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Mag. rer. nat. Christoph Jindra

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Abstract

Cervical cancer is the second largest cause of cancer deaths in women worldwide. Persistent infection with one of the about 15 known high-risk mucosal human papilloma virus (HPV) types, in particular HPV16 and HPV18, cause all cervical, most anal and a subset of penile, vulvar, vaginal and oropharyngeal cancers. The viral major oncogenes E6 and E7 are maintained and expressed in HPV-induced intraepithelial neoplasia and invasive cancer. Although prophylactic vaccines are a major accomplishment to protect against new infections, the high prevalence of HPV in the general population suggests the need for therapeutic vaccines to efficiently diminish viral burden and to treat patients with prevalent disease.

Here we show the generation of a live vector-based therapeutic HPV16 vaccine candidate. For this purpose we used a ΔNS1 influenza virus system which has the advantages of high immunogenicity, availability of different serotypes (H1N1, H3N2) for prime and booster immunizations and lack of DNA intermediates as important safety feature.

As target immunogen we generated an HPV16 E6-E7 fusion construct fused to an affinity (FLAG octapeptide) tag (16E6E7FL), which was cloned into the ΔNS1 locus. Two different serotypes of recombinant influenza viruses were generated by reverse genetics and transfection of Vero cells. For safety concerns a second fusion of the E6 and E7 oncogenes was generated, with short deletions that abrogate biological functions (16E6E7mFL). To enhance protein expression the splice donor site for the fusion was further modified. Following viral infection of Vero cells for 12 hours (h), mRNA and fusion protein expression were verified by RT-PCR and Western Blot, respectively. Protein expression was only detected in the presence of the proteasome inhibitor MG-132, suggesting rapid degradation of the artificial E6-E7 fusion protein due to impaired folding.

The immune response to recombinant viruses was characterized by subcutaneous (s.c.) infection of C57BL/6 mice followed by HPV16 E6 and E7 peptide ELISA, IFN-γ ELISpot and T-cell proliferation experiments. In general the vaccines were well tolerated and mice showed no detectable signs of toxicity. Immune characterization showed peptide specific IFN-γ production by cytotoxic T lymphocytes of vaccinated mice and proliferation of antigen specific $CD4^+$ T cells.

ELISA experiments detected antibodies to either influenza A serotype but not to HPV16 E6 or E7 peptides.

Therapeutic and prophylactic vaccine efficacy was analysed in an in vivo mouse challenge model using HPV16-transformed TC-1 tumour cells. Animals that were vaccinated twice s.c. and challenged with TC-1 cells 10 days after the boost either did not develop any tumours or showed significantly decreased tumour growth rates as compared to mock or empty influenza vector treated animals. Similar results were obtained in a more stringent therapeutic setting, in which animals were primed s.c. with H1N1 recombinant virus inoculated s.c. with TC-1 the same day, and boosted with the recombinant H3N2 serotype 10 days later. Additional vaccine administration routes were analysed using intranasal and intratumoral application, with the latter inducing complete tumour regression in a subset of mice and significantly decreasing tumour growth rates in additional animals.

These results suggest recombinant E6E7 influenza viruses as a promising new tool to develop therapeutic vaccination strategies against HPV-induced high-grade intraepithelial lesions.

Zusammenfassung

Das Zervixkarzinom ist weltweit die zweithäufigste Todesursache durch Krebserkrankungen der Frau. Die persistierende Infektion mit einem von etwa fünfzehn hoch-Risiko humanen Papillomviren (HPV), insbesondere HPV 16 oder 18, ist für die Entwicklung aller Zervix- und der meisten Analkarzinome, sowie eines Teils der Penis-, Vulva-, Vagina- und Oropharynxkarzinome verantwortlich. Obwohl die zugelassenen prophylaktischen HPV Vakzinen mit hoher Effizienz neue Infektionen verhindern, besteht kein therapeutischer Effekt auf bestehende Infektionen und induzierte Läsionen. Aufgrund der hohen Prävalenz (ca. 10%) von genitalen HPV Infektionen in der Bevölkerung sind effiziente therapeutische Impfstoffe wünschenswert, um bestehende Infektionen und daraus resultierende intraepitheliale Neoplasien zu reduzieren. HPV-induzierte neoplastische Zellen exprimieren die viralen Onkogene E6 und E7, welche daher mögliche Zielantigene für die Induktion einer effizienten Immunantwort darstellen.

In dieser Arbeit wurde eine therapeutische HPV 16 Vakzine auf Basis attenuierter Influenza A Virus-Vektoren generiert welche über Vorteile wie hohe Immunogenität, verschiedene Serotypen für multiple Immunisierungen, sowie das Fehlen von DNS Zwischenstufen verfügt. Letzteres wird als wichtiger Sicherheitsfaktor für eine Lebendvakzine angesehen.

Es wurde ein HPV 16 E6E7 Fusionskonstrukt (16E6E7FL) generiert und in den ΔNS1 Locus des Influenza A Genoms kloniert. Wegen möglicher Sicherheitsbedenken wurde eine weitere Fusion generiert, in der kurze Sequenzen von E6 und E7 deletiert wurden (16E6E7mFL), um die biologische Aktivität dieser Onkogene zu unterbinden. Um die Expression der Fusionsproteine zu steigern, wurde außerdem die ‚donor' Spleißstelle von NS1 modifiziert. Daraus resultierten insgesamt vier verschieden Influenza A Viren, da jeweils zwei unterschiedliche Serotypen, H1N1 und H3N2, als Vakzine generiert wurden.

Nach Infektion von Vero-Zellen für 12 Stunden mit rekombinanten Influenzaviren konnte die Expression von Transgen-mRNS mittels RT-PCR, und von Protein im Western Blot nachgewiesen werden. Die Fusionsproteine konnten erst durch die Verwendung eines Proteasomblockers (MG-132) detektiert werden, was auf eine rasche Degradation des Fusionsproteins durch inkorrekte Proteinfaltung hinweist.

Nachfolgend wurden C57BL/6 Mäuse mit hochtitrigen Influenzaviren mittels prime-boost Schema subkutan (s.c.) vakziniert und die generierte Immunantwort durch Peptid-ELISA, IFN-γ ELISpot und Proliferationsassays untersucht. Es konnte Peptid-spezifische IFN-γ Produktion in Splenozyten von vakzinierten Tieren nachgewiesen werden. Ebenso konnte Proliferation von Antigen-spezifischen $CD4^+$ T-Zellen detektiert werden. Im ELISA konnten Antikörper gegen beide Influenza A Serotypen, aber nicht gegen HPV16 E6 oder E7 nachgewiesen werden. Allgemein wurden die Vakzinen gut vertragen und keines der Tiere zeigte erkennbare Zeichen von Toxizität.

Die prophylaktische und therapeutische Wirksamkeit der Vakzinen wurde anhand eines in vivo Mausmodells analysiert, bei dem HPV16-transformierte TC-1 Tumorzellen für den Challenge verwendet wurden.

Tiere, die zweimal s.c. immunisiert und anschließend mit Tumorzellen s.c. injiziert wurden, entwickelten keine Tumoren oder wiesen deutlich verringertes Tumorwachstum auf, verglichen mit Placebo- oder Leervektor-behandelten Tieren. Ähnliche Ergebnisse wurden erzielt, wenn Mäusen TC-1 Zellen s.c. injiziert wurden, diese am selben Tag mit rekombinanten H1N1 Viren geimpft und zehn Tage darauf mit dem analogen H3N2 Serotyp geboostet wurden. Es wurden auch unterschiedliche Administrationsrouten der Vakzine, im speziellen intranasal (i.n.) und intratumoral (i.t.), näher analysiert. Intratumorale Applikation induzierte vollständige Tumorregression in 25% der Tiere sowie eine reduzierte Wachstumsrate der TC-1 Tumoren in den weiteren Tieren, während die i.n. Route wegen Nebenwirkungen nicht weiter verfolgt wurde.

Diese Ergebnisse unterstützen rekombinante Influenzaviren als eine vielversprechende, neue Strategie, um therapeutische Impfstoffe gegen HPV Infektionen und dadurch induzierte Erkrankungen zu entwickeln.

Abbrevations

aa	amino acid
APC	antigen presenting cell
ATM	Ataxia telangiectasia mutated
BM	basement membrane
bp	base pairs
CBP	CREB-binding protein
CFS	common fragile site
CHK2	checkpoint kinase 2
CIN	cervical intraepithelial neoplasia
CR	conserved region
CsCl	cesium chloride
CTL	cytotoxic T lymphocyte
CxC	cervical cancer
DC	dendritic cell
ds	double stranded
DSB	double strand break
DMSO	dimethyl sulfoxide
E6AP	E6 associated protein
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immuno spot
EV	Epidermodysplasia verruciformis
HA	hemaglutinin
hDlg	human disc large
HLA	human leukocyte antigen
HPV	human papillomavirus
hr	high risk
HRP	horse radish peroxidase
hScrib	human scribbled
HSIL	high-grade squamous intraepithelial lesions
hsp	heat shock protein
HSPG	heparan sulfate proteoglycans
IFN	interferon
Ig	immunoglobuline
IL	interleukin
i.n.	intranasal
IRF	interferon regulatory factor
i.t.	intratumoral
kD	kilodalton
LCR	long control region
lr	low risk
M1	matrix 1
M2	matrix 2
mAb	monoclonal antibody
MAGI	membrane-associated guanylate kinase protein
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
moi	multiplicity of infection
MPL	3-O-desacyl-4'-monophosphoryl lipid
NA	neuraminidase
NEP	nuclear export protein
NFκB	nuclear factor κ B
NLS	nuclear localisation signal
NMSC	non melanoma skin cancer
NS1	non-structural protein 1
OAS	2'-5'-oligoadenylate synthetase
ORF	open reading frame
PA	polymerase acidic protein
PAP	Papanicolaou
PB	polymerase basic protein
PcG	polycomb group complex
PD-1	programmed death 1
Pfu	plaque forming units
PKR	protein kinase R
pRb	Retinoblastome protein
PsV	pseudovirion
PV	papillomavirus
RIG-1	polymerase acidic protein
RNP	ribonucleoprotein
s.c.	subcutaneous
STAT	Signal Transducer and Activator of Transcription
TCID50	50% tissue culture infectious dose per millilitre
TEM	transmission electron microscope
TLR	toll-like receptor
Th	T helper
URR	upstream regulating region
VLP	virus-like particle
vRNP	viral ribonucleoprotein
WHO	World Health Organization

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1 Introduction

Cervical cancer (CxC) is the second largest cause of cancer deaths in women worldwide. Nearly half a million women are diagnosed each year and about 250,000 die from the disease [1]. The institution of Papanicolaou (PAP) cytological screening programs to detect precursor lesions, high-grade cervical intraepithelial neoplasia, and the ablation of such pre-invasive lesions, has dramatically reduced the incidence of CxC in developed countries. Thus the majority of CxC diagnoses and deaths worldwide occurs in developing countries which cannot afford PAP screening. Nevertheless, approximately 33,000 CxC cases occur in Europe every year and approximately 4% will die. Persistent infection with high-risk (hr) human papillomavirus (HPV) is the necessary cause for development of CxC [2] although additional genetic changes are required for disease progression. The World Health Organization (WHO) estimates that more than 500 million people are infected with genital HPV, a worldwide prevalence of 9-13% [3]. The risk of acquiring this most common sexually transmitted viral disease during a lifespan is at least 50% [3], and socioeconomic status, number of lifetime sexual partners, being unmarried, immuno-suppression, long-term use of oral contraceptives or tobacco are major risk factors [4-6]. So far there is no specific antiviral treatment for HPV infections available. Thus cervical high-grade squamous intraepithelial lesions (HSIL) are treated by cone biopsy or large loop excision [7], and other anogenital HSIL and benign genital warts by destructive or ablative methods, like laser vaporisation or cold coagulation. However, invasive treatments carry the risk of complications and recurrence and at the cervix increase the risk for preterm labour [8].

1.1 Human papillomaviruses

Over 190 HPV genotypes have been completely characterized (http://www.hpvcenter.se) and are classified into five genera (alpha, beta, gamma, mu and nu), about forty of which (mucosal types) are known to infect the mucosa of the anogenital tract and oropharynx [9]. Within the α-HPV, fifteen hr mucosal types (most often HPV16, 18, 31 and 45) are of particular importance

because of their causal association with virtually all cervical cancers [10], most anal cancers, and up to 50% of vulvar, vaginal, penile and oropharyngeal cancers, whereas infection with mucosal low-risk (lr) types, most often HPV 6 or 11 causes genital warts (condylomata acuminata) [11].

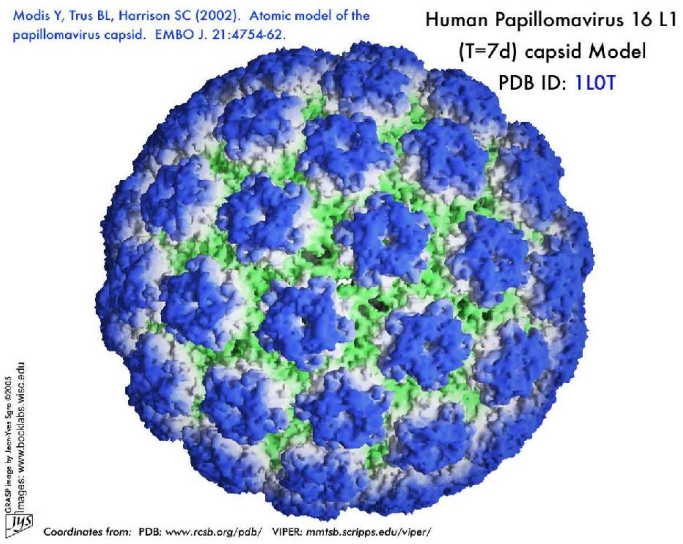

Figure 1: Schematic illustration of the HPV 16 capsid. Adapted from Modis Y, Trus B, Harrison SC (2002) EMBO J 21:4754-62

Cutaneous HPV types infect the cornified skin and cause common and palmo-plantar warts (most often HPV1, 2, 4, 27, 57) or flat warts (HPV3 or 10). Another large group of at least 25 HPV types belongs to the genus beta. The carcinogenic potential of beta-HPV has first been observed in patients with Epidermodysplasia verruciformis (EV), a rare autosomal recessive genetic disease. EV-patients develop non melanoma skin cancer (NMSC) at young age at sun exposed areas and HPV DNA of types 5 and 8 have been identified in the majority of these cancers [12] suggesting a causal role in addition to UV irradiation. More recent epidemiological and experimental evidence suggests that beta-types, in particular HPV5, 8, 15, 20, 24, 36 and 38 might play an adjunct carcinogenic role in NMSC of the general population, the most common cancer in caucasian populations, and in particular in immune-suppressed patients [13].

Papillomaviruses (PV) are a large family of highly species-specific DNA tumour viruses of the taxonomic family *Papillomaviridae* that infect a wide array of vertebrates (e.g. humans, cattle, dogs, dolphins, etc.) [14]. More than 240 distinct types have been described, that are classified into 37 genera. The non-enveloped ('naked') virion shell of about 60 nm diameter is composed of two structural proteins, the major capsid protein L1, which is highly immunogenic [15], and the less abundant L2 minor capsid protein [16]. Both are encoded in the late (L) region of the HPV genome. The capsid consists of 72 capsomeres, each a pentamer of 5 L1 monomers, (Fig. 1) with T=7 icosahedral symmetry [17]. Highly

variable loops of L1 are exposed on the virion surface that contain the immuno-dominant neutralization epitopes that determine PV serotypes. The L2 minor capsid protein associates with L1 capsomeres, and up to 60 L2 copies are found incorporated into the native virion [18, 19], although their exact location is not totally clear.

1.1.1 HPV genome

Papillomaviruses contain a circular, double-stranded DNA genome of about 7-8 kb in size condensed around cellular histones as a minichromosome [20]. The genome can be assigned to a long control region (LCR) or upstream regulating region (URR), an early (E) region and an L region (Fig. 2), the latter two being separated by an early and a late polyadenylation site [21]. The LCR contains the origin of replication (Ori) and multiple transcription factor binding sites which regulate transcription of the viral E and L open reading frames (ORFs) [22]. The E2 protein can inhibit transcription of viral genes through interaction with certain E2 binding sites by steric hindrance [23]. For HPV16 the two promoters are termed P97 (pEarly) which lies just upstream of the E6 ORF and P670 (pLate) which is located within the E7 ORF respectively.

The early promoter contains a TATA box, the enhancer region and 4 conserved upstream E2-binding motifs [20, 24]. Early primary transcripts are either bi- or polycistronic, which for HPV16 allows for at least 14 different mRNA transcripts containing 2 introns [25]. Intron 1 is located in the E6 ORF, and intron 2 in the E1 ORF. Transcription of E6 therefore causes retention of intron 1 in the primary mRNA whereas intron 2 is retained in E1 transcripts respectively. Transcription of oncogene E7 needs splicing of intron 1 to efficiently progress to protein

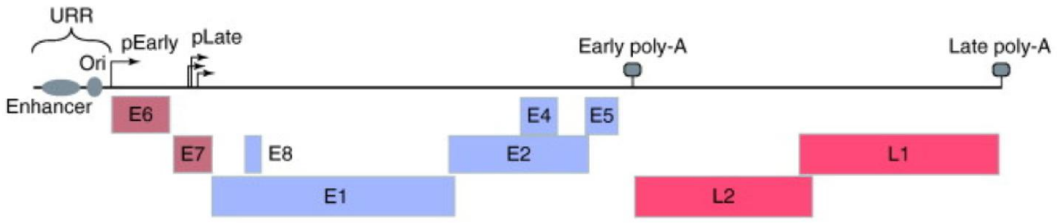

Figure 2: The genome of HPV16. Adapted from Bodily J , Laimins LA (2011) Trends Microbiol. 19(1): 33-39

translation [26]. Alternative splicing needed for efficient gene expression is not yet fully understood. Factors involved include E2 and E6 which have been shown to directly suppress splicing [27] and E5 which affects the cellular splicing machinery by altering the epithelial growth factor (EGF) pathway [28].
The productive PV life cycle is strictly linked to the differentiation state of the stratified epithelia. The late promoter P670 e.g. can only be induced in differentiated keratinocytes [20] and thus capsid protein production and assembly of infectious virions only takes place in highly differentiated upper layers of stratified epithelia.

1.1.1.1 Genome integration

Since productive infections with genital lr HPV (condylomata) do not pose a carcinogenic threat to the squamous epithelium, neoplastic progression requires more than “normal” expression of viral genes [29]. Expression of the oncogenes of hr HPV in basal epithelium induces genomic instability and blocks cell differentiation which can expedite malignant progression [30]. In most CxC truncated viral genomes are integrated into the host genome causing deregulated oncogene expression [31], mostly due to loss of the viral E2 gene [32]. Integration is not part of the viral life cycle. and the frequency of integration itself correlates with the presence of DNA double-strand breaks (DSBs) which can be induced by E6 or E7 or common fragile sites (CFSs) [29]. No predominant specific host sequence motif for integration has been identified although many occur within CFSs [33].

1.1.2 HPV life cycle

The investigation of the infectious life cycle using native HPV virions harbours difficulties, since in vitro production of native virus is only possible in organotypic cultures due to the strict linkage of HPV to keratinocyte differentiation. An alternative is isolation of HPV directly from patient wart material. Since these methods reveal relatively few virions only for common cutaneous or lr mucosal

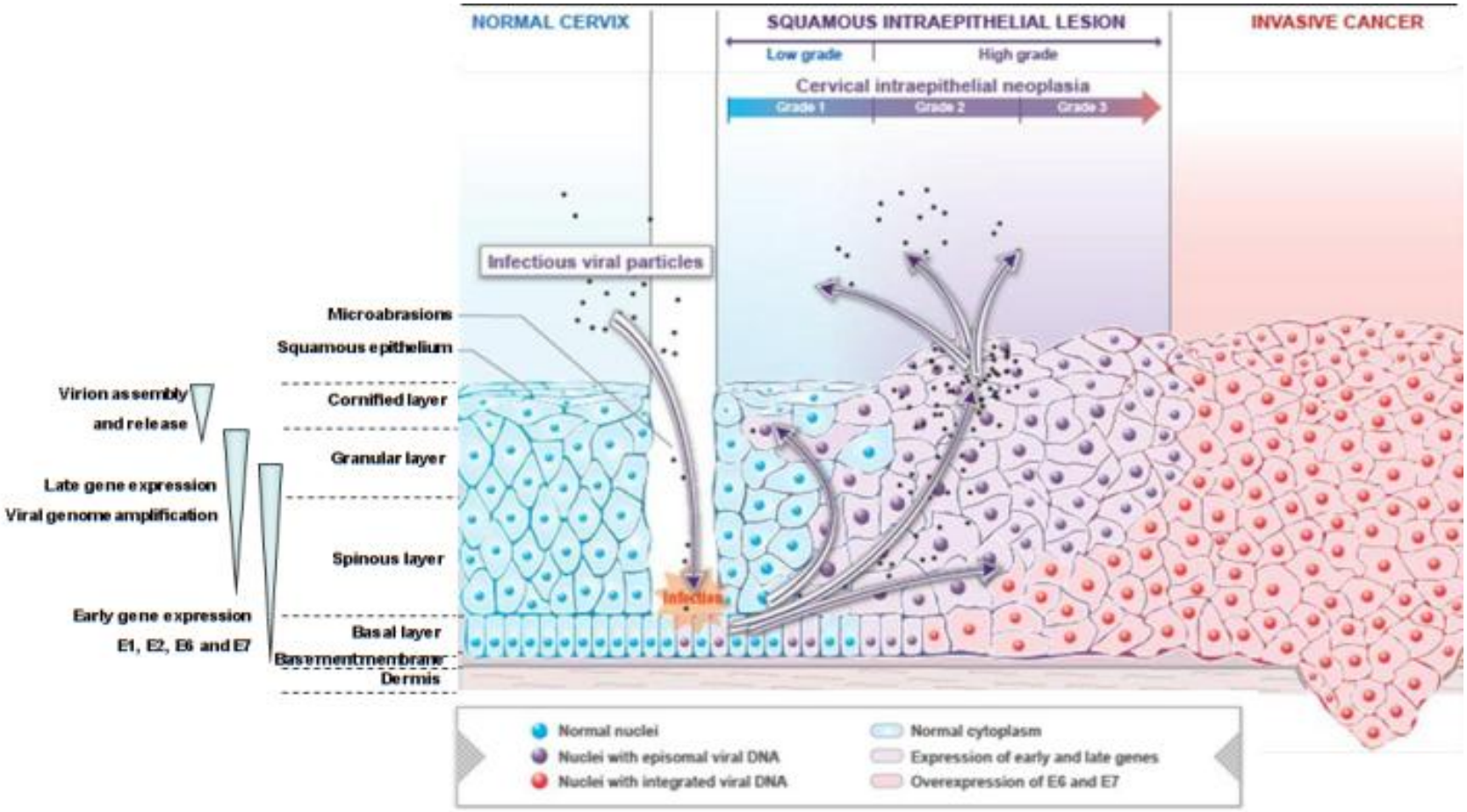

Figure 3: The life cycle of HPV. Adapted from Genticel; Vienna 2011.02.24; Next Generation of Vaccines Symposium

HPV, alternative methods were established to allow analysis of early events in HPV infection such as cell binding and entry, immunology and seroepidemiology. The major capsid protein L1 alone assembles readily into empty capsids, when expressed in mammalian or insect cells [34]. Those virus-like particles (VLPs) have become the basis for prophylactic vaccines currently available. Co-expression of L1 and L2 generates VLPs harbouring both capsid proteins. In vitro assembled capsids termed pseudovirions (PsV) are composed of the viral capsid packaging a reporter gene [35], expression of which is used as read out for infection or internalization studies. Infectious PV particles are thought to gain access to the basal layer of keratinocytes through microabrasions (Fig. 3) in the epithelium to initiate infection in vivo. Attachment to host structures was shown to be similar when VLPs consisted either of L1 alone or L1 and L2 suggesting that the capsid protein L1 is the major viral component for attachment. Heparin sulphate proteoglycans (HSPGs) appear to play an essential role for primary binding of PV to the basement membrane (BM), an important first step for subsequent viral transfer to the migrating keratinocytes and productive infection [36]. Virion binding to the BM causes a conformational change of the capsid that unmasks the N-terminus of L2, which then becomes cleaved by a cellular protease furin [37]. This cleavage is essential for viral infection, since furin inhibition prevents binding to keratinocytes in vivo [38]. Subsequent binding to the cell surface triggers a variety of different pre-entry steps. Viral uptake into the epithelial cell takes up to 24 h [38] which is unique compared to other viruses.

Although internalization and intracellular trafficking of PV have been extensively studied, little consensus has emerged [39]. Besides clathrin-mediated endocytosis, also caveolae-mediated pathways and tetraspanin-associated internalization have been associated with viral cell entry. The use of VLP, PsV or virion and their respective maturation stage (conformational changes) might explain observed differences in obtained data [38]. Upon internalization L2 plays a critical role in escape from the endosome into the cytoplasm, and L2 and the viral genome remain in a complex [40, 41]. How the genome gets located into the nucleus is controversially discussed. One possibility is that syntaxin-18 transports the L2-genome complex to a perinuclear site [42] upon which the whole complex translocates into the nucleus via interaction of the nuclear localisation signal (NLS) of L2 with the nuclear import machinery [43, 44]. Also recent research

suggests that cell cycle progression through mitosis is critical for infection arguing that degradation of the nuclear membrane is required for translocation [38].

Completion of the PV life cycle is strictly linked to the keratinocyte differentiation program [44, 45] and virion production is restricted to highly differentiated epithelial layers. HPV establishes its genome as extrachromosomal element or episome [46], and infection can persist over years in basal keratinocytes. E1 and E2 are the first viral genes to be expressed and both can either bind to the viral genome either singly, E1 via A/T rich sequences [47], E2 via E2-binding sites [48], or by forming a complex thereby increasing binding affinity [49]. E2 then gets released by the complex, E1 recruits cellular replication machinery, acts as a helicase [50] and the viral genome becomes amplified resulting in 20 to 100 episomal copies per basal cell [51, 52]. Copy number though is tightly controlled by both E1 and E2, the latter binding at the early promoter when abundantly available [45, 53].

Following mitosis each daughter cell harbours equal amounts of viral episomes. One cell remains attached to the basal layer whereas the other starts to migrate through the suprabasal layers thereby entering terminal differentiation. There is need for HPV to retain an active cell cycle for its own replication. E6 and E7, the actions of which will be discussed in more detail later, accomplish this task by blocking p53 and Retinoblastoma protein (pRb) function respectively [21]. Another protein, E5, is also known to facilitate hyperproliferation of infected cells [54]. By these mechanisms viral amplification and late gene transcription are ensured and production of high levels of viral proteins is restricted to suprabasal layers that are less susceptible to immune surveillance [55].

Cell differentiation triggers activation of the viral late promoter resulting in a large increase in viral transcripts and genome copy number. Genes expressed are E1, E2, E1^E4 and E5, the latter two contributing to a variety of late viral functions [56, 57]. Upon keratinocyte terminal differentiation the viral capsid proteins L1 and L2 are produced in the superficial layers of the epithelium [58] and virions are assembled with the assistance of host chaperones [35]. Virion assembly at sites separated from the systemic immune system is regarded as a viral strategy to evade an effective immune response [44, 59]. It is believed that the E1^E4 protein causes the collapse of host keratin networks, thereby shedding mature

HPV into the environment in the absence of cell lysis, inflammation or necrosis [60, 61].

1.1.3 HPV oncogenes/oncoproteins

One of the major functions of E6 and E7 in the viral life cycle is to ensure that differentiating keratinocytes retain an active cell cycle to support viral replication [21, 62]. Transcription of both oncogenes is highly regulated by E1 and E2. In CxC the viral genome of hr HPV is often integrated into the cellular genome. Integration is regularly accompanied with loss of L1, L2 and E2, whereas E6 and E7 genes are invariably retained. Loss of E2 leads to de-repression and thus overexpression of E6 and E7. Both oncoproteins co-operate, providing the major transforming capacity in cells through multiple steps [63]. Although E6 and E7 are sufficient to immortalize keratinocytes in vitro, for cells to become tumorigenic [64] additional mutations are required [65]. Tumour cells go into apoptosis or cellular senescence when E6 and E7 are silenced, showing that both oncogenes are necessary to maintain an immortalized and malignant phenotype.

1.1.3.1 E6 oncoprotein

HPV16 E6, a small protein of about 18 kilodalton (kD) of size, locates in the nucleus and the cytoplasm and is not capable to immortalize primary keratinocytes on its own following transfection. Three functional domains have been described for this protein, 2 zinc finger motifs which mediate binding of its major cellular target, the tumour suppressor p53 [66], and interactions with other proteins harbouring α-helical domains [67], and a C-terminal PDZ binding domain [68]. The major function of E6 is the abrogation of growth arrest which is primarily accomplished by degradation of p53. E6 recruits the cellular ubiquitin ligase E6-associated protein (E6AP) leading to subsequent ubiquitnylation and proteasomal degradation of p53 [69]. E6 can also directly bind p53 thereby interfering with its DNA-binding activity [46].

Interestingly, the other p53 family members, p73 and p63, are not targeted by E6 [70, 71]. Interference with p53 function is also observed through E6 binding to CREB-binding protein (CBP)/p300 thus blocking the histone acetyltransferases and inhibiting the ability of p53 to activate transcription [72].

Besides p53 inhibition, E6 of hr HPV has additional important targets which add up to its immortalizing capacity. It binds to the pro-apoptotic factor Bak and increases its proteolytic turnover rate by E6AP recruitment thus inhibiting apoptosis significantly [73].

One important domain of hr E6 is its C-terminal PDZ domain which interacts with a variety of cellular proteins [74] via PDZ-binding motifs [75, 76] targeting these for degradation. These include human Disc large (hDlg) [77], human Scribbled (hScrib) [78], membrane-associated guanylate kinase protein (MAGI)-1, -2 and -3 and others [79], causing deregulation of cell polarity and junction integrity. The importance of the PDZ domain of E6 was shown in E6-transgenic mice, in which

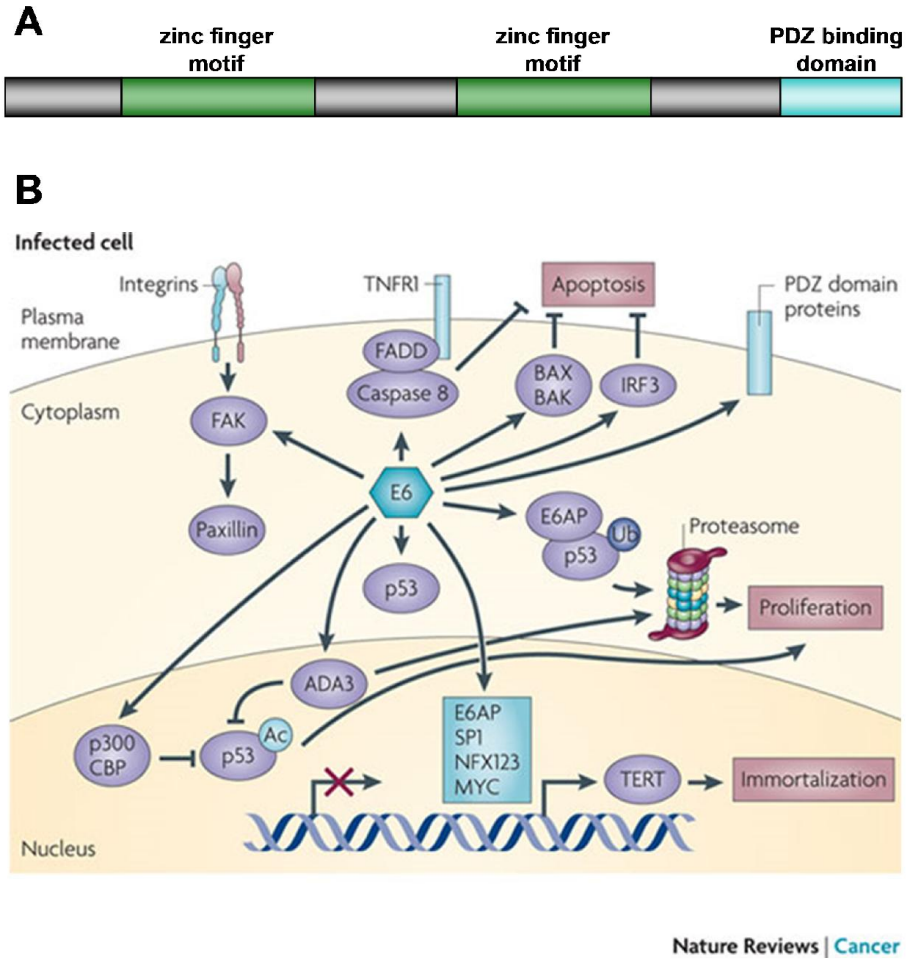

Figure 4: The E6 oncogene of HPV. **A** Domains in the HPV E6 oncogene **B** Cellular pathways and proteins affected by HPV E6 Adapted from Moody CA, Laimins LA (2010) Nat. Rev. Can. 10: 550-**560**

deletion of the C-terminus of E6 did not result in epithelial hyperplasia upon radiation compared to wt E6 transgenic animals [68].

Immune system evasion by HPV is facilitated by direct interaction of E6 with IFN regulatory factor 3 (IRF3), thereby blocking IFN-responsive gene expression [80], and E6 binding to the double-stranded RNA dependent serine/threonine protein kinase R (PKR), re-localizing this factor to cytoplasmic P-bodies [81], sites of mRNA degradation and storage. Binding of E6 to a complex of p53 and CBP/p300 also facilitates escape from immune surveillance and enhances viral long-term persistence [46].

1.1.3.2 E7 oncoprotein

The E7 oncoprotein is smaller than E6, with 11 kD of size, but with more potent transforming function considering that E7 expression alone is sufficient to

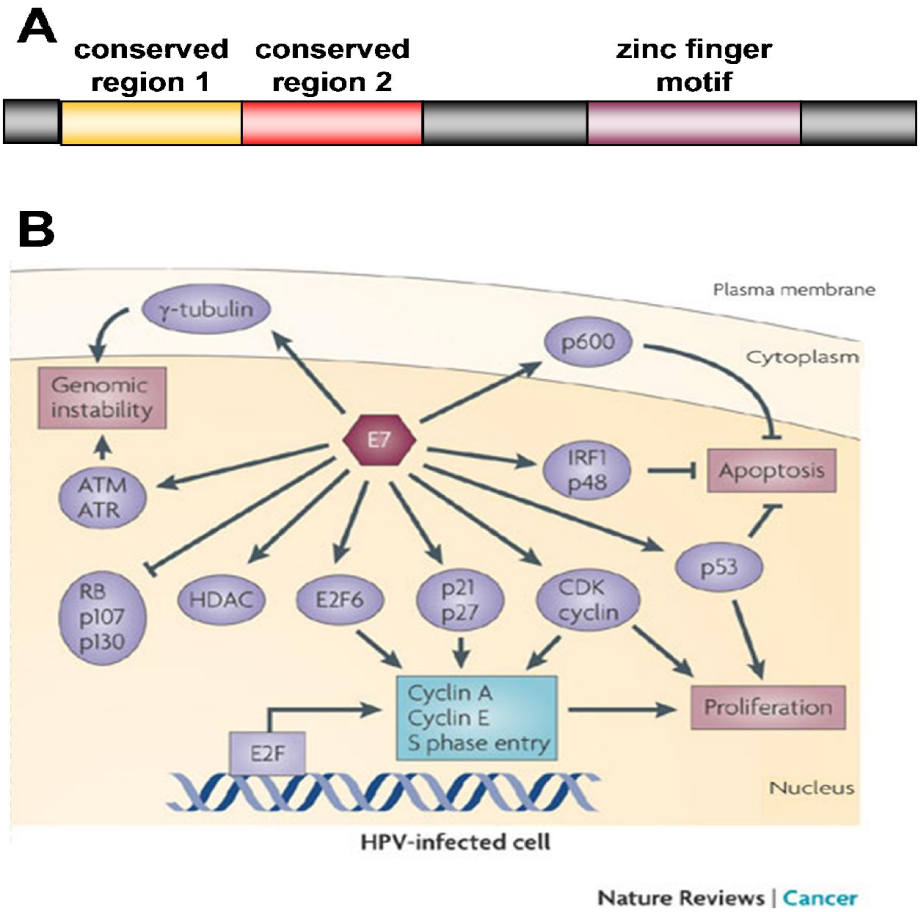

Figure 5: The E7 oncogene of HPV. **A** Domains in the HPV E7 oncogene **B** Cellular pathways and proteins affected by HPV E7 Adapted from Moody CA, Laimins LA (2010) Nat. Rev. Can. 10: 550-560

immortalize primary keratinocytes [62]. Two conserved regions (CR1, CR2) near the N-terminus and a zinc finger motif close to the C-terminus of the protein have been described [82]. Similar to E6, its major function during the life cycle of HPV is to maintain an active cell cycle to allow for viral genome replication and transcription. In particular hr E7 binds and degrades the tumour suppressor pRb through the proteasome [83]. The E7 oncoprotein interacts by its conserved LXCXE motif, which is located in CR2 [74], with the pocket proteins of the Rb-family, which include p107 and p130 [84].

E7 only targets unphosphorylated, and thus active, pRb by forming an ubiquitinylation complex [85], resulting in deregulation of the cell cycle, loss of checkpoint controls, and uncoupling of keratinocyte differentiation from cell cycle progression.

Besides degradation of Rb-family members to trigger E2F mediated transcription, E7 also blocks E2F6, which recruits polycomb group (PcG) complexes, and acts as a transcriptional repressor [86]. Other targets of hr E7 include histone deacetylases to directly influence keratinocyte differentiation. Furthermore E7 can interact with the tumour suppressors p21 and p27 and block their inhibitory capacity against cyclin/cdk complexes [87, 88].

The N-terminus of E7 binds the pRb associated factor p600, allowing cells to grow anchorage-independently [89].

To overcome the host's immune system E7 can bind to p48 and IRF1 [90, 91], abrogating translocation of Signal Transducer and Activator of Transcription (STAT)1-STAT2 heterodimers to the nucleus.

As important mechanism for carcinogenic progression, E7 also induces DNA damage thus activating the Ataxia telangiectasia mutated (ATM) repair pathway with its downstream target checkpoint kinase 2 (CHK2). The latter was shown to be important for viral genome amplification but not for viral episome maintenance [92]. This observation suggests that E7 induces genomic instability while ensuring HPV genome amplification.

1.1.3.3 E5 oncoprotein

The E5 of hr HPV is a less potent oncogene than hr E6 and E7, but may augment their function and enhance tumour progression [93]. E5 is a small hydrophobic, membrane associated protein with 9 kD of size. It localizes to the endoplasmic reticulum of host cells where it exerts a variety of functions and is believed to be active in transporting proteins into the cytoplasm [94]. High-level expression in mouse models causes hyperproliferation in the epithelium and spontaneous malignant progression [46, 95].

E5 is also known to activate the epidermal growth factor receptor (EGFR). HPV E5 decreases ligand-induced down-regulation of EGFR leading to increased recycling of the receptor to the cell surface [54]. E5 also binds directly to the vacuolar ATPase and interferes with acidification of the endosomal compartment [94].

Another effect of E5 is the reduction of cell surface major histocompatibility complex class I (MHC I) proteins through direct interaction between its first hydrophobic domain and human leukocyte antigen (HLA) heavy chains, thus enabling cellular evasion of immune surveillance by the innate immune system [96]. The modulation of caveolin 1 levels and of the mitogen activated protein kinase (MAPK) signalling pathway are further functions [46].

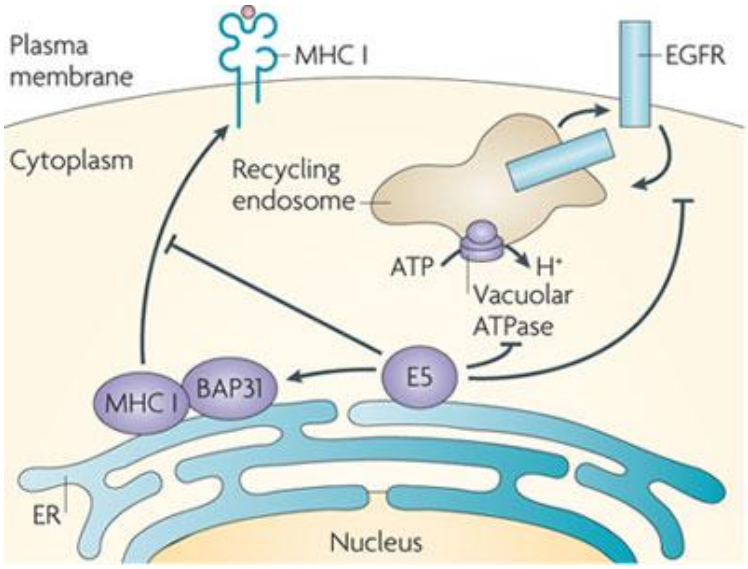

Figure 6: The E5 oncogene of HPV. Cellular pathways and proteins affected by HPV E5 Adapted from Moody CA, Laimins LA (2010) Nat. Rev. Can. 10: 550-560

1.1.4 HPV vaccines

Persistent infection with hr HPV is the necessary cause for developing CxC, most anal cancers and a subset of vulvar, vaginal, penile and oro-pharyngeal carcinomas [97-99]. Implementation of cervical screening programs has reduced CxC incidences and fatal outcome. Still the overall viral burden is not affected by these secondary prevention measures. Unfortunately there exists no effective anti-viral therapy to clear infection and HPV-induced lesions are removed by surgery or other ablative or destructive measures. Prophylactic vaccination against HPV as primary prevention measure has been shown to decrease new infections and development of disease. Preliminary data from population wide vaccination in Australia has shown a dramatic decline in genital warts and a tendency towards reduced incidence of cervical intraepithelial neoplasia (CIN) after few years in vaccinated young females [100], but not in older, unvaccinated women.

Therapeutic vaccination targets patients already infected and suffering from HPV associated pre-invasive disease, whereas prophylactic immunization provides type-restricted protection against infection with the types included in the vaccine, which is mainly antibody mediated, but has no direct effect on prevalent infection or disease. Furthermore the high cost of HPV vaccination hinders population-wide vaccination programs in low (and even high) income countries. Estimates suggest that it may take decades until HPV associated invasive cancers will decrease in vaccinated populations.

Therapeutic vaccination has the theoretical advantage of an immediate effect on viral burden and disease by inducing an effective cell-mediated immune response to clear infected host cells. Ideally target antigens are of viral origin, exclusively expressed in infected cells, play an essential role throughout transformation and immortalization, and are not subject to immunological tolerance. Furthermore the most efficient immunization route needs to be considered and whether important cells of the immune system like dendritic cells (DC), which are potent inducers of adaptive immune responses, are efficiently targeted. Finally safety issues are of concern, like genome integration for DNA vaccines or for certain live-based vector vaccines.

1.1.4.1 Prophylactic HPV vaccines

Molecular studies analysing the biology and pathogenesis of HPV have identified viral targets for prophylactic vaccination to prevent viral infection. The capsid proteins of HPV are suited as antigens to induce sterilizing immunity.

Currently two prophylactic vaccines are commercially available, composed of VLPs self-assembled from recombinantly expressed major capsid protein L1 [101]. The bivalent Cervarix® contains VLP of types 16 and 18, which together cause 70% of all CxC diagnosed worldwide, whereas quadrivalent Gardasil® includes VLPs of HPV 6, 11, 16 and 18. HPV 6 and 11 are two lr types responsible for 90% of genital warts [101]. Both vaccines utilize adjuvant: Cervarix® contains aluminium hydroxide and 3-O-desacyl-4'-monophosphoryl lipid (MPL), a toll like receptor (TLR) 4 agonist, whereas Gardasil® uses amorphous aluminium hydroxyphosphate sulphate. Both adjuvants are reported to stimulate a humoral immune response in patients together with specific interleukin (IL) 10 secreting $CD4^+$ T cells which have been shown to dampen potential cytotoxic T cell (CTL) responses [102]. Both vaccines induce systemic neutralizing immunoglobuline (Ig) G antibodies [101] with 50- to 100-fold higher titre than those observed in natural infections [103, 104]. These antibodies can also be detected in the vaginal fluid of vaccinated patients via active transport (exsudation) or passive transudation [101].

The major capsid protein L1 of PV is highly conserved among different types. However, regions of L1 that form outer loops in the assembled capsid remain highly variable between types and harbour conformational epitopes detected by neutralizing antibodies thus minimizing cross-neutralization between different genotypes. Thus each genotype generally comprises a distinct serotype. L1 VLPs are very immunogenic due to their closely packed array of neutralization epitopes, which allows B-cell receptor cross-linking and induction of high-avidity antibodies. VLPs are also readily taken up by antigen presenting cells (APCs), like myeloid or plasmacytoid DC, which facilitates presentation via MHC I and MHC II. Both prophylactic vaccines induce high-titre neutralizing antibodies and confer protection against the hr vaccine-types HPV16 and 18. Limited cross-protection can be found against closely related hr HPV31 and 45, respectively. However, with antibody titres being much lower, duration of cross-protection is

unsure [105]. To expand protection against additionally hr types causing the remaining 30% of CxC cases, clinical trials with a nona-valent vaccine including seven different hr HPV VLPs (16, 18, 31, 33, 45, 52 and 58) are ongoing. Possible disadvantages of this new vaccine are that production is technically demanding and high cost may further aggravate introduction in low economy settings.

Therefore other options to increase the vaccine spectrum are being exploited. The minor capsid protein L2 is of particular interest since it harbours conserved cross-neutralization epitopes (e.g.: a 20 aa peptide recognized by monoclonal antibody (mAb) RG1 which is essential for viral infection) in particular near its N-terminus [106]. However L2 is sub-dominant in co-assembled L1/L2 VLPs, as L1 is present in excess compared to L2 (ratio of 1:30 – 1:5). Our lab has recently shown that by genetic insertion and multimeric surface display of RG1, chimeric L1 VLPs induce a potent immune response against a broad variety of different HPV types [16, 107] suggesting the feasibility to develop a second generation broad-spectrum HPV vaccine.

1.1.4.2 Therapeutic HPV vaccines

A cell-mediated immune response is regarded essential to eradicate HPV infection and induced neoplasia, whereas a humoral immune response is not therapeutically effective. Consistent with this notion, HPV-induced dysplasias are more frequent in patients with suppressed cell-mediated immunity (e.g.: HIV, transplantation), in contrast to patients with B-cell deficiencies. Although VLP-vaccination induces robust T cell responses, these are insufficient to establish therapeutic activity, as expression of the viral capsid proteins L1 and L2, and HPV virion production are confined to highly differentiated cells of the superficial layers which are difficult for T cells to reach. Thus cellular immune responses to L1/L2 do not target persistently infected basal (stem) cells [108].

For therapeutic vaccines the oncoproteins E6 and E7 appear as suitable targets, since both are retained and expressed in infected benign and neoplastic cells, are essential for retaining a malignant phenotype, and are of viral origin which lowers the possibility of immunological host tolerance.

Various strategies of therapeutic immunization have been employed, utilizing different bacterial, viral or DNA vectors, purified proteins or whole cells for antigen delivery. To mention them all is beyond the scope of this work, but the most promising approaches have been summarized in this excellent review [109]. Yet representative examples will be given and both their advantages and downsides discussed. Although type-specific cell-mediated immune responses have been induced, efficacy has not been reported in prospective controlled studies.

1.1.4.2.1 DNA based therapeutic HPV vaccines

DNA based therapeutic HPV vaccination has become an attractive strategy. DNA vaccines are temperature stable and have a long shelf life, which facilitates storage and transport [110]. Furthermore repeated vaccine application does not reduce efficacy and adverse effects are rare [111]. They are well suited for large scale production at high purity and can consist of sequences coding for multiple characterized epitopes of the immunogen of choice [111].

On the other hand DNA vaccines lack the ability to spread widely in the host, and APCs, which are the major target cells to induce an immune response, are not specifically targeted. To overcome these shortcomings, different strategies have been tried with variable success, either using various administration routes like intradermal gene gun injection or electroporation for enhancing intrinsic specificity [112, 113] through Langerhans cell-uptake, or linking the antigen-encoding part to a transport molecule sequence like herpes simplex virus 1 protein VP22 to increase vaccine spreading [114, 115]. The possibility of DNA integration into the genome is of further concern.

Several DNA based HPV vaccines have been tested in clinical trials. A Phase I trial tested a DNA vaccine encoding HPV16 E7 (with the pRb binding site abolished) linked to the heat shock protein (hsp) 70 of *Mycobacterium tuberculosis*. E7 specific CTL responses were detected in patients receiving the highest doses [116], and the vaccine was well tolerated.

ZYC-101© is a microencapsulated DNA vaccine coding for E7 derived epitopes in the context of HLA-A2. When tested in patients with CIN2/3, one third showed

histological responses, and two thirds mounted specific T cell responses against the encoded epitopes [117].

1.1.4.2.2 Protein based therapeutic HPV vaccines

Short peptide vaccines have limited capacity to induce cell-mediated immunity, since immune responses are restricted by the patient's HLA type. Larger protein based vaccines may circumvent this problem. They become processed and presented by APCs and thus contain all possible MHC related epitopes of the antigen used, and are easily produced, stable and safe.

A drawback for protein vaccines is their generally low immunogenicity, which led to creation of fusion proteins and adjuvant strategies to overcome this limitation. Since proteins are exogenously administered the probability to encounter APCs to establish an immune response is low. To increase protein uptake by APC, fusion proteins have been generated, for example using adenyl cyclase of *Bordatella pertussis*, or the translocation domain of *Pseudomonas aeruginosa* exotoxin A, both known to target APCs [118, 119], or hsp [116]. Further, protein based vaccines are better suited to elicit humoral compared to cell-mediated immune responses, the latter of which is regarded essential for therapeutic efficacy against infected cells. Thus different adjuvants have been tested like ISOCOMATRIX [120] or liposome-polycation-DNA adjuvant [121] to induce preferentially cell-mediated immune responses.

Various therapeutic HPV protein vaccines have translated into clinical trials. For example immunization with an HPV16 E6E7 fusion protein plus ISOCOMATRIX adjuvant was shown to elicit increased CTL responses to E6 and E7 compared to placebo groups [122]. Another vaccine, termed PD-E7, consisting of HPV16 E7 fused to the protein D fragment of *Haemphilus Influenzae* was administered with adjuvant AS02B to CIN-1 and CIN-3 patients, and induced significant E7-specific CTL responses [123]. An HPV16 L2-E6-E7 fusion-protein, termed TA-CIN, elicited robust neutralizing antibody titres and IFN-γ producing CTLs only when administered subcutaneously (s.c.) with the adjuvant GPI-0100 [124].

1.1.4.2.3 Viral vectors used for therapeutic HPV vaccines

Live vectors used to deliver therapeutic HPV vaccines include both viral vectors and bacterial vectors like attenuated *Listeria monocytogenes* or *Salmonella*. For the latter see [125] for review.

Viral vaccine vectors usually provide high immunogenicity to the transgene and facilitate vaccine antigen spreading. Vectors that have been commonly used include vaccinia virus, adenovirus, or sindbis virus [126]. Because live vectors are immunogenic by themselves, the induction of neutralizing antibodies may reduce efficacy of repeat immunizations. Furthermore attenuated life vectors may replicate uncontrolled in immunosuppressed patients and, for viruses with DNA stages during their life cycle, the viral genome may integrate into the host chromosome risking tumorigenesis.

In patients with CIN clinical studies have been performed with a recombinant vaccinia virus, termed TA-HPV, expressing fusion proteins of HPV16 E6E7 and HPV18 E6E7. HPV specific CTLs and serological responses were already found after the first vaccination [127]. Another recombinant vaccinia vector, encoding a fusion of HPV16 E2 and E7, was tested in patients with CIN, who developed E7-specific CTL responses and antibodies [128]. Preclinical studies have been performed with replication-deficient adenovirus delivering a CRT/E7 fusion-protein. In vaccinated mice a therapeutic effect against TC-1 tumours and long-term immunologic memory was induced [129].

Although not a live vector approach, VLPs have been utilized as delivery system for therapeutic vaccination. For example, recombinant L1-E7 fusion-protein assembles into VLPs that induce E7 specific CTL responses [130]. In another VLP-based approach animals were immunized with HPV16 L1/L2-E7 VLPs, in which E7 was fused to the minor capsid protein L2. Vaccinated mice produced strong CTL responses against E7 and were protected against challenge with E7 harbouring tumour cells [131]. VLPs have also been used as delivery tools for therapeutic DNA vaccines [132].

1.2 Influenza A virus

In this thesis work a live viral vector-based approach was used utilizing influenza A virus as therapeutic vaccine delivery system. Influenza A virus belongs to the family of *Orthomyxoviridae* which also includes influenza genera B and C [133]. Whereas types B and C are mostly isolated from humans, influenza A viruses also circulate in avian, horses, dogs, cats and swine [134]. Interest in influenza A research has been raised after the latest pandemic flu outbreaks in 2003 (avian-flu H5N1) [135] and in 2009 (swine-flu H1N1) [136]. Influenza virus is enveloped by a lipid membrane derived from host cells in which the virus replicates. The viral shell consists of the matrix protein M1 and three viral proteins, hemagglutinin (HA), neuraminidase (NA) and matrix 2 (M2), embedded into it [133]. Its genome consists of eight single stranded, negative sense RNA segments, each of which encode for one or two proteins [137]. Each viral RNA segment is encapsidated by ribonucleoproteins forming ribonucleoprotein complexes (vRNPs) [138]. Nuclear export protein (NEP) also is present in the virion in small amounts. Influenza A viruses are further typed by their respective HA and NA surface glycoproteins. There are 16 different HA (1-16) and 9 different NA (1-9) known [133]. Host specificity is obtained by HA, which is the primary recognition receptor of influenza A. HA targets cell surface sialic acids, which are nine-carbon acidic monosaccharides linked via its carbon-2 either to the carbon-3 or carbon-6 of galactose forming an α-2,3- or an α-2,6-linkage. Whereas the α-2,3-linkage is more common in avian species, the α-2,6-linkage predominates in human tracheal epithelium. HA molecules 1, 2 and 3 bind preferentially to α-2,6-sialic acid whereas the remaining types are restricted to α-2,3-sialic acid [139]. Since swine epithelium expresses both types of cell surface sialic acids, both avian and human types can infect the animal at the same time, which can lead to rearrangement of viral RNA

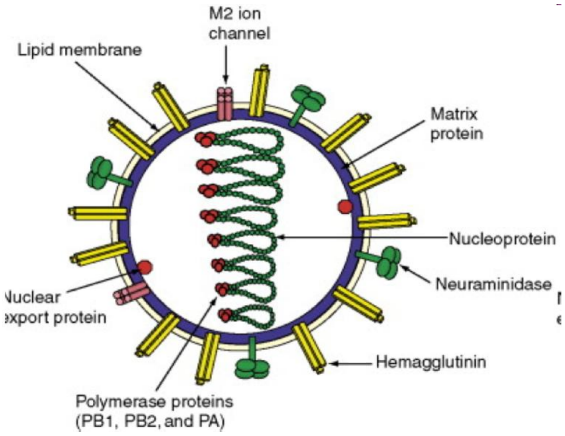

Figure 7: Structure of the Influenza A particle Adapted from Shaw M.L., Palese P. (2008) Orthomyxoviruses: Molecular Biology/Encyclopedia of Virology

segments generating viruses with completely novel antigenic properties and pandemic potential [140]. This event is termed antigenic shift. Another known host range determinant is the viral polymerase [141]

1.2.1 Influenza A genome

Eight viral negative strand RNA segments, numbered in the order of decreasing length, encode at least 10 different genes (Table 1). HA is encoded on segment 4, NA on segment 6, nucleoprotein (NP) on segment 5, non-structural protein1 (NS1) and NEP (also known as NS2) on segment 8, M1 and M2 are encoded on segment 7 by RNA splicing, polymerase acidic protein (PA) on segment 3, polymerase basic protein 1 (PB1) on segment 2 and polymerase basic protein 2 (PB2) is encoded on segment 1 [137]. Two additional viral proteins are polymerase basic protein 1-F2 (PB1-F2), which is encoded on segment 2 via a +1 alternate reading frame, and polymerase basic protein 1-N40 (PB1 N40) on segment 1, although it has been shown that not all influenza A viruses encode these [142, 143]. The extreme ends of all segments are highly conserved and partially complementary to each other, thus allowing base pairing which serves as promoter for viral RNA (vRNA) replication [144]. Viral transcription depends on base pairing of vRNA and host mRNA. Each vRNA segment has non-coding sequences of varying length, which include packaging signals and a stretch of five to seven uridines located approximately 16 nucleotides from the 5' end,

Segment	Length in nucleotides	Encoded protein(s)	Protein function
1	2341	PB2	Polymerase subunit, cap recognition
2	2341	PB1	Polymerase subunit, endonuclease, RNA elongation
		PB1-F2	Pro-apoptotic activity
3	2233	PA	Polymerase subunit, protease
4	1778	HA	Surface glycoprotein, receptor binding, membrane fusion
5	1565	NP	RNA binding protein, nuclear import, replication
6	1413	NA	Surface glycoprotein, virus release
7	1027	M1	Matrix protein, viral budding, nuclear export
		M2	Ion channel, virus assembly
8	890	NS1	Interferon antagonist protein
		NEP	Nuclear export of RNA

Table 1: The gene segments of influenza A PR8/34 and their encoded proteins [138].

which act as polyadenylation signals [145]. The vRNA is encapsidated with NP, which interacts with its phosphate backbone, with the nucleotide bases being exposed and the whole ribonucleoprotein (RNP) forming a double helical structure with a stem loop [146]. RNPs consist of the RNA segments, which form helical hairpin loops, bound by the heterotrimeric RNA polymerase complex consisting of PA, PB1 and PB2 [147]. The remaining vRNA is coated with the positively charged NP protein [147].

1.2.2 Influenza A as vaccine vector

Reverse genetic techniques have allowed the rescue of influenza A virus from plasmid DNA and to insert mutations into its genome [148]. For influenza to serve as a safe vaccine vector the virus needs to be attenuated without impairing infectivity and antigen expression. Of all viral proteins, only two, namely NS1 and PB1-F2, are absent in virions and become exclusively expressed in infected cells [149]. Two major functions of NS1, a 237 aa non-structural protein, are to enhance viral replication and, more importantly, to interfere with the cellular immune system. The latter is accomplished through various effector functions. NS1 antagonizes IFN-β induction, which normally enables the cell to mount an antiviral response, by preventing activation of transcription factors IRF-3, NFκB and c-Jun/ATF-2 [150, 151]. Furthermore NS1 binds to the pathogen sensor polymerase acidic protein (RIG-1) in a viral RNA-dependent mechanism [152]. By complexing with nuclear export factors, like NXF1, p15 or Nup98, NS1 inhibits nucleo-cytoplasmic transport of host mRNAs [153]. Other host sensors for dsRNA that are targeted by NS1 include dsRNA-dependent PKR and 2'-5'-oligoadenylate synthetase (OAS) [154, 155]. Many other effector functions have been described for NS1 (see [156] for review). Partial deletion of NS1 (ΔNS1) thus leads to attenuation of influenza A virus in an IFN-β competent background without impairing infectivity and viral gene transcription in such cells [149]. In addition attenuated viruses prove to be more immunogenic because NS1 is also known to impair maturation, migration or cytokine production of DC, besides impairment of induction of type 1 IFNs [157]. Safety of viruses with truncated NS1 was analysed in various models like mice [158], swine [159], horses [160], birds

[161], a primate model [162] and in humans [163]. To generate an influenza A based vaccine vector the reading frame of NS1 can be partially replaced by foreign gene of interest, without disrupting expression of the NEP gene, located on the same segment as NS1. In contrast to NS1, NEP is essential for virus infection. Influenza A virus offers several advantages compared to other live-based vectors. It induces strong cell-mediated immune responses and belongs to the family of RNA viruses that do not have any DNA intermediates during their life cycle, thus excluding viral integration into the host genome. The challenge one has to overcome when using live-based vectors is the immune response against the carrier itself attenuating booster immunizations. For influenza A different serotypes for multiple vaccine administrations are available. The feasibility of this system has been demonstrated in studies against cancer [164, 165], tuberculosis [166, 167] and HIV-1 [168]. Influenza vectors that express immunostimulatory cytokines are alternative options of optimized vector usage [169].

2 Aim of this study

Although licensed prophylactic HPV vaccines are highly effective against infection and associated disease for naïve patients, reducing the incidence of genital neoplasia in the population will take years and will be restricted to the two hr types included in the licensed vaccines (and additionally two lr types included in the quadrivalent vaccine). Therapeutic HPV vaccination could benefit patients already infected and with associated neoplasia. Despite considerable efforts no effective therapeutic vaccinations have been reported. To induce regression of HPV-induced epithelial neoplasia, vaccines need to induce potent cell-mediated immune responses directed specifically against HPV infected cells. The early genes E6 and E7 encode the major viral oncoproteins that are retained and expressed in epithelial lesions and invasive cancers and thus may serve as appropriate target antigens for immuno-therapeutic strategies.

Influenza A virus infection is known to induce robust CTL responses, thus we decided to produce attenuated, recombinant influenza A vectors expressing a fusion protein of either wt or mutated HPV16 E6 and E7 oncogenes. To enhance transgene expression the splice-donor site in the remaining coding part of NS1 was mutated. Following functional verification of four recombinant viruses, therapeutic and prophylactic vaccine efficacies were analysed in a mouse tumour model. Prior to or after vaccination, syngenic TC-1 cells stably transformed by and expressing HPV16 E6 and E7 (and h-ras) were injected s.c. into C57BL/6 mice, and tumour size and growth kinetics were used as primary endpoints. As secondary endpoint the in vivo immune response was analysed by peptide based IFN-γ ELISpot, proliferation studies and peptide based ELISA.

3 Materials and methods

3.1 Cells

The murine TC-1 tumour cell line, which was generated through stable transfection of C57BL/6 lung cells with HPV16 E6, E7 and h-ras, was a kind gift from T.C. Wu (Johns Hopkins, University of Baltimore) [170], and was maintained in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin and 0,4 mg/ml G418. The C3 cell line, a tumorigenic HPV16 EJ-ras transformed C57BL/6 mouse embryo cell line, was a kind gift from Peter Öhlschläger (University Konstanz) and was cultivated in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, 0.8 mg/ml G418 and 0.1 mg/ml kanamycin. The type I IFN deficient Vero cell line, derived from kidney epithelial cells of the African green monkey, was provided by Avir Greenhills Biotech Austria and was cultivated in serum-free Opti-Pro supplemented with penicillin, streptomycin and L-glutamine. The human embryonic kidney cell line 293TT, generated by stable transfection of whole SV40 genome and a SV40 large T antigen cDNA cassette, was cultivated in DMEM supplemented with 10% FCS, penicillin and streptomycin. The human cervical carcinoma cell line CaSki was maintained in RPMI 1640 supplemented with 10% FCS, penicillin and streptomycin. The human melanoma cell line UACC62 was kindly provided by G. Pathria (Medical University Vienna) and cultivated in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin and 0.4 mg/ml G418. All mammalian cell lines were cultivated at 37°C, 5% CO_2 and 95% humidity. Sf9 insect cells were cultivated with Grace's medium supplemented with 5% FCS and 0.5% Pluronic at 27 °C. All cell culture reagents by Gibco Life Technologies.

3.2 Generation of HPV16 E6E7FL and E6E7mFL fusion genes

An HPV16 E6E7FL fusion gene with wt ORF's and a flag tag (FL) was designed and synthesized (GeneArt, Life Technologies). For safety considerations a similar fusion-construct with mutated E6 and E7 genes was also generated (HPV16

E6E7mFL). For the latter codons encoding HPV16 E6 amino acids (aa) 73-77 and aa 146-151, and for HPV16 E7 aa 21-24, were deleted, with all deletions reported to abrogate biological activities of these oncoproteins [68, 82, 171]. The synthetic fusion genes were subcloned into a pHW2000 derivative [172], containing a mutated NS segment under the control of a Pol I and Pol II promoter for vRNA and mRNA expression respectively. The NS1 reading frame was truncated to 14 N-terminal aa, thus resulting in a fusion gene encoding NS1-14, HPV16 E6, E7 (either wt or mutated) and FL.

3.3 Generation and isolation of recombinant influenza A viruses

Recombinant influenza A viruses were obtained as previously described [173]. The internal segments have been derived from the IVR-116 vaccine strain (WHO) that originated from A/Puerto Rico/8/34 for PA, PB2, NP and M, and A/Texas/1/77 for PB1. The HAs and NAs used were A/New Caledonia/20/99 for H1N1 and A/Aichi/2/68 for H3N2. Ninety percent confluent Vero cells were co-transfected with 0.5 µg of plasmid pHW-delNS-16E6E7FL or pHW-delNS-16E6E7mFL, expressing both vRNA and mRNA of the recombinant NS gene with either wt or mutated HPV16 E6 and E7, and each 0.5 µg of bidirectional plasmids encoding the remaining seven segments, using the Nucleofection technique (Amaxa) according to the manufacturer's instruction manual. Recombinant influenza A viruses were then collected with the supernatant after cultivation for 4-5 days.

3.4 Transgene stability

To verify transgene stability (in the ΔNS1 reading frame), recombinant influenza viruses were passaged in Vero cells for at least five cycles, viral RNA isolated using the QIAamp viral RNA mini kit (Qiagen) and subjected to RT-PCR using the uni-12 primer: 5'-AGC AAA AGC AGG-3' (VBC Biotech) for cDNA generation of viral RNA. To amplify the wt and mutated NS1-16E6E7 fusion genes in recombinant viruses the following primers for PCR were used after cDNA

generation: Forward: 5´-AGC AAA AGC AGG GTG ACA AAG-3', Reverse: 5´-CTC TTG CTC CAC TTC AAG C-3'.

3.5 Modification of the viral NS1 splice donor site

To enhance transgene expression the splice donor site was mutagenized according to [174], and pHW-delNS-16E6E7FL or pHW-delNS-16E6E7mFL plasmids were used as template for PCR directed mutagenesis, respectively.
Primers used were: Forward: 5'-TGT GTC AAG CTT TCA GGT ATT TGC CGC AAT-3', Reverse: 5'-CTG TGT TAT CAT TCC ATT CAA GTC C-3' (VBC Biotech). To minimize mutations during elongation, phusion™ proofreading polymerase (Finnzymes) was used according to manufacturer's advice. The amplimer was purified via agarose gel electrophoresis, isolated using the Qiaquick kit (Qiagen) and blunt end subcloned into pCR2.1 using the TOPO-cloning procedure (Invitrogen) generating pCR2.1-16E6E7FLsplice and pCR2.1-16E6E7mFLsplice vectors. Digestion of pHW-delNS-16E6E7FL and both pCR2.1-16 E6E7FLsplice and pCR2.1-16E6E7mFLsplice with *Xcm I* (New England Biolabs) and *Hind III* (Roche) and subsequent purification was performed to receive sticky ends, fragments were gel purified and ligated into digested pHW-delNS-16E6E7FL using T4 DNA ligase (Roche). The final products pHW-delNS-16E6E7FLsplice and pHW-delNS-16E6E7mFLsplice were then transformed into bacteria, plasmids isolated and used for recombinant influenza virus generation resulting in 6 recombinant influenza A viruses: H1N1 ΔNS1 16E6E7FLsplice (H1N1 16E6E7), H1N1 ΔNS1 16E6E7mFLsplice (H1N1 16E6E7m), H1N1 ΔNS1 (parental virus), H3N2 ΔNS1 16E6E7FLsplice (H3N2 16E6E7), H3N2 ΔNS1 16E6E7mFLsplice (H3N2 16E6E7m) and H3N2 ΔNS1 (parental virus).

3.6 Bacterial transformation, plasmid purification and nucleotide sequencing

BL21 competent *E. coli* (Merck) were transformed by incubating with the desired plasmid on ice, heat shock for 30 seconds at 42 °C and plated with antibiotic

selection on lucia broth agar plates. Plasmid preparations of cultures were done using the Plasmid mini and maxi kits (Qiagen). DNA sequence analysis was performed by VBC Biotech using the following primers: Forward: 5'-GTT AGA TTT GCA ACC-3', Reverse: 5'-GTT CTA ATG TTG TTC C-3'.

3.7 Production of high titre recombinant influenza A virus stocks

Vero cells were seeded at a density of 30.000 cells /cm² in 125 cm² tissue culture flasks, cultivated for 24 h and infected with recombinant virus at multiplicity of infection (moi) of 0.001 in Opti Pro supplemented with 20 mM L-glutamine, 5 µg/ml trypsin and 0,5 µg/ml amphotericin B (both Sigma) in a total volume of 5 ml at 37 °C for 1 ½ h. Culture medium was added to a final volume of 25 ml and infected cells cultivated for 48 h observing cell lysis after 24 h. Virus titers were determined in Vero cells in medium containing Opti-Pro, 20 mM L-glutamine, 5 µg/ml trypsin and 0,5 µg/ml amphotericin B (Sigma) by endpoint dilution assay. Vero cells were seeded at a density of $1{,}5 \times 10^4$ cells/well the day before infection. Viruses were diluted in the range of 10^{-3} to 10^{-9}, cells infected for 3 days and analysed for cytopathic effects using a confocal light microscope. Virus titres were calculated as 50% tissue culture infectious dose per millilitre ($TCID_{50}$/ml) by means of the Reed and Muench method [175].

3.8 Transgene expression

To verify transgene mRNA expression, Vero cells were grown to subconfluency in 6 well plates, infected with recombinant influenza A viruses (10^5 plaque forming units (pfu)) and incubated for 12 hours. Cells were lysed using TRI-Reagent (Sigma Aldrich) and total RNA was isolated performing phase separation by adding chloroform. RNA in the aqueous phase was precipitated with isopropanol, washed with 70% ethanol, resuspended in RNase free water and subjected to RT-PCR analysis. For cDNA generation of mRNA first strand cDNA synthesis kit (Roche) was used with poly dT primers, thus transcribing only mRNA since the viral RNA-genome lacks a polyA tail. Subsequently PCR for amplification of the HPV16 E6E7 fusion was performed using primers: Forward:

5'-ATG TTT CAG GAC CCA CAG GAG CGA-3', Reverse: 5'-TTT ATC GTC ATC GTC CTT GTA GTC-3' (VBC Biotech).

For protein analysis, Vero cells were grown to subconfluency in the presence of 10 µM MG-132 dissolved in dimethyl sulfoxide (DMSO) for 12 hours, infected with recombinant influenza A viruses (10^5 pfu) and incubated for 12 hours in the presence of MG-132. Cells were harvested, lysed in laemmli-sample buffer and boiled for 5 min.

3.9 Oncogene expression and stability in TC-1 cells

TC-1 cells, or Caski, C3 and 293TT as controls were grown in 60 mm cell culture dishes until confluent and lysed with TRI-Reagent. RNA extraction was performed as described above. To verify HPV16 E6 and E7 expression RT-PCR as described was performed with isolated RNA using the following primers: Forward: 5'-GGA CCC ACA GGA GC-3', Reverse: 5'-CCG GTC CAC CGA CC-3' for E6; Forward: 5'-GCA ACC AGA GAC AAC TG-3', Reverse: 5'-CAC AAT TCC TAG TGT GCC C-3' for E7. For DNA isolation DNA was precipitated in the phenol-chloroform phase by the addition of pure ethanol pro analysis. After centrifugation DNA was washed two times with 0.1 M trisodium citrate in 10% ethanol and once with 70% ethanol. The pellet was briefly air-dried, resuspended in nuclease free water and subjected to PCR methods. To analyse transgene stability cellular DNA was used as template for PCR. Amplification of HPV16 E6 and E7 was performed using Taq polymerase (Promega) and the respective primer pairs: Forward: 5'-GGA CCC ACA GGA GC-3', Reverse: 5'-CCG GTC CAC CGA CC-3' for E6; Forward: 5'-GCA ACC AGA GAC AAC TG-3', Reverse: 5'-CAC AAT TCC TAG TGT GCC C-3' for E7

3.10 Generation of chimeric HPV16 L1/L2-E7 recombinant baculovirus

The double expression plasmid encoding the wt HPV16 L1 and L2-E7 fusion proteins was a kind gift of John T. Schiller (National Cancer Institute, Bethesda).

Recombinant baculovirus stocks were generated by co-transfection of Sf9 cells with baculovirus DNA (Baculo-Gold, BD Biosciences) using Lipofectin (Invitrogen). Plaque purification and high titre production was performed as previously described [34].

3.11 Co-expression of HPV16 L1 and chimeric L2-E7 proteins in insect cells

Sf9 cells were infected at a moi of ~10 with recombinant HPV16 L1+L2-E7 double expressing baculoviruses. After 72 hours cells were lysed by boiling in SDS sample buffer and analysed by SDS/PAGE and Coomassie stain or western blotting.

3.12 Purification of chimeric VLPs and transmission electron microscopy

Purification of VLP by cesium chloride (CsCl) gradient has been described previously [34]. In brief $1{,}5x10^8$ Sf9 cells were infected with recombinant baculovirus at high moi, seeded in 10 245x245 mm tissue culture dishes, harvested after 72 hours, shock frozen with liquid nitrogen, resuspended in PBS containing 0.8 M NaCl, 2 mM $CaCl_2$ and 1 mM PMSF, homogenized by sonication and incubated over night at 4 °C after the addition of Brij58 (Sigma Aldrich) to 0.5% final concentration. The suspension was then centrifuged at 12,000 g, 4 °C for 40 min, the supernatants were loaded onto 35% (wt/vol) sucrose/PBS cushions supplemented with 0.5 M NaCl and 0.1% Brij58, and centrifuged for 2.5 h at 110,000 g at 4 °C. The resulting pellet was resuspended in 29% (wt/wt) CsCl/PBS supplemented with 0.05% Brij58, sonicated and ultracentrifuged (Beckmann) at ~300,000 g at 4 °C for 48 hours. The resulting visible band was collected and again centrifuged in 29% (wt/wt) CsCl/PBS supplemented with 0.05% Brij58 at ~270,000 g at 4 °C for 72 hours. The band containing VLP was again collected and dialyzed over night against PBS containing 0.5 M NaCl, 1 mM $CaCl_2$ and 0.01% Tween80 (Thermo Scientific).

Particles were then adsorbed to glow-discharged carbon-coated copper grids, fixed with 2.5% glutaraldehyde, negatively stained with 1% uranyl acetate and analysed on a JEOL 1010 transmission electron microscope (TEM) at 80 kV with 30,000 fold magnification.

3.13 Antibodies

Antibodies used in this study were mouse α-Influenza A NP blend clone A1/A3 (Millipore, 1:1,000 dilution), mouse α-Flag clone M2 (Sigma, 1:5,000 dilution), mouse α-HPV16 E7 ED17 (Santa Cruz, 1:200 dilution), mouse α-HPV16 E7 8C9 (Invitrogen, 1:150 dilution), mouse α-HPV16 E6 6F4 (Euromedex, 1:200 dilution), rabbit α-HPV16 L2 $_{(11\text{-}200)}$ (described in [107], 1:5,000 dilution), mouse α-HPV16 L1 Camvir-1 (BD Biosciences, 1:5,000 dilution), mouse α-pRb (1:500 dilution), rabbit α-GAPDH (polyclonal, Santa Cruz, 1:1,000 dilution), goat α-rabbit horse radish peroxidase (HRP) and goat α-mouse HRP (both Biorad,1:25,000).

3.14 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Proteins were boiled for 5 min in Laemmli-sample buffer supplemented with 2% β-mercaptoethanol (Sigma), separated on a 10 to 15% gradient SDS-tris-glycine gel (Biorad) using the Tris-glycine buffer system and western blotting was performed by electrophoretic transfer of proteins to a polyvinyl difluoride membrane (Millipore) for 1 hour at 45 V. The blot was incubated with 5% milk, 0.1% Tween20 (both Biorad) in PBS for 1 hour at room temperature, rinsed once with PBS 0.1% Tween20 and incubated with primary antibody, diluted in 3% BSA, 0.1% Tween20 in PBS, over-night at 4 °C. The blot then was washed three times with PBS 0.1% Tween20 and incubated with the corresponding HRP conjugated secondary antibody diluted in 5% milk, 0.1% Tween20 in PBS for 1 hour at room temperature, washed three times and the membrane developed by using chemiluminescent substrate (Thermo Scientific Pierce) and exposed to x-ray film (Amersham Hyperfilm™ ECL, GE-Healthcare).

3.15 Animals

Six to eight weeks old C57BL/6 female mice were obtained from Charles River Laboratories (Germany) and used for ELISPOT assay, proliferation studies, ELISA studies and vaccination studies. Animals were kept under conventional conditions at the Institute of Biomedical Research of the Medical University Vienna and were provided with a standard diet and water ad libitum approved by the ethics committee of the Medical University Vienna, and Federal Ministry of Science and Research (*GZ66.0099/0289-II/10b/2008, GZ66.0099/0057-II/3b/2013*).

3.16 Prophylactic immunization studies in mice

Animals were randomly divided into 5 groups of 8. Group 1 and 2 were primed s.c. at the skin fold of the neck with H1N1 16E6E7 or 16E6E7m (3.75 log $TCID_{50}$ in 500 µl) respectively, group 3 with the same quantity parental H1N1 virus, group 4 with 125 µg HPV 16 L1/L2-E7 VLPs and group 5 with PBS on day 0. Mice were boosted s.c. on day 20 with H3N2 16E6E7 (3.75 log $TCID_{50}$ in 500 µl/mouse) for group 1 and H3N2 16E6E7m for group 2, parental H3N2 virus for group 3, 60 µg HPV16 L1/L2-E7 VLPs for group 4 and PBS for group 5. Animals were challenged on day 30 with 5_x10^4 TC-1 cells s.c. into the right flank and tumour growth was monitored once per week, diameters measured and volumes calculated according to the formula $V=4/3*\pi*r^3$; $(r=d/2)$. Tumour size of mice, that had to be euthanized due to severe side effects of TC-1 tumour burden, prior to the study endpoint, was calculated as tumour growth rate per day using the formula: Mean tumour growth/day = (tumour diameter measurement 1/elapsed days + tumour diameter measurement 2/elapsed days + tumour diameter measurement 3/elapsed days)/3.

3.17 Therapeutic immunization studies in mice

Animals were randomly divided into 5 groups of 8 and s.c. challenged with 5_x10^4 TC-1 tumour cells into the right flank on day 0. Mice were primed s.c. at the skin fold of the neck on the same day with either H1N1 16E6E7 or 16E6E7m for groups 1 or 2 (3.75 log $TCID_{50}$ in 500 µl/mouse), parental H1N1 virus for group 3, 125 µg HPV16 L1/L2-E7 VLP (group 4) or PBS (group 5). Animals of groups 1-3 were boosted with the corresponding H3N2 serotypes (3.75 log $TCID_{50}$ in 500 µl/mouse), or 60 µg VLP for group 4, or PBS for group 5 on day 10 after TC-1 inoculation. Tumour diameters were measured once per week and volumes calculated as in 3.16. Tumour size of mice that had to be euthanized prior to study endpoint was calculated as in 3.16.

3.18 Therapeutic immunization studies with established TC-1 tumours in mice

Animals were randomly divided into 5 groups of 8, s.c. challenged with 5_x10^4 TC-1 tumour cells into the right flank on day 0 and tumour development monitored twice per week. As soon as more than 50% of animals of each group had developed palpable tumours, mice were primed s.c. at the skin fold of the neck with either H1N1 16E6E7 or 16E6E7m for groups 1 or 2 (3.75 log $TCID_{50}$ in 500 µl/mouse), parental H1N1 virus for group 3, 125 µg HPV16 L1/L2-E7 VLP or PBS. Ten days after priming animals of groups 1-3 were boosted with the corresponding H3N2 serotypes (3.75 log $TCID_{50}$ in 500 µl/mouse), or 60 µg VLP for group 4, or PBS for group 5. Tumour growth was monitored once per week and volumes calculated from tumour diameter as in 3.16. Tumour size of mice that had to be euthanized prior to study endpoint was calculated as in 3.16.

3.19 Intranasal (i.n.) and intratumoral (i.t.) therapeutic vaccination studies in mice

Animals were randomly divided into 4 groups of 8, s.c. challenged with 5_x10^4 TC-1 tumour cells into the right flank on day 0 and tumour development monitored twice per week. As soon as more than 50% of animals of each group had developed palpable tumours mice were primed with either H1N1 16E6E7 or 16E6E7m for groups 1 or 2, parental H1N1 virus for group 3, or PBS for group 4 i.t. or i.n.. Due to constraints of application volume mice received twice 250 µl (3.75 log $TCID_{50}$ in 500 µl) of recombinant influenza viruses i.t. for two days, resulting in a total application volume of 500 µl/mouse, or 3 times 50 µl/per day (3.75 log $TCID_{50}$ in 500 µl) for two days i.n. resulting in a final application volume of 300 µl/mouse. Ten days after priming animals of groups 1-3 were boosted with the corresponding H3N2 serotypes, or PBS for group 4, in the same application manner as for priming. Tumour growth was monitored once per week and volumes calculated from tumour diameter as in 3.16. Tumour size of mice that had to be euthanized prior to study endpoint was calculated as in 3.16.

3.20 Immunization studies in mice

Animals were randomly divided into 5 groups of 4. Group 1 was primed s.c. at the skin fold of the neck with H1N1 16E6E7 (3.75 log $TCID_{50}$ in 500 µl/mouse), group 2 with H1N1 16E6E7mFL (3.75 log $TCID_{50}$ in 500 µl/mouse), group 3 with (3.75 log $TCID_{50}$ in 500 µl/mouse) parental, group 4 with 125 µg HPV16 L1/L2-E7 VLPs and group 5 with PBS on day 0. Mice were boosted s.c. on day 10 with H3N2 16 E6E7 (3.75 log $TCID_{50}$ in 500 µl/mouse) for group 1, H3N2 16E6E7m for group 2 (3.75 log $TCID_{50}$ in 500 µl/mouse), H3N2 parental virus for group 3, 60 µg HPV 16 L1/L2-E7 VLPs for group 4 and PBS for group 5. Animals were sacrificed by heart puncture under anaesthesia (6 mg/ml Ketamin, 1 mg/ml Rompun, 3 mg Ketamin, 0.5 mg Rompun per mouse) on day 20 or day 40 (for long term assessment) after priming and serum and spleens isolated for further experiments.

Mice immunized i.t. and i.n. were immunized as described in 3.19. and sacrificed 10 days after boosting by heart puncture under anaesthesia and serum and spleens isolated for further experiments.

3.21 Enzyme-linked immune spot (ELISpot) assay

The E6/E7-specific CTL response in mice was tested ten days after the second immunization. Spleens were collected and dissociated into single cell suspension using cell strainers (Falcon). Erythrocytes were lysed with 150 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA pH 7.4 and the splenocytes washed and resuspended in RPMI 1640 containing 10% FCS (Sigma Aldrich), penicillin, streptomycin, non-essential amino acids (Gibco Life Technologies), sodium-pyruvate and 50 µM β-mercaptoethanol (Gibco Life Technologies). Cell suspensions of 10^5, 5_x10^5 and 10^6 cells/well in a final volume of 250 µl were added to 96-well plates with nitrocellulose base (Millipore), which were pre-wetted with 30% ethanol and pre-coated with IFN-γ capture antibody according to manufacturer's advice (eBioscience). Cells were incubated for 24 hours either in presence or absence of 5 µM of peptides HPV16 $E6_{41-50}$, HPV16 $E6_{91-100}$, HPV16 $E6_{131-140}$ [176], HPV16 $E7_{36-62}$ [177], comprising characterized H-2^b restricted $CD4^+$ ($E6_{41-50}$, $E6_{91-100}$) or $CD8^+$ ($E6_{131-140}$, $E7_{36-62}$) T cell epitopes, scrambled control peptides, 5 µM influenza A H1N1 $NP_{311-325}$, HPV16 $L1_{165-173}$ [178] (all Severn Biotech Ltd.) or 2 µg/ml final concentration of *S. aureus* enterotoxin A (SEA, Sigma Aldrich). Biotinylated anti-IFN-γ monoclonal antibodies (eBioscience) were then used as conjugate, and plates incubated with avidin-HRP (eBioscience). Spots representing IFN-γ producing cells were developed utilizing 3-amino-9-ethylcarbazole containing hydrogen peroxide in 0.1 M sodium acetate, pH 5.0 (Dako). Spots were counted using a dissecting microscope and results are expressed as mean number of spot forming cells ± SD of triplicate cultures.

3.22 Proliferation assay

Proliferation of T cells was analysed ten days after the second immunization. Splenocytes isolated as described above, were incubated with or without 5 µM of peptides HPV16 $E6_{41-50}$ (CD4), HPV16 $E6_{91-100}$ (CD4), HPV16 $E6_{131-140}$ (CD8), HPV16 $E7_{36-62}$ (CD4/CD8), scrambled control peptides, or 2 µg/ml SEA for 72 hours. Proliferation was assessed by incorporation of [^{3}H]TdR (Hartman Analytic), added during the final 12 hours of culture. Data are expressed as mean cpm ± SD of triplicate cultures.

3.23 Enzyme-linked immunosorbent assay (ELISA)

To characterize the humoral immune responses to influenza vaccination, mouse blood was collected by heart puncture 10 days after booster immunizations, sera isolated and ELISA conducted with peptides harbouring known B cell epitopes of both HPV16 E6 [179, 180] and E7 [181, 182] as antigen. Peptides used were HPV16 $E6_{11-20}$, HPV16 $E6_{86-107}$, HPV16 $E6_{111-120}$, HPV16 $E6_{136-145}$, HPV16 $E7_{36-62}$, a scrambled peptide as negative control (all Severn Biotech). Whole H1N1 and H3N2 recombinant influenza A virus and HPV16 L1 VLP served as a positive controls. One µg per well of peptides in PBS was coated onto Maxisorp® flat-bottom 96 well plates (Nunc) for 12 hours at 4 °C. Plates were washed and blocked for 1 hour with 0.5% milk – PBS at 4 °C. Plates were washed and sera were diluted 1:5, 1:50 in 0.5% milk – PBS and incubated for 1 hour at room temperature under constant shaking. Subsequently plates were washed, incubated with HRP conjugated goat α-mouse (Biorad 1:10,000) for 45 min at room temperature, ABTS – substrate (Millipore) added and OD at 405 nm analysed using an ELISA-reader.

3.24 Statistics

Statistical analysis for ELISpot, proliferation and ELISA experiments were conducted as 1-way ANOVA followed by Dunnet's multiple comparison test. For TC-1 tumour cell challenge experiments 1-way ANOVAs were calculated followed

by Dunnett's multiple comparison tests between control and treated groups. For statistical analysis of survival curves a Mantel-Cox test was performed.

4 Results

4.1 Recombinant 16E6E7 and 16E6E7m influenza A viruses are stable after several passages

Recombinant influenza viruses of both H1N1 and H3N2 serotypes were generated (Fig. 8) expressing either a fusion protein of wild type HPV16 E6 and E7, or a mutated form in which aa 73-77 and aa 146-151 of HPV16 E6, and aa 21-24 of HPV16 E7 were deleted. All four viruses grew to high titres in Vero cells (data not shown). As control, parental viruses expressing only truncated NS1 (ΔNS1) were generated for both serotypes. To ensure that fusions of E6/E7 with the truncated ΔNS1 gene were genetically stable, recombinant viruses were passaged several rounds in Vero cells and NS1-E6E7 transcripts analysed by RT-PCR and sequencing (data not shown). Results verified, that the E6E7 fusion

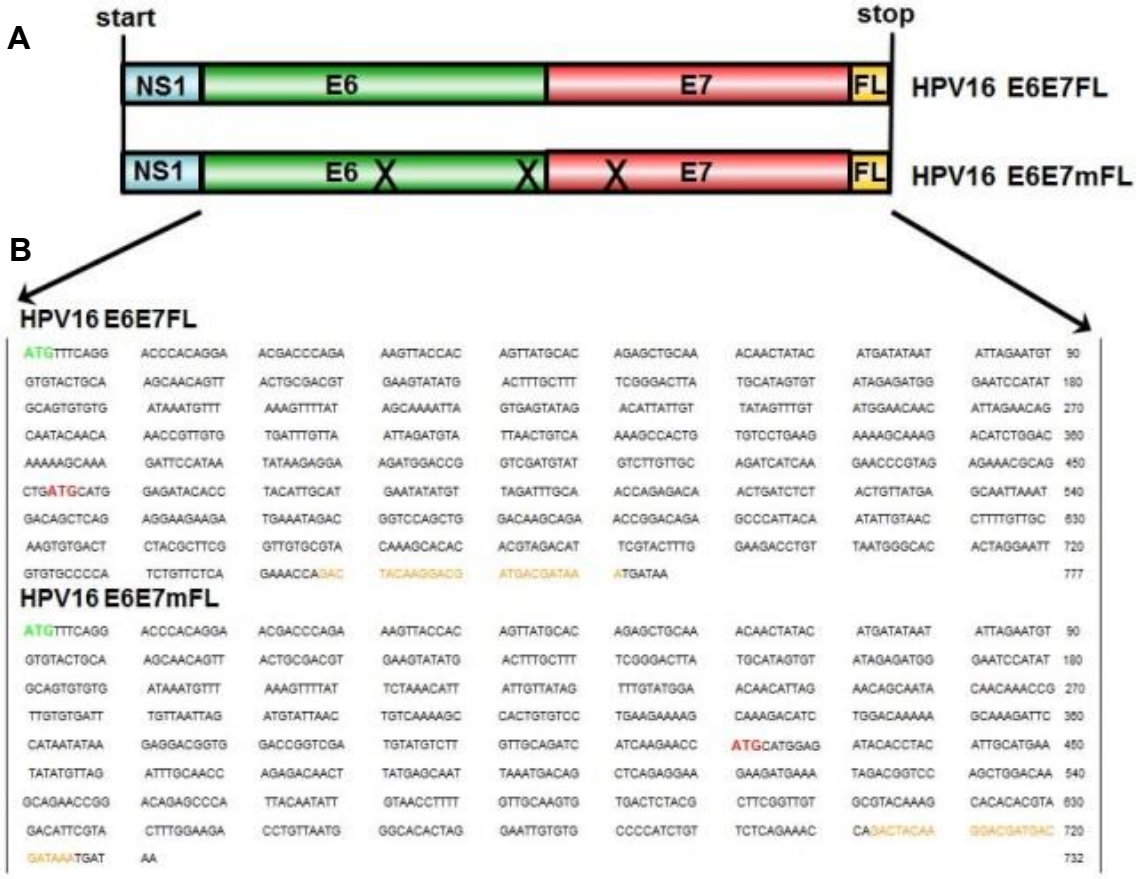

Figure 8: Illustration (A) and sequence analysis (B) of the HPV 16 E6E7FL and HPV16 E6E7mFL fusion-genes Starting ATG for HPV16 E6 is shown in green, starting ATG for HPV16 E7 is shown in red, orange determines FLAG-tag.

ORF was retained and expressed after at least five passages. Titres of recombinant influenza virus were comparable to that of parental viruses. The viral genome segment that codes for ΔNS1 also harbours the NEP reading frame, which is expressed through alternative splicing and essential for the viral life cycle. To favour transgene stability the splice donor site was constructed for high homology to human U1snRNA which is a facilitator for mRNA splicing [183]. Thus enhanced levels of NEP mRNA were produced. However fusion gene expression can be slightly favoured by mutating the splice donor site located in the NS1 reading frame to a lesser homology grade, similar as in wt influenza A virus [174]. To optimize transgene expression we mutated the sequence CAGGTAAGTCT to CAGGTATTTGC by PCR. This splice mutation did not negatively affect fusion gene stability (Fig. 9A) and viral infectivity (data not shown). Since expression of the fusion genes was slightly enhanced (data not shown), these splice-optimized viruses were used for all further experiments.

4.2 Vero cells infected with recombinant viruses express 16E6E7 or 16E6E7m mRNA and fusion protein

Infectivity of recombinant viruses was verified by analysis of expression of the viral non-structural protein NP in target cells, demonstrating that cells were effectively infected by all 4 recombinant viruses (Fig. 9B, lanes 4-7) similar to parental strains (Fig. 9B, lanes 2, 3). Expression of E6E7 mRNA was detected in cells infected with H1N1 16E6E7, H3N2 16E6E7 (~744 bp), H1N1 16E6E7m, or H3N2 16E6E7m (~732 bp) viruses (Fig. 9C, lanes 2, 3 for H1N1 and 5, 6 for H3N2), but not in cells infected with parental virus or cultivated in medium alone (Fig. 9C, lanes 4, 7 and 8). By western blotting, fusion protein expression was seen in cells infected with H1N1 and H3N2 16E6E7 and E6E7m viruses (Fig. 9D, lanes 3-6), but not in cells receiving parental virus or medium alone (lanes 1 and 2). Recombinant proteins were detected as ~38 kD bands (with 16E6E7m migrating slightly faster than 16E6E7), only when cells were treated with proteasome inhibitor MG132 before and during infection. This suggests rapid proteasomal degradation of the artificial fusion-protein, presumably due to irregular folding caused by steric hindrance. Three of the four components of the

fusion protein comprising truncated NS1, HPV16 E6, HPV16 E7 and the flag tag were detected using specific antibodies, suggesting full length translation. Slight molecular weight differences between wild type (38 kD) and mutated (36 kD) forms were recognizable on mRNA and protein level. Transgene stability was verified prior to (Fig. 9A) infection experiments for all recombinant influenza viruses.

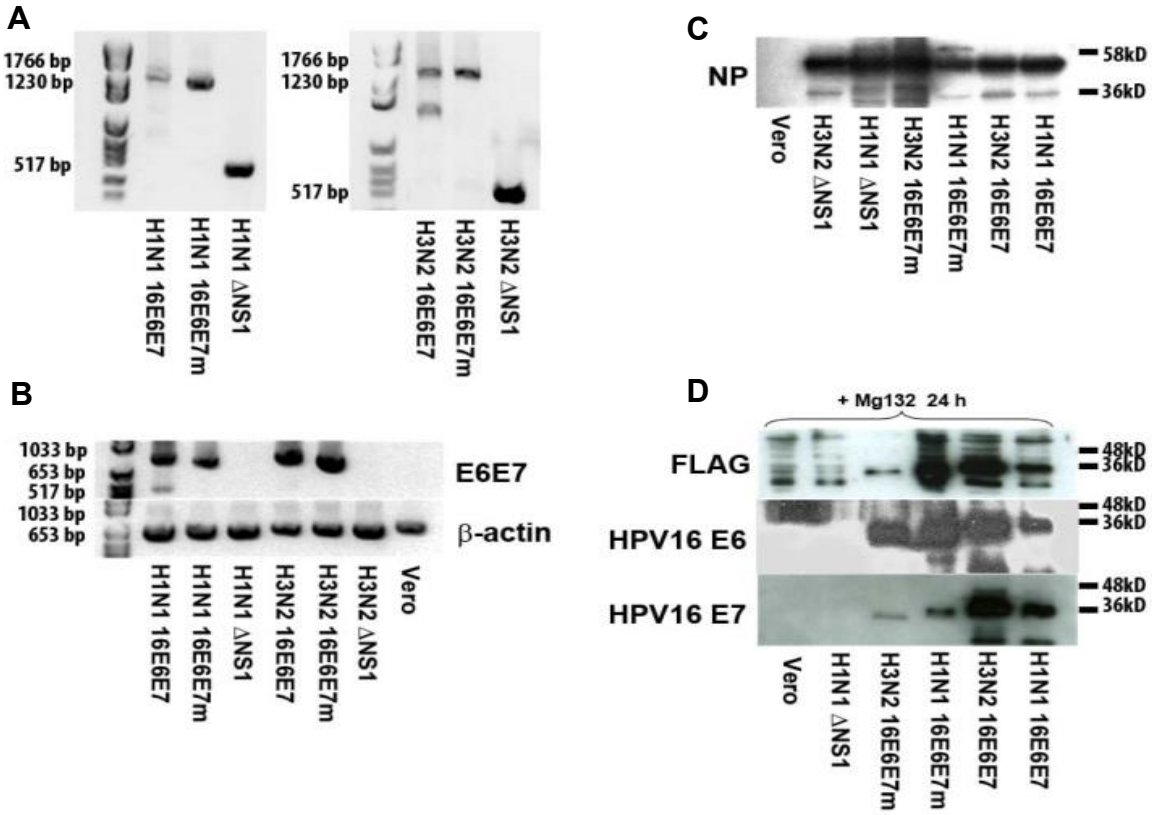

Figure 9: Recombinant 16E6E7 and 16E6E7m viruses are stable and express their transgenes **A** RT-PCR of the ΔNS1 16 E6E7 or E6E7m fusion after 5 passages in Vero cells. Viral RNA was isolated, and the fusion-genes amplified by RT-PCR. **B** Vero cells were infected with recombinant viruses as indicated for 12 h and protein samples prepared to detect viral NP protein by Western blot. **C** Vero cells were infected with recombinant virus as indicated for 12 h and RNA isolated and subjected to RT-PCR using 16E6E7specific primers for fusion-gene detection (or β-actin as a control). **D** Vero cells were cultivated in the presence of proteasomal inhibitor MG-132 for 12 h, infected with recombinant viruses as indicated and incubated for further 12 h with MG-132 present. Cells were lysed, extracts separated by SDS-PAGE, and fusion-proteins detected by Western blot using mAbs against FLAG, HPV16 E6, or HPV16 E7, respectively.

4.3 Infection of IFN competent cells with recombinant 16E6E7 or 16E6E7m virus leads to fusion gene expression without reduction of pRb

The oncoproteins E6 and E7 of hr HPV are potent driving factors for cell transformation. To analyse biological activity of non-mutated HPV16 E6E7 fusion

protein we investigated whether infection with recombinant influenza viruses causes degradation of the tumour suppressor pRb, and thus may potentially become harmful in patients. For this purpose the human melanoma cell line UACC62 was utilized, expressing pRb and type I IFNs. UACC 62 cells were infected with recombinant viruses and pRb levels analysed. To more closely simulate the in vivo situation no proteasome inhibitor was used, contrary to previous experiments in Vero cells. Detection of viral NP by western blot verified efficient infection of UACC 62 cells with recombinant viruses of both serotypes (Fig. 10B, medium panel). By RT-PCR mRNA expression of the HPV16 E6E7 fusion gene was detected in cells infected with 16E6E7 and 16E6E7m viruses of both serotypes (Fig. 10A). Western blot analysis of cells infected with recombinant viruses revealed no significant degradation of pRb (Fig. 10B, lanes 4-7, upper panel) compared to either uninfected cells or cells infected with parental virus (Fig.10B, lanes 1-3, upper panel). GAPDH served as a loading control (Fig. 10B, lower panel). This suggests that probably due to the observed rapid degradation of the 16E6E7 fusion protein, the cellular tumour suppressor pRB (and presumably also p21 and p107) is not degraded by recombinant 16E6E7 viral infection.

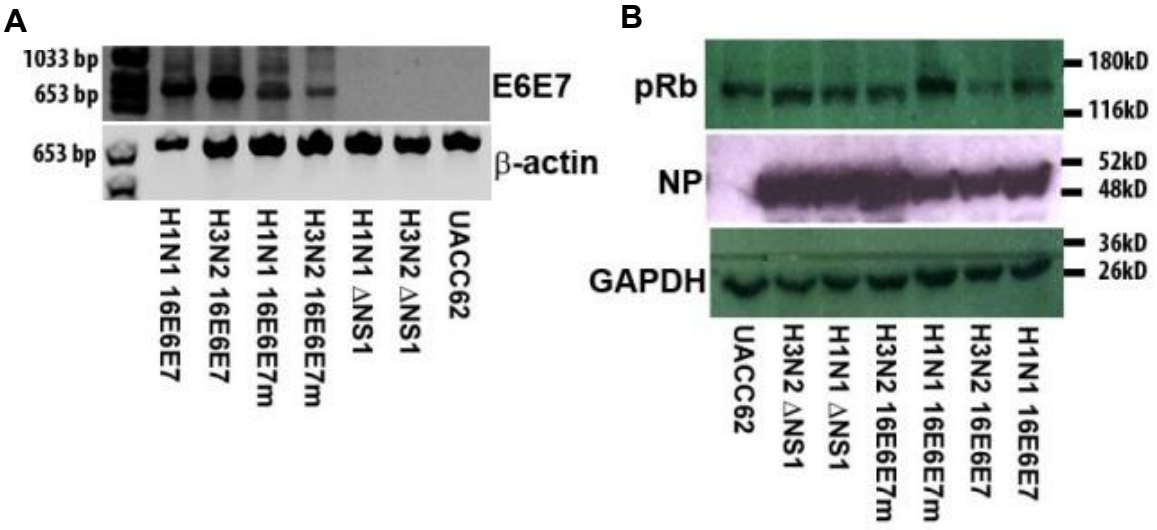

Figure 10: Infection with recombinant 16E6 and 16E6E7m viruses does not cause degradation of the cellular tumoursuppressor pRb **A** UACC62 cells were infected with recombinant viruses as indicated for 12 h and RNA isolated and subjected to RT-PCR to detect HPV16 E6E7 or β-actin mRNA expression, or **B** protein samples prepared and cellular pRb, viral NP, or GAPDH as loading control detected by western blot.

4.4 Production of recombinant HPV16 L1 and L2-E7 VLP by co-expression in Sf-9 insect cells

Greenstone et al. have described previously that vaccinations with HPV16 L1/L2-E7 VLP elicit efficient cellular immune responses against TC-1 tumours in mice which are predominantly CTL driven and perforin dependent [131]. Therefore we decided to use this system as control in our vaccination studies. For this purpose recombinant baculovirus was generated from a DNA double-expression-vector provided by J. Schiller (NIH). HPV16 L1 and a fusion of HPV16 L2-E7 were co-expressed in Sf-9 insect cells and co-assembled VLP purified by sucrose cushion and CsCl density gradient centrifugation. Analysis of infected crude Sf-9 cell lysate by SDS-PAGE and Coomassie staining showed the L1 protein as a prominent band of the expected size around 55 kD (Fig. 11A, lane 2). Following VLP purification a characteristic double band was resolved, the smaller of which may result from partial proteolysis of L1 (Fig. 11A, lane 3). Western blot using mAb Camvir showed double bands for HPV16 L1 in purified L1/L2-E7 VLP and a single band for crude lysate at 55 kD and smaller degradation products (Fig. 11B, lanes 4 and 5, upper panel). As positive controls, HPV16 L1 only and HPV16 L1/L2 VLP were positive for L1 as expected (Fig.11B, lanes 3 and 2, upper panel). HPV16 L2 reactivity was detected in purified 16 L1/L2-E7 VLP and

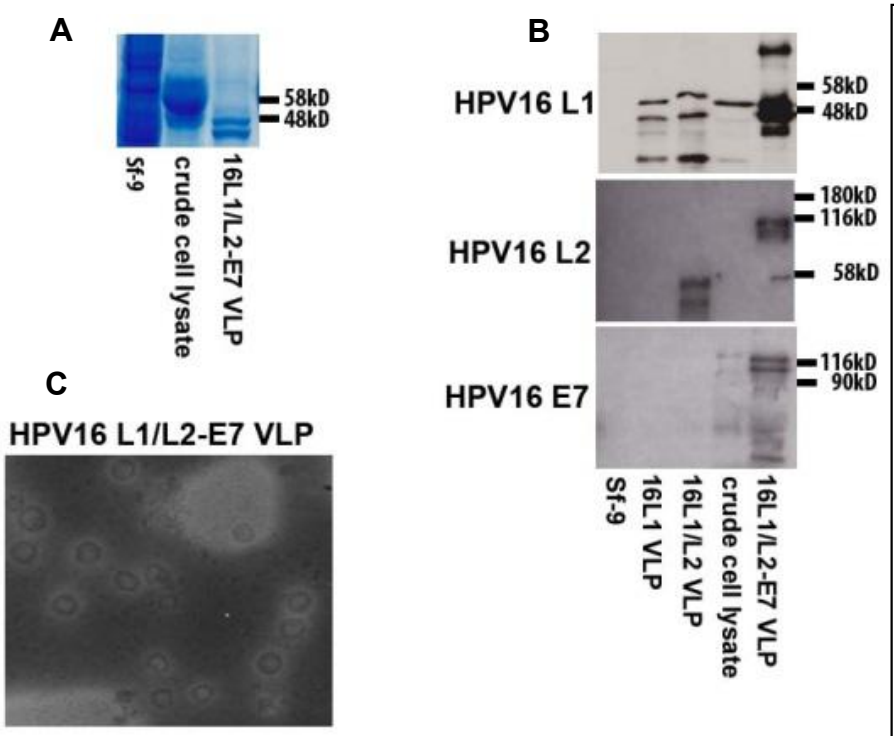

Figure 11: HPV16 L1/L2-E7 VLP
A SDS-PAGE and Coomassie stain of HPV16 L1/L2-E7 VLP. Sf-9 cells were infected with recombinant baculovirus and VLPs purified by sucrose cushion and 2x CsCl gradient centrifugation. VLPs, crude lysate prior to purification and Sf-9 cells alone were analyzed as indicated. **B** Antigenicity of individual proteins of HPV16 L1/L2-E7 particles was detected by Western blot using antibodies to HPV16 L1, HPV16 L2, or HPV16 E7. As controls HPV16 L1 and HPV16 L1/L2 VLPs were used. **C** Transmission electron microscopy of HPV16 L1/L2-E7 VLP by negative staining ($_x$30.000).

HPV16 L1/L2 VLP only (Fig. 11B, lanes 3 and 5, middle panel). For HPV16 L1/L2-E7 VLP, the L2-E7 fusion-protein migrated at approximately 116 kD (Fig. 11B, lane 5, middle panel). In comparison wild type L2 migrated at about 60 kD (Fig. 11B, lane 3, middle panel). An explanation for the negative L2 staining in crude cell lysate may be unproportionally low levels of L2 in the lysate (Fig. 11B, lane 4, middle panel). The HPV16 E7 antiserum strongly reacted with purified 16L1/L2-E7 VLP and slightly with the crude cell lysate (Fig. 11B, lower panel, lanes 5 and 4), but not with controls as expected (Fig. 11B, lower panel, lanes 1-3). These results verified antigenicity and suggested efficient co-assembly of HPV16 L1 and HPV16 L2-E7 fusion protein into VLP. To examine VLP morphology transmission electron microscopy was performed that revealed large numbers of well-formed spherical viral capsids (Fig. 11C).

4.5 HPV16 E6- and E7-specific IFN-γ producing CTLs are generated in C57BL/6 mice by vaccination with recombinant 16E6E7 or 16E6E7m influenza virus

Having ensured infectivity and stability of transgene and fusion protein expression by recombinant viruses in vitro, C57BL/6 mice were primed with either PBS as mock control, recombinant H1N1 16E6E7 or H1N1 16E6E7m viruses, parental H1N1 virus, or HPV16 L1/L2-E7 VLP, and boosted with PBS the corresponding H3N2 viruses, or VLP, respectively (Fig. 12). The live and subunit vaccines showed no serious side effects and were well tolerated in all mice comparable to sham treated animals. To characterize the elicited cellular immune response, mice were sacrificed 10 or 30 days after the boost, splenocytes were isolated, stimulated with peptides harbouring known CTL epitopes of HPV16 E6, E7, or combinations thereof, and specific IFN-γ production was analysed by ELISpot. As controls, *S. aureus* enterotoxin A (SEA) and a peptide harbouring known CTL epitopes of influenza A NP were used for stimulation. As expected splenocytes of mice vaccinated with influenza A vectors showed extensive IFN-γ production when stimulated with NP peptide (Fig. 12A, group 3), and similar production was observed for all groups when SEA was used as stimulus (Fig. 12A, group 4). Cells from mice treated with recombinant 16E6E7 or 16E6E7m

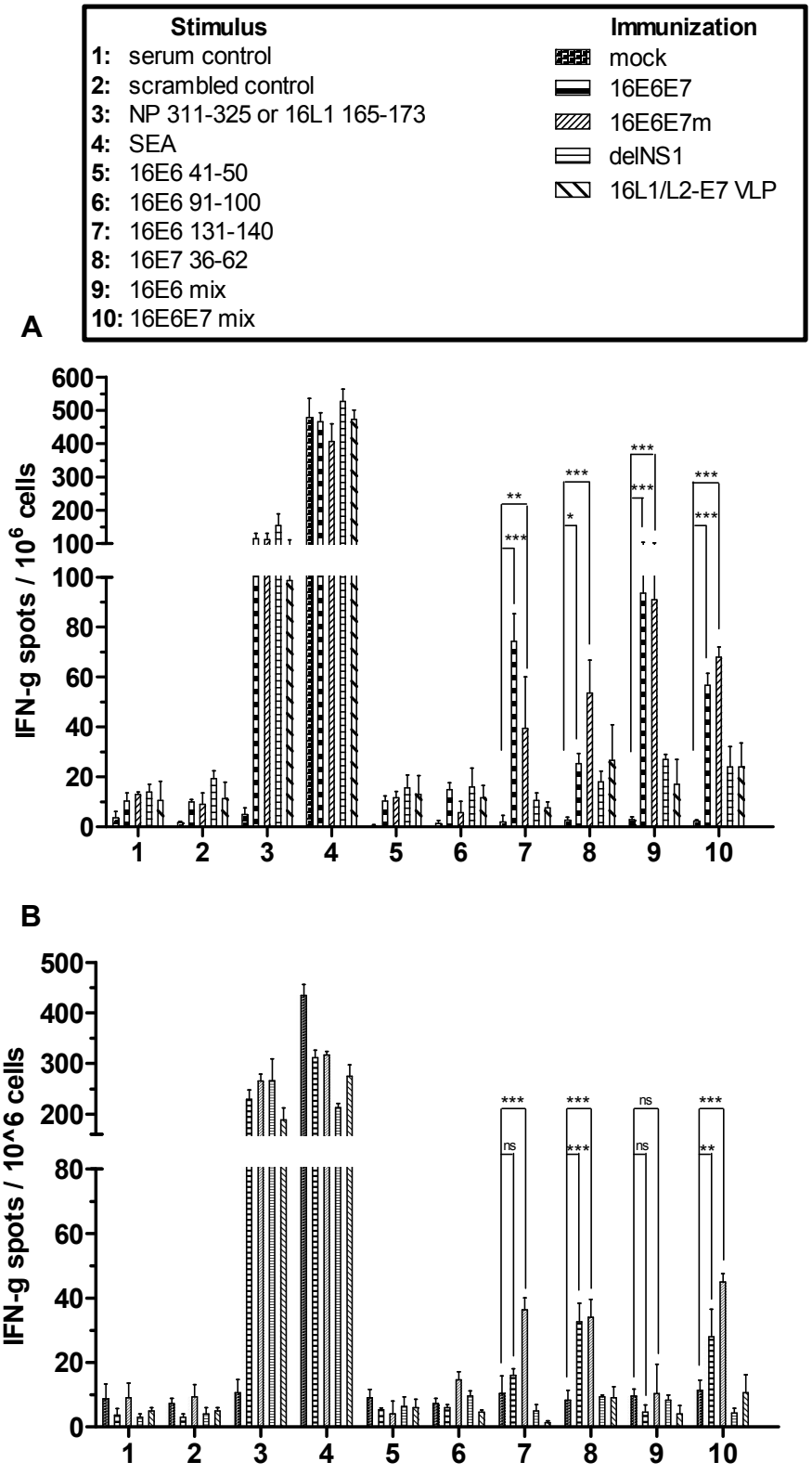

Figure 12: Recombinant 16E6E7 and 16E6E7m influenza viruses elicit HPV-specific CTL responses in mice. 4 mice were primed either with H1N1 16E6E7, or 16E6E7m viruses, or parental virus (delNS1), 16L1/L2-E7 VLP, or PBS (mock) and boosted 10 days later with the corresponding H3N2serotypes, or VLP or PBS as indicated. Mice were sacrificed **A** 10 days or **B** 30 days after boosting, splenocytes isolated and stimulated in triplicates for 24 h with indicated peptides, SEA or medium alone. For animals vaccinated with recombinant influenza A viruses, $NP_{311-325}$ peptide served as a positive control, for mice immunized with 16L1/L2-E7 VLP, $16L1_{165-173}$ peptide was used as positive control. IFN-γ spots were counted under a light microscope and plotted as mean number ± SD. One representative experiment of two is shown. Statistical p-values of 16E6E7 or 16E6E7m compared to mock are indicated as asterisks (*** p<0,001, ** p<0,01, * p<0,05, ns not significant).

influenza viruses that were stimulated with HPV16 $E6_{131-140}$ and HPV16 $E7_{36-62}$ peptides (Fig. 12A, groups 7,8), in contrast to peptides HPV16 $E6_{41-50}$ or $E6_{91-100}$, specifically elicited a statistically significant IFN-γ response compared to mock (Fig. 12A groups 5,6). In contrast, cells from mock or parental virus treated mice did not show specific IFN-γ production compared to medium or scrambled peptide controls (Fig. 12A groups 1,2). A mixture of E6 and E7 peptides did statistically significant increase IFN-γ production of cells from mice immunized with recombinant influenza viruses (Fig. 12A, groups 9, 10) compared to mock control. Interestingly for animals vaccinated with HPV16 L1/L2-E7 VLPs the CTL response against E7 peptides was lower compared to recombinant influenza A vaccinated animals (Fig. 12A, group 8) suggesting either less efficient priming or boosting of the immune system against HPV16 E7 by chimeric VLP vaccination. Alternatively, immune responses against VLP may be primarily of Th-2 type, contrary to Th1-driven immunity induced by recombinant influenza viruses. However IFN-γ production was observed upon stimulation with HPV16 $L1_{165-173}$ peptide for animals vaccinated with HPV16 L1/L2-E7 VLPs, thus the L1 capsid appears immunodominant over the less abundant minor capsid L2-E7 fusion protein component. To further assess whether specific immunity was maintained over a longer time frame, splenocytes were isolated from vaccinated animals 30 days after the boost and ELISpot experiments similar to those described above were conducted (Fig. 12B). Overall, CTL responses against peptides of HPV16 E6 and E7 decreased approx. 2.5-fold (Fig. 12B, groups 7-10) and no synergistic stimulation by the mixture of three E6 peptides was observed in splenocytes from mice vaccinated with either recombinant influenza viruses (Fig. 12B group 9). An explanation may be that tolerogenic cells in splenocytes are activated by HPV16 $E6_{41-50}$ or $E6_{91-100}$ peptides which do not harbour CTL but T-helper cell epitopes and thus dampen the cellular immune response. Nevertheless the IFN-γ response remained detectable over the longer time course in vaccinated mice and was statistically significant compared to mock control. In contrast, in control animals no specific CTL responses to HPV16 E6 or E7 were detected.

4.6 Proliferation of splenocytes of vaccinated animals in response to stimulation with peptides harbouring HPV16 E6 and E7 T-helper cell epitopes

To analyse whether vaccinations with recombinant influenza A virus elicit a specific T-helper cell response, animals were immunized as in 4.5 and splenocytes were isolated and stimulated with peptides harbouring known T-helper cell epitopes of HPV16 E6 or E7 (Fig. 13A). Control animals were mock treated or immunized with parental influenza virus or HPV16 L1/L2-E7 VLPs. Proliferation assays were performed and specific T cell proliferation detected by H^3-thymidine incorporation. Cells of all animals showed strong proliferation of responsive T cells when stimulated with SEA (Fig. 13A, group 4) which served as positive control. Due to high levels of unspecific background proliferation by splenocytes of parental and recombinant influenza A immunized animals (Fig. 13A, groups 1,2) it was difficult to assess whether HPV16 E6 or E7 specific T cells proliferated specifically in response to peptide stimuli. When a mixture of all HPV16 E6 peptides was used as stimulus, a slight increase of proliferation was measurable from splenocytes of animals immunized with recombinant influenza A viruses (Fig. 13A, group 9) compared to parental virus vaccinated mice. Interestingly we did not detect specific proliferation against NP peptide (Fig. 13A, group 4). This might be due to CD8 restriction of epitopes in the peptide. Alternatively, we may speculate that specific proliferation has been masked due to high background proliferation of splenocytes.

4.7 Vaccination with 16E6E7 and E6E7m influenza viruses does not elicit antibodies to HPV16 E6 and E7

To address the question whether recombinant influenza A vaccinations induce a humoral immune response against HPV16 E6 and E7, C57BL/6 mice were immunized twice (with 10 days interval) with PBS, or either serotype of recombinant or parental influenza A viruses, or HPV16 L1/L2-E7 VLP. Ten days after the boost, sera were isolated and a peptide based ELISA performed (Fig. 13B). Peptides harbouring previously described humoral epitopes of HPV16 E6 or E7, scrambled control peptides, or whole H1N1 or H3N2 viruses were used as

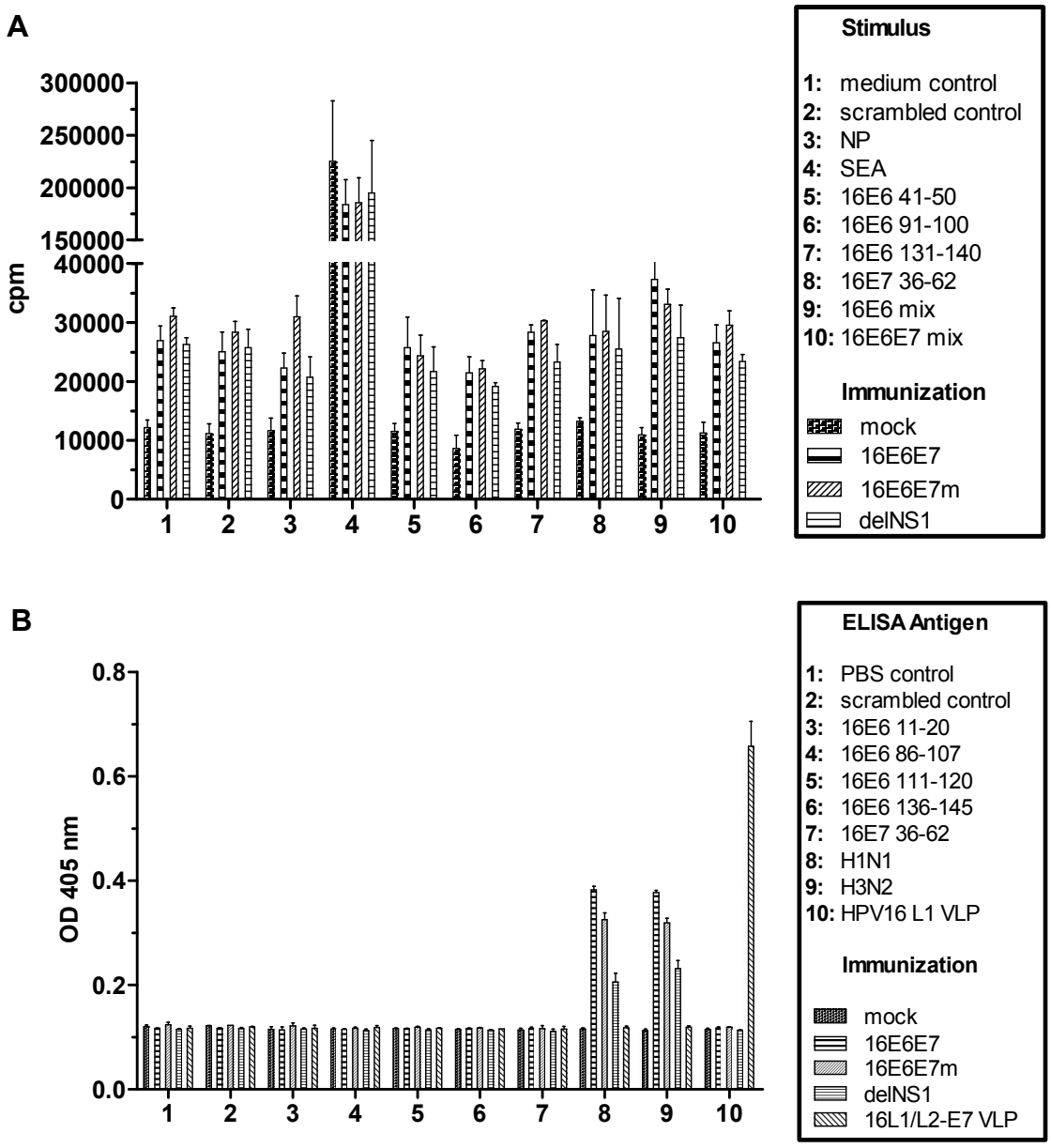

Figure 13: HPV-specific T helper cell proliferation and humoral immunity to vaccination with recombinant 16E6E7 and 16E6E7m influenza viruses. A Mice were primed s.c. with indicated recombinant or parental H1N1 viruses, or PBS (mock) and boosted 10 days later with the corresponding H3N2 serotype. Splenocytes were isolated 10 days after boosting and stimulated in triplicates with indicated peptides. H^3-tymidine was added for the final 12 h incubation and incorporation measured using a liquid scintillation beta counter. Results are expressed as CPM mean number ± SD. One representative experiment of two is shown. **B** Mice were immunized as in A and sera isolated 10 days after the final immunization. Sera were diluted at 1:50 and incubated with peptides harbouring known antibody epitopes of HPV16 E6 and E7, whole H1N1 or H3N2 attenuated influenza A virus, or HPV16 L1 VLP attached to ELISA plates for 1 h. Secondary HRP-conjugated antibody was added for 45 min and ELISA was developed using ABTS-substrate. Colour change was monitored at 405 nm. Triplicate results are expressed as mean number ± SD. One representative experiment of two with a statistical confidence level of $p < 0.001$ is shown.

ELISA antigen, respectively, and HPV16 L1 VLP served as control antigen for sera of VLP immunized animals. Antibodies to HPV16 E6 or E7 peptides could not be detected in sera of mice vaccinated with recombinant 16E6E7 and 16E6E7m viruses (Fig. 13B, groups 3-7). In contrast, antibodies directed against influenza A (Fig. 13B, groups 8-9) were detected in sera from mice vaccinated with recombinant or parental influenza A viruses. Sera of mice immunized with HPV16 L1/L2-E7 VLP also contained high levels of HPV16 VLP reactive antibodies (Fig. 13B, group 10). These results suggest, the humoral immune response is induced predominantly by influenza A virion proteins whereas the fusion protein becomes preferably presented in an endogenous cytokine environment supporting cell-mediated immunity. Furthermore due to the rapid degradation of the recombinant fusion protein, antigen presentation via MHC class I may be favoured compared to MHC class II. Interestingly sera from mice vaccinated with HPV16 L1/L2-E7 VLP also lacked detectable antibodies against HPV16 E7 (Fig. 13B, group 7). This suggests that E7 serves as a poor immunogen for humoral immune responses or is subdominant to the highly immunogenic HPV16 L1 of VLP-based papillomavirus vaccines. Another possible explanation is the use of C57BL/6 mice intended to primarily analyse cell-mediated immune responses, since this mouse strain tends to have lower humoral immune responses compared to e.g. BalbC mice.

4.8 Mice vaccinated with recombinant 16E6E7 or 16E6E7m viruses are partially protected from challenge with TC-1 tumour cells

Given the E6/E7-specific cell-mediated immune response generated by recombinant influenza A we addressed the question whether vaccinations protect

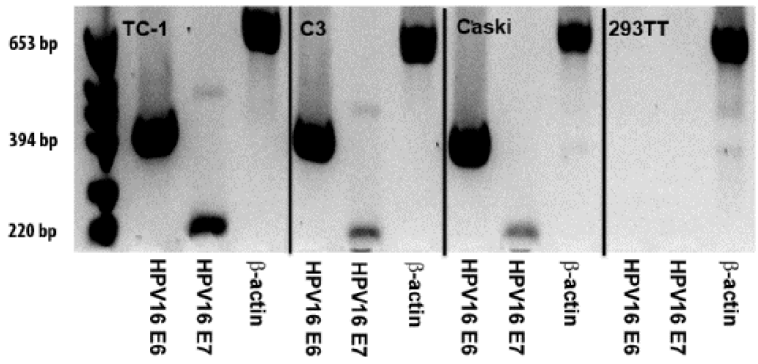

Figure 14: Verification of HPV16 E6 and E7 transgenes in TC-1 cells Cells were cultivated in their respective medium, DNA was isolated and subjected to PCR using specific primers for 16E6 and 16E7 and β-actin. 293TT cells served as negative control, C3 and Caski cells were used as positive control

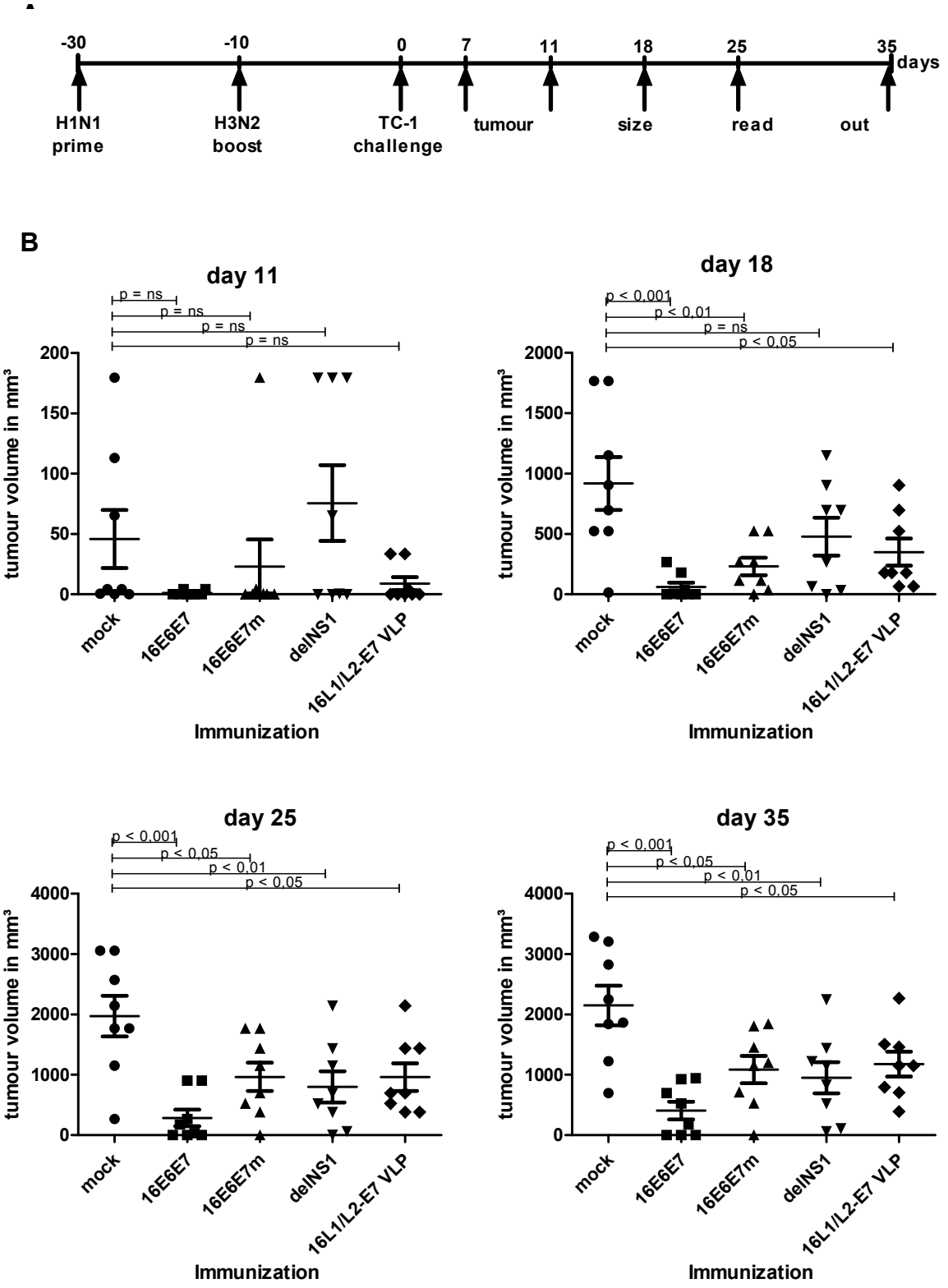

Figure 15: Mice vaccinated with recombinant 16E6E7 or 16E6E7m influenza viruses are partially protected from TC-1 induced tumours. A Schematic illustration of the experimental set up **B** C57BL/6 female mice (n=8 per group) were vaccinated s.c. on day 0 with PBS (mock), H1N1 16E6E7, H1N1 16E6E7m, parental virus (delNS1) or HPV16 L1/L2-E7 VLP, boosted either with PBS, the according H3N2 influenza serotype or VLP on day 20 as indicated on the x axis and challenged with $5x10^4$ TC-1 cells on day 30. Tumour diameter was monitored once per week and shown are calculated mean tumour volumes ± SD and individual animals of one representative of two independent experiments. Day indicates days after TC-1 tumour inoculation.

animals from challenge with HPV16 E6- and E7-positive TC-1 tumour cells. Prior to inoculation, E6/E7 oncogene presence and expression by TC-1 tumour cells was verified by PCR (Fig. 14, lanes 5-7) and RT-PCR (data not shown). DNA from HPV16 positive cell lines C3 and Caski served as positive controls, and DNA of 293TT cells as negative control.

Subsequently, groups of C57BL/6 mice (n=8) were vaccinated s.c. into the skin fold of the neck with either PBS as mock control, recombinant H1N1 16E6E7 or 16E6E7m or parental viruses, or HPV16 L1/L2-E7 VLP. Booster immunizations with PBS, the respective H3N2 serotypes, or 16 L1/L2-E7 VLPs were performed on day 20, and ten days later TC-1 cells were injected s.c. into the right flank of each animal and tumour growth monitored over 35 days (Fig. 15A). In PBS (mock) immunized mice tumours were detectable as soon as seven days after TC-1 inoculation (data not shown) compared to eleven days in vaccinated animals (Fig. 15B, day 11). Tumour growth from day 11 to day 18 was most profound in animals immunized with PBS or parental virus (delNS1). At day 18 after TC-1 inoculation 8/8 animals of the mock treated group and of the parental virus treated group harboured palpable tumours, with volumes ranging between 14 to 1,700 mm^3 for mock treated animals and 33 to 1,150 mm^3 for parental virus treated mice, whereas 4/8 animals vaccinated with 16E6E7 virus showed no distinguishable tumour growth (Fig.15B, day 18). Mice treated with 16E6E7m virus showed similar results from day 11 to day 18 after inoculation with overall slower tumour growth compared to mock and parental virus treated animals. However in 16E6E7m vaccinated mice enhanced tumour progression was observed from days 18 to 35 (Fig.15B) compared to mice vaccinated with 16E6E7 virus, which were significantly ($p < 0.001$) protected compared to mock vaccinated animals (Fig.15B, lower panels). This difference in protective efficacy between 16E6E7 and 16E6E7m viruses could be due to less efficient induction of cell-mediated anti-tumour responses by 16E6E7m in vivo, possibly due to deletion of crucial rejection determinants by the introduced mutations. Interestingly animals vaccinated with parental ΔNS1 viruses also seemed to mount an anti-tumour response. The biological mechanisms of oncogene-independent rejection remains unclear and will be topic of further investigation, e.g. by screening for HPV16 E6 and E7 specific T cells by tetramer FACS staining, in combination with analysis of T cell activation markers. Alternatively,

influenza vaccination per se may enhance innate and adaptive immune surveillance recognizing TC-1 tumour cells. In mice immunized with HPV16 L1/L2-E7 VLP, tumour cell growth was delayed initially (Fig.15B, day 11), but eventually mice were not protected against tumour development compared to 16E6E7 vaccinated animals, similar to mice treated with 16E6E7m and parental influenza virus (Fig.15B, lower panels), indicating a possible advantage of the live viral vaccine approach over subunit VLP vaccination.

4.9 Vaccination with recombinant 16E6E7 or 16E6E7m influenza A viruses delays the growth of TC-1 tumour cells in mice

To further evaluate the therapeutic capacity of recombinant influenza A vaccines groups of C57BL/6 mice (n=8) were inoculated with TC-1 tumour cells and immunized the same day with PBS as mock control, recombinant 16E6E7 or 16E6E7m influenza, parental virus, or HPV16 L1/L2-E7 VLP (Fig.16A). Booster immunizations were performed on day 10 with PBS, H3N2 serotypes, or VLP respectively (Fig.16A). Palpable tumours were detected as early as day 11 in mock treated animals whereas all other mice showed no sign of tumour development (data not shown). At day 18 after tumour inoculation palpable tumours were detected in all groups: mock 2/8, 16E6E7 3/8, 16E6E7m 2/8, parental vector 7/8 and VLP 4/8 (Fig.16B, day 18). TC-1 tumours developed rather slowly during the first 18 days after TC-1 inoculation in all groups except for animals immunized with parental virus, in which 7/8 animals had already developed tumours and increased tumour volumes between 33 to ~1000 mm^3 were observed. In contrast to prophylactic vaccinations (Fig. 15B, day 18), immunization with recombinant 16E6E7 virus did not delay tumour development as efficiently, since 6/8 animals had developed palpable tumours during day 18 to day 25, with volumes ranging from 4 to ~500 mm^3, whereas only 4/8 mock control animals had TC-1 tumours (Fig. 16B, day 25). In contrast, only 2/8 animals immunized with 16E6E7m vaccines developed tumours, whereas 6/8 VLP treated mice had tumours with volumes between 4 and 33 mm^3 at day 25. Animals vaccinated with parental influenza A virus had significantly increased tumour burden compared to 16E6E7m and VLP immunization groups (Fig. 16B, day 25).

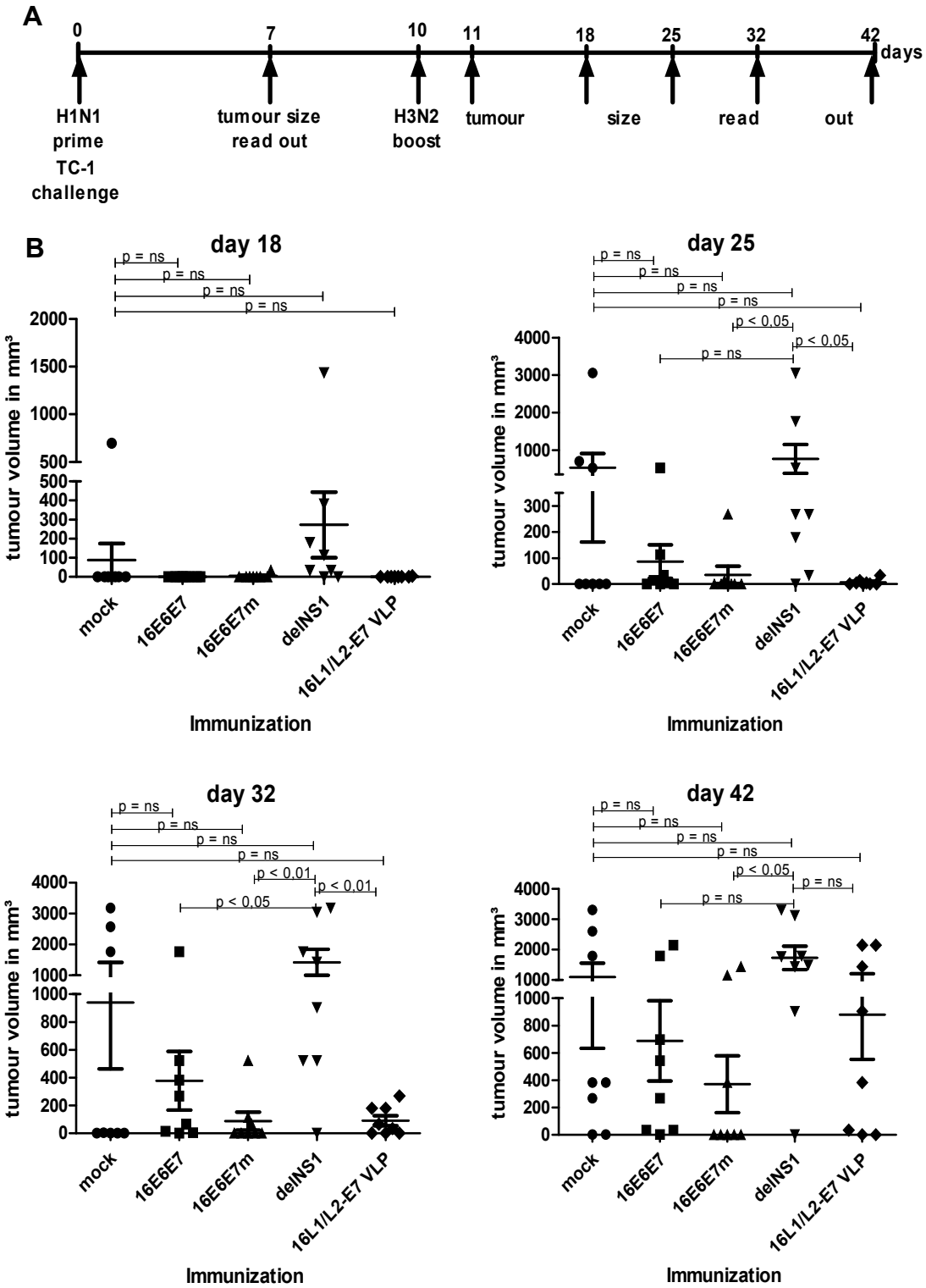

Figure 16: Vaccination with recombinant 16E6E7 and 16E6E7m influenza viruses delays the growth of TC-1 tumour cells **A** Schematic illustration of the experimental set up **B** C57BL/6 female mice (n=8 per group) were vaccinated on day 0 with PBS (mock), H1N1 16E6E7, H1N1 16E6E7m, parental virus (delNS1) or HPV16 L1/L2-E7 VLP, challenged with 5x104 TC-1 cells on the same day and boosted either with PBS, the according H3N2 influenza serotype or VLP on day 10 as indicated on the x axis. Tumour diameters were monitored once per week and tumour volumes calculated. Mean ± SD tumour volumes of individual animals of one representative of two independent experiments are shown. Day indicates days after tumour inoculation and prime.

At day 32 post tumour inoculation 5/8 mock treated mice had tumours with volumes between 0.5 to ~3,000 mm³ (Fig. 16B, day 32). Animals vaccinated with recombinant 16E6E7 virus had lower tumour burden compared to mock treated animals with volumes ranging between 4 to ~1,500 mm³, although 7/8 animals had developed palpable tumours (Fig. 16B, day 32). Remarkably, mice immunized with 16E6E7m influenza A vaccines had the lowest mean tumour volumes of all groups, with 5/8 mice remaining tumour free after 32 days and volumes ranging from 65 to 523 mm³ (Fig. 16B, day 32). VLP treated animals had similar tumour volumes as 16E6E7m treated mice in the range of 33 to 268 mm³, and 5/8 animals had developed palpable tumours after 32 days (Fig.16B, day 32). Parental virus treated mice had the highest tumour burden ranging from 500 to ~3,000 mm³, with 7/8 animals being positive. This result seemed to be specific since 16E6E7 and 16E6E7m vaccinated animals had significantly lower tumour volumes ($p < 0.05$ for 16E6E7; $p < 0.01$ for 16E6E7m) compared to ΔNS1 immunized mice. Also VLP treated animals had a significantly lower tumour burden ($p < 0.01$) at that time point (Fig. 16B, day 32). 42 days post TC-1 inoculation 6/8 mock treated animals had solid tumours compared to 7/8 16E6E7 and 3/8 16E6E7m treated mice (Fig. 16B, day 42). Tumour volumes had increased also in VLP immunized animals with 6/8 mice having solid tumours in the range of 33 to ~2,000 mm³ (Fig. 16B, day 42). Only one parental virus treated mouse did not develop any palpable tumour during the whole experiment compared to 5 tumour free mice immunized with recombinant 16E6E7m ($p < 0.05$) and 1 with 16E6E7 influenza A viruses. Although not statistically significant when compared to mock treated animals, a clear trend of decreased tumour growth rates was observed in mice treated with recombinant 16E6E7 or 16E6E7m influenza A viruses.

4.10 Vaccination with recombinant 16E6E7 or 16E6E7m influenza viruses delays growth of established TC-1 tumours and prolongs survival

To address whether vaccinations with recombinant 16E6E7 and 16E6E7m influenza viruses induce regression of established TC-1 tumours, C57BL/6 mice were inoculated with TC-1 cells and monitored for tumour development. After 12 days at least 50% of animals had developed palpable tumours on the right flank (Fig.17 B, day 12). Groups of mice (n=8) were then vaccinated with PBS, recombinant 16E6E7, 16E6E7m, or parental viruses, or HPV16 L1/L2-E7 VLP, and boosted with PBS, the corresponding H3N2 serotypes, or VLP 10 days after priming (Fig. 17A). Tumour volumes did differ slightly between groups vaccinated with 16E6E7 and 16E6E7m viruses compared to control groups 19 days after inoculation (Fig.17B, day 19), with mean tumour volumes in mock treated animals around 179 mm^3 whereas 16E6E7 treated animals had mean tumour volumes of 65 mm^3, and 16E6E7m treated mice a mean of 113 mm^3. Tumour volumes were highest for parental virus and VLP treated animals at day 19 (Fig.17B, day 19). At day 26, TC-1 tumour expansion was reduced in both 16E6E7 and 16E6E7m influenza A virus vaccinated groups with decreased tumour volumes observed in 2/8 mice immunized with recombinant 16E6E7m virus compared to day 19, whereas no regression was seen in all other groups (Fig.17B, day 26). The trend of reduced TC-1 growth rates was preserved from day 26 to day 33 and most prominent in 16E6E7 vaccinated followed by 16E6E7m immunized mice (Fig.17B, day 33). Contrary, in mock control animals stable TC-1 tumour growth was observed and mean tumour volumes appeared larger compared to recombinant influenza vaccinated mice, although differences were not statistically significant. Animals receiving parental influenza virus also showed decreased mean tumour volumes compared to mock controls. From day 33 to day 42 tumour growth increased rapidly in all groups suggesting that efficacy of recombinant influenza vaccination was not sustained (Fig.17B, day 42). Nevertheless, 16E6E7 vaccinated mice showed a trend for lower mean tumour volumes compared to all other vaccination groups. Mice treated with subunit VLPs had mean tumour volumes of 11 mm^3 before priming (Fig. 17B, day 12) with one animal showing no signs of tumour development at day 26 (Fig. 17B,

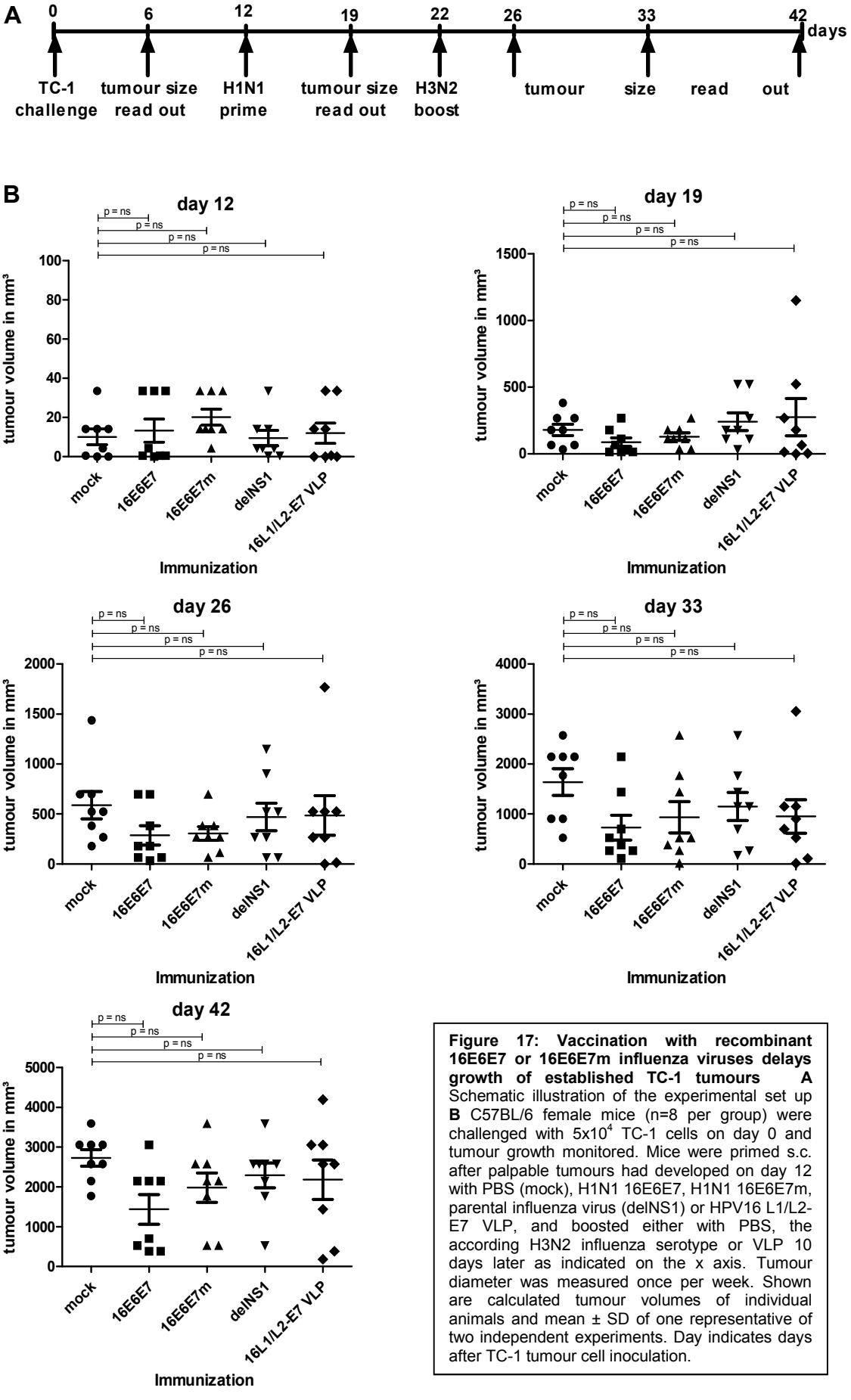

Figure 17: Vaccination with recombinant 16E6E7 or 16E6E7m influenza viruses delays growth of established TC-1 tumours **A** Schematic illustration of the experimental set up **B** C57BL/6 female mice (n=8 per group) were challenged with $5x10^4$ TC-1 cells on day 0 and tumour growth monitored. Mice were primed s.c. after palpable tumours had developed on day 12 with PBS (mock), H1N1 16E6E7, H1N1 16E6E7m, parental influenza virus (delNS1) or HPV16 L1/L2-E7 VLP, and boosted either with PBS, the according H3N2 influenza serotype or VLP 10 days later as indicated on the x axis. Tumour diameter was measured once per week. Shown are calculated tumour volumes of individual animals and mean ± SD of one representative of two independent experiments. Day indicates days after TC-1 tumour cell inoculation.

day 26). After 6 weeks all mice had developed solid tumours with tumour volumes lowest in 16E6E7 followed by all other groups (Fig. 17B, day 42). Statistical analysis showed no significant differences between groups of mice compared to mock treated animals, but a clear trend of decreased tumour growth was observable in 16E6E7 and 16E6E7m immunized mice over time. Furthermore, survival rates of vaccinated mice compared to mock controls were analysed (Fig. 18). At pre-specified termination points mice were sacrificed, i.e. when rapid loss of weight (>50%), tumour diameters >16 mm, scrappy fur, diarrhoea, or tumour ulceration occurred. Mock treated animals had to be euthanized as soon as day 27 after TC-1 inoculation. After 29 days, less than 50% of control mice survived compared to 100% for recombinant 16E6E7 and 16E6E7m virus treated animals. After 36 days all mock treated animals and 50% of mice vaccinated with parental influenza virus fulfilled termination criteria, whereas 75% of 16E6E7 and 62,5% of 16E6E7m treated mice survived. On day 44 survival rates decreased for 16E6E7m immunized animals to 25% whereas 50% of 16E6E7 vaccinated animals did not fulfil termination criteria. Also 12,5% of parental virus treated mice and 50% of VLP immunized animals survived on day 44 after tumour inoculation. At day 51 all remaining mice had to be euthanized due to tumour size, ulcerations and weight loss. The observed survival rates for vaccine groups were statistically significant compared to mock controls, when analysed using a Mantel-Cox-Test.

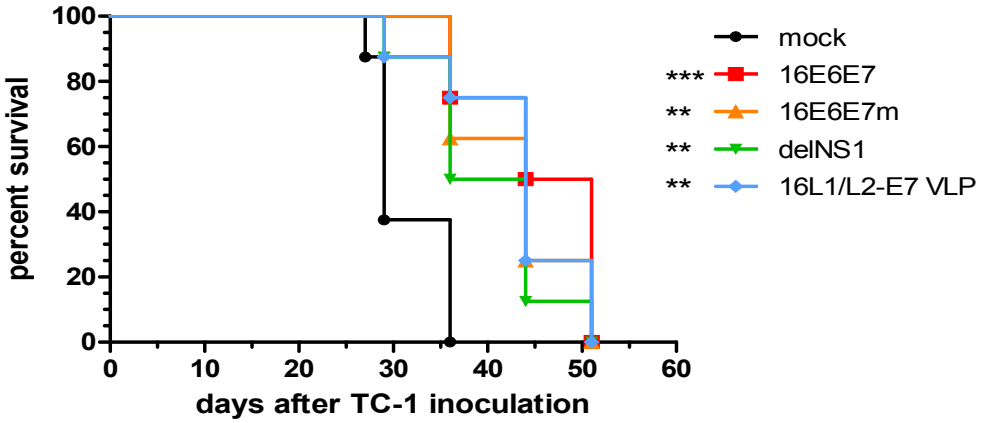

Figure 18: Vaccination with recombinant 16E6E7 and 16E6E7m influenza viruses prolongs survival. C57BL/6 mice were challenged with $5x10^4$ TC-1 cells on day 0 and tumour growth monitored. Mice were primed after solid tumours had developed on day 12 with PBS (mock), H1N1 16 E6E7, H1N1 16E6E7m, parental H1N1 influenza virus (delNS1), or HPV16 L1/L2-E7 VLP, and boosted either with PBS, the according H3N2 influenza serotype or VLP 10 days later. Predefined termination points were rapid weight loss, tumour diameters >16 mm, ulceration, scrappy fur and/or diarrhoea. Days indicate time after TC-1 tumour inoculation. One representative experiment of two is shown. Asterisks indicate p-values compared to mock control mice (*** $p<0.001$, ** $p<0.01$) using Mantel-Cox-Test.

4.11 Intratumoral but not intranasal application of recombinant 16E6E7 and 16E6E7m influenza viruses leads to reduced TC-1 tumour growth and regression in C57BL/6 mice

One possible advantage of using influenza A virus as a live vaccine vector is the opportunity to use different application routes that might affect vaccine efficacy. Thus two further application routes, besides s.c. injection, were investigated, namely intratumoral (i.t.) and intranasal (i.n). C57BL/6 mice were inoculated with $5x10^4$ TC-1 tumour cells and monitored for tumour development. After 13 days at least 50% of animals of each group (n=8) had developed palpable tumours (Fig. 19B, day 13). The next day mice were vaccinated with PBS, or recombinant 16E6E7, 16E6E7m, or parental virus. Vaccination volumes had to be restricted due to application routes, thus to inoculate similar amounts of virus as in previous s.c. experiments, a second immunization was performed on day 15. Booster immunizations with PBS, or the corresponding H3N2 influenza serotypes were again performed in two stints on day 24 and 25 (Fig. 19A, 20A). Mice that had not yet developed palpable tumours were immunized at the site of TC-1 inoculation. Overall slight differences in mean tumour volumes among groups existed but were not statistically significant. At day 19 after TC-1 inoculation mean tumour volumes increased in mock treated mice (Fig. 19B, day 19) with individual volumes ranging between 4 to 381 mm^3. Parental virus treated animals had slightly higher mean tumour volumes compared to recombinant vaccine groups (Fig. 19B, day 19). Tumours in mice receiving recombinant 16E6E7 or 16E6E7m influenza virus grew slower compared to both mock and parental virus groups (Fig.19B, day 19). Unfortunately 2 mice vaccinated with 16E6E7 and one mouse receiving 16E6E7m recombinant influenza viruses died when boosted on day 25. One possible explanation for the fatal outcome may be vascular shock since TC-1 tumours were well established and heavily vascularized and high injection volumes might be less tolerable under these conditions. However, remaining animals did not show any side effects during and after i.t. vaccinations. On day 26 after TC-1 inoculation mock treated mice had palpable tumours, with mean volumes 400 mm^3 whereas parental virus treated mice had the highest mean tumour volume compared to all other vaccination groups (Fig. 19B, day 26). Complete tumour regression was observed in 3 mice immunized with 16E6E7m

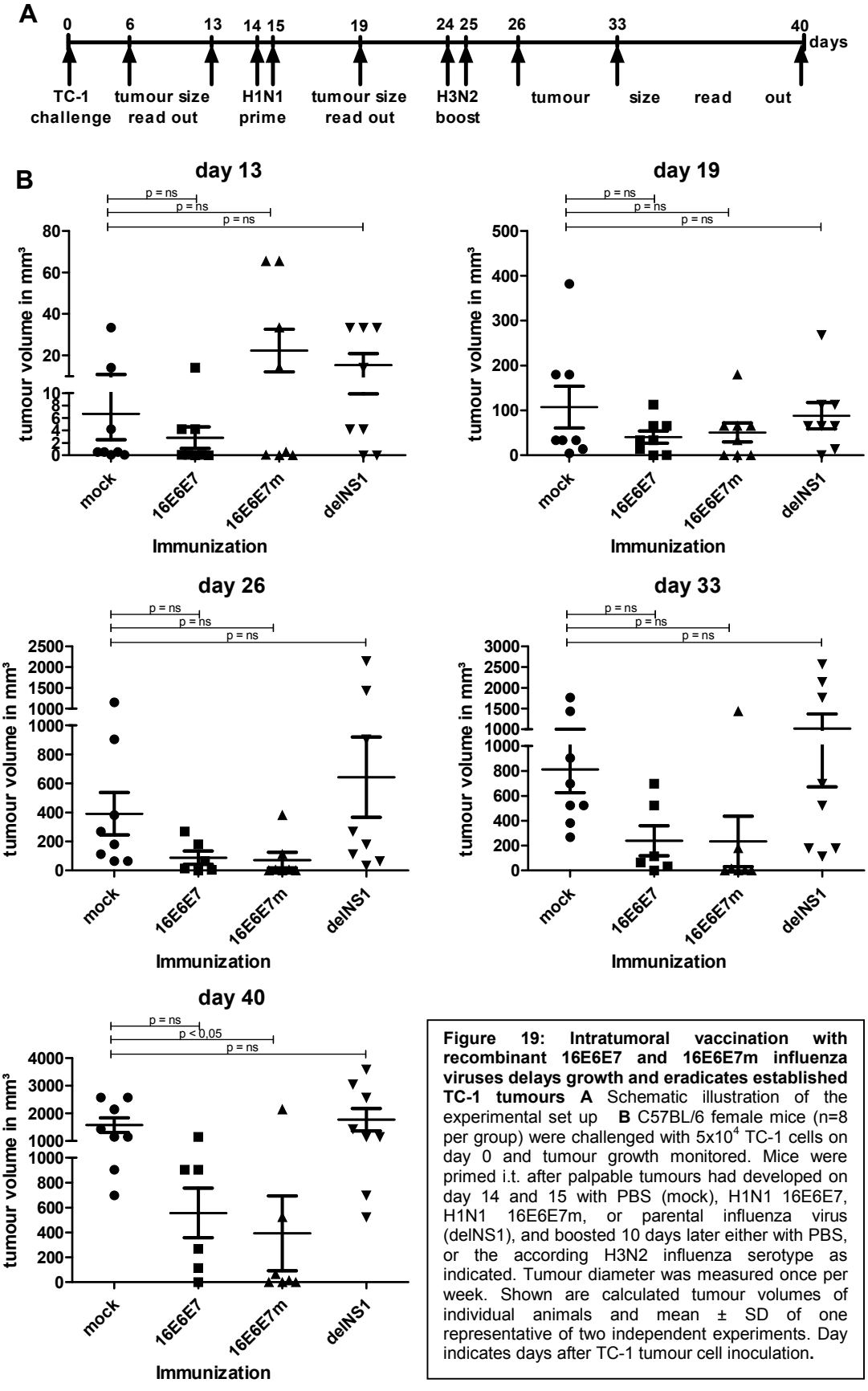

Figure 19: Intratumoral vaccination with recombinant 16E6E7 and 16E6E7m influenza viruses delays growth and eradicates established TC-1 tumours A Schematic illustration of the experimental set up **B** C57BL/6 female mice (n=8 per group) were challenged with $5x10^4$ TC-1 cells on day 0 and tumour growth monitored. Mice were primed i.t. after palpable tumours had developed on day 14 and 15 with PBS (mock), H1N1 16E6E7, H1N1 16E6E7m, or parental influenza virus (delNS1), and boosted 10 days later either with PBS, or the according H3N2 influenza serotype as indicated. Tumour diameter was measured once per week. Shown are calculated tumour volumes of individual animals and mean ± SD of one representative of two independent experiments. Day indicates days after TC-1 tumour cell inoculation.

viruses and tumour volumes of all other animals ranged between 4 and 381 mm³ (Fig. 19B, day 26). In 16E6E7 vaccinated mice tumour growth was retarded from day 19 to day 26 compared to mock and parental virus treated animals (Fig. 19B, day 26). On day 33 post TC-1 inoculation the mean tumour volume in mock treated mice had doubled (Fig. 19B, day 33) and only parental virus treated mice had a higher mean tumour volume than mock treated animals (Fig. 19B, day 33). Of the 16E6E7m vaccination group 3 animals remained tumour free, and tumours in all other mice had progressed less (Fig. 19B, day 33) compared to mock and parental groups. In recombinant 16E6E7 virus vaccinated mice tumour volumes ranged between 33 to 696 mm³ (Fig.19B, day 33). On day 40 parental virus treated mice had the largest mean tumour volume followed by mock treated animals (Fig. 19B, day 40). Complete tumour regression was observed in one mouse immunized with recombinant 16E6E7 influenza viruses (Fig.19B, day 49) and a trend of decreased tumour growth rate was observed compared to both mock and parental virus groups. In 16E6E7m vaccinated mice 3 animals remained tumour free, and mean tumour volume was lowest compared to all other groups and were statistically significant compared to mock treated animals with $p < 0.05$. All mice with complete tumour regression remained tumour free 70 days post TC-1 inoculation (data not shown). The observed cases of tumour regression and overall delayed tumour growth in 16E6E7 and 16E6E7m vaccinated mice may indicate the potential for i.t. application of this recombinant live vector vaccine.

In a second experiment mice were inoculated similarly with TC-1 and primed i.n. with PBS, or recombinant 16E6E7, 16E6E7m, or parental virus on day 14/15 (Fig. 20A). All groups of mice primed i.n. had similar mean tumour volumes, with the exception of mice receiving parental influenza virus which had an almost 4 times higher mean tumour volume compared to all other groups (Fig. 20B, day 13). Unexpectedly, mice vaccinated i.n. developed rhinitis, showed less physical fitness compared to animals immunized by s.c. or i.t. vaccination routes, and developed noticeable weight losses even for mock treated mice. Altogether no trend of a reduced tumour growth rate or mean tumour burden was observed in 16E6E7 and 16E6E7m treated mice compared to mock vaccinated animals over

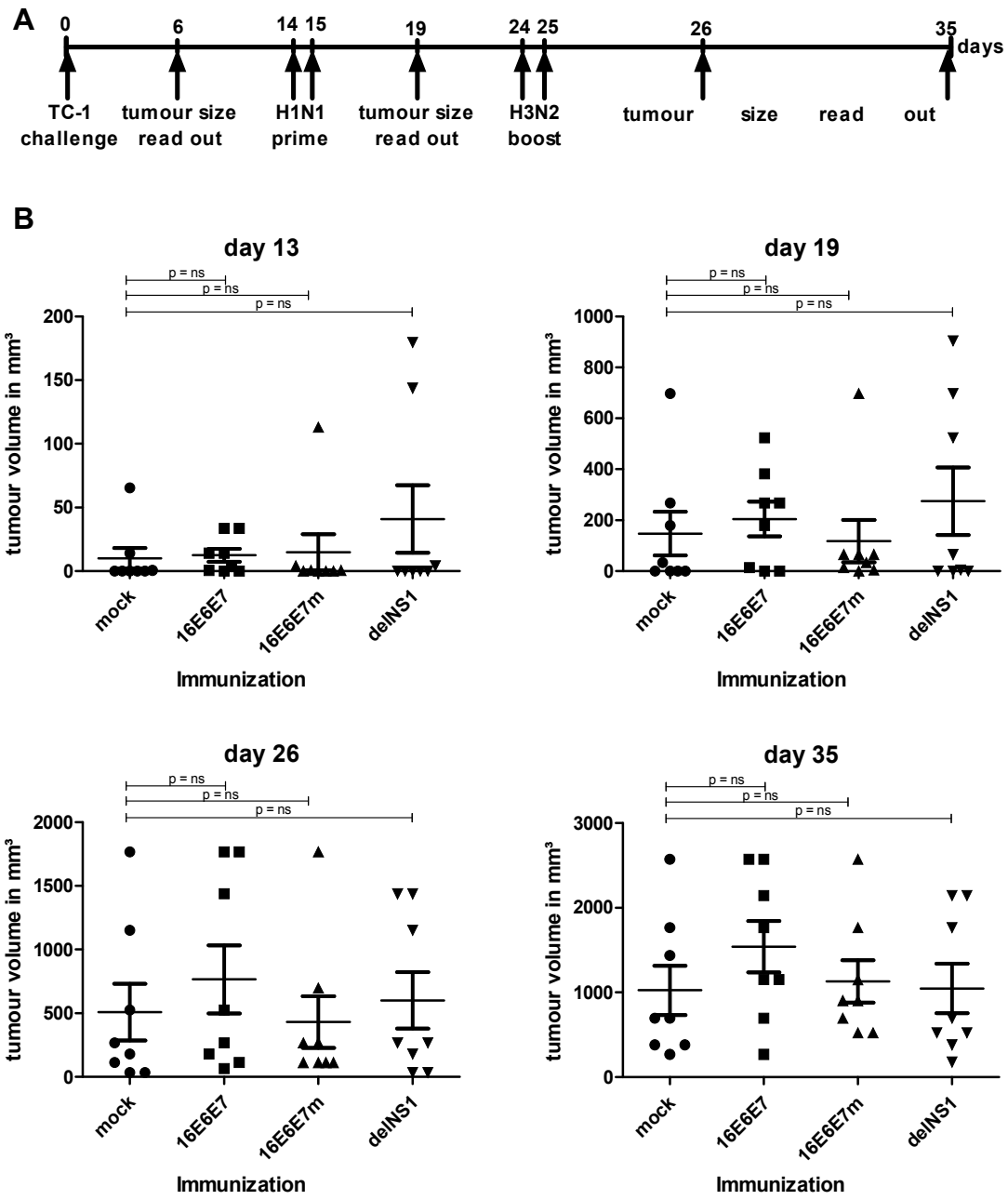

Figure 20: Intranasal vaccination with recombinant 16E6E7 and 16E6E7m influenza viruses does not influence TC-1 tumour growth A Schematic illustration of the experimental set up **B** C57BL/6 female mice (n=8 per group) were challenged with $5x10^4$ TC-1 cells on day 0 and tumour growth monitored. Mice were primed i.n. after palpable tumours had developed on day 14 and 15 with PBS (mock), H1N1 16E6E7, H1N1 16E6E7m, or parental influenza virus (delNS1), and boosted 10 days later either with PBS, or the according H3N2 influenza serotype as indicated. Tumour diameter was measured once per week. Shown are calculated tumour volumes of individual animals and mean ± SD of one representative of two independent experiments. Days after TC-1 tumour cell inoculation are indicated.

the whole time frame of the experiment (Fig. 20B). These results suggest, that mice receiving recombinant influenza A viruses i.n. are unable to mount an effective cell-mediated immune response against HPV16 E6 and E7. This could be either due to induction of rhinitis interfering with effective immune response and/or due to lower vaccination volume because of application constraints. Overall i.n. vaccine inoculation in mice appeared less suitable as application route to induce TC-1 tumour regression due to volume constraints and severe side effects.

4.12 Intratumoral and intranasal vaccination with recombinant 16E6E7 or 16E6E7m influenza A viruses induces HPV16 E6 and E7 specific IFN-γ secreting cells

To address whether i.t. and i.n. application would induce an HPV16 E6- and E7-specific cell-mediated immune response, mice were vaccinated with recombinant 16E6E7, or 16E6E7m influenza viruses, parental virus, or PBS on two consecutive days (13/14) after TC-1 challenge to apply similar volumes as in s.c. experiments and boosted with the corresponding H3N2 influenza serotypes, or PBS 10 days later (days 23/24). Mice were sacrificed 10 days after the boost, splenocytes were isolated, stimulated with peptides harbouring known CTL epitopes of HPV16 E6, E7, or combinations thereof, and specific IFN-γ production was analysed by ELISpot. As controls, SEA and a peptide harbouring known CTL epitopes of influenza A NP were used. Splenocytes of mice immunized either i.t. or i.n. with recombinant or parental influenza A viruses showed IFN-γ production when stimulated with NP-peptide (Fig. 21A, B, groups 3) and cells of all groups had extensive IFN-γ production when stimulated with SEA (Fig. 21A, B, groups 4). In accordance with previous results obtained by s.c. application, cells from mice treated i.t. or i.n. with recombinant 16E6E7 or 16E6E7m influenza viruses produced IFN-γ when peptides $16E6_{131-140}$ or $16E7_{36-62}$, or a mixture thereof were used as a stimuli. Spleen cells from i.n. vaccinated animals revealed about half absolute spot counts (Fig. 21A, groups 7 – 10) compared to i.t. immunized mice (Fig. 21B, groups 7 – 10), suggesting less efficient stimulation by the i.n. application route. In contrast to results obtained

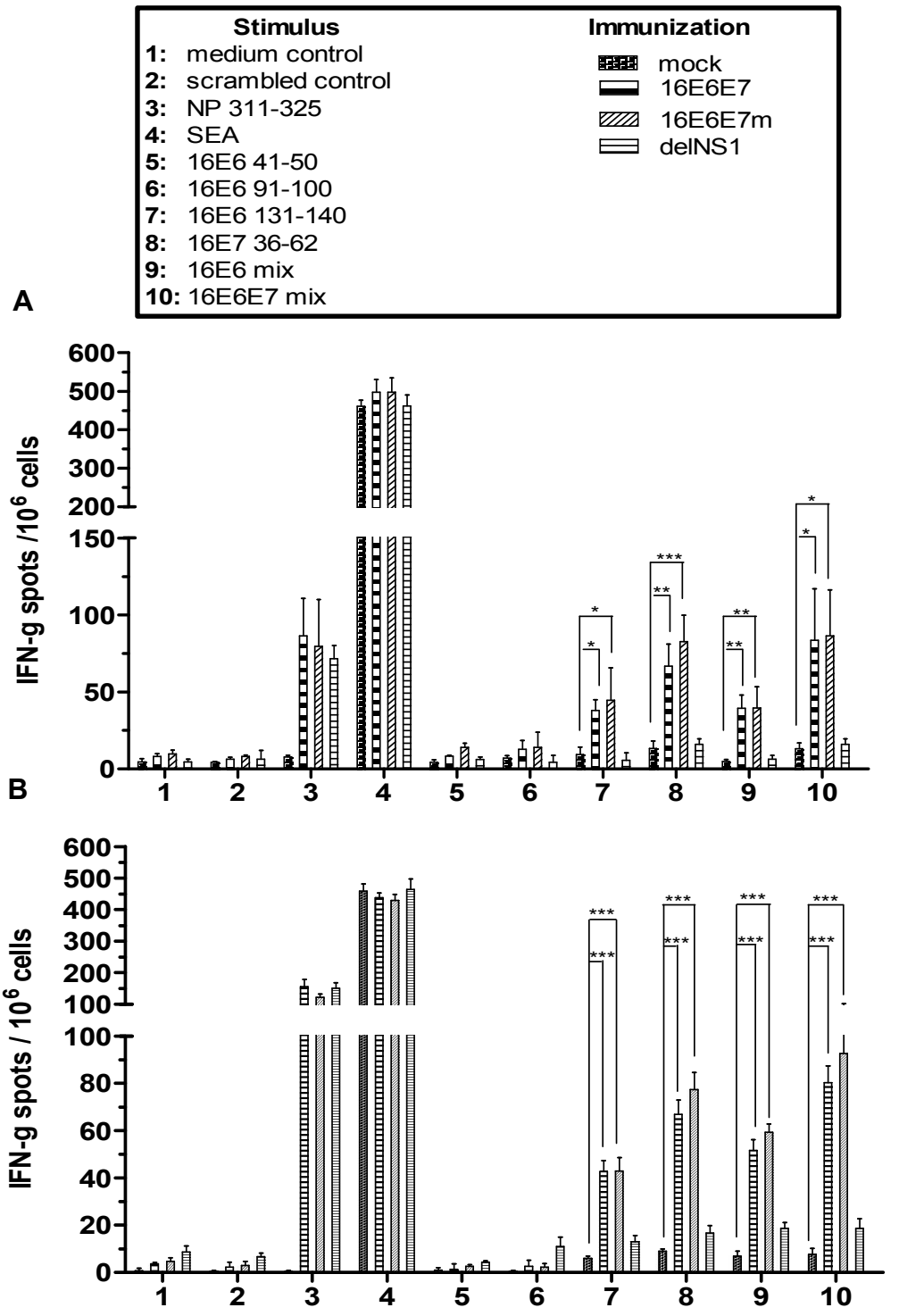

Figure 21: **Intratumoral and intranasal administration of recombinant 16E6E7 or 16E6E7m influenza viruses elicits HPV-specific CTL responses in mice.** C57BL/6 mice (n=4 per group) were inoculated with $5x10^4$ TC-1 cells, primed either **A** i.n. or **B** i.t. with H1N1 16E6E7, 16E6E7m viruses or parental viruses (delNS1), or PBS (mock) as soon as palpable tumours were detectable and boosted 10 days later with the corresponding H3N2 serotypes, or PBS as indicated. Mice were sacrificed 10 days after boosting, splenocytes were isolated and stimulated in triplicates for 24 h with indicated peptides, SEA or medium alone. For animals vaccinated with recombinant influenza A viruses, NP peptide served as a positive control. IFN-γ spots were counted under a light microscope and plotted as mean ± SD. One representative experiment of two is shown. Statistical p-values for 16E6E7 or 16E6E7m vaccination compared to mock are indicated as asterisks (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).

with mice vaccinated s.c., splenocytes of mice immunized with 16E6E7 or 16E6E7m viruses i.t. and i.n. showed higher IFN-γ production when stimulated with $16E7_{36-62}$ (Fig. 21A, B, groups 8) peptides compared to $16E6_{131-140}$ (Fig. 21A, B, groups 7) peptides. When using a mixture of all three 16E6 peptides, no synergy was observed but rather a slight decrease of specific IFN-γ producing cells (Fig. 21A, group 9). This latter observation could be due to suppression by regulatory T cells reactive to $16E6_{45-50}$ and $16E6_{91-100}$ peptides which harbour Th-cell epitopes. A slight increase of IFN-γ spots was seen with splenocytes of mice immunized i.t. with 16E6E7 or 16E6E7m viruses using a mixture of 16E6 and E7 peptides as stimulus (Fig. 21B, group 10), compared to single peptide stimulation with either E6 or E7 alone. No synergy was observable in cells of 16E6E7 and 16E6E7m i.n. vaccinated animals (Fig. 21A, group 10) compared to single E7 peptide stimulation.

4.13 Recombinant 16E6E7 and 16E6E7m influenza A viruses are capable to infect TC-1 tumour cells

Intratumoral application of recombinant Influenza viruses was capable to induce complete TC-1 tumour regression in several animals. We speculated that one possibility for this result might be that TC-1 cells themselves were efficiently infected by recombinant influenza viruses and thereby influenza A specific CTLs were attracted which subsequently, through tumour cell lysis, might enhance the HPV16 E6 and E7 specific immune response due to increased epitope levels of viral oncoproteins expressed in lysed cells. To support this hypothesis TC-1 cells were infected with either 16E6E7, 16E6E7m, or parental viruses of both serotypes for 12 h, mRNA and proteins isolated and subjected to RT-PCR or Western blot, respectively. As control uninfected TC-1 cells were used. RT-PCR revealed bands for both E6E7 and E6E7m fusion mRNAs in recombinant influenza infected cells (Fig. 22A, upper panel lanes 2, 3, 5 and 6), but not in either parental or mock infected TC-1 cells. By Western blot the viral NP protein was observed in all influenza A virus infected cells but not uninfected TC-1 cells (Fig. 22B, upper panel lanes 2 – 7). Cells inoculated with H1N1 16E6E7 virus showed a particular strong band for NP which was due to a higher pfu used for infection. The HPV16 E7 oncoprotein which is stably expressed in TC-1 cells

served as loading control (Fig. 22B, lower panel). These results suggest that recombinant influenza viruses are capable to efficiently infect TC-1 tumour cells which might have altering effects on immune evasion of tumour cells by inducing an antiviral state thus attracting immune cells by modulation of the cytokine microenvironment.

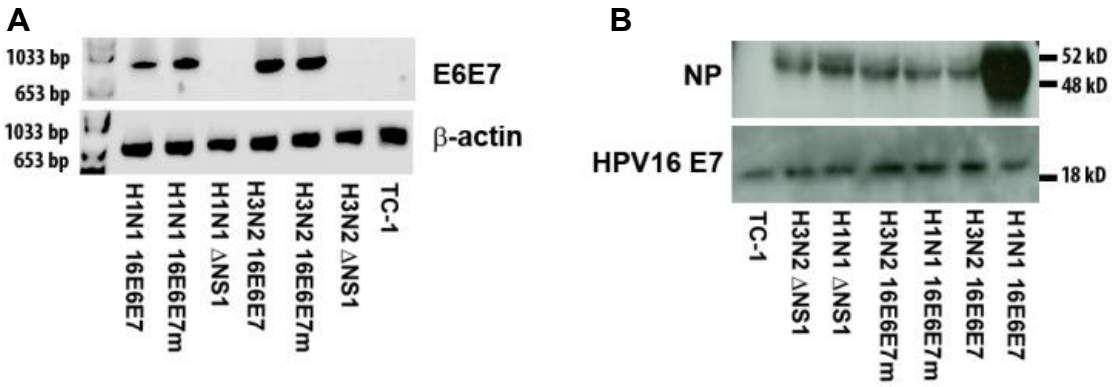

Figure 22: Recombinant 16E6E7 or 16E6E7m influenza viruses infect TC-1 tumour cells **A** TC-1 cells were infected with indicated recombinant viruses for 12 h. RNA was isolated and subjected to RT-PCR to detect HPV16 E6E7 or β-actin mRNA expression, or **B** protein samples were separated by 10% SDS-PAGE and viral NP, or cellular 16E7 as loading control detected by Western blot.

5 Discussion

Driven by the worldwide high incidence of HPV infections and induced disease, two prophylactic vaccines, bivalent Cervarix® and quadrivalent Gardasil®, that target the two most important hr mucosal types HPV16/18 (and also lr HPV6/11 for the quadrivalent vaccine) have been licensed. HPV16 is the most important oncogenic type, present in about 50% CxC, 80% anal carcinomas, and even at higher percentage in the subset of HPV-induced vulvar, vaginal, penile, or oro-pharyngeal cancers. In many countries HPV vaccination coverage is low, primarily due to financial constraints, thus the prevalence of genital HPV is expected to decrease only slowly. Given the lifetime cumulative incidence of genital HPV infection is >50%, HPV associated diseases are expected to remain a significant problem in women and men, especially in high risk groups such as immunosuppressed patients, or homosexual men. For prevalent HPV infection and overt disease there is no specific antiviral or anti-tumour treatments, other than ablative, destructive, or immunomodulatory, and therapeutic vaccination strategies with proven efficacy are still lacking. HPV induced genital neoplasias invariably retain and express the viral E6/E7 oncogenes, which allows using these non-self (viral) components as therapeutic immune target thus avoiding the danger of inducing autoimmunity.

Different approaches, like DNA- or protein-based immunizations have been undertaken for therapeutic vaccination against genital dysplasia or cancer without proven success. This lack of efficacy is probably at least in part due to insufficient targeting of APC and/or induction of cytotoxic immunity against target antigens. Live viral vectors that express the viral oncoproteins E6 and E7 are alternative options to develop therapeutic HPV vaccines. One principle drawback for most live viral vector approaches is the immune response against the vector itself that can lower efficiency of booster immunizations. Attenuated influenza A offers a variety of different serotypes that can be employed in prime-boost vaccination protocols to circumvent previously generated host immune response and therefore allows efficient boosting against target proteins.

Furthermore a strong cell-mediated immune response is induced by influenza A virus vaccination, which may be enhanced even further by use of suitable adjuvant. As important safety feature this RNA virus replicates without any DNA

intermediates, thus avoiding recombination events between the viral and host genomes which alleviates the theoretical concern of E6/E7 oncogenes integrating into the host genome. Furthermore influenza A viruses can be easily propagated in cell culture and thus produced economically in large quantities. By partial deletion of the NS1 ORF, influenza A becomes type I IFN sensitive and thereby attenuated, and offers the opportunity to express non-viral proteins in frame with the NS1 ORF. Previously this attenuated ΔNS1 influenza A vector has been used to express either lymphocyte stimulating cytokines like IL-2 as immune response enhancer or the bacterial antigen ESAT-6 to induce protective immunity against tuberculosis [166, 169].

To develop a therapeutic vaccine against persistent HPV infection and induced disease, recombinant influenza A virus expressing a fusion of the oncoproteins E6 and E7 of HPV16 was generated. Considering patient safety in future clinical trials, in addition to the wt oncogene sequences a transgene with mutationally inactivated HPV16 E6 and E7 sequences was generated, whereby biological activity of the encoded fused proteins was abrogated through informed deletions. Transgene expression was observed after 5 rounds of viral passaging which indicates stability of recombinant viruses.

Following infection of Vero cells, immuno-detection of HPV16 E6, E7, and the FLAG tag located at the C-terminus suggested complete translation of fusion-proteins. Interestingly fusion-proteins were only detected when the proteasomal machinery was inhibited, whereas mRNA expression could readily be observed. These results suggest rapid intracellular degradation of the artificial HPV16 E6/E7 fusion-protein due to improper folding. Therefore it appears unlikely that either the wt or the mutated form of the HPV16 E6/E7 fusion-proteins are sufficiently stable to exert potentially oncogenic biological activity and thus may be regarded principally safe for application in patients. Degradation itself serves favourable for CTL induction since antigen presentation via MHC I complexes is connected to proteasomal degradation of endogenous proteins [184].

In order to eradicate (pre-) cancerous HPV-induced lesions by therapeutic vaccination, a robust cell-mediated immune response is considered necessary. Many approaches like DNA- or protein-based vaccines require strong adjuvants to direct the immune system towards a Th1 response [110, 116]. In contrast attenuated influenza A can serve as its “own adjuvant” to generate robust cellular

immunity. Cells infected with influenza virus attempt to produce type I IFN to obtain an antiviral state that blocks viral replication, and to create a cytokine environment that promotes a cell-mediated immune response against virally infected cells. The viral protein NS1 detains this cellular reaction. By partial genetic deletion of NS1 (and insertion of HPV16 E6 and E7 genes), recombinant ΔNS1 influenza A viruses cannot avoid the host immune system as effectively since they are confronted with the effects of type I IFNs restricting replication and thus become replication deficient in mice [185]. Nevertheless infected cells can express and present epitopes of HPV16 E6 and E7 in an environment that promotes Th1 mediated immunity. The production of IFN-γ serves as a read out for this type of immune response. Herein, in vivo and ELISpot experiments demonstrate that recombinant influenza A viruses are capable to induce HPV16 E6 and E7 specific CTLs that produce IFN-γ in response to stimulation with short peptides of these viral oncoproteins, regardless of application route (s.c., i.t., i.n.). Importantly, CTL reactivity was retained over 5 weeks.

Recent research revealed, that despite high levels of IFN-γ production, HPV16 E7 expressed in skin cells is capable to act immunosuppressively e.g. by expression of IDO1 [186, 187]. It was shown that mice, that received skin grafts expressing HPV16 E7 under a K14 promoter control, were able to reject these grafts when IDO1 activity was inhibited. Interestingly the enzyme was induced by IFN-γ and invariant natural killer T cells (iNKT). By analysis of IFN-γR expression it was shown that DCs in murine K14E7 skin were one resource of IDO1 expression. The possible relevance for humans was shown by analysis of IDO expression levels, which were significantly elevated in epithelia of CIN2/3 patients compared to healthy mucosa.

Besides IFN-γ production by CTL, an additional criterion for effective therapeutic immunity is specific T cell proliferation. T helper (Th) cells are the main effectors for (mounting an immune response and) producing a cytokine environment that promotes either humoral or cell-mediated immunity. However, weak HPV-specific proliferation was observed in vaccinated animals, when splenocytes were stimulated with a mixture of different HPV16 E6 peptides, which may be at least in part due to low sensitivity of the H^3-thymidine incorporation assay used. To increase assay sensitivity carboxyfluorescin succinimidyl ester (CFSE) staining could be used to assess Th-cell proliferation upon peptide stimulation.

Many therapeutic vaccine approaches use adjuvant that direct the immune response towards Th1. To evaluate whether recombinant ΔNS1 influenza A viruses would preferentially induce a (preferable) Th1, rather than Th2 immune response, sera of vaccinated mice were screened for antibodies reactive against HPV16 E6 and E7. By ELISA, sera were negative for E6/E7 Ab reactivity, suggesting that immunity induced by recombinant ΔNS1 influenza A viruses is primarily cell-mediated (Th1) rather than humoral (Th2). Presumably this effect might be further enhanced through the addition of suitable adjuvant to the vaccine formulation. A variety of adjuvant-formulations like ISOCOMATRIX, poly(I:C), which is a toll-like receptor (TLR) 3 ligand, or monophosphoryl lipid A (MPLA), a TLR 4 ligand, in combination with alum might be suitable. TLR3 agonists are especially interesting since influenza reacts predominantly with TLR 7 [188], pattern-recognition-receptors (PRRs) like dsRNA-dependent protein kinase (PKR), and retinoic acid inducible gene-1 (RIG-1). Furthermore TLR3 knock-out mice have similar immune responses and type I IFN production as wild-type control mice when infected with influenza [189]. Therefore induction of type I IFN by different signalling pathways and TLRs might act synergistically to induce a robust cell-mediated immune response against HPV infected cells.

Interestingly control mice immunized with HPV16 L1/L2-E7 VLP were also seronegative for antibodies against HPV16 E7. We may speculate that the amount of HPV16 L2-E7 in the VLP was too low for efficient antibody generation against E7. Vaccination experiments with VLP have shown that predominantly Th2 immune responses are induced. L1 is the major structural protein with a regular repetitive array in the assembled VLP that displays many described epitopes for antibodies. L1 has been shown to act immunodominant over L2 in co-assembled L1/L2 capsids, and masking of E7 by L1 due to its sheer abundance may have driven the humoral immune response primarily against HPV16 L1 rather than E7 as shown by ELISA (Fig. 13B).

The TC-1 mouse challenge model is a well-established system to evaluate therapeutic papillomavirus vaccination. Herein, efficacy of HPV16 E6/E7 influenza vaccination to provide immune protection and tumour rejection was evaluated. In a prophylactic setting 50% of mice vaccinated with recombinant 16E6E7 virus were protected against subsequent challenge with TC-1 tumour cells (Fig. 15). In contrast animals immunized with 16E6E7m virus carrying the

mutated E6/E7 could not sustain protection, as 7/8 animals had developed palpable tumours 35 days after TC-1 inoculation. Overall tumour growth was significantly decreased in animals receiving recombinant 16E6E7- or 16E6E7m-expressing viruses compared to PBS treated animals. Unexpectedly mice vaccinated with parental virus also carried significantly lower mean tumour volumes compared to PBS-immunized control animals (Fig. 14), in the absence of E6/E7-specific immune responses (Fig. 12A, groups 7-10). One hypothesis for this observation might be an enhanced surveillance by stimulation of the innate immune system due to encounter with influenza A viruses. The high unspecific splenocyte proliferation levels observed in virally infected mice may support this assumption (Fig. 13A) as do results obtained by Bergmann et al. who have found unspecific high IFN-γ production by a variety of innate and adaptive immune cells [190].

To analyse the therapeutic capacity of recombinant 16E6E7 and 16E6E7m viruses four different experiments were designed. At first TC-1 inoculation and prime vaccination were performed on the same day. This was done considering that HPV16 associated lesions normally are treated at pre-cancer stages like CIN 2-3 which are less transformed lesions than invasive cancer (or established TC-1 tumours). Even high-grade squamous intraepithelial lesions in humans may be cleared spontaneously and thus may need a less extensive immune response to regress.

By vaccinating mice the same day as tumour inoculation occurred, 62.5% (5/8) animals receiving recombinant 16E6E7m virus were completely protected from tumour development (Fig. 16B, day 42): Mice vaccinated with recombinant 16E6E7 or 16E6E7m viruses or HPV16 L1/L2-E7 VLP showed decreased tumour growth over time compared to mock and parental virus treated animals (Fig. 16B, day 42). Interestingly, in this setting the largest mean tumour volume was observed in animals receiving parental influenza A viruses. These results suggest that therapeutic vaccination with recombinant 16E6E7 and 16E6E7m influenza A inducted specific HPV16 E6/E7 immune responses which significantly reduced growth of TC-1 tumour cells.

In a second even more stringent therapeutic setting TC-1 cells were inoculated and vaccination started when at least 50% of mice had developed palpable tumours. Tumour cell growth tended to be delayed and slower in mice immunized

with recombinant 16E6E7 and 16E6E7m viruses compared to PBS-immunized animals (Fig. 17B), the suppressive effect being stronger in 16E6E7 virus vaccinated animals. Importantly decrease of tumour diameters was observed in 2 mice receiving recombinant 16E6E7m virus (not shown). Furthermore, animals receiving either recombinant, but also parental influenza viruses showed significantly prolonged survival compared to mock treated animals (Fig. 18). This may suggest that vaccination provided a putative cell-mediated immune response in mice that was not sustained over time, or circumvented by tumour escape mechanisms,. Therefore strategies to enhance Th1 mediated immune responses should be an important next step for improvement of influenza-based vaccine efficacy. One strategy envisaged might combine live-based and protein- or peptide-based vaccination, either concomitantly or sequentially. Another approach could combine in vitro matured syngeneic dendritic cells loaded with peptides of either HPV16 E6 or E7 as immune cell-therapy. Previous studies with a DNA-based therapeutic HPV vaccine showed that boosting with DC which had been pulsed with the epitope RAHYNIVTF of HPV16 E7 induced a strong cell-mediated immune response against TC-1 tumour cells in mice [191]. A recent study used human peripheral blood derived DC pulsed with either HPV16 E7 protein or peptide as stimulators for allogeneic proliferation assays. Potent proliferation levels of antigen-specific T cells were found and cytotoxicity assays verified HPV16 E7 specific CTLs. Furthermore in vivo experiments in a immune reconstitution SCID mouse model showed enhanced IFN-γ levels of HPV16 E7 specific T cells and size-reduction of HPV16 E7 expressing tumours [192]. Further options include inactivation of HPV16 E6/E7-specific tolerogenic immune cells like $CD25^+$ $FoxP3^+$ regulatory T cells, or the addition of the universal T cell epitope PADRE to the HPV16 E6E7 fusion-protein, to enhance T helper cell proliferation. Influenza A viruses engineered to express cytokines stimulating the adaptive immune system or directing immune responses towards a Th1 type could be applied simultaneously in alternative vaccination regimens that might be applicable in humans.

Furthermore a third therapeutic vaccination scheme was performed to analyze the effectiveness of i.t. or i.n. administration and whether tumour regression could be achieved with efficacy similar or even superior to s.c. injection. For this setting animals were inoculated with TC-1 cells and mice primed as soon as at least

50% of animals of each immunization group had developed palpable tumours. One restriction of i.t. and i.n. application is the reduced vaccine volume that can be applied. Therefore i.t. and i.n. vaccination had to be splitted into two immunizations, with reduced total vaccine volume for i.n. administrations (see methods). Vaccination by both administration routes induced E6/E7 specific IFN-γ production by $CD8^+$ T cells (Fig.21, groups 7-10). More importantly i.t. vaccination with 16E6E7 viruses caused tumour regression in 25% of recombinant influenza A virus immunized mice (Fig. 19B) and generally delayed tumour progression compared to mock and parental virus treated animals. One may speculate that recombinant influenza viruses are capable to infect TC-1 tumour cells as possible explanation for the observed complete tumour regression. Infection of TC-1 cells in vitro was indeed verified by detection of viral NP (Fig. 22B) and mRNA of the E6E7 fusion-gene (Fig. 22A). HPV16 E6 is known to interfere with IFN-β and the transcription factor IRF-3 whereas HPV16 E7 blocks IFN-α inducible genes [193]. Thus we may speculate that infection with recombinant influenza A viruses forces tumour cells to produce large amounts of type-I IFNs like IFN-α or -β. As a consequence this increased cytokine production could overcome the effects of the oncoproteins E6 and E7 and cause induction of an antiviral state of the cell and expression of pro-inflammatory genes, thereby attracting cell-mediated immunity to the tumour site [194].

In contrast to i.t. application, i.n. administration of recombinant influenza viruses did not have any therapeutic effect. Although a HPV specific CTL response was detectable in mice immunized i.n with recombinant 16E6E7 or 16E6E7m influenza viruses (Fig.21A, groups 7-10) IFN-γ spot counts were lower compared to s.c. and i.t. vaccinated animals. This could be due to lower vaccine volumes (and thus influenza inoculum) due to administration restrictions, or less efficient induction of cell-mediated immunity since i.n. treated mice suffered from rhinitis and significant weight loss. Interestingly even PBS-treated mice showed these side effects suggesting that the TC-1 tumour challenge mouse model may not be suitable to analyse the efficacy of i.n. vaccination for technical reasons.

Further research will focus to elucidate antigen presentation efficacy upon s.c. immunization. For example, histological preparations of TC-1 tumours of s.c. vaccinated mice will be evaluated for tumour infiltrating $CD8^+$ T cells, DCs, and infiltrates as compared to i.t. vaccinated mice. The combination of different

vaccination approaches like protein- and live-vector-based prime-boost regimens will be another topic of research. Most importantly tumour escape mechanisms may need to be addressed to increase therapeutic vaccine efficacy. Due to the close linkage of the life cycle of HPV to epithelial cell differentiation viral antigen exposure to immune cells is minimized. Recently CxC cells were shown to produce high levels of IL-6 which suppressed up-regulation of the chemokine receptor CCR7 but did not alter myeloid-derived DC maturation or the matrix metallo-proteinase 9 (MMP-9) which is necessary for DC migration to lymph nodes. Thus CxC cells may impair DC egress to peripheral lymph nodes while using DC-derived MMP-9 for tumour growth and angiogenesis [195]. Another example is naturally occurring $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells, the immune-modulatory role of which in tumours could be therapeutically targeted. The programmed death 1 (PD-1) and its ligand PDL-1 are in particular interesting. In hr HPV positive women with CIN increased expression of both surface receptors on cervical T cells and DC has been observed. Expression increases with increasing CIN grade whereas co-stimulatory molecules CD80 and CD86 are reduced on DC [196].

Another field of future research will focus on the oncolytic capacity of recombinant influenza A viruses. Besides a previous IL-15 expressing oncolytic influenza virus, it was shown that a variety of tumours, like prostate [197] or colon [198] cancer, can be lysed or driven into apoptosis by influenza A virus infection. Further analysis of the role of innate immunity will address the function of natural killer (NK) cells, which have been shown to efficiently lyse tumour cells infected by attenuated influenza A virus [199].

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Curriculum vitae

Christoph Jindra, Mag. rer. nat.
Nationality: Austria
E-mail: christoph.jindra@gmx.at

Work experience:

02. 2004 – 04. 2004 Intern, Novartis Research Corporation, Vienna
>Dr. T. Schweighoffer
> Co-worker on the project „Characterization of the surface marker EWI-2”

11. 2002 – 07. 2003 Scientific associate, Dep. of oral surgery, Medizinische Universität Wien
> Dr. R. Gruber
> Research on bone formation and degradation

07. 2002 – 08. 2002 Intern, Dep. of obstetrics and gynecology, Medical University Vienna
> Prof. Dr. M. Hengstschläger
> Role of tuberin and harmatin on the cell cycle

Education:

06. 2008 – to date PhD studies in molecular biology: Laboratory for viral oncology, Div. of Immunology, Allergy and Infectious Diseases (DIAID), Dept. of Dermatology, Medical University Vienna
> Prof. Dr. R. Kirnbauer
> Topic: „Generation of an Influenza A based therapeutic HPV16 vaccine“

09. 2000 – 09. 2007 Master studies in molecular biology: University Vienna
Diploma thesis: Institute of immunology
Prof. Dr. J. Stöckl
> Topic: „Characterization of novel surface markers on human naturally occurring regulatory T cells“

1999 – 2000 Civil service: Treatment of handicapped school-children

1991 – 1999 Erich Fried Bundesrealgymnasium Vienna IX

Advanced training:

> FELASA B certificate, University of veterinary medicine Vienna

> Biosafety Level 2 training, Medical University Vienna

> Quality Control for working with radioactive materials

Qualifications:

Languages	> German (native speaker)
	> English (fluently)
	> Italian (basic knowledge)
Computer skills	> MS Office (excellent)
	> Image – video – and audio editing(good)
	> System administration (basic knowledge)

Publications:

Gruber R, **Jindra C**, Kandler B, Watzak G, Fischer MB, Watzek G: Proliferation of dental pulp fibroblasts in response to thrombin involves mitogen-activated protein kinase signalling. Int Endod J. 2004 Feb;37(2):145-50.

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submitted

Jindra C, Huber B, Shafti-Keramat S, Wolschek M, Ferko B, Muster T, Brandt S, Kirnbauer R: Attenuated recombinant Influenza A virus expressing HPV16 E6 and E7 as a novel therapeutic vaccine approach.
submitted

Presentations:

Identification of a novel cell surface marker on CD4+ CD25+ regulatory T cells
Oral presentation, Annual Meeting ÖGAI; Graz, Austria, 2005

Identification of a novel cell surface marker on CD4+ CD25+ regulatory T cells
Poster-Presentation, 16th European Congress of Immunology; Paris, France, 2006

Generation of an Influenza A based therapeutic human papillomavirus vaccine
Poster- Presentation, 28th International Papillomavirus Conference; San Juan, Puerto Rico, 2012

Generation of an Influenza A based therapeutic human papillomavirus vaccine
Poster- Presentation, 40th Annual Meeting ADF; Dessau, Germany, 2013

Recombinant, attenuated Influenza A virus expressing HPV16 E6 and E7 elicits cell-mediated immune responses and induces regression of TC-1 tumours in mice
Oral presentation, 29th International Papillomavirus Conference; Seattle, USA, 2014

Funding:

ECI-2006/EFIS scholarship travel grant, 2006
ÖGAI travel grant, 2006