Immunotherapy based on influenza virosomes and recombinant Semliki Forest virus

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CHAPTER 3

RECOMBINANT ALPHAVIRUSES AS VECTORS FOR ANTI-TUMOR AND ANTI-MICROBIAL IMMUNOTHERAPY

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Abstract

Background: Vectors derived from alphaviruses are gaining interest for their high transfection potency and strong immunogenicity.

Objectives: After a brief introduction on alphaviruses and their vectors, an overview is given on current preclinical immunotherapy studies using vector systems based on alphaviruses. The efficacy of alphavirus vectors in inducing immune responses will be illustrated by a more detailed description of immunization studies using recombinant Semliki Forest virus for the treatment of human papillomavirus-induced cervical cancer.

Results: Immunization with recombinant alphavirus results in the induction of humoral and cellular immune responses against microbes, infected cells and cancer cells. Preclinical studies demonstrate that infectious diseases and cancer can be treated prophylactically as well as therapeutically.

Conclusions: Alphavirus-based genetic immunization strategies are highly effective in animal model systems, comparing quite favourably with any other approach. Therefore, we hope and expect to see an efficient induction of tumour- or microbial immunity and a positive outcome in future clinical efficacy studies.
**Introduction**

Vectors based on alphaviruses are gaining increasing interest for their superiority over other viral vectors with respect to the induction of cellular and humoral immune responses. Currently, prophylactic and therapeutic vaccines for infectious diseases and cancer based on these vectors are being developed. The prototypic vectors are derived from Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus. Recombinant alphavirus particles carry the RNA that codes for the replicase and the heterologous gene but lack the RNA that codes for the structural proteins. Consequently, infection of cells with recombinant alphavirus vectors will not result in productive replication and the virus will not spread beyond initially infected cells.

Before giving an overview of the current (pre)clinical studies using alphavirus vectors we will briefly introduce alphaviruses and their derived vector systems. We will illustrate the high efficacy of alphavirus vectors in inducing immune responses by a more detailed description of our immunization studies using recombinant Semliki Forest virus for the treatment of human papillomavirus-induced cervical cancer.

**Alphaviruses**

Alphaviruses are small, enveloped, positive strand RNA viruses belonging to the family Togaviridae. The alphavirus genus comprises 27 different members including Semliki Forest virus (SFV), Venezuelan Equine Encephalitis virus (VEE) and Sindbis virus (SIN), from which vector systems have been developed. SFV and SIN were originally isolated from mosquitoes. SFV is named after the Semliki Forest (Uganda) and SIN after the Egyptian village Sindbis [1]. VEE was first recognized as the causative agent of infectious equine encephalomyelitis in Venezuela.

Alphaviruses are naturally transmitted by mosquitoes to vertebrates, and in turn back to mosquitoes [1]. In vertebrate cells, virus infection results in the rapid shutoff of host mRNA translation, take over of the translational machinery by viral mRNAs, production of high titres of infectious virus and eventually cell death by apoptosis. In mosquito cells virus replication is slower and often has minimal effects on the cell. Natural vertebrate hosts are avian and mammalian species. Although there is a risk of infection, alphaviruses are not major pathogens to humans. The spectrum of alphavirus disease in humans ranges from silent asymptomatic infections or undifferentiated febrile illness (SFV) and mild polyarthritis (SIN) to encephalitis (VEE). The incidence in humans is very low. Nonetheless, especially for vectors derived from VEE, bio-
safety features in the vector system are essential to prevent formation of infectious virus. Yet, also for vector systems derived from SFV and SIN these biosafety aspects are incorporated, as will be explained below.

**Alphavirus structure**

As the structure of SIN and SFV has been studied in considerable detail, the information in this and the following paragraph is based on these two type-specific members of the alphaviruses [2]. Alphaviruses are spherical particles with a diameter of 65-70 nm. The viral genome consists of a single stranded RNA genome surrounded by a capsid, together forming the nucleocapsid. The capsid is formed by a regularly arranged icosahedral (20-faced) shell composed of 240 copies of one protein: the capsid protein. The nucleocapsid is enveloped by a lipid bilayer derived from the host-cell plasma membrane into which 240 copies of the glycoproteins, E1, E2 for SIN and E1, E2 and E3 for SFV, are inserted. The glycoproteins form 80 hetero-oligomeric spikes. Each spike consists of a trimer of E1/E2 heterodimers (SIN) or E1/E2/E3 heterotrimers (SFV) [3]. These polypeptide chains span the lipid bilayer and interact with the C protein. The spike-forming trimers projecting outward from the surface of the virus can be seen by electron microscopy (Figure 1). The alphavirus genome consists of a single

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**Figure 1: The SFV structure.**

RNA molecule of positive polarity which is capped and polyadenylated and serves directly as an mRNA once introduced in the host cell. It consists of two open reading frames (ORF). The first ORF codes for the four non-structural proteins of the virus that form the replicase complex (nsP1-4). The second ORF encodes the structural proteins of the virus: the capsid (C) protein and the envelope proteins E2 (synthesized as a larger precursor P62 (SFV) or PE2 (for SIN)), 6K and E1, as shown in Figure 2.

Alphavirus life-cycle and replication

Alphaviral infection is initiated by binding of the viral envelope protein to a cell surface protein that serves as its receptor on the host-cell plasma membrane [2]. For SFV several proteins have been suggested as functional receptors and consistent with its broad host range, SFV is probably able to utilize a variety of surface receptors with varying affinity. After binding, the virus enters the cell by clathrin-mediated endocytosis and is transported to endosomes. The acidic pH within the endosomal compartment causes the viral spikes to mediate fusion between the viral and the endosomal membranes [4;5]. The nucleocapsid then is released into the cytosol, where uncoating of the nucleocapsid by ribosomes is followed by release of the viral RNA into the cytoplasm. After fusion with the endosomal membrane the viral glycoproteins are transported to lysosomes to be degraded [6]. Recently, the regulation of this intracellular transport mechanism was further characterized demonstrating that Rab7 (a small GTPase) is recruited to early endosomes, where it forms distinct domains that mediate cargo sorting as well as the formation of late-endosome-targeted transport vesicles [7].

Alphavirus infection results in the shutoff of host proteins synthesis in favour of viral translation. Recently, McInerney et al [8] demonstrated that for SFV the inhibition of host protein synthesis is due to the activation of the cellular stress response resulting in the formation of stress granules [9]. Stress granules (SG) are cytoplasmic domains into which mRNAs are sorted in response to phosphorylation of eukaryotic initiation factor eIF2-alpha, a key regulatory step in

![Figure 2.](image)

The wild-type SFV genome consists of two open reading frames; the first of which encodes the four non-structural proteins that form the replicase complex, the second codes for the structural proteins.
translation initiation. This mechanism enables stressed cells to shut down the expression of normal house-keeping genes to allow the selective expression of stress response factors. The mRNA is believed to be stored, pending either degradation or resumption of normal translation in the absence of stress. During SFV infection, SG formation is transient and occurs at the time of host shutoff, i.e., when the profile of protein production changes from cellular to viral [8]. SFV-induced SGs dissolve in the vicinity of viral RNA as replication progresses.

Viral replication begins with translation of the first ORF, which codes for the four non-structural proteins nsP1, nsP2, nsP3 and nsP4 that make up the viral replicase. This replicase initially catalyses the formation of a full-length negative strand intermediate – the 42S RNA – from which more genomic RNA is produced. These genomic positive-strand RNA molecules are capped by the replicase at the 5′-end. Secondly, the replicase catalyses transcription of the second ORF to form a subgenomic 26S RNA molecule that codes for the structural proteins. This subgenomic RNA is replicated to large amounts, leading to a high production of the structural proteins. The newly synthesized capsid and envelope proteins follow separate pathways through the cytoplasm. The capsid protein, like the cytosolic proteins of the cell, is synthesized by ribosomes that are not membrane-bound. The newly synthesized genomic RNA and capsid protein are rapidly associated into new nucleocapsids in the cytosol. In contrast, the envelope proteins, like the plasma membrane proteins of the host cell, are synthesized by ribosomes that are bound to the ER. Rapidly after their synthesis P62 and E1 (SFV) or PE2 and E1 (SIN) form heterodimers. These future envelope proteins are inserted into the membrane of the ER, where they are glycosylated, transported to the Golgi apparatus, and then delivered to the plasma membrane. At a compartment after the trans-Golgi network, but prior to appearance at the cell surface, the P62 or PE2 precursor is cleaved into the mature moieties E2 and E3. The viral nucleocapsids and envelope proteins finally meet at the plasma membrane. As a result of an interaction between the C proteins in the nucleocapsid and the cytoplasmic tail of E2, the nucleocapsid forms a bud whose envelope contains the envelope proteins embedded in host-cell lipids. Finally, the bud pinches off and a free virus particle is released on the outside of the cell. The clustering of envelope proteins as they assemble around the nucleocapsid during viral budding excludes the host plasma membrane proteins from the final virus particle.

Recombinant vector system based on alphaviruses

Alphavirus-based expression vectors have been constructed according to two fundamental designs. On the one hand replicon vectors, generating recombinant replicon particles that are
limited to one round of infection and on the other hand double-genomic vectors that generate replicating recombinant virus. In both designs the recombinant RNA is self-replicating and expresses the foreign gene(s) at high levels. Yet, in the replicon system transgene expression is transient while in the replicating system transgene expression is more sustained. Next to replicon particles, alphavirus vaccines are being developed consisting of naked DNA/RNA replicons.

**Replicon vectors**

Using SIN, Xiong et al [10] were the first to develop a replicon expression vector based on an alphavirus. Later on replicon vectors were developed using SFV and VEE virus [11;12]. The principles of the SIN and VEE replicon systems are in essence similar to the vector system based on SFV, which is described below.

Liljeström and Garoff [11] developed the SFV-based replicon vector system. The full-length cDNA copy of the viral genome was cloned in a bacterial plasmid including a prokaryotic DNA-dependent RNA polymerase such that viral RNA can be transcribed *in vitro*. These RNA transcripts are fully infectious, i.e. introduction into cells suffices to initiate replication and a full infection cycle, resulting in virus formation. Next the alphavirus RNA replication and packaging machinery was adapted for expression of heterologous RNAs and proteins in animal cells. The structural proteins of SFV have been deleted and replaced with a polylinker into which foreign genes can be inserted (Figure 3). As the RNA is self-amplified by the replicase complex,

![Wild type SFV and Recombinant SFV vector](image)

**Figure 3: Schematic presentation of the wild-type SFV genome and the recombinant SFV vector.**

For use of alphaviruses as recombinant vector systems, the structural proteins are deleted and a gene of interest can be inserted.
high level expression of the foreign gene is obtained. The helper vector(s) codes for the capsid and spike proteins (Figure 4). Recombinant SFV (rSFV) virus-like particles can be generated by cotransfection of cells with the recombinant RNA vector and a helper RNA vector (Figure 5). The RNA packaging signal is located in the non-structural region of the vector and absent on the helper vector. Thus, only the recombinant RNA is packaged into newly generated virus particles that are released from the packaging cell. This helper system provides a first line of biosafety in that virus particles are formed that lack the genes encoding the structural proteins. For use in humans, increased biosafety can be obtained by the split helper system (Figure 4) [13]. Splitting the helper plasmid in two helper plasmids decreases the probability of formation of infectious, replication competent virus, as recombination between the two helper and the vector plasmids is highly unlikely. Since the RNA encoding the structural proteins is not encapsidated, the recombinant particles that are generated undergo only one round of infection, being unable to produce progeny virus. These rSFV particles are therefore also termed “suicide” particles. Upon infection, the recombinant RNA is expressed to high levels, including the gene of interest that is inserted into the multiple cloning site. Expression is transient as infected cells undergo apoptotic cell death.

More recently it was shown that by the introduction of a translational enhancer element in the SFV vector system the expression of the foreign gene can be strongly enhanced. This enhanced vector contains the first 34 amino acids of the SFV capsid gene and the foot and mouth disease virus (FMDV) 2A protease downstream of the 26S promoter. Foreign genes are cloned in frame with this translational enhancer element, which results in enhanced translation of the gene. The FMDV protease is included so that the enhancer element is cotranslationally removed from the foreign protein. Expression levels in cells transfected with these “enhanced” rSFV particles are up to 10-fold higher than those transfected with the standard rSFV particles [14]. Although the mechanism of the enhancement is not entirely clear it was demonstrated that translation is only enhanced in the presence of intact eIF2-alpha (transla-
tion initiation factor) phosphorylation; these cells form stress granules upon SFV infection (see above). The authors suggest that the SFV translational enhancer counteracts the translation inhibition imposed by eIF2-alpha phosphorylation [8].

**Replicating vector systems**

Next to these suicide replicon vectors, replicating vectors based on alphaviruses are being developed that allow more prolonged gene expression. In the first type of replicating vectors the transgene is placed under the transcriptional control of a duplicate 26S promoter inserted within the 3' nontranslated region of the viral genome or within the short nontranslated region located just upstream of the native 26S promoter [15]. Since double-subgenomic vectors retain all viral genes, they are capable of multiple rounds of infection and result in a more sustained transgene expression. Yet, eventually also with these vectors gene expression is
transient as, similar to the replicon particles, cells infected with these replicating virus particles die and the virus is cleared by the immune system. As replicating vectors may be of great value for future (immuno)therapy of cancer or infectious diseases several other strategies are being investigated to generate replicating alphavirus vectors [16-19]. Yet, as the number of studies using replicating virus is limited so far, we will, in the next paragraphs, focus on the use and applications of replication-defective replicon particles.

Immunization strategies based on alphavirus vectors

Prophylactic vaccination against infectious diseases in general aims at the induction of humoral immune responses to prevent infection. This humoral immune response is mediated by plasma cells, i.e. antibody-producing B cells. On the other hand, therapeutic immunisation against infected cells and tumour cells requires the induction of cytotoxic T lymphocytes (CTL) that can specifically recognise and lyse infected cells or tumour cells. For the differentiation, expansion and memory induction of plasma cells and tumour- or microbe-specific CTLs, T helper cells (Th cells) are required. And, as key orchestrators in these processes of both humoral and cellular immune responses, properly activated antigen-presenting cells, dendritic cells (DCs) in particular, are essential.

Characteristics of alphavirus-based immunization strategies

Vectors based on alphaviruses are gaining increasing interest because of their superiority over other viral vectors with respect to the induction of both humoral and cellular immune responses. This superiority can be ascribed to several features of alphavirus-based immunization strategies but presumably lies in the combined effects of these features. Characteristics that make alphaviruses attractive candidates for development of replication-defective viral vectors for application in humans are that (i) recombinant alphaviruses induce high-level expression of encoded proteins, (ii) after 48-72 h of protein expression infected cells die by apoptosis resulting in apoptotic bodies containing high levels of the transgene protein which may be very beneficial for the induction of immune responses via cross-priming [20], (iii) recombinant alphaviruses activate both the innate and the adaptive immune system. Infection of cells results in dsRNA intermediates that are known for their immunopotentiating capacity [21]. dsRNAs can be recognized by innate immune receptors such as Toll-like receptor 3 and trigger production of Interferon type I, while, in addition, dsRNAs activate and induce maturation of DCs
[22], (iv) humans in general do not carry neutralizing antibodies against SIN or SFV that may decrease the efficacy of the immunization. In addition, Berglund et al [23] demonstrated that upon immunization of mice with rSFV the immune responses against the SFV vector itself did not disable boost responses by subsequent immunizations with the same vector.

With respect to the delivery of encoded protein antigen to DCs for MHC class I and MHC class II processing and presentation one can envision two alternative mechanisms: (i) recombinant alphaviruses transfect DCs directly thereby inducing synthesis of the encoded antigen in the cytosol followed by MHC processing and presentation (direct priming) or (ii) the recombinant virus particles transfect other cell populations. When these cells, due to the infection, undergo apoptotic cell death they could serve as a source of apoptotic bodies containing substantial amounts of the expressed antigen. Dendritic cells have been shown to take up apoptotic bodies and to efficiently present the enclosed antigens on MHC class I molecules in a process of so-called cross-priming [24]. In this respect rSFV, rVEE and rSIN differ in their natural cell tropism. While rVEE and rSIN readily transfect murine DCs [25-27], rSFV does not [20;28]. Studies with monocytes, macrophages and DCs from various origins, including human and murine DCs, revealed that rSFV has a very limited capacity to transfect these cell types in vitro [20]. To further investigate whether rSFV in vivo transfect professional antigen-presenting cells directly or whether the antigens reach these cells via cross-priming we compared the immunological effects of rSFV-constructs encoding different forms of Human Papillomavirus (HPV) E6 and E7 or influenza nucleoprotein [20;29]. These constructs differed in the amount of protein produced per cell or in the stability of the protein. We found that the induction of CTLs appeared to benefit from a large amount of stable antigen. In contrast, rapid antigen degradation, and thus availability of antigenic peptides in the transfected cell, was found to be disadvantageous. Based on these in vitro and in vivo results, we hypothesize that antigen presentation after SFV-based immunization proceeds via a mechanism in which antigen-presenting cells are not transfected directly but acquire antigen from other transfected cells and present it to CTLs in a process of cross-priming (Figure 6). Recently, Chen et al [30] confirmed that infection of DCs with SFV in vitro is very inefficient. Interestingly, in this study on the role of MyD88 on the presentation of antigen derived from virally infected cells, these authors provide further proof that cross-priming indeed is the main mechanism by which immunity to an SFV replicon is generated.

Despite the difference in tropism for DCs between SIN and VEE, on the one hand and SFV on the other hand, immune responses elicited upon immunization with these recombinant alphaviruses in general is comparably efficient, yielding high levels of antigen-specific CTL and antitumour or anti-viral responses. As it is to be expected that rVEE and rSIN, similar to rSFV also
infect cells other than DCs resulting in cross-priming of antigen, it remains to be established if the immune response elicited with rVEE and rSIN is a consequence of direct priming of DCs or of cross-priming or perhaps of a combination of both routes.

Wahlfors et al [31] evaluated the utility of both SIN and SFV vectors in comparison to each other and to other vector types on different target cells. In general, SFV appeared to have a higher transduction efficiency than SIN. However, high transduction efficiency turned out to be not necessarily accompanied by a high transgene expression: the rate of transgene expression was identical for both viruses. These authors also compared alphaviral (SIN) vectors with adenoviral and retroviral vectors by transduction of several cell lines with these vectors carrying human clotting factor IX cDNA. The concentration of hFIX in the cell medium was followed for four days after transduction. SIN expression climbed rapidly to high level, followed by a

Figure 6: Schematic presentation of our hypothesis on the mechanism of antigen presentation after SFV-based immunization.

Antigen-presenting cells (DC) are not transfected directly but acquire antigen from other transfected cells and present it to CD4+ and CD8+ T cells in a process of cross-priming. This process results in the generation of CTLs that can recognize tumor cells or virally infected cells expressing peptide epitopes of the recombinant protein as produced by rSFV.
quick drop, whereas retroviral and adenoviral expression initiated later and remained constant for a longer period of time (48h). This suggests that alphaviruses may be particularly valuable in situations that require rapid high-level but transient gene expression such as certain cancer gene therapies and vaccination.

**Immunization strategies against infectious disease**

The efficacy of rSFV expressing viral antigens has been evaluated in immunization studies in mice, guinea-pigs, monkeys and even in chicks. The antigens most extensively studied are the nucleoprotein and haemagglutinin of influenza virus [23], several HIV and SIV antigens [32;33], Human Papilloma virus (HPV) E6 and E7 protein [29;34-37], antigens from Louping Ill virus [38], Respiratory Syncytial virus [39;40], Tickborne Encephalitis virus [39], Hepatitis C virus [41] and Infectious Bursal Disease virus in chicks [42]. So far only a few studies have been reported on the use of rSFV immunizations against bacterial (Chlamydia pneumoniae [43] and Brucella Abortus [44]) and parasitic diseases (Plasmodium Falciparum [45;46]). Similarly, rSIN and rVEE have been studied as vectors for the induction of immune responses against HIV and SIV [47-50], HPV E7 [51-54], Norwalk virus [55], Equine Arteritis virus in horses [56] Anthrax [57], Staphylococcus [58] and Mycobacterium tuberculosis [59].

These immunizations aim to induce sterilizing and long-lasting immunity against the microbe and/or eradication of infected cells by inducing micro organism-specific antibodies and/or specific CTL responses. Although in general T cell responses, including CTL responses, are readily induced against antigens encoded by recombinant alphaviruses, humoral responses against the antigens are not always induced. Humoral responses have been reported against rSFV-encoded nucleoprotein and hemagglutinin protein of influenza virus, [23] and spike proteins of Louping ill virus [38;60]. Yet, in our own studies on rSFV expressing HPV16 E6E7, in which we aim to induce strong CTL responses against HPV-infected cells, immunizations never resulted in detectable humoral responses against the E6 and E7 protein, using various E6E7 constructs and routes of immunization (as determined for us by Michael Pawlita, German Cancer Centre, Heidelberg, Germany). In a Chlamydia pneumoniae study [43] in which SFV-MOMP and SFV-Omp2 were evaluated, immunization resulted in detectable systemic Omp2 antibody levels while no MOMP-specific antibodies were induced. Despite this difference in humoral response, both constructs induced similar antigen-specific T cell responses and similar levels of (partial) protection against a challenge with Chlamydia pneumoniae. Similarly, although T cell responses and (partial) protection were induced, no antibodies could be raised against the non-structural protein 3 of hepatitis C virus [41], SIV antigens [33] and Brucella abortus
superoxide dismutase [44] encoded by rSFV and HIV-1 Gag encoded by rSIN [47].

Although for several immunization strategies the induction of humoral responses is not essential to confer protection as long as cellular immunity is induced, in the malaria studies of Chen et al [46] the aim is to specifically induce antibodies that disrupt rosettes and protect against the sequestration of Plasmodium falciparum-infected erythrocytes. Therefore, these authors generated an SFV construct which generates proteins that are expressed extra-cellularly but anchored to the cell membrane by a transmembrane domain. In this way the antigen is displayed at the eukaryotic cell surface as is the native protein on the infected RBC surface. A prime-boost immunization regime of rSFV (prime) and recombinant protein (boost) resulted in antibody levels with rosette-disrupting activity.

In conclusion, the induction of humoral responses upon immunizations with alphaviral vectors varies a great deal depending on the antigen, the processing and presentation of the antigen and the immunization route. Nonetheless, in most studies, strong cellular immune responses are induced that result in (partial) protection against specific microorganisms. Further studies will have to elucidate if the humoral responses that in some models are induced against alphavirus-encoded antigens are indeed responsible for the observed protection or if cellular immunity that in general is also induced is the main effector mechanism.

**Immunization strategies against tumors**

Tumor vaccines based on alphaviruses are in general designed to stimulate or augment an immune response against existing tumor cells. The efficacy of rSFV has been evaluated in a limited number of preclinical tumor models, including melanoma (MAGE-3, [61] and mammary tumor (Neu) [62]). Yet, most of the work on the use of SFV vectors as tumor vaccines has concentrated on two mouse models of human tumors, which both are weakly antigenic and express known tumor-associated antigens. The first tumor model tested was the P815 mastocytoma tumor in mice [63;64], which expresses a weak tumor rejection antigen. Administration of rSFV expressing variants of the P815 antigen resulted in induction of strong cell-mediated immune responses against the tumor antigen. Vaccinated mice were protected against tumor challenge, or, when vaccinated therapeutically, showed inhibition of tumor growth or even total regression of the tumor. The second tumor model used in alphaviral immunization studies is HPV-induced cervical cancer. In the next paragraph we will focus on our own studies using rSFV for therapeutic immunization against cervical cancer. Similar studies are being performed using rVEE, and rSIN as vectors [51;52].
Cervical cancer is the third most common cancer among women worldwide. It is caused by infection with high-risk Human Papillomavirus (HPV), in particular types 16, 18, 31, 33 or 45. High-risk HPVs have the capacity to transform cervical epithelial cells by integrating the open reading frames encoding the viral early proteins E6 and E7 into the host cell genome. This integration may lead to constitutive overexpression of E6 and E7, mediating transformation of the cells to a malignant phenotype. Since the continued production of E6 and E7 is required for the maintenance of the transformed phenotype, E6 and E7 in fact represent tumor-specific antigens in cervical carcinoma and premalignant HPV-transformed cells. As a consequence, E6 and E7 are potential targets for immunotherapeutic intervention strategies involving induction or stimulation of cytotoxic T lymphocyte (CTL) activity against HPV-transformed cells.

We initially demonstrated that immunization of mice with rSFV particles encoding HPV16 E6 and E7 resulted in a potent HPV16-specific CTL and anti-tumor response [34]. However, we were unable to induce full tumor protection. We next generated a construct in which the stop codon between E6 and E7 was removed and one base pair was inserted between the genes of E6 and E7 resulting in a construct that codes a stable fusion protein of E6 and E7. In addition, a translational enhancer was included to induce a high production of the fusion protein. The CTL response and anti-tumor activity induced by this so-called SFV-enhE6,7 virus appeared much stronger compared to the responses induced with rSFV, producing the separate E6 and E7 proteins [29]. Tumor treatment experiments, clearly demonstrated the high potency of the

![Figure 7: Efficacy of SFV-enhE6,7 immunization on regression of established tumors.](image)

Mice were inoculated s.c. in the neck with $2 \times 10^4$ TC-1 cells. Subsequently, mice were immunized and boosted i.v. with $5 \times 10^6$ SFV-enhE6,7 at days 7, 14 and 21 ($n = 7$; panel B), at days 14, 21, 28 ($n = 7$; panel C) after tumor inoculation. As control, mice were injected i.v. with PBS ($n = 10$, panel A) at days 7, 14 and 21. Tumor measurements were initiated 10–14 days after tumor challenge and subsequently measured twice weekly. Given is the tumor volume of individual mice. The percentages indicate the percentage of tumor-free mice for each treatment at day 70 after tumor inoculation.
vector (Figure 7). Exponentially growing tumors of approximately 500 mm$^3$ in size were seen to completely resolve and even some tumors as large as 1500 mm$^3$ decreased to one third of their size [35;36]. Considering that a tumor nodule of 1000 mm$^3$ contains approximately $10^9$ cells, this implies that in the latter situation, i.e. a tumor decreasing 1000 mm$^3$ in volume, the CTLs generated, manage to kill $10^9$ cells in a one-week period. An other important aspect of our immunization approach is the induction of a long-term immune response, i.e. a memory response is induced which can even eradicate s.c. inoculated tumors half a year after the mice were immunized.

Enhancement of CTL induction upon immunization with a vector encoding a more stable protein may seem inconsistent with several excellent studies in which MHC class I presentation has been demonstrated to be potentiated by enhanced degradation of antigen. However, the explanation lies in the cross-priming pathway through which antigen is presented upon injection of rSFV, as described in a previous section. For cross-priming, the recombinant proteins to be presented should be stable for the time that is required for the entire process of infection of cells through uptake by APCs. Although RNA replication and translation occur within 6 hr after infection, dying of the infected cells by apoptosis takes another 24 to 72 hr. It therefore takes at least 24 hr after production before the protein gradually becomes available for APCs to be presented. Thus, the balance between stability and rate of degradation of the protein appears to determine the efficiency of antigen presentation.

In cervical cancer patients, HPV-specific CTL activity is generally low (Visser JTJ et al 2005 Int J Cancer, in press), suggesting that they have mounted a certain degree of immunological tolerance or ignorance for the HPV-derived antigens. We recently studied whether SFV-expressing HPV16 E6 and E7 is potent enough to also prime a cellular immune response in immune-tolerant HPV-transgenic mice, in which CTL activity can not be induced using protein or DNA vaccines. We demonstrated that, depending on the route of immunization, SFV-enhE6,7 indeed has the capacity to induce HPV16 E7-specific cytotoxic T cells in HPV-transgenic mice [37]. Clearly, although the mechanism and kinetics of tolerance in this mouse model differ from that in humans, these studies demonstrate the potency of alphaviral vectors for immunization purposes.

**Clinical studies using alphavirus vectors**

Until recently, alphavirus vector systems had not been used in human clinical studies. In 2003,
a human trial among 40 volunteers has been performed in the area of HIV vaccine development (HIV Vaccine Trials Network (HVTN)). This study involved the use of a vector system based on VEE. The vaccine was well tolerated and no serious adverse events have been identified. In 2004/2005 another 96 volunteers were included in a multi centre dose-escalation study. Several rSFV applications will be evaluated in clinical trials in the near future. The European Vaccine Effort Against HIV/AIDS (Eurovac) will conduct human clinical studies using rSFV vectors encoding HIV-1 subtype C gag, pol, nef and env genes.

Reference List


[10] Xiong C, Levis R, Shen P, Schlesinger S, Rice CM, Huang HV. Sindbis virus:


danger motifs bridge innate and adaptive immunity and are potent adjuvants

[23] Berglund P, Fleeton MN, Smerdou C, Liljestrom P. Immunization with
recombinant Semliki Forest virus induces protection against influenza

cross-priming to virus-infected cells. Nature 2005 Feb 24;433(7028):887-
92.


sindbis virus replicon vector is determined by a single amino acid

[27] Ryman KD, Klimstra WB, Nguyen KB, Biron CA, Johnston RE. Alpha/beta
interferon protects adult mice from fatal Sindbis virus infection and is an
important determinant of cell and tissue tropism. J Virol 2000 Apr;74(7):3366-
78.

[28] Chen M, Cortay JC, Logan IR, Sapountzi V, Robson CN, Gerlier D. Inhibition
of ubiquitination and stabilization of human ubiquitin E3 ligase PIRH2 by

[29] Daemen T, Regts J, Holtrop M, Wilschut J. Immunization strategy against
cervical cancer involving an alphavirus vector expressing high levels
of a stable fusion protein of human papillomavirus 16 E6 and E7. Gene Ther

expression is required for efficient cross-presentation of viral antigens from

recombinant alphaviruses as vectors in gene therapy. Gene Ther 2000

using an alphavirus replicon DNA vaccine against human immunodeficiency


[51] Lin CT, Hung CF, Juang J, et al Boosting with recombinant vaccinia increases HPV-16 E7-Specific T cell precursor frequencies and antitumor effects of HPV-16 E7-expressing Sindbis virus replicon particles.


[61] Ni B, Gao W, Zhu B, et al Induction of specific human primary immune responses to a Semliki Forest virus-based tumor vaccine in a Trimera mouse


