Alphavirus Vectors in Vaccine Development

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Abstract
The high-level heterologous gene expression provided by alphavirus vectors has accelerated their applications in vaccine development. The versatility of alphavirus vectors has allowed their use in the form of recombinant viral particles, naked RNA and layered DNA molecules for immunization. The most commonly used alphaviruses have been Semliki Forest virus, Sindbis virus and Venezuelan Equine Encephalitis virus. Numerous viral structural proteins have been used as antigens to generate neutralizing antibodies in immunized animals. Vaccination has demonstrated protection against challenges with lethal doses of viruses. Moreover, vaccination with tumor antigens has demonstrated prophylactic protection against cancer. Novel approaches include the application of RNA interference and microRNA. The other side of the coin is the development of vaccines against alphaviruses themselves, and typically the Chikungunya virus.

Keywords: Alphaviruses; Viral vectors; DNA vaccines; Neutralizing antibodies; Protection against viral; Tumor challenges

Introduction
The members of alphaviruses belong to the Togaviridae. They possess a single stranded RNA genome, which together with the capsid forms the nucleocapsid surrounded by membrane proteins embedded in a liposome envelope structure [1]. A number of alphaviruses has demonstrated pathogenicity among them recently Chikungunya with global fever epidemics [2]. Although Semliki Forest Virus (SFV) has been associated with an outbreak of febrile illness in Central Africa [3] and Venezuelan Equine Encephalitis (VEE) Virus with an epidemic in horses and humans in South America [4], attenuated strains has been the basis for the development of safe and efficient expression vectors. In this context, SFV [5], Sindbis virus (SIN) [6] and VEE [7] vectors have been engineered for heterologous gene expression.

The most common approach has been to generate replication-deficient alphavirus vectors. These can be used for vaccine development in three variations (Figure 1). Naked RNA consisting of the nonstructural replicase genes and the antigen generating gene downstream of the strong subgenomic promoter can be administered as such. Moreover, replication-deficient recombinant alphavirus particles providing infection capacity of a broad range of host cells without any further production of virus progeny are potential vehicles for immunization. Finally, layered DNA vectors, which provide the replicase genes and antigen of interest in DNA form, can be applied. In all approaches advantage is taken of the extremely efficient RNA replication of some 200,000 RNA copies from each RNA molecule due to the presence of the alphaviral replicase genes. The most significant differences are related to delivery and safety aspects described later in the review. A number of topologically different recombinant proteins have been expressed from particularly SFV vectors [8]. Typically, high expression levels of integral membrane proteins have been obtained in various mammalian host cell lines [9], in primary neurons [10], and in vivo [11]. In the context of vaccine development, the three main vector systems (SFV, SIN and VEE) have been applied in the forms of naked RNA, recombinant virus particles and layered DNA vectors [12]. As described below viral and tumor antigens have been administered in various animal models to elicit neutralizing antibodies and protection against challenges with tumor cells or lethal doses of viruses.

Viral Vaccine Approaches
The obvious targets for vaccine development have been viral structural proteins [12] (Table 1). For instance, influenza nucleoprotein (NP) and hemagglutinin (HA) have generated strong immune responses in rodents [13] and even protection against challenges with H5N1 virus in chicken [14]. Moreover, several studies have been conducted on HIV targets (env, gp41, MA/CA) in attempts to elicit antibody responses in mice [15-17]. Additionally, immunization

Figure 1: Alphavirus vectors applied in vaccine development. A. Naked RNA vector: In vitro transcribed RNA is directly injected into animals. B. Replication-deficient recombinant particles: Replicon particles are obtained from in vitro transcribed RNA from expression and helper vectors after co-electroporation of BHK-21 cells. C. Layered DNA vectors: Plasmid DNA can be used directly for immunization.
studies with VEE vectors in mice and guinea pigs demonstrated that protection could be achieved against challenges with some of the most feared viruses such as Ebola [18] and Lassa [19]. More recently, an alphavirus replicon (VEE) particle vaccine expressing the cluster IV H3N2 swine influenza HA gene demonstrated protection against challenges with homologous influenza virus [20]. In another study, VEE particles expressing the human influenza HA protein was demonstrated to generate high antibody titers in swine illustrating their potential use in vaccine development [21]. In attempts to improve the immunogenicity the herpes simplex virus type 1 (HSV-1) VP22 protein was fused to influenza HA from the H5N1 subtype [22]. Immunization studies demonstrated that both interleukin-4 (IL-4) of CD4+ T cells and interferon-gamma (IFNγ) of CD8+ T cells in vaccinated mice suggesting a promising approach for vaccine development against human-avian influenza viruses. Among the newly emerging viruses, the severe acute respiratory syndrome coronavirus (SARS-CoV) has

| Virus          | Target                  | Vector/Delivery | Immunization | Response | Reference |
|---------------|-------------------------|-----------------|--------------|----------|-----------|
| NS3 (p80)     | SFV / DNA               | Mouse           | CTL          | CMI      | [63]      |
| CSFV          | E2                     | SFV / DNA       | Swine        | Neutralizing Abs | [24] |
| Ebola         | NP, GP                  | VEE / Particles | Mouse        | Guinea pig | Ebola protection | [65] |
| VP24, 30, 35, 30 | VEE / Particles | Mouse        | Ebola protection | [66] |
| Hepatitis B   | cAg                    | SIN / DNA       | Mouse        | Specific Abs | [67] |
|               | sAg                    | SIN / DNA       | Mouse        | Specific Abs | [67] |
| Hepatitis C   | cAg                    | SFV / Particles | Mouse        | CTL       | [68] |
| NS3           | SFV / Particles         | Mouse          | Cellular     | [69] |
| HeV           | Glycoprotein            | VEE / Particles | Mouse        | Neutralizing Abs | [25] |
| HIV-1         | env                    | SFV / Particles | Mouse        | Humoral   | [15] |
|               | gp11                   | SFV / Particles | Mouse        | Monoclonal Abs | [16] |
| MA/CA         | VEE / Particles         | Mouse          | Humoral, CTL | [17] |
| HPV           | 16E7                   | SFV / DNA      | Mouse        | CTL       | [70] |
|               | 16E7-VP22               | SIN / Particles | Mouse        | CDB+ T cell response | [71] |
| HSV-1         | gpB                    | SIN / Particles | Mouse        | CTL       | [72] |
|               | gpB                    | SIN / DNA      | Mouse        | CTL, protection | [74] |
| IBDV          | VP2                    | SFV / Particles | Chicken     | Specific Abs | [75] |
| Influenza     | HA                     | SFV / Particles | Mouse        | Systemic response | [13] |
|               | HA                     | SFV / DNA      | Mouse        | Humoral, cellular | [76] |
|               | HA                     | VEE / Particles | Chicken      | Influenza protection | [14] |
|               | HA                     | VEE / Particles | Swine        | Influenza protection | [20] |
|               | HA                     | VEE / Particles | Swine        | Specific Abs | [21] |
|               | NP                     | SFV / Particles, RNA | Mouse | Humoral, CTL | [77] |
| Measles       | HA, F Ud               | SIN / DNA      | Mouse        | Measles protection | [29] |
|               | HA, F Ud               | SIN-VEE / Particles | Macaques | Measles protection | [30] |
| MVE           | prME, E                | SFV / Particles | Mouse        | Neutralizing Abs | [84] |
| NIV           | Glycoproteins          | VEE / Particles | Mouse        | Neutralizing Abs | [25] |
| NLV           | VLP                    | VEE / Particles | Mouse        | Immune response | [85] |
| Rabies        | G                      | SIN / DNA      | Mouse        | Rabies protection | [32] |
| RSV           | F, G                   | SFV / DNA, RNA | Mouse        | RSV protection | [86] |
|               | F, G                   | SFV / Particles | Mouse        | RSV protection | [87] |
| SARS-CoV      | Glycoprotein           | VEE / Particles | Mouse        | SARS-CoV protection | [23] |
| SEOV          | M, S                   | SIN (Particles, DNA) | Hamster | SEOV protection | [86] |
| SHIV          | env                    | SFV / Particles | Macaques     | T cell prolif. Response | [89] |
| Vaccinia      | A33R, B5R              | VEE / Particles | Mouse        | Vaccinia protection | [31] |

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| Vaccinia      | A33R, B5R              | VEE / Particles | Mouse        | Vaccinia protection | [31] |
been targeted for vaccine development applying VEE replicon particles [23]. VEE vaccinated aged mice showed protection against challenges with SARS-CoV. Moreover, a combined vaccine approach with SFV DNA vectors and recombinant adenovirus expressing the Classical Swine Fever Virus (CSFV) E2 glycoprotein demonstrated higher titers of neutralizing antibodies in pigs [24]. Challenges with the virulent CSFV Shimen strain showed no symptoms of viremia, for the combined vaccine, whereas immunization with adenovirus alone resulted in viremia in one pig of five. Glycoproteins from the zoonotic pathogenic Hendra Virus (HeV) and Nipah Virus (NiV), which can cause fatal infections in both animals and humans, have been expressed from VEE particles to generate neutralizing antibodies [25]. Preliminary results indicated the approach enhanced the induction of cross-reactive neutralizing antibodies. In a study on Rift Valley Fever Virus (RVFV) both DNA plasmids and aliphavirus replicons expressing the RVFV glycoprotein Gn fused to the C3d complement protein were applied for the vaccination of mice [26]. This strategy resulted in generation of neutralizing antibodies and provided protection against RVFV challenges. The plasmid DNA and aliphavirus replicon approaches as well as the combined DNA prime/replicon boost strategy therefore shows great promise for valid RVFV vaccine development.

Sequential immunization with SIN and VEE replicon particles encoding the type 1 HIV gp140 envelope (Env) and trimeric Env protein in MF59 adjuvant resulted in partial protection against high-dose intravenous challenge with simian-human immunodeficiency virus (SHIV) in macaques [27]. More recently, it was shown that antibody-mediated protection could be extended to intramuscular and mucosal routes of delivery [28]. The immunization resulted in different degrees against subsequent mucosal SHIV challenge, but interestingly those macaques that were vaccinated intramuscularly with aliphavirus replicon particles and boosted with Env protein were completely protected. Two SIN DNA vaccines expressing the hemagglutinin (pMSIN-H) and fusion proteins (pMSINH-FdU) elicited neutralizing antibodies, mucosal and systemic antibody-secreting cells, memory B cells and IFNγ secreting T cells in cotton rats [29]. A one hundred percent protection against pulmonary measles was obtained after priming with pMSIN-H. In contrast, pMSINH-FdU priming gave protection only after live measles virus vaccine boost. Moreover, chimeric VEE/SIN replicon particles have been applied for the expression of hemagglutinin (H) and fusion (F) proteins of measles virus [30]. Intradermal vaccination of macaques resulted in high-titer neutralizing antibody and IFNγ-producing T cells. Challenges with wildtype measles virus 12-17 months after vaccination showed protection from rash and viremia. In the need of safe and more efficient smallpox vaccines, VEE particles expressing the vaccinia virus A33R, B5R, A27L and L1R genes elicited protective immunity in vaccinated mice [31]. Likewise, immunization of macaques generated efficient antibody response and was able to neutralize and inhibit the spread of vaccinia and monkeypox viruses. Interestingly, a rabies virus vaccine study where SIN-based DNA encoding rabies glycoprotein (G) was compared to a conventional rabies DNA vaccine and to Rabipur vaccine [32]. The replicon-based DNA vaccine induced better humoral and cell mediated immune responses than the conventional DNA vaccine in immunized mice. Moreover, complete protection was demonstrated against challenge with rabies virus CVS strain.

Non-viral Targets

In addition to viral targets a number of other infectious pathogens have been addressed as targets for vaccine development (Table 2). In this context, mice immunized with SFV vectors expressing the Plasmodium falciparum Pf32 antigen elicited immunological memory [33]. In another approach, SIN-based plasmid DNA vaccination with the Mycobacterium tuberculosis 85A antigen (Ag85A) provided strong immunity and resulted in long-term protection against M. tuberculosis challenges in mice [34]. Moreover, SFV DNA replicons were applied to express the botulinum neurotoxin A Hc gene (BoNTA-Hc) [35]. Both antibody and lymphoproliferative responses were obtained in BALB/c mice. Co-expression of the granulocyte-macrophage colony-stimulating factor (GM-CSF) as an adjuvant enhanced immunogenicity. Replication-deficient SFV particles have also been used for immunization experiments in BALB/c mice for the Brucella abortus translation Initiation Factor 3 (IF3) [36]. It was demonstrated that mice challenged with the virulent B. abortus strain 2308 exhibited a significant level of resistance. Alternative forms of the protective antigen (PA) for Bacillus anthracis were expressed from SIN vectors [37]. Vaccination of Swiss Webster mice induced PA-specific IgG and neutralizing antibodies and also offered some protection against challenges with a lethal Ames strain.

Tumor Vaccine Approaches

Additional aliphavirus applications are related to tumor vaccines (Table 3). One of the basic studies involved the immunization of mice with naked SFV RNA carrying the LacZ gene, which resulted in therapeutic efficacy [38]. Other vaccine approaches with SFV particles expressing the P1A gene [39] and VEE vectors carrying the Human Papilloma Virus (HPV) E7 gene [40] resulted in protection against further tumor development. In attempts to enhance the efficacy of HPV vaccines the adjuvant effect of interleukin-12 expressed from SFV vector was evaluated in mice [41]. Even a low dose of SFV-IL12 stimulated antigen-specific CTL responses and anti-tumor activity after SFV-based HPV16-E6E7 immunization. However, increased dosages did not improve these activities. Recently, aliphavirus replicon-based expression of Melanoma Differentiation Antigen (MDA) tyrosine managed to prevent the growth of B16 transplantable melanoma [42]. It was demonstrated that the vaccine encoding tyrosine related protein 2 (TRP-2) relied on a novel immune mechanism, which required activation of both IgG and CD8+ cell effector responses.

### Table 2: Vaccine development for non-viral infectious agents.

| Agent | Target | Vector/Delivery | Immunization | Response | Reference |
|-------|--------|-----------------|--------------|----------|-----------|
| B. antracis | PA | SIN / Particles | Mouse | B. antracis protection | [37] |
| B. abortus | IF3 | SFV / Particles | Mouse | Brucella protection | [36] |
| C. botulinum | BoNTA-Hc | SFV / DNA | Mouse | Abs, lymphoproliferation | [35] |
| Malaria | CS | SIN / Particles | Mouse | Malaria protection | [90] |
| M. tuberculosis | Ag85A | SIN / DNA | Mouse | Protection | [34] |
| P. falciparum | Ag P1332 | SFV / Particles-RNA | Mouse | Immunological memory | [33] |
| Prior | NP | SFV / Particles | Mouse | Monoclonal Abs | [91] |
| Staphylococcus enterotox B | VEE / Particles | Mouse | Protection | [92] |

Abs: Antibodies; SFV: Semliki Forest virus; SIN: Sindbis virus; VEE: Venezuelan equine encephalitis virus.
**Clinical Trials for Alphavirus Vaccines**

Despite the numerous studies conducted in various animal models very few evaluations have been carried out with alphaviruses in humans. The first clinical trial for SFV relates to intravenous administration of liposome encapsulated particles in melanoma and kidney carcinoma patients [43]. The first vaccine-related alphavirus study was a Phase I randomized, double-blind clinical trial for cytomegalovirus (CMV) [44]. A two component vaccine expressing CMV gB or pp65/1E1 fusion protein was administered intramuscularly or subcutaneously in CMV seronegative adult volunteers. The vaccine was well tolerated showing only mild to moderate local reactogenicity and no clinical important changes. The immunization induced neutralizing antibody and multifunctional T cell responses against CMV antigens. In another study, it was shown that alphavirus particles, which efficiently infect dendritic cells, could be repeatedly administered to patients with metastatic cancer expressing the Carcino Embryonic Antigen (CEA) [45]. Moreover, CEA-specific antibodies were capable of mediating antibody-dependent cellular cytotoxicity against tumor cells from human colorectal cancer metastases. Most encouragingly, patients with T cell responses against CMV antigens. In another important changes. The immunization induced neutralizing antibody showing only mild to moderate local reactogenicity and no clinical important changes. The immunization induced neutralizing antibody and multifunctional T cell responses against CMV antigens. In another study, it was shown that alphavirus particles, which efficiently infect dendritic cells, could be repeatedly administered to patients with metastatic cancer expressing the Carcino Embryonic Antigen (CEA) [45]. Moreover, CEA-specific antibodies were capable of mediating antibody-dependent cellular cytotoxicity against tumor cells from human colorectal cancer metastases. Most encouragingly, patients with CEA-specific antibodies showed extended overall survival.

**Recent Vector Development**

It is appropriate in this review to describe some recent development of alphavirus vector applications. It was demonstrated that when VEE replicon particles without any transgene were used as adjuvant for an inactivated influenza vaccine in rhesus monkeys, the influenza-specific CD4+ T cell responses were 4.4 fold higher and the virus-specific IFNγ and IL-2 producing CD4+ T cells were enhanced 7.6 and 5.3 fold, respectively [46]. In summary, the VEE replicon particles used as adjuvant dramatically improved the immunogenicity and protection against challenges with the human seasonal influenza isolate A/Memphis/7/2001 (H1N1). In another study, alphavirus adjuvants were co-administered with mouse-tropic norovirus (MNV)-like particle vaccines [47]. These multivalent vaccinations significantly reduced the viral load of MNV suggesting that the humoral immunity may protect against challenges with heterologous noroviruses. Furthermore, VEE replicons were shown to possess adjuvant activity and induced an increased and balanced IgG subtype response, which also increased augmented systemic and mucosal antigen-specific CD8+ T cell responses [48]. This approach provides the potential molecular basis for alphavirus-induced immunity and improvement in alphavirus-based vaccines.

Chimeric VEE-SIN vectors for the expression of measles virus hemagglutinin (VEE/SIN-H) have been compared to a non-formalin-inactivated alum-precipitated measles vaccine (FI-MV) [49]. Although the MV-specific IgG levels were similar, the VEE/SIN-H antibodies showed neutralizing activity. Spontaneous ex vivo production of IFNγ and IL-4 was observed in induced T cells after immunization with VEE/SIN-H, whereas vaccination with FI-MV-induced T cells generated IL-4 only after stimulation. In another approach vaccine vectors were constructed based on live recombinant Vesicular Stomatitis Virus (VSV) and an SFV replicon that propagates through expression of the VSV glycoprotein (G) [50]. Applying these vectors for the expression of Simian Immunodeficiency Virus (SIV) gag and env proteins in vaccinated macaques resulted in protection against challenges with lethal doses of SIV. Recently, it was demonstrated that a heterogeneous prime-boost approach with recombinant SFV encoding a HPV E6-E7 fusion protein and virosomes containing HPV E7 resulted in higher numbers of antigen-specific CTL in mice than applying homologous protocols [51]. However, the higher frequency of central memory T cells after homologous immunization, which is crucial for cancer vaccines, indicates that the superiority in number of antigen-specific CTL observed after heterologous prime-boost immunization should not be overestimated.

**Vaccines against Alphaviruses**

In the context of alphavirus vaccines a large portion has dealt with generating vaccines against various alphaviruses (Table 4). For instance, BALB/c mice vaccinated with an attenuated VEE strain resulted in protection against airborne virus [52]. Furthermore, the live attenuated V3526 VEE vaccine showed improved protection against VEE challenges [53]. Likewise, C57BL/6 mice demonstrated complete protection against lethal challenges with a virulent Eastern Equine Encephalitis (EEE) virus strain after vaccination with a chimeric EEE and Western Equine Encephalitis (WEE) virus [54]. A live Chikungunya (CHIK) tested in a human Phase II trial demonstrated generation of neutralizing antibodies [55].

A new approach for designing attenuated alphaviruses has been

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**Table 3: Vaccine development for cancer targets.**

| Target            | Gene          | Vector/ Delivery | Immuno- | Response | Reference |
|-------------------|---------------|------------------|---------|----------|-----------|
| Brain tumor       | IL-12         | SFV / Particles  | Mouse   | Mouse    | [93]      |
| Cervical cancer   | HPVE6E7       | SFV / Particles  | Mouse   | Mouse    | [94]      |
| Glioma            | B16, 203      | SFV / Particles  | Mouse   | Mouse    | [95]      |
| Melanoma          | MDA/trp-2     | VEE / Particles  | Mouse   | Mouse    | [96]      |
| Tumor             | G-galactosidase| SFV / RNA       | Mouse   | Mouse    | [38]      |
| Tumor             | HPV/E7        | VEE / Particles  | Mouse   | Mouse    | [40]      |
| Tumor             | HPV/E6E7+IL12 | SFV / Particles  | Mouse   | Mouse    | [41]      |
| Tumor             | HPV/E7-VP22   | SIN / Particles  | Mouse   | Mouse    | [96]      |
| Tumor             | IL-12         | SFV / Particles  | Mouse   | Mouse    | [97]      |
| Tumor antigen     | MHC class II  | SFV / Particles- DNA | Mouse   | Mouse    | [98]      |
| Tumor antigen     | P185          | SFV / Particles  | Mouse   | Mouse    | [39]      |
| Tumor antigen     | trp-1         | SIN / DNA       | Mouse   | Mouse    | [99]      |

**Table 4: Vaccine development against alphaviruses.**

| Virus      | Gene       | Vector Delivery | Immuno- | Response | Reference |
|------------|------------|-----------------|---------|----------|-----------|
| CHIK       | TSI-GSD-218| CHIK Infection  | Human   | Neutralizing Abs | [55]      |
| CHIK       | Glycoprotein| CHIK Infection  | Macaques| Neutralizing Abs | [58]      |
| EEE        | EEE/EWE    | EEE Infection   | Mouse   | EEE protection | [54]      |
| VEE        | VEE att    | VEE Infection   | Mouse   | VEE protection | [100]     |
| VEEc       | VEE V5526  | VEE V5526       | Mouse   | VEE protection | [101]     |
| VEE        | VEE TC-83  | VEE Infection   | Mouse   | VEE protection | [102]     |
| VEE        | VEE 265    | VEE Infection   | Macaques| VEE protection | [59]      |
| WNV        | WNV att    | WNV Nanopatch   | Mouse   | Abs | [60]      |

Abs: Antibodies; att: attenuated; CHIK: Chikungunya virus; EEE: Eastern equine encephalitis virus; SFV: Semliki Forest virus; SIN: Sindbis virus; VEE: Venezuelan equine encephalitis virus; WNV: West Nile virus
to tackle the mechanisms of replication and virus-host interaction. Variants of CHIK envelope lacking important contributors to viral pathogenesis were made incapable of transmission by mosquito vectors by making their replication dependent on internal ribosome entry sites (IRES) [56]. This engineering prevented replication on cells of mosquito origin, whereas the replication occurred efficiently in Vero cells. In another study, chimeric vaccine candidates were engineered applying the non-structural genes of either the attenuated VEE strain TC-83 or a naturally attenuated EEV strain and the structural genes of CHIK [57]. The vaccines showed significantly lower infection of *Aedes aegypti* and *A. albopictus*, the common urban vectors for CHIK, which suggested a low risk of transmission. A synthetic DNA vaccine expressing a component of the envelope glycoprotein was engineered based on a new CHIK virus isolated from an acutely infected human patient [58]. In vivo electroporation induced robust antigen-specific cellular and humoral immune responses and provided protection against CHIK challenge in mice. Additionally, studies in macaques showed induction of neutralizing antibodies similar to those found in convalescent human patient sera.

Nonhuman primates were subjected to a VEE DNA vaccine in an aerosol model, where the VEE 26S structural genes were expressed from a DNA vector [59]. No viremia was detected in two out of three vaccinated macaques, while one animal showed low viremia. In contrast, control animals demonstrated high viremia.

In the area of vaccine delivery, the Nanopatch comprised of arrays of densely packed projections has been applied for skin vaccination of West Nile Virus and CHIK in mice [60]. The goal was to target epidermal and dermal antigen presenting cells (APCs). The efficiency of Nanopatch delivery was demonstrated using an inactivated whole CHIK vaccine and a DNA-based attenuated West Nile Virus vaccine. This approach offered needle-free, highly effective and inexpensive vaccine delivery.

The discovery of gene silencing as a common phenomenon in biology has had a major impact on all areas of drug discovery. Not surprisingly, efforts to apply RNA interference have also reached vaccine development. In this context, the efficiency of small interfering RNAs (siRNAs) against CHIK replication has been investigated in Vero cells [61]. Two siRNAs against the conserved regions nsF3 and E1 genes showed a reduction of virus titer up to 99.6%. The effect was most prominent at 24 h (99%) and still significant at 48 h (65%) and might present a new therapeutic approach. Moreover, microRNA (miRNA) specific target sequences have been introduced into alphavirus helper RNAs used for replicon particle production (see Figure 1) [62]. Interestingly, particles were efficiently produced when miRNA-specific inhibitors were present. However in their absence, cellular miRNAs down-regulated helper RNA replication in vitro. When replicon RNA with miRNAs incorporated into the sequence was administered in mice, cellular miRNAs were able to prevent the replication of replicon RNA. These results suggest the feasibility of potentially using miRNA for the inhibition of viral replication as a therapeutic approach.

**Conclusions**

As described above a number of vaccine development studies have been carried out using mainly the three most commonly applied alphavirus vectors, SFV, SIN and VEE. Interestingly, these vectors have been used as replicon particles, naked RNA and layered DNA vectors. The results indicate that each approach has generated responses in the form of cellular or humoral responses and in many cases protection against challenges with lethal doses of virus (Table 1) and other non-viral agents (Table 2). Furthermore, protection against tumor challenges has been successfully achieved (Table 3), which bodes well for future preventive vaccination against cancer. Obviously, as alphaviruses themselves are pathogens causing epidemics [2-4], they are credible targets for vaccine development. A number of studies have been conducted, particularly for VEE and CHIK (Table 4), which have indicated the feasibility of generating efficient vaccines providing protection against challenges with virulent alphavirus strains.

Application of alphavirus vectors for vaccine development requires the addressing of biosafety issues. Although certain alphaviruses have been the cause of global fever epidemics [2-4] the strains used in vaccine development have most commonly been attenuated. Furthermore, second generation helper vectors [103] or split helper systems [104] have been used for the generation of replication-deficient alphavirus particles to ensure that no wild-type like replication-proficient particles are produced through homologous recombination. The safe application of SFV vectors in humans was first demonstrated in a phase I trial in melanoma and kidney carcinoma patients [43]. Repeated intravenous administration showed no SFV related toxicity or adverse reactions. Obviously, the use of naked RNA or layered DNA vectors presents no biosafety risk as no infectious viral particles are produced at any stage of the immunization procedure. Most encouragingly, the positive outcome of the first clinical trials with alphavirus replicons will further boost additional studies.

In summary, the ease of generating naked RNA, layered DNA vectors and recombinant particles are great assets in vaccine development. Alphavirus vectors provide rapid transgene expression of a transient nature, which makes them attractive as efficient gene delivery vehicles. However, their full potential has not yet been employed and future development will provide excellent opportunities for the generation of new and efficient vaccines.

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