times at a 1-week intervals by a gene gun with 2 μg of plasmids and one week after the third immunisation, pooled lymphocyte cultures were prepared from splenocytes. Lymphocytes producing IFN-γ were detected after overnight incubation with the $E6_{48-57}$ peptide by an ICS assay. (b) Mice ($n = 5$) were immunised with three 2 μg doses of plasmid DNA by a gene gun and one week after the last immunisation were challenged s.c. with 3×10^4 TC-1 tumour cells. No. of mice with a tumour/no. of mice in the group is indicated. Asterisks, statistical significance ($^*P < 0.05$, $^{***}P < 0.001$) of the mean tumour size in comparison with the pBSC negative control.

3.2. Abolition of 5′ the splice site eliminated the expression of truncated E6 transcripts but did not enhance the immunogenicity of DNA vaccines

The alternative splicing in the HPV16 E6 oncogene results in the production of truncated forms of the E6 protein. Moreover, the most abundant transcript is not mRNA for the full-length E6 but the truncated E6*I transcript. To increase the production of the full-length E6 protein and subsequently the immunogenicity of DNA vaccines, we abolished the 5′ splice site in the E6 gene. The site-directed mutagenesis substituting two nucleotides resulted in the E6cc gene, from which a protein with the V49L mutation is produced (Fig. 1). The RT-PCR amplification of the E6 transcripts from 293T cells transfected with pBSC-derived plasmids demonstrated that the abolition of the 5′ splice site in the E6 gene completely eliminated the expression of the truncated E6*I and E6*II transcripts. Alternative splicing was also abolished in the modified E6cc.GUS and GUS.E6cc fusion genes. Moreover, the expression of the full-length mRNA was profoundly enhanced (Fig. 4).

Densitometric analysis of the amplified transcripts of the unmodified genes revealed that the fusion of the E6 gene with GUS increased the production of the E6*II transcript at the expense of the E6*I mRNA expression (Table 1). However, the most importantly, we found a markedly increased level of the full-length E6 transcript generated in the cells transfected with the GUS.E6 gene.

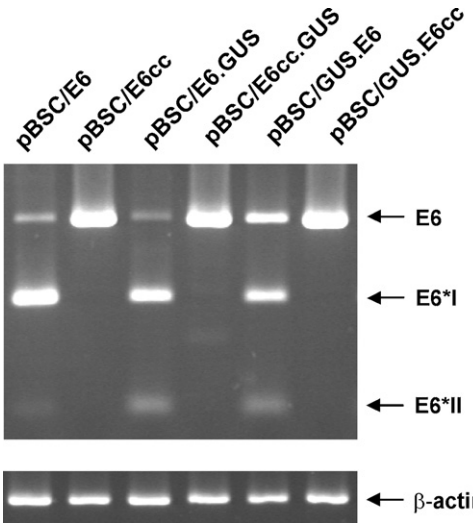

Fig. 4. Detection of E6 mRNA by RT-PCR. Total RNA was isolated from transfected 293T cells and treated with DNase I. Then RNA was utilised for cDNA synthesis and PCR. Amplification of β-actin served as an internal control.

Table 1
The quantification of E6 mRNA.

Plasmid	E6	E6*I	E6*II	E6*I+E6*II	E6/(E6*I+E6*II)
pBSC/E6	0.34	1.52	0.15	1.67	0.20
pBSC/E6.GUS	0.24	1.25	0.43	1.68	0.14
pBSC/GUS.E6	1.21	1.27	0.42	1.69	0.72

The quantification of the PCR products was preformed in the gel shown in Fig. 4 using ScanPack 3.0 software. Intensity values were normalised to β-actin expression.

The production of the E6 proteins expressed from the genes modified by elimination of the splice site was tested by immunoblotting staining after transfection of 293T cells. The genes without this modification were used for comparison. Immunoblotting staining with E6- and GUS-specific antibodies confirmed the production of the expected proteins (Fig. 5). A considerably enhanced E6 protein production was detected with anti-E6 monoclonal antibody after transfection of the cells with the plasmid expressing the modified E6 protein alone (pBSC/E6cc; Fig. 5a). However, this effect was not recorded for the GUS fusion genes (Fig. 5b). The difference between the cells transfected with the genes GUS.E6 and GUS.E6cc suggests that the middle band of the GUS.E6 sample detected with anti-GUS antibodies represents the product of the alternative splicing (Fig. 5c). Densitometric analysis of the full-length fusion proteins detected with anti-E6 antibody indicated a higher production of proteins after the fusion of the E6 gene with the 3′ terminus of GUS (Table 2).

Examination of the immunogenicity of the genes with the abolished E6 splicing site by an *ex vivo* ELISPOT assay showed that this modification moderately decreased the number of IFN-γ positive cells when compared with the unmodified genes (Fig. 6a). The

Table 2
The quantification of GUS-fused E6 proteins.

Plasmid	Intensity values		Ratio E6 + GUS/ tubulin
	E6 + GUS	Tubulin	
pBSC/E6.GUS	1963	1991	0.99
pBSC/E6cc.GUS	1594	1886	0.85
pBSC/GUS.E6	2820	1887	1.49
pBSC/GUS.E6cc	3221	1847	1.74

The quantification of the proteins was preformed with the membrane shown in Fig. 5b using ScanPack 3.0 software.

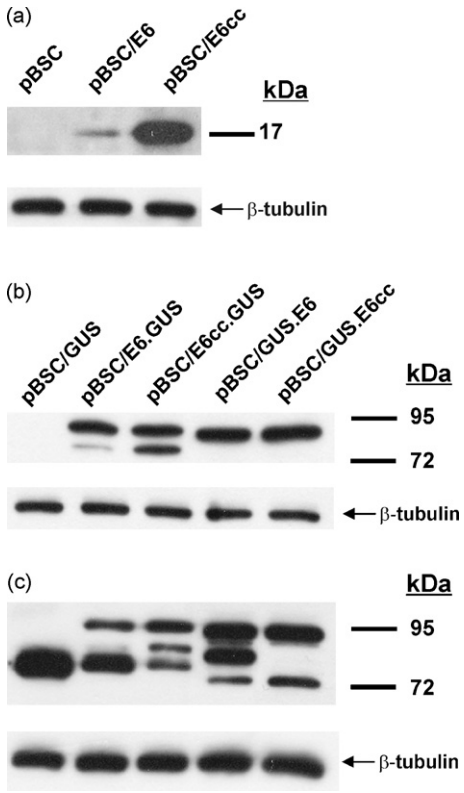

Fig. 5. Immunoblotting analysis of proteins expressed from the genes with the abolished 5′ splice site. 293T cells were transfected and lysed in GUS buffer 2 days thereafter. (a) Proteins were separated in a 12% gel, transferred onto a PVFD membrane and detected with anti-E6 monoclonal antibody. (b, c) Protein lysates were analysed by 7% SDS-PAGE, electroblotted onto a PVDF membrane and detected with anti-E6 monoclonal antibody (b) or anti-GUS polyclonal antibody (c). The lower panels (a, b, and c) show reprobing of the membranes with anti-β-tubulin antibody as a protein loading control.

reduced immunogenicity of the modified fusion genes was confirmed after challenging the immunised mice with TC-1 tumour cells (results non-significant; Fig. 6b). In conclusion, the abolition of the E6 splicing site did not enhance the immunogenicity of the fusion genes but rather slightly decreased it.

3.3. *Modifications reducing E6-mediated p53 degradation slightly decreased the immunogenicity of the 3′ GUS fusion gene*

The oncogenicity of the E6 protein consists in several close-knit interactions; however the key role is ascribed to its induction of p53 degradation. In an effort to decrease the oncogenicity of E6 and thus enhance the safety of the DNA vaccine, we altered the E6 protein with two substitutions reported to reduce E6-mediated p53 degradation [14,16]. The mutations C70G or/and I135T were introduced as described under Section 2 and the resulting genes were designated E6G, E6T, and E6GT (Fig. 1). The ability of these constructs to induce degradation of p53 was tested by an *in vivo* p53 degradation assay after cotransfection of p53-null cells with plasmids encoding p53 and the mutated E6 gene (Fig. 7). The transfection efficiency was monitored by cotransfection with a GFP expression plasmid. The altered E6 proteins with the single mutations (E6G or E6T) were still able to induce the p53 degradation like the wt E6 protein. However, the introduction of the double mutation into the E6 protein (E6GT) led to the loss of this ability. Therefore, we fused the E6GT gene with the 3′ terminus of GUS and verified the expression of the fusion gene by immunoblotting staining. The steady-state level of the GUS.E6GT protein produced by 293T cells was similar to that of GUS.E6 (data not shown).

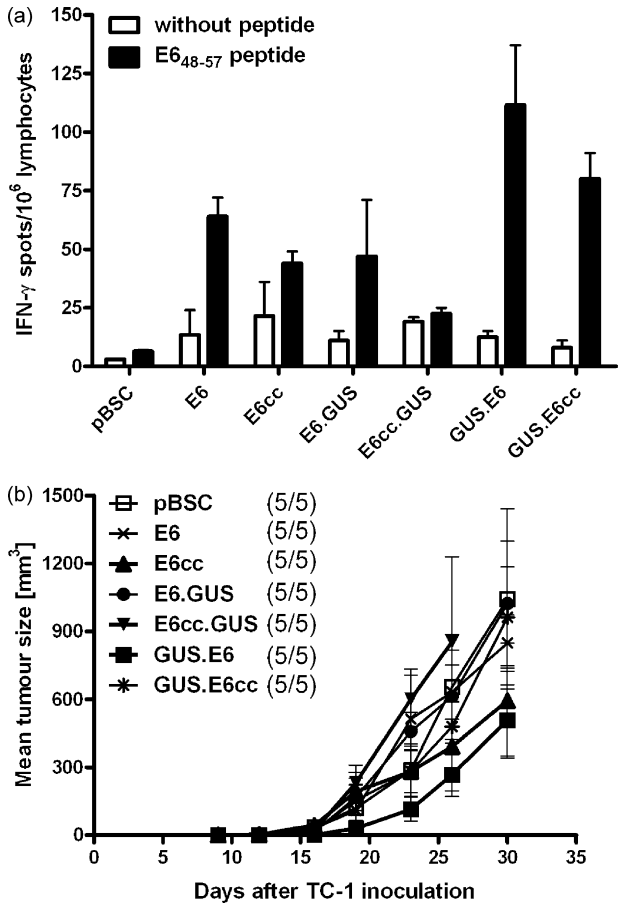

Fig. 6. Immunogenicity of the genes with the abolished E6 splicing site. Mice ($n = 8$) were immunised at 1-week intervals by a gene gun with three 2 μg doses of DNA. (a) Pooled lymphocytes isolated from the spleen of mice ($n = 3$) one week after the last immunisation were incubated overnight with the E6$_{48-57}$ peptide and IFN-γ-producing cells were detected by an ELISPOT assay. (b) Mice ($n = 5$) were challenged s.c. with 3×10^4 TC-1 tumour cells one week after the last immunisation and monitored for the tumour growth. No. of mice with a tumour/no. of mice in a group is indicated.

We compared the immunogenicity of the E6GT, GUS.E6GT and unmodified genes by an ICS assay. Vaccination with the mutated genes resulted in a slightly decreased level of E6-specific CD8$^+$ IFNγ$^+$ lymphocytes compared to the respective genes without the "GT" modification (Fig. 8a). Challenging the animals with TC-1 tumour cells confirmed the lower immunogenicity of the mutated genes (Fig. 8b). This effect was more evident for the E6GT gene. Similar to the GUS.E6 gene, tumour development in animals immunised with GUS.E6GT was significantly slower than in the pBSC control group ($P < 0.001$); however, vaccination with the GUS.E6 protected some mice from tumour formation. These findings show that the combination of the C70G and I135T mutations substantially inhibited the E6-induced p53 degradation, while only slightly reduced the immunogenicity of the GUS.E6 gene.

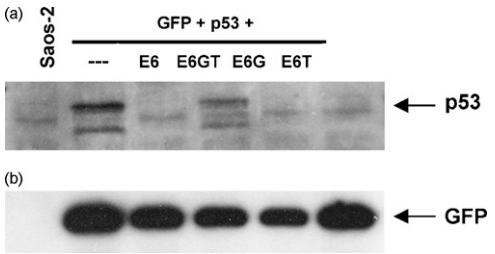

Fig. 7. *In vivo* p53 degradation assay. Saos-2 (p53-null) cells were cotransfected with plasmids expressing wt p53 and the wt or modified E6 protein at a ratio of 20:1. The cells were also cotransfected with the GFP expressing plasmid to monitor the transfection efficiency (the ratio of GFP to p53 plasmids was 1:6). After 48 h, the cells were harvested and the protein production was detected by immunoblotting staining with anti-p53 monoclonal antibody (a) or anti-GFP monoclonal antibodies mixture (b). Non-transfected and GFP + p53-transfected Saos-2 cells were used as a negative and positive controls, respectively.

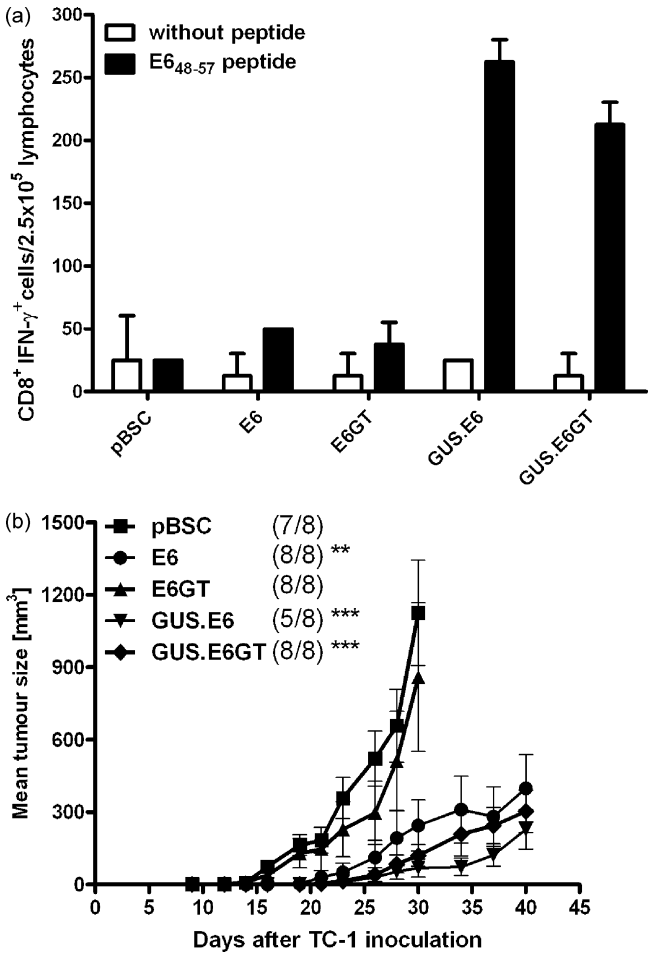

Fig. 8. Immunogenicity of E6GT and GUS.E6GT genes. Mice ($n = 11$) were immunised at 1-week intervals by a gene gun with three 2 μg doses of DNA. (a) Pooled lymphocytes isolated from the spleen of mice ($n = 3$) one week after the last immunisation were incubated overnight with the E6$_{48-57}$ peptide and IFN-γ producing cells were detected by ICS. (b) Mice ($n = 8$) were challenged s.c. with 3×10^4 TC-1 tumour cells one week after the last immunisation and monitored for the tumour growth. No. of mice with a tumour/no. of mice in the group is indicated. In the E6GT group, one animal with a tumour died 26 days after TC-1 inoculation. Asterisks, statistical significance (**$P < 0.01$, ***$P < 0.001$) of the mean tumour size in comparison with the pBSC negative control.

4. Discussion

We constructed several DNA vaccines against the HPV16 E6 protein with expectations of increased immunogenicity and decreased oncogenicity of E6. The prepared plasmid constructs encode the GUS-fused E6 gene with one or two mutational modifications to meet these demands.

We showed previously that the fusion of the modified E7 protein with the 5′ or 3′ terminus of GUS (E7GGG.GUS, GUS.E7GGG) substantially enhanced the efficacy of gene gun DNA immunisation, the 5′ fusion being slightly more potent [8,23]. The mechanism(s) responsible for this effect could be stabilisation of the E7 antigen, modification of its cellular localisation and/or helper epitopes

present in the GUS protein. Accordingly, we fused the E6 gene to both termini of GUS, but the 3′ fusion (GUS.E6) was markedly more immunogenic. The stronger immunogenicity of GUS.E6 could be caused by the more efficient production of the full-length E6 transcript that resulted in the higher production of the fusion protein.

Checking the sequence of the E6 gene and its surroundings in our plasmid DNA, we found that the translation from several potential initiation codons may lead to the production of proteins with different molecular weights visible after immunoblotting analyses. As the GUS protein alone was expressed at a high level after the fusion of the E6 gene to the 5′ terminus of GUS (E6.GUS), we abolished the GUS initiation codon in this construct and thus eliminated GUS production. However, similar to the corresponding mutagenesis in E7GGG.GUS [23], this modification presumably resulted in the E6.EGUS protein with low stability and with the immunogenicity comparable to that of E6.GUS.

The generation of E6*I and E6*II alternative spliced forms of mRNA that give rise to truncated E6 proteins has been reported for the high-risk E6 genes. The most abundant transcript in HPV16 infected cells is E6*I [18]. In order to increase the production of the full-length E6 protein, we abolished the 5′ splice site in the E6 gene by two point mutations ("cc" constructs) that completely eliminated the production of the E6*I and E6*II truncated mRNA. Nevertheless, the non-fused or fused proteins with this modification did not induce a stronger immune response, but on the contrary, the immunogenicity was moderately decreased. As the elimination of the splice site resulted in the substitution of valine by leucine at position 49 (V49L) included in the H-2K^b immunodominant epitope of E6 (aa 48–57) [26], we considered whether this alteration of the epitope could influence the immune response. Using the Bioinformatics & Molecular Analysis Section (BIMAS) for HLA peptide binding predictions (www-bimas.cit.nih.gov/molbio/hla_bind) and the SYFPEITHI database of MHC ligands and peptide motifs (www.syfpeithi.de), we compared the EVYDFAFRDL and ELYDFAFRDL epitope sequences and did not find any significant difference in predicted binding to H-2K^b molecules. Furthermore, it has been reported that the region aa 50–57 of E6, presented by H-2K^b, represents the minimal core sequence required for the activation of E6-specific CD8$^+$ T lymphocytes [26]. However, our comparison of immunisation with synthetic peptides delivered together with immunostimulatory CpG motifs (ODN 1826) by a tattoo device showed a markedly decreased immunogenicity of the peptide carrying the V49L mutation (data not shown). Therefore, we suppose that the elimination of the 5′ splice site which led to the V49L substitution in the E6 protein probably impaired the epitope immunodominant in C57BL/6 mice and thus decreased the immunogenicity of the DNA vaccines altered by this modification.

High-risk HPV E6 proteins in association with the E6AP cellular ubiquitin ligase play a significant role in p53 degradation, regulation of apoptosis and overall in cell transformation. Several mechanisms are known to be involved in the E6-mediated inhibition of p53 signalling, including those independent of p53 protein degradation due to site specific binding of the E6 protein to p53 resulting in masking the nuclear localisation signal on p53 or conformational change in p53 leading to inhibition of p53 binding to DNA and abrogation of the transactivation of p53 responsive genes [27]. We introduced two mutations (C70G, I135T) into the E6 protein to enhance the safety of DNA vaccines against E6. The C70G substitution was not able to induce p53 degradation *in vitro* at 37 °C and was defective for immortalization of mammary epithelial cells (MECs) *in vivo* [14]. The I135T mutant did not induce the degradation of p53 *in vivo* and its oncogenic potential was substantially reduced in transgenic mice [16,28]. Interestingly, our constructs with single mutations (C70G or I135T) were able to induce p53 degradation *in vivo*, while double mutation (C70G and I135T) eliminated this ability. Our findings correspond with the previous observations suggesting that the E6AP binding surface of E6 consists of residues from both the N- and C-termini of the E6 protein [29].

Recently the whole E6 protein structure was proposed from the results of nuclear magnetic resonance (NMR) analysis of the C-terminal half of the HPV16 E6 protein. The proposed structure indicates that many of the mutations performed to map the functions of E6 might have indirectly altered these as a result of structure destabilization [29]. Mutations performed in our laboratory (V49L, C70G, and I135T), chosen according to articles published prior to these new structural findings, are all located at the key buried positions of the N- or C-termini of the E6 protein and thus might deform the protein structure. Such conformational changes, induced particularly by the C70G and I135T mutations, might affect the antigen processing and presentation and thus moderate the immunogenicity of DNA vaccines.

In conclusion, we performed some modifications of the E6 oncogene to adapt it for DNA immunisation. Based on the results with the modified E7GGG gene, we fused the wt or modified E6 gene with the 5′ or 3′ terminus of GUS and demonstrated a superior immunogenicity of the GUS.E6 fusion gene. In order to increase the production of the full-length E6 protein, we abolished the 5′ splice site in the E6 gene, but this modification did not improve the efficacy of DNA vaccines. Finally, we reduced the oncogenic potential of E6 by two substitutions (C70G and I135T) that, together, profoundly reduced the degradation of the p53 protein, while only slightly diminishing the immunogenicity of the E6 protein.

Acknowledgments

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4. DISCUSSION

The submitted PhD. thesis represents a work focused on therapeutic DNA vaccines against HPV 16, especially on problems with low immunogenicity of the vaccines. The prepared DNA constructs were designed in an effort to induce strong immune responses targeted against the E7 or E6 oncoproteins. A gene gun delivery method, which belongs to the main administration methods of DNA vaccines, was chosen for immunisation. As immunoresistance was observed when immunisation against E7 was accomplished, a tumour escape mechanism was discovered and described. Moreover, immunodominant epitopes from the E6 and E7 proteins were employed in vaccination using a tattoo device.

4.1 Construction of plasmid DNA vaccines encoding fusion genes

The low immunogenicity belongs to important limitations of DNA vaccines. Their efficacy can be enhanced by using several strategies as described under Chapter *2.3.3 Enhancement of DNA vaccine potency*. The strategy of modification of the antigen was preferred in this work. However, this type of modification may lead to alterations in protein synthesis, protein stability and/or its cellular localisation (Manoj *et al.*, 2004).

Previously, in order to enhance the immunogenicity of the modified E7 protein (E7GGG), several plasmid DNA vaccines encoding E7 fusion genes were constructed in our laboratory. We fused the E7GGG gene with LAMP-1 (Smahel *et al.*, 2003), GUS (Smahel *et al.*, 2004) and Hsp70 (Pokorna *et al.*, 2005). Overall, the highest anti-tumour immune responses were recorded after immunisation with the GUS-fused constructs. Therefore, in the following experiments, the E7GGG.GUS was further modified to enhance even more its immunogenicity. *Paper II* demonstrates the performed modifications (see also below).

The steady-state level of antigen production that may be influenced by the sequence of the gene encoding the antigen, and the stabilisation of the antigen contribute to DNA vaccine efficacy (Manoj *et al.*, 2004; Bins *et al.*, 2007). Hence, to increase the steady-state level of resultant proteins expressed from plasmid DNA, the following modifications were performed with the E7GGG.GUS fusion gene: i) As the fusion of the full-length E7GGG with GUS evokes a decreased steady-state level of GUS antigen (Smahel *et al.*, 2004), portions of amino

acids from the C-terminus of the E7GGG were deleted (E7GGG41.GUS, E7GGG60.GUS and E7GGG75.GUS), ii) the GUS initiation codon was eliminated to abolish the production of GUS alone (E7GGG.EGUS), iii) E7GGG codon adjustment for enhanced expression in human cells was realized (hE7GGG.GUS), and iv) the E7GGG was fused also to the 3'-terminus of GUS (GUS.E7GGG) as the stability of the fusion proteins can be influenced by the position of fusion partners (Li *et al.*, 2006). The production of fusion proteins was compared *via* measuring the enzymatic activity of GUS and immunoblotting staining (*Paper II, Figures 2A, B and 4A, B*). The results showed that only the deletion mutants and the GUS.E7GGG had an increased antigen production when compared with E7GGG.GUS. Examination of protein stability by pulse-chase labelling followed by immunoprecipitation indicated that the stability of the proteins corresponded with their steady-state levels (*Paper II, Figure 7*).

Targeting the antigens to certain cellular location or compartment is another important factor of enhancing the DNA vaccine potency. However, it is dependent on the nature of the antigen. A signal sequence (SS) targets the antigen to the ER from where it may be retro-translocated to the cytosol for proteasome degradation (Bonifacino & Weissman, 1998; Golovina *et al.*, 2002) and subsequently, successful antigen presentation and a strong antigen-specific immune response may be induced (Leifert *et al.*, 2004). Fusion of the signal sequence from the adenoviral E3 gene to GUS.E7GGG led to the accumulation of the SS.GUS.E7GGG protein in the ER. The detection of GUS activity showed that the protein was not secreted from cells (*Paper II, Figures 4 and 5*).

According to the results achieved with the GUS-fused E7GGG protein, the E6 gene was joined to both termini of GUS (E6.GUS, GUS.E6; *Paper IV*). The expression of fusion proteins was verified by immunoblotting staining (*Paper IV, Figure 2*) and measuring the enzymatic activity of GUS (data not published). The unidentified bands found after the immunoblotting detection are supposed to be the products of expression from different initiation codons or the products of degradation or alternative splicing as it was found out after further modifications were performed with the E6 gene.

And how these results finally influence the immune responses induced after the immunisation with the corresponding constructs? These findings are described under Chapter *4.3.*

4.2 Modifications of the HPV 16 E6 oncogene

A low expression of the full-length E6 protein has been reported for the HR E6 genes as an alternative splicing occurs from the one 5' (donor) splice site and two 3' (acceptor) splice sites. The production of the spliced E6*I and E6*II transcripts give rise to two truncated proteins beside the full-length E6. Moreover, the E6*I transcript is the most abundant E6 mRNA in HPV-associated premalignant and malignant lesions (Smotkin *et al.*, 1989; Cornelissen *et al.*, 1990; Griep *et al.*, 1993). The fusion of the E6 gene with the 3'-terminus of GUS (GUS.E6) led to markedly increased level of the full-length E6 mRNA as the RT-PCR amplification and densitometric analysis of the amplified transcripts showed while the fusion to the 5'- terminus (E6.GUS) merely increased the production of the E6*II at the expense of the E6*I mRNA *(Paper IV, Figure 4, Table 1*).

To further increase the production of the full-length E6 protein and thus to enhance the immunogenicity of DNA vaccines, the 5' splice site was abolished in the E6 gene. From the resultant E6cc gene with two substituted nucleotides, a protein with the V49L mutation was produced (*Paper IV, Figure 1*). RT-PCR amplification demonstrated that the abolition of the donor splice site completely eliminated the expression of E6*I and E6*II. The corresponding spliced transcripts were also not expressed from the modified E6cc.GUS and GUS.E6cc fusion genes *(Paper IV, Figure 4*). Immunoblotting staining revealed substantially enhanced modified-E6cc-protein production when compared to the production of the unmodified E6 protein (*Paper IV, Figure 5A*). Densitometric analysis demonstrated also the difference for GUS fusion proteins – a higher production of proteins was detected after the fusion of the E6 or E6cc genes to the 3'- terminus of GUS (*Paper IV, Figures 5B, C and Table 2*). As it was predicted, the middle band of the GUS.E6 sample represents the product of the alternative splicing since this band is missing after the abolishment of the splice site (*Paper IV, Figure 5C*).

Chapter *2.1.3.1 Oncoproteins of HPVs* describes in details the role of the E6 protein in HPV associated carcinogenesis. When designing a DNA vaccine based on the expression of a protein that is originally an oncoprotein (wild-type E6), it is necessary to take in consideration the safety of the vaccine, especially when a clinical trial is expected.

One of the possible ways to decrease the oncogenic feature of the E6 protein is to reduce its ability to induce the degradation of the p53 tumour suppressor protein. Several studies reported mutations that affect the binding of the E6 protein with p53 or its α-helix partners, including E6AP (Smotkin *et al.*, 1989; Dalal *et al.*, 1996; Nguyen *et al.*, 2002). In

this work the E6 protein was altered with one or two substitutions to decrease its oncogenicity. The introduced C70G and I135T mutations (*Paper IV, Figure 1*) were reported to reduce E6-mediated p53 degradation (Dalal *et al.*, 1996; Nguyen *et al.*, 2002; Shai *et al.*, 2007). The ability of the mutated proteins (E6G, E6T, and E6GT) to induce degradation of the p53 protein was tested by an *in vivo* p53 degradation assay. Cotransfection of p53-null cells with plasmids encoding p53 and the modified E6 genes led to the following results: The proteins with a single mutation (E6G or E6T) were still able to induce p53 degradation similar to the wt E6 protein. Interestingly, the E6 protein modified with both mutations (E6GT) eliminated the degradation of p53 (*Paper IV, Figure 7*). These results suggest that the E6AP binding surface of E6 consists of residues from both the N- and C- termini (Nomine *et al.*, 2006). As the modified E6GT protein was the only one unable to induce degradation of p53, solely this protein was fused with GUS (GUS.E6GT) for immunisation experiments. The expression of the fusion protein was verified with immunoblotting staining and by measuring the GUS activity (data not published).

4.3 Immunisation with the prepared DNA vaccines

The prepared plasmid DNA constructs, containing the non-fused and fusion genes with or without modifications, were used in immunisation experiments to verify the vaccine efficacy. C57BL/6 female mice were immunised with DNA vaccines utilising a gene gun device. Administration of DNA vaccines by a gene gun is one of the main delivery methods of these vaccines that leads to effective induction of antigen-specific immune responses (see Chapter *2.3.1 Immune responses and vaccine delivery*). The immunisation scheme of animals, the doses of plasmid DNA and the inoculation of tumour cell lines are described under *Materials and methods of Papers II* and *IV*. To pursue the immunogenicity of the DNA vaccines, two methods were used for the detection of antigen-specific T cells – the intracellular cytokine staining (ICS), when IFN-γ was stained in $CD8^+$ T lymphocytes, and the more sensitive ELISPOT assay to detect IFN-γ-producing cells. The production of antibodies was examined by ELISA.

DNA vaccines against the E7 protein:

Comparison of the production of E7-specific antibodies after immunisation with the truncated E7GGG.GUS genes revealed a high production of antibodies against E7 while the primary E7GGG.GUS construct induced a weak humoral immune response (*Paper II, Figure 3A*). Furthermore, the level of antibody production correlated with the steady-state levels of the fusion proteins (*Paper II, Figure 2A*). On the contrary, about a twice higher cell-mediated immunity was observed by the ELISPOT assay after immunisation with E7GGG.GUS than with the truncated fusion genes (*Paper II, Figure 3B*). Immunisation with GUS.E7 and the truncated fusion genes showed a low number of the IFN-γ-producing splenocytes after restimulation with the immunodominant H-2D^b E7$_{49-57}$ epitope, despite the fact that the high steady-state level of the modified fusion proteins predicted a higher amount of the released E7 antigen and thus suggested an enhanced cross-priming. Moreover, the results from preventive immunisation against TC-1/A9 tumour cells with reduced surface expression of MHC class I molecules corresponded with the highest efficiency of the E7GGG.GUS gene in the ELISPOT assay: all mice developed a tumour except of two animals immunised with the E7GGG.GUS construct (*Paper II, Figure 3C*). The therapeutic immunisation against TC-1 cells led to elimination of tumour cells in about half of mice induced by both the original E7GGG.GUS and also the newly constructed GUS fusion genes (*Paper II, Figure 3D*).

Immunisation with the other modified genes, SS.GUS.E7GGG, E7GGG.EGUS and hE7GGG.GUS showed that none of the vaccines induced significant production of E7-specific antibodies and only immunisation with SS.GUS.E7GGG demonstrated higher antigen-specific CTL immune response in comparison to the original E7GGG.GUS fusion gene. Furthermore, the tumour development was significantly slower after immunisation with SS.GUS.E7GGG in a therapeutic manner of vaccination (*Paper II, Figures 6A, B*). As mentioned above, the SS.GUS.E7GGG protein was not secreted (*Paper II, Figure 4D*) and accumulated in the ER (*Paper II, Figure 5*). This vaccine might have induced the highest cell-mediated immune response due to the retrograde transport of the fusion protein from the ER into cytosol and its degradation with proteasomes as reported by Golovina *et al.* (Golovina *et al.*, 2002). The immunogenicity of the fusion gene with codon optimisation in the E7GGG part (hE7GGG.GUS) and also its protein production were comparable to E7GGG.GUS (*Paper II, Figures 4A and 6A*) though the hE7GGG gene alone produced about a 6-fold higher amount of the E7GGG protein when compared to the E7GGG gene (data not published). A reasonable assumption for this unaltered immunogenicity of the hE7GGG.GUS

fusion protein may be the fusion with the 6-fold longer GUS. This non optimised part may suppress the contributory effect of E7GGG codon optimisation.

The cell-mediated immunity may be influenced by the antigen stability. It has been reported that the destabilisation of the E7 antigen, for instance with a mutation, enhanced the CTL responses (Shi *et al.*, 1999). However, vaccination with fusion proteins with higher (E7GGG60.GUS) or lower (E7GGG.EGUS) stability than the original E7GGG.GUS protein did not enhance the $CD8^+$ T cell immune responses (*Paper II, Figures 3B, 6A and 7*). This may be in accordance with the data reported by Golovina *et al.* showing that mutations or deletions causing misfolding of the proteins did not impact on the epitope production (Golovina *et al.*, 2005).

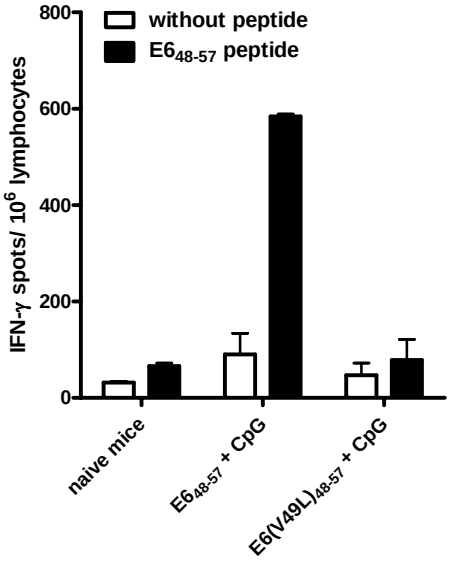
DNA vaccines against the E6 protein:

The fusion of the E6 gene with both termini of GUS produced a bit different immunisation results than the fusion of the GUS with the E7 gene. IFN-γ-producing $CD8^+$ T cells were detected by ELISPOT or ICS assays after overnight incubation with the H-$2K^b$ $E6_{48-57}$ epitope. While the E7GGG.GUS induced the strongest immune response after immunisation with E7-derived plasmid DNAs, the immunogenicity of the E6 fused to the 5'-terminus of GUS (E6.GUS) was comparable with that of the E6 gene alone. The highest number of E6-specific T cell was recorded after immunisation with the GUS.E6 construct (*Paper IV, Figure 3A*). Moreover, using a preventive immunisation scheme against TC-1 tumour cells the immunisation with GUS.E6 resulted in a significantly lower tumour growth (*Paper IV, Figure 3B*). The high efficacy of the GUS.E6 vaccine could be caused by the higher production of the unspliced E6 transcript (*Paper IV, Figure 4, Table I*) and subsequently the high full-length E6 fusion protein production.

The cell-mediated immunity was moderately decreased after immunisation with plasmids carrying genes with the abolished E6 splicing site (E6cc constructs) – a decreased number of E6-specific T cells was recorded by ELISPOT assay when compared with the unmodified genes (*Paper IV, Figure 6A*). Immunisation of animals against TC-1 cells also confirmed the reduced immunogenicity of the modified fusion genes (*Paper IV, Figure 6B*). An acceptable explanation could be the substitution of valine by leucine at position 49 (V49L) in the H-$2K^b$ immunodominant epitope of E6 (aa 48-57) as a result of the eliminated splice site. However, the comparison of the original EVYDFAFRDL epitope sequence with the mutated ELYDFAFRDL sequence by computer analysis did not reveal any significant difference in predicted binding to H-$2K^b$ molecules. For the analysis, two databases of MHC

ligands and peptide motifs were used, the BIMAS (www-bimas.cit.nih.gov/molbio/hla_bind) and the SYFPEITHI (www.syfpeithi.de). Furthermore, Peng *et al.* reported that the minimal core sequence required for the activation of $CD8^+$ T cells is the aa 50-57 region of the E6 protein (Peng *et al.*, 2004). Nevertheless, immunisation with synthetic peptides delivered together with CpG motifs (ODN 1826), using a tattoo device confirmed the reduced immunogenicity of the E6 peptide carrying the V49L mutation (data not published, Fig. 9). Supposedly, the abolishment of the splicing site, which led to the V49L mutation in the E6 protein impaired the immunodominant epitope for C57BL/6 mice and thus decreased the immunogenicity of the modified DNA vaccines.

Figure 9. Immunogenicity of the $E6_{48-57}$ and $E6(V49L)_{48-57}$ peptides



Mice (n=3) were immunised three times at a 1-week interval with the $E6_{48-57}$ or $E6(V49L)_{48-57}$ peptides (100 μg) supplemented with CpG motifs (50 μg) using a tattoo device. Splenocytes were isolated one week after the last immunisation, incubated overnight with the $E6_{48-57}$ peptide and IFN-γ-producing cells were detected by an ELISPOT assay.

Mutations C70G and I130T that were performed to reduce the E6-mediated p53 degradation evoked a slightly decreased immune response detected by ICS assay after vaccination with the mutated genes (*Paper IV, Figure 8A*). This moderate distinction was also confirmed after challenging the animals with tumour cells (*Paper IV, Figure 8B*). Moreover, the immunisation with plasmid DNA encoding the fusion protein with both types of modifications, the one eliminating the donor splice site in the E6 gene and that reducing the p53 degradation ability of the E6 protein (GUS.E6ccGT), did not lead to satisfactory results

(data not published). A recent proposal of the structure of the HPV 16 E6 protein indicates that many of the mutations made to find out the functions of this protein resulted in structure destabilisation (Nomine *et al.*, 2006). As the mutations performed in this work were chosen prior to these new structural findings, they are unfortunately all located at the key buried positions of the E6 protein. Therefore, the potential conformational changes induced particularly by the C70G and I130T mutations might have influenced the antigen processing and presentation that consequently moderated the immunogenicity of DNA vaccines.

4.4 HPV 16 E7 mediated mechanism of tumour escape

Chapter *2.2.2.2 Molecular mechanisms leading to tumour escape* describes the importance of the impaired presentation of the antigen epitopes by MHC class I molecules in tumour evasion from the immune system. The down-regulation of MHC class I and/or antigen expression or alterations in the epitope sequence can result in tumour escape. All these modifications may represent an obstacle in anti-tumour vaccine designing.

Recently, after vaccination of animals, we derived immunoresistant clones from their tumours developed from the challenged mouse TC-1 cells producing E6 and E7 oncoproteins. The clones were resistant to immunisation with a DNA vaccine against E7. The immunoresistance correlated with the N53S mutation in the immunodominant epitope of the E7 protein (Smahel *et al.*, 2005). *Paper I* demonstrates that this mutation is responsible for the evasion of TC-1 clones from the antigen-specific immune responses induced by vaccination.

The RANKPEP computer analysis for the prediction of peptide binding to MHC molecules revealed decreased binding of the mutated peptide to H-2D^b molecules. This prediction was confirmed after testing the ability of the E7 peptides (aa 49-57) to stabilise the H-2D^b molecules on the RMA-S cell surface (*Paper I, Figure 1*). The incubation of the cells with the RAHYSIVTF peptide carrying the N53S mutation showed the same result as the control RMA-S cells (with empty H-2D^b) while the original RAHYNIVTF peptide prolonged the presence of MHC I molecules on the cell surface. This finding suggested a reduced immunogenicity of the mutated $E7_{49\text{-}57}$ epitope as the immunogenicity of peptides correlates with their ability to stabilise surface MHC class I expression (Lipford *et al.*, 1995). Therefore, the N53S mutation was introduced to the E7GGG.GUS fusion gene (E7GGGS.GUS) to test its immunogenicity. The production of the fusion protein was verified by immunoblotting

staining and measuring the enzymatic activity of GUS (*Paper I, Figure 2A*). After the immunisation of animals with E7GGG.GUS and E7GGGS.GUS by a gene gun, the immunogenicity of the genes was detected by ELISPOT assay and tetramer staining after restimulation of the splenocytes with the RAHYNIVTF peptide (*Paper I, Figures 2B, C*). In both cases, a loss of immunogenicity of the E7GGGS.GUS gene was demonstrated even after restimulation with the mutated RAHYSIVTF peptide (data not published). Moreover, after the preventive immunisation against the TC-1 tumour cells, all mice vaccinated with E7GGGS.GUS developed a tumour (*Paper I, Figure 2D*). These findings show that the N53S substitution eliminates the $E7_{49-57}$ immunodominant H-$2D^b$ epitope. Furthermore, the immunoresistance of the TC-1/F9 and TC-1/C6 clones, producing the E7 protein with the mutated epitope, is strictly E7-specific as the immunisation of mice against E6 prevented the growth of tumours from these clones (*Paper I, Figure 3*).

To prove finally that the N53S mutation was responsible for the immunoresistance of the TC-1 clones, the TC-1/F9 clone was transduced with the wt E7 signed with FLAG and the TC-1/F9/C1 clone was derived. The TC-1/F9/B5 control clone is a result of E7S.FLAG transduction. The clones produced the E7.FLAG or E7S.FLAG protein at a level similar to that of the E7 in TC-1 cells (*Paper I, Figure 4*). To test the immunosensitivity of the clones, mice were vaccinated with the E7GGG.GUS and E7GGGS.GUS gene and challenged with the appropriate TC-1 clones. Tumours developed in all immunised mice after the challenge with TC-1/F9/B5 or the parental TC-1/F9 cells. A reduced tumour growth was observed after the challenge with the TC-1/F9/C1 cells (*Paper I, Table 1*). The inhibition of the tumour development, though, was not significant as the tumour growth after the challenge with the TC-1/F9/C1 cells was also partly reduced in the pBSC- and E7GGGS.GUS-immunised animals. Furthermore, the oncogenicity of four TC-1/F9/C1-derived clones (A7, H10, G4, E12) was also decreased when compared with the TC-1/F9 cells while their immunosensitivity was high (*Paper I, Table 1*). This phenomenon of reduced oncogenicity of the tumour-cell-derived clones has been described previously. The transcriptional analysis of the examined cells revealed variability in the expression of immunomodulatory cytokines, namely MCP-1, osteopontin and midkine (Smahel *et al.*, 2005). These cytokines might influence the oncogenicity of TC-1 clones. Nevertheless, the summarised results showed significant inhibition of tumour development after the immunisation with the E7GGG.GUS gene and the challenge with TC-1/F9/C1 cell line or its clones and thus confirmed the responsibility of the N53S mutation for immunoresistance of TC-1/F9 cells.

Unfortunately, the frequency of the N53S escape mutation in the E7 oncogene was rare. The sequence analysis of the E7 gene in 52 clones derived from 10 cell lines isolated from TC-1-induced tumours did not detect any mutation in E7. However, the tested cell lines had reduced expression of MHC class I molecules (data not published). The previous study also showed reduced H-2D^b and H-2K^b molecules on TC-1 cells after immunisation against E7 (Smahel *et al.*, 2003). These data indicate that the principal escape mechanism of TC-1 cells from the host's immune system was the down-regulation of MHC class I molecules. However, the mutations in the HPV oncoproteins can contribute to the evasion from immune surveillance. For instance, the L83V substitution in a HPV 16 E6 protein variant can alter the activities of the protein important for its oncogenic potential (Lichtig *et al.*, 2006).

4.5 Vaccination against HPV 16 using a tattoo device for administration of E7- and E6-derived peptide vaccines

The $E7_{49-57}$ and $E6_{48-57}$ epitopes are considered to be the immunodominant H-2^b CTL epitopes of the appropriate HPV 16 oncoproteins (Feltkamp *et al.*, 1993; Peng *et al.*, 2004). Therefore, these epitopes may represent the key peptides employed in peptide-based vaccines against HPV 16. As described under the *Introduction* of *Paper III*, one of the disadvantages of peptide vaccines, similarly to the DNA vaccines, is their low immunogenicity. Several strategies were developed to enhance their efficacy like the modification of epitopes, administration with adjuvants, conjunction with helper proteins and different ways of application. Comparison of the administration methods of DNA vaccines revealed that tattooing induces higher cellular and humoral immune responses than i.m. needle injection (see Chapter *2.3.1 Immune responses and vaccine delivery*). The mechanical injuries caused by the tattoo procedure non-specifically stimulate the immune system and may partially compensate the need of adjuvants. As adjuvants approved for humans are rare and may cause adverse effects (Israeli *et al.*, 2009), a delivery method providing efficient immunisation without adjuvants is desirable. Tattooing for delivery of peptide vaccines and its comparison with s.c. needle injection of peptides was examined for the first time in our laboratory.

The immunisation experiments revealed that the cellular immune responses induced with the $E7_{49-57}$ peptide delivered by a tattoo device were comparable to those after gene gun DNA vaccination with pBSC/E7GGG.GUS (*Paper III, Figure 1A*). Further, all mice

immunised against TC-1 tumour cells with the E7 peptide in a preventive manner remained tumour free, which indicates a high immunogenicity of the tattooed peptide vaccine (*Paper III, Figure 1B*).

Subsequently, the ability of two different delivery methods for induction of efficient immune responses was compared using E7-derived peptide vaccines. The $E7_{49-57}$ and the $E7_{44-62}$ (carrying epitopes for CTL, $CD4^+$ T cells and B cells) peptides, supplemented with or without CpG motifs, were administered to animals by a tattoo device or s.c. needle injection. The highest cellular immune response was detected after immunisation with the $E7_{49-57}$ peptide and CpG motifs by a tattoo device. Similarly, high amount of E7-specific CTLs was detected in mice tattooed with the $E7_{44-62}$ peptide and CpG motifs (*Paper III, Figures 2A, B*). Humoral immune responses were examined only after immunisation with the $E7_{44-62}$ peptide (*Paper III, Figures 2C, D*). The production of E7-specific Abs was again higher after tattoo administration of the peptide in combination with or without CpG motifs than after s.c. injection of the same vaccine. Moreover, the results obtained from preventive immunisation of mice against TC-1 tumour cells showed that the protection of animals from tumour development corresponded with the immune responses induced by the peptide vaccines (*Paper III, Figure 2E*).

Surprisingly, the addition of CpG motifs to the s.c. injection of $E7_{49-57}$ peptide decreased the induction of cellular immune responses and the protection against tumour cells. The reason could be the mild precipitation of the water-dissolved peptide after addition of PBS. The peptide aggregation further increased after CpG motifs were added. The precipitation may influence the presentation of the peptide by APCs and may lead to decreased immune responses after s.c. delivery. On the contrary, the tattoo procedure may mechanically disrupt the peptide aggregates and then the induction of the immune system remains non-affected. In summary, tattoo delivery of peptide vaccines induced higher cell-mediated and humoral immune responses when compared with the s.c. injection, and the addition of CpG motifs further enhanced the efficacy of the vaccines. The comparison of these results with the previous results achieved with peptide immunisation used in other laboratories (Dileo *et al.*, 2003; Gendron *et al.*, 2006) where the vaccines did not protect the animals against tumour formation, indicates that the higher dose of the vaccines and the number of immunisations used in our laboratory and also the administration of lower dose of the challenging tumour cells could be the reason of the breaking through the detection limit.

In order to examine whether the tattoo delivery method is efficient also for peptide vaccines carrying different CTL epitopes, immunisation with the $E6_{48-57}$ peptide was

performed. The E6 peptide was administered to animals by tattooing or s.c. injection and the vaccines were supplemented with CpG motifs. Only the tattoo-delivered E6 peptide vaccine was able to induce CTL-mediated immune response (*Paper III, Figure 3C*) and to slow down the TC-1 induced tumour growth (*Paper III, Figure 3B*). However, all E6-tattooed mice developed tumours while two animals immunised subcutaneously remained protected against tumour formation (*Paper III, Figure 3A*).

Moreover, *Paper III* also demonstrated that the tattoo immunisation with KLH-conjugated $E7_{49-57}$ peptide (with or without CpG motifs) induced higher $CD8^+$ T cell immune responses in comparison with the s.c. injection of the same vaccine (*Paper III, Figure 4A*). However, the s.c. immunisation with the KLH-conjugated E7 peptide induced higher number of KLH-specific Abs than the vaccine administered *via* the tattoo device (*Paper III, Figure 4B*). This might be the result of the aggregation of the KHL-conjugated peptide that could have been less detrimental to the induction of humoral responses after s.c. delivery. Furthermore, the addition of CpG motifs enhanced the levels of KLH-specific Abs after immunisation by both delivery methods.

In conclusion, the comparison of the two delivery methods, tattoo device *vs*. s.c. needle injection, revealed that the administration of the E7- and E6-derived peptide vaccines by tattooing induced higher cellular and humoral immune responses than the s.c. injection.

5. SUMMARY

DNA vaccination has become an effective strategy for the development of vaccines against cancer including cervical carcinoma associated with HPV infection. DNA vaccines are proved to induce both humoral and cellular immunity. However, an enhancement of their low efficacy is required. The therapeutic DNA vaccines against HPV 16 are targeted against the viral E7 and E6 oncoproteins. In order to design safe vaccines, the oncogenicity of these targets has to be eliminated or properly reduced.

In this work, an enhanced immunogenicity of DNA vaccines against HPV 16 delivered by the gene gun was demonstrated after the fusion of the E7 and E6 genes with GUS.

DNA vaccines against the E7 protein:

The increased steady-state level of the E7GGG.GUS deletion mutants and the GUS.E7GGG fusion protein enhanced the production of E7-specific antibodies after immunisation with these vaccines but did not improve the CTL response. Joining of the signal sequence with GUS.E7GGG led to ER-localisation of the SS.GUS.E7GGG fusion protein, enhancement of the cell-mediated immune responses and slower tumour growth in immunised mice.

DNA vaccines against the E6 protein:

Enhanced immunogenicity was showed after immunisation with the E6 gene fused to the 3'-terminus of the GUS (GUS.E6). The abolishment of the splice site in the E6 gene resulted in complete elimination of the expression of the truncated E6 transcripts. However, this modification moderately reduced the immunogenicity of the non-fused (E6cc) or fused (GUS.E6cc) genes probably as a consequence of the V49L substitution in the immunodominant E6 epitope. The oncogenicity of the E6 protein was reduced by two point mutations and the modified E6GT protein was unable to induce p53 degradation. These substitutions in the E6 protein did not substantially influence the immunogenicity of the vaccines.

The infection with HPV can lead to development of malignant tumours. Several mechanisms may be responsible for the immune escape of the tumour cells infected with HPV. This work demonstrates one of the possible ways of this tumour escape. The N53S substitution in the RAHYNIVTF immunodominant epitope (aa 49-57) of the E7 protein was responsible for the immunoresistance of TC-1 clones derived from tumours of immunised

mice. The resistance of the clones was E7-specific as the tumour growth was inhibited after immunisation against E6. Transduction of the immunoresistant clones with the wt E7 gene restored their sensitivity to immunisation against E7.

Tattooing is another efficient delivery method of DNA vaccines beyond the gene gun administration and i.m needle injections. Nevertheless, it has not yet been tested for administration of peptide vaccines. This thesis reports the comparison of tattooing with s.c. injection using E7- and E6-derived peptide vaccines. Higher peptide-specific immune responses were observed after immunisation with the $E7_{49-57}$, $E7_{44-62}$ or $E6_{48-57}$ peptides administered by a tattoo device than after their s.c. inoculation. The addition of CpG motifs enhanced the induced immune responses after both types of vaccination.

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