Chapter 3
Alphavirus Replicon Vectors for Prophylactic Applications and Cancer Intervention

Peter Pushko and Irina Tretyakova

Abstract Alphavirus replicons represent self-replicating RNA molecules resembling alphaviral genomic RNA, except replicons encode antigen(s) of interest in place of an alphaviral structural polyprotein. Because viral structural genes are missing, replicon RNA cannot initiate replication of an alphavirus. However, due to the presence of intrinsic RNA-dependent RNA polymerase activity, replicons are capable of self-amplification in vitro and in vivo resulting in high levels of expression of antigen of interest. For vaccination or therapeutic purposes, replicons can be delivered in vivo by replicon particles. The latter represent viruslike particle vectors (VLPVs) that encapsidate replicon RNA and deliver it into target cells for antigen expression. The viruslike nature and self-replicating RNA features ensure efficient priming of innate immunity and adjuvant effect, while high-level expression provides antigen for induction of cell-mediated and humoral immune responses. Replicon vectors have been developed from several alphaviruses including Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), and Sindbis virus (SINV). Applications of replicon particles included prophylactic and therapeutic vaccines for infectious diseases and cancer, as well as adjuvants for enhancement of immune responses. In several preclinical models including nonhuman primates, alphavirus replicons have shown exceptional promise as safe and effective vaccines and adjuvants. Experimental replicon vaccines included vaccines against influenza, Ebola, Marburg, and Lassa viruses. Bivalent vaccines protecting from both Ebola and Lassa viruses have been also described. Protective effects have been reported for cancer indications after therapeutic vaccination with replicon vaccines expressing tumor-associated antigens. Clinical trials involving alphavirus replicons are underway. In this review, an attempt is made to summarize the state of the art of the alphavirus replicon-based technology for prophylactic and therapeutic applications. The advantages and challenges of the replicon technologies are presented, and the future of this promising platform is discussed.

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3.1 Introduction

During recent years, several alphavirus-based vectors have been developed as experimental vaccines for infectious diseases and cancer, as well as novel adjuvants. Alphavirus replicons represent self-replicating RNA molecules resembling alphaviral genomic RNA, except replicons encode antigen(s) of interest in place of an alphaviral structural polyprotein. Replicon vectors have been generated from several alphaviruses. Historically, the first alphaviral replicon systems have been derived from Sindbis virus (SINV), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEEV). Replicon vectors derived from SINV, SFV, and VEEV have been configured as vaccines for human and/or veterinary applications. Search of PubMed database conducted on May 1, 2014 by using two keywords (alphavirus replicons) resulted in retrieving 377 published articles. Search by using three keywords (alphavirus replicon vaccine) resulted in 214 articles. Finally, four keywords search (alphavirus replicon clinical trials) retrieved 16 articles from PubMed database. Thus, alphavirus replicons seem to be actively developed for vaccine applications. Alphavirus replicon vectors have shown exceptional promise in preclinical trials including nonhuman primates (Geisbert et al. 2002; Hevey et al. 1998). Human clinical trials were also conducted (Slovin et al. 2013; Wecker et al. 2012; Bernstein et al. 2009). This review represents an attempt to summarize available information on alphavirus replicon vectors for the development of prophylactic and therapeutic vaccines for infectious diseases and cancer.

In this review, the term "replicon" will be used to indicate alphaviral genome-derived RNA molecule that encodes RNA replicase and is therefore capable of self-amplification (replication). Replicons are generally engineered to express foreign gene of interest in eukaryotic cell lines. Chemical instability of replicon RNA prevents the broad use of "naked" RNA replicons for in vitro and in vivo applications. Therefore, for vaccination or therapeutic purposes, replicons can be encapsidated into viruslike particle vectors (VLPVs), or "replicon particles," by using alphavirus structural proteins supplied in trans. The genetic constructs for expression of alphavirus structural proteins and encapsidation of replicons are named "helpers." Encapsidated replicons are often termed replicon particles, for example, VEEV replicon particles (VRPs) (Schafer et al. 2009; Kamrud et al. 2008). Obviously, the term "VRP" is not applicable to replicon particles that are made from SINV or SFV. Since replicon particles essentially represent VLPs encapsulating replicon RNA, a broader functional term of "VLP vectors" (VLPVs) may be suitable. The VLPVs are capable of delivering replicon RNA into target cells in vitro and in vivo for antigen expression. In this review, we will use terms "replicon particles," VRPs, and "VLPVs" interchangeably to indicate alphavirus-like particles that encapsulate replicon RNA and are capable of delivering replicon RNA to target cells. Replicon particles, or VLPVs, cannot replicate beyond the initially infected target cells because replicons do not encode alphaviral structural proteins. The concept of VLP vectors has been also used in other viruses, such as polyomavirus (Eriksson et al. 2011; Tegerstedt et al. 2005; Chang

et al. 2011). For example, prior research suggested that murine polyomavirus VLPs can be useful vectors for gene therapy and immune therapy and for vaccine applications (Tegerstedt et al. 2005).

3.2 Biology of Alphaviruses

As mentioned earlier, replicons have been derived from several representatives of the *Alphavirus* genus. *Alphavirus* is one of two genera in the viral family Togaviridae. The other Togaviridae genus is *Rubivirus* with rubella virus (Strauss and Strauss 1994). Alphaviruses are classified according to antigenic characteristics, as they have antigenic sites on the capsid and on the envelope glycoproteins. Viruses can be differentiated by serological tests, particularly neutralization assays. Many alphaviruses can cause disease in people and can also be identified by clinical manifestations. Therefore, it is critically important that live alphavirus does not regenerate during preparation of VLPVs, or replicon particles (Pushko et al. 1997). There are approximately 30 alphavirus species capable of infecting invertebrates (mosquito) as well as vertebrates such as humans, rodents, fish, birds, and larger mammals including horses (Strauss and Strauss 1994). Transmission between species and individuals occurs mainly via mosquitoes, which places the alphaviruses into the group of arboviruses—or arthropod-borne viruses. Viruses are maintained in nature by mosquito-vertebrate-mosquito cycles. The mosquito borne pathogenic arboviruses include Venezuelan (VEEV), western (WEEV), and eastern equine encephalitis (EEEV) viruses, chikungunya virus (CHIKV), and others (Strauss and Strauss 1994; Schwartz and Albert 2010). Restricted interactions between viruses, invertebrate vector species, and vertebrate hosts tend to confine the geographic spread of alphaviruses. Occasionally, a virus may escape its usual ecological niche and cause widespread epizootics (VEEV) or urban epidemics (CHIKV). Human infections are seasonal and are usually acquired in endemic areas. For example, recently, a 17-year-old female traveled to Central America and developed clinical symptoms including fever, headaches, and myalgias. Laboratory tests revealed infection with VEEV (Muniz 2012).

Alphaviruses often are medically relevant as they can cause human disease. Clinical disease occurs in either of two general forms, depending upon the virus: one is typified by fever, malaise, headache, and/or symptoms of encephalitis (e.g., EEEV, WEEV, or VEEV viruses), while the other by fever, rash, and arthralgia (e.g., CHIKV, Ross River, Mayaro, and SINV). For example, VEEV, which is endemic in South and Central America, can cause severe neurological disease. In contrast, CHIKV, an etiological agent of chikungunya fever, an emerging global infectious disease, is characterized by severe arthralgia. Infection is transmitted via infected mosquitoes. Various mosquito species are known to be potential vectors for different arboviruses. For example, *Culex* spp. mosquitoes are considered a possible vector for SINV or Ockelbo virus (Werblow et al. 2013). CHIKV is transmitted by mosquitoes of *Aedes* spp. (Chen and Wilson 2012). In the vertebrate

host including humans, transient viremia and dissemination occur as virus is released from cells during lytic infection cycle. Infection with seroconversion in the absence of clinical disease is common, but severe disease can also occur and can be either incapacitating or, in cases of encephalitis, occasionally fatal. Virus is eliminated by the immune system; however, central nervous system pathology or arthritis may persist for weeks. Initial resistance is conferred by unspecific defense mechanisms including interferon. Antibodies play a critical role in recovery and resistance, and T-cell responses are also important (Strauss and Strauss 1994). Long-lasting protection is mostly restricted to the same alphavirus and is dependent on the presence of neutralizing antibodies. Diagnosis is suggested by clinical manifestations and by known risk of exposure to virus. Confirmation is typically by virus isolation and identification, or by a specific rise in IgG antibody, or the presence of IgM antibody. Disease surveillance and virus activity in natural hosts are used to determine if any control measures are needed to reduce populations of vector mosquitoes or to vaccinate hosts, especially horses. There are no FDA-approved human vaccines for alphaviruses (Strauss and Strauss 1994; Tretyakova et al. 2013). However, experimental live attenuated vaccines TC-83 and 181/25 for VEEV and CHIKV, respectively, do exist (Tretyakova et al. 2013). These vaccines are used under the Investigational New Drug (IND) Protocols in individuals at particularly high risk of exposure, such as laboratory or medical workers. Generally, the use of live attenuated strains such as TC-83 for preparation of alphavirus replicon and helper systems can significantly alleviate biosafety concern that pathogenic alphavirus can regenerate during VLPV preparation.

3.3 Genome and Structure of Alphaviruses

For preparation of alphavirus replicon and helper systems, understanding of the alphavirus life cycle (Fig. 3.1), genome, and virion structure is critically important. Alphavirus genome inside the virion is a positive-sense, monopartite, single-stranded RNA genome, approximately 12 kb in length. Genomic RNA is capped and polyadenylated. After introduction into permissive eukaryotic cell, alphaviral genomic RNA serves as mRNA for translation of nonstructural proteins (NSPs) that function as RNA-dependent RNA polymerase, or replicase (Strauss and Strauss 1994), as depicted in Fig. 3.1. The NSPs are encoded in the 5′ two-thirds of the genome (Fig. 3.2a). After NSPs are made in the cells, they direct synthesis of a complementary antisense (−) RNA by using genomic RNA as a template. In turn, the newly synthesized (−) strand RNA serves as a template for the synthesis of progeny genomic (+) strand RNA (Fig. 3.1). This process is mediated by the viral NSP proteins (replicase complex). In addition, the (−) strand RNA serves as a template for synthesis of subgenomic (+) strand RNA, which functions as mRNA for synthesis of structural proteins. The subgenomic RNA covers approximately the 3′ one-third of the genome. In the alphaviruses, subgenomic RNA encodes the structural proteins that interact with genomic RNA and form progeny virions

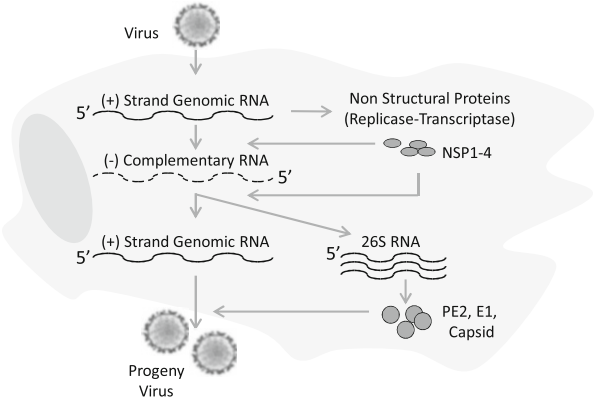

Fig. 3.1 Alphavirus life cycle. Indicated are (+) and (−) polarities of RNA during replication, as well as subgenomic RNA for expression of structural proteins PE2 and E1

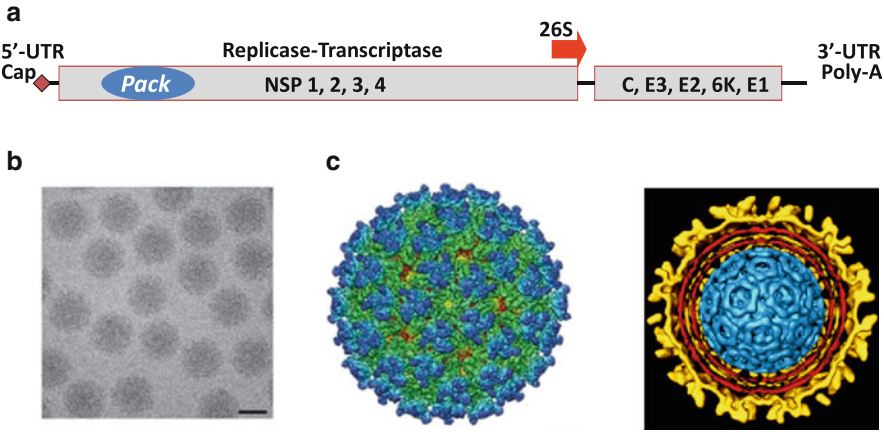

Fig. 3.2 Alphavirus genome and virion particle structure. (**a**) Alphavirus RNA genome. Indicated are nonstructural proteins (NSP), structural proteins (C, E3, E2, 6K, E1), 26S subgenomic promoter, as well as 5′ and 3′ untranslated regions (UTR) and genome packaging signal. (**b**) Alphavirus particles, by high-resolution cryoelectron microscopy. VEEV TC-83 strain embedded in vitreous ice is shown (Zhang et al. 2011). (**c**) Image reconstruction of alphavirus virion particle (Paredes et al. 1993; Zhang et al. 2011)

(Fig. 3.1). In contrast, in engineered RNA replicons, subgenomic RNA encodes the gene of interest in place of alphavirus structural proteins, as described below. Accordingly, in order to encapsidate replicons into VLPVs, or replicon particles, structural proteins are provided in trans from the helper constructs.

From the structural perspective, alphavirus virions are spherical, enveloped particles 60–70 nm in diameter and icosahedral symmetry (Fig. 3.2b, c). The lipid-containing envelope generally contains two surface glycoproteins E1 and E2 that mediate virus attachment, fusion, and penetration. The E1 and E2

glycoproteins are arranged into trimers of E1/E2 heterodimers (Strauss and Strauss 1994). The icosohedral nucleocapsid contains capsid protein and genomic RNA. Alphavirus virions mature by budding through the plasma membrane. Within the recent years, considerable knowledge was generated regarding the structure and functional organization of alphavirus particles (Vaney et al. 2013; Paredes et al. 1993). This includes the crystal structures of the envelope glycoprotein complexes at neutral and at acid pH, as well as image reconstructions of intact virions at neutral pH to resolutions of 4–7 Å. For example, structure of live attenuated vaccine strain TC-83 of VEEV was determined by using cryo-electron microscopy (cryo-EM) at 4.4 Å resolution. Density map clearly resolved regions (including E1 and E2 transmembrane helices and cytoplasmic tails) that were missing in the crystal structures of domains of alphaviruses. Interestingly enough, E3 protein was observed on mature TC-83 virions (Zhang et al. 2011). The new data provided unprecedented detail in the understanding of the alphavirus virion structure as well as improved understanding of the biology of the virus and the process of the alphavirus particle assembly during maturation and disassembly during cell entry.

Generally, the alphavirus infection cycle begins with E2 protein binding to host receptors on the surface of a host cell. This is followed by internalization of the virus and its transport into the acidic intracellular vesicles. The low pH induces a rearrangement of the E2/E1 dimer, thus activating fusion activity of E1 (Wahlberg and Garoff 1992). E1 inserts its hydrophobic fusion loop into the membrane of the host cell vesicle, forms E1 trimers, and refolds to pull the host cell and viral membranes together, thus causing membrane fusion and virus infection (Gibbons et al. 2004). Alphavirus genome delivery occurs directly at the plasma membrane in a time- and temperature-dependent process. Upon attaching to the cell surface, intact RNA-containing viruses become empty shells, which could be identified by antibody labeling. The rate at which full particles were converted to empty particles increased with time and temperature (Vancini et al. 2013).

In addition to binding the host cell receptor, E2 protein participates in other processes throughout the virus life cycle. During viral replication, this protein is synthesized as a precursor, PE2, and acts to facilitate the folding of its E1 protein. Furthermore, similarly to the vesicular entry pathway, the exit pathway also involves transport through cellular compartments that have an acidic pH. Interestingly enough, the PE2/E1 pair is more acid resistant than the E2/E1 dimer, and this feature likely protects E1 from random fusion during transport through the exit pathway (Wahlberg et al. 1989). Late in transport, the cellular enzyme furin cleaves PE2 to produce the mature E2 protein plus a small peripheral protein, E3 (Zhang et al. 2003). Finally, the trimers of E1/E2 heterodimers are transported to the plasma membrane, where they interact with the nascent nucleocapsid cores in the cytoplasm to form the intact progeny viruses that bud out of the host cell for the next round of infection. The virus then exits by budding from the cell surface, with some alphavirus species retaining E3 and others releasing it.

Thus, the alphavirus gains entry into cells by a process of receptor-mediated endocytosis followed by membrane fusion in the acid environment of the endosome. Cryoelectron micrograph analyses and 3D reconstructions showed that SINV virus retains its overall icosahedral structure at mildly acidic pH, except in the

membrane-binding region, where monomeric E1 associates with the target membrane and the E2 glycoprotein retains its original trimeric organization (Cao and Zhang 2013). CHIKV infection of susceptible cells is also mediated by E1 and E2. Glycoprotein E2, derived from furin cleavage of the PE2 precursor into E3 and E2, is similarly responsible for receptor binding, while E1 is responsible for membrane fusion. Glycoprotein organization of CHIKV particles was revealed by X-ray crystallography (Voss et al. 2010). The structures of SINV and CHIKV alphaviruses show that the mature E2 protein is an elongated molecule containing three domains with immunoglobulin-like folds: the amino-terminal domain A, located at the center; domain B at the tip; and the carboxy-terminal domain C, located close to the viral membrane. The structures of the PE2/E1 and E2/E1 pairs suggest specific residues that may control their dissociation at low pH and explain how PE2 and E2 regulate virus fusion (Kielian 2010).

3.4 Preparation of Replicon RNA

Alphaviruses, including attenuated strains of VEEV, have been configured as vaccine vectors (Strauss and Strauss 1994; Frolov et al. 1996). The availability of the full-length infectious clone technology greatly facilitated the development of vectors. Using infectious clone technology, alphavirus RNA can be easily made by using transcription in vitro and then transfected into permissive cells to initiate replication (Strauss and Strauss 1994; Davis et al. 1989, 1994). Generally, two types of VEEV vectors were prepared and evaluated: (1) double-promoter and (2) replicon vectors (Fig. 3.3). In the double-promoter vectors, a gene of interest is introduced into the full-length viral RNA downstream from a duplicated 26S

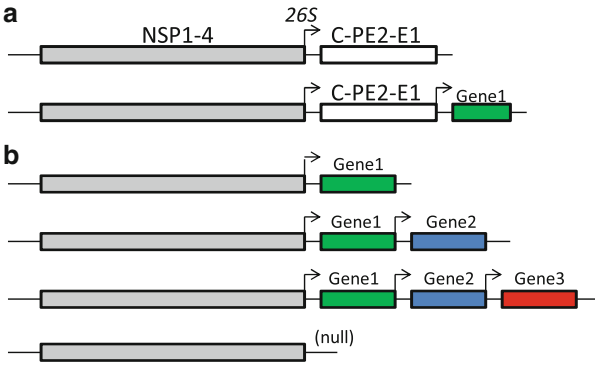

Fig. 3.3 Alphavirus genomic RNA and alphavirus vectors. (**a**) Alphavirus genomic RNA (*top*) and double-promoter vector (*bottom*). Indicated are locations of nonstructural proteins (NSP), 26S promoter, and structural proteins (C-PE2-E1), and location of foreign gene of interest. (**b**) Alphavirus replicon vectors. Locations of foreign gene(s) are indicated. 26S promoter is indicated with an *arrow*. Null replicon does not encode any foreign gene

promoter, resulting in independent transcription of two subgenomic mRNAs for both the viral structural proteins and the gene of interest (Hahn et al. 1992; Davis et al. 1996). This is depicted in Fig. 3.3a, along with parental genomic alphaviral RNA. These double-promoter vectors assemble into infectious, replication-competent viruses that are capable of propagating and expressing gene of interest through multiple rounds of replication in vitro and in vivo. However, during multiple rounds of replication in vitro or in vivo, the foreign gene of interest within the double-promoter vector tends to be deleted, which limits applications of this type of vector.

In the other type of alphavirus vectors, replicon vectors, the structural genes were deleted, and a gene of interest was introduced in place of the structural protein genes downstream from a single viral 26S promoter (Pushko et al. 1997; Xiong et al. 1989; Liljestrom and Garoff 1991; Frolov et al. 1997; Zhou et al. 1995; Mossman et al. 1996; Seregin et al. 2010). As with the double-promoter vectors, RNA replicons retained the nonstructural protein (NSP) genes and cis-acting elements (Fig. 3.3b) and were capable of RNA replication, transcription, and high-level expression of heterologous genes in the cell cytoplasm. However, when introduced into cells, replicons are restricted to a single cycle of replication as the lack of the structural proteins precludes alphavirus particle assembly and spread to uninfected cells. The lack of expression of structural protein genes also minimizes anti-vector immune responses in vivo (Pushko et al. 1997). It has been shown that VEEV- and EEEV-based replicons appear to be less cytopathic than Sindbis virus-based constructs and they can readily establish persistent replication in BHK-21 cells (Petrakova et al. 2005).

Double-promoter or bicistronic replicons have also been described (Carrion et al. 2012; Pushko et al. 2001). In these vectors, one heterologous gene of interest was placed downstream from the first 26S promoter, while the second heterologous gene was placed downstream from the second 26S promoter (Pushko et al. 2001). Such replicons expressed both genes in vitro and induced immune response in vivo to both expressed heterologous proteins (Pushko et al. 2001). Potentially, three or more genes can be expressed from independent 26S promoters (Fig. 3.3b). However, the number of genes can be limited by the size of the genes, as well as by other factors, such as vector capacity, genetic stability of the vector expressing multiple genes, the effects of expressed proteins on host cell metabolism, and others.

In recent years, replicons that contain no foreign GOI (null replicons, Fig. 3.3b) have been made as a novel type of adjuvant (Thompson et al. 2006). For example, co-inoculation of null replicon particles (not expressing any transgene) with inactivated influenza virions, or ovalbumin, resulted in a significant increase in antigen-specific systemic IgG antibodies, compared with antigen alone. Pretreatment of replicon particles with UV light largely abrogated adjuvant effect. These results demonstrated that VEEV alphavirus replicon particles possess intrinsic adjuvant activity and suggest that vector RNA replication may be responsible for this activity (Thompson et al. 2006).

3.5 Replicon Vector Delivery In Vivo

For vaccination, alphavirus replicons (Fig. 3.3b) can be delivered in permissive cells in vivo by using several methods. For example, replicons can be delivered in vivo as naked RNA (Johanning et al. 1995) (Fig. 3.4) or by plasmids directing the synthesis of replicons in vivo (Dubensky et al. 1996; Berglund et al. 1998). For example, intradermal electroporation of SFV naked replicon RNA elicited strong immune responses in mice (Johansson et al. 2012). Unfortunately, instability of naked RNA limits its use for in vivo applications. Improved T-cell responses to conserved HIV-1 regions were observed by electroporating SFV replicon DNA compared to that with conventional plasmid DNA vaccine (Knudsen et al. 2012). Tetravalent replicon vaccines against botulinum neurotoxins were prepared by using DNA-based SFV replicon vectors (Yu et al. 2013). However, there are technical and regulatory difficulties associated with plasmid DNA transfections in vitro and in vivo. Recombinant viruses were also used to prepare replicon vaccines (Vasilakis et al. 2003; Sun et al. 2013). Adenovirus-vectored SFV replicon construct expressing the E2 glycoprotein from classical swine fever virus (CSFV), rAdV-SFV-E2, induced immunity against a lethal CSFV challenge (Sun et al. 2013). Furthermore, transfection-independent system for packaging alphavirus replicon vectors was generated by using modified vaccinia virus Ankara (MVA) vectors to express all of the RNA components necessary for the production of VEEV replicon particles. Infection of mammalian cells with these recombinant MVA vectors resulted in robust expression of VEEV structural genes, replication of the alphavirus replicon vector, and high titers of replicon particles. Interestingly

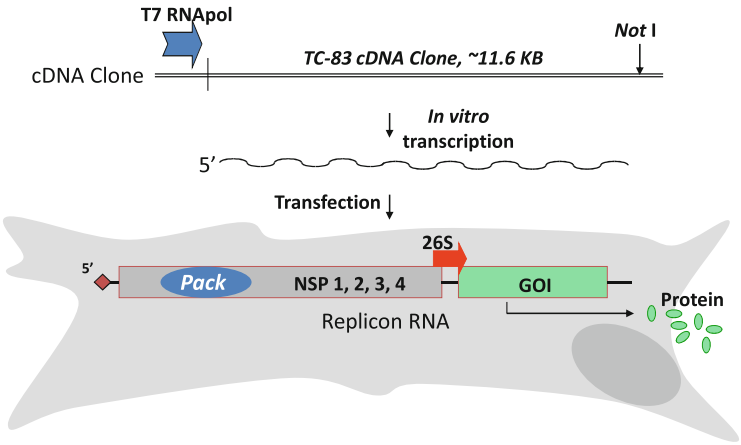

Fig. 3.4 Preparation of replicon RNA vector from the cDNA clone and expression of foreign gene of interest (GOI) in eukaryotic cell. The cDNA clone of TC-83 replicon is shown on *top* including T7 RNA polymerase promoter and NotI restriction site, which are used for runoff in vitro transcription. Replicon RNA that is made in vitro is transfected into permissive eukaryotic cell by electroporation or other method

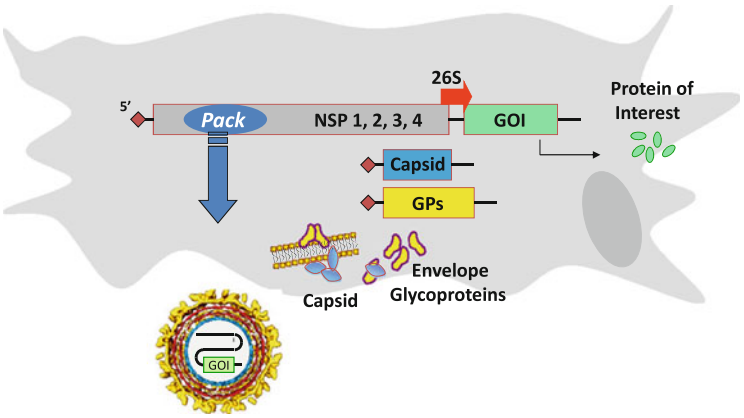

Fig. 3.5 Encapsidation of alphavirus RNA replicons into replicon particles in eukaryotic cells. Replicon particles represent viruslike particle vectors (VLPVs) that encapsulate replicon RNA. VLPVs are made in eukaryotic cells that are cotransfected with replicon RNA, capsid helper RNA, and GP helper RNA. Capsid and GP helpers express alphavirus capsid and glycoproteins, which recognize packing signal within the replicon RNA and form replicon particles

enough, replicon packaging was achieved in a cell type (fetal rhesus lung) that has been approved for vaccine manufacturing for human use (Vasilakis et al. 2003). There are challenges for virus-based replicon encapsidation process, including the need for live virus clearance from replicon particle preparations.

In spite of the fact that various vaccination strategies exist that utilize alphavirus replicons for vaccination purposes, preparation of alphavirus-like VLPVs, or replicon particles by using packaging RNA helpers (Fig. 3.5), remains the workhorse of alphavirus vector technologies (Pushko et al. 1997; Zhou et al. 1995; Mossman et al. 1996; Carrion et al. 2012). The process of preparation of replicon particles by cotransfection of replicon and helper RNAs is described below in more detail.

3.6 Preparation of Replicon Particles by Using RNA Helper Systems

One of the first replicon-helper vaccine vector systems was developed from an attenuated strain of VEEV (Pushko et al. 1997). The replicon RNA consisted of the cis-acting 5′ and 3′ ends of the VEEV genome, the complete NSP gene region, and the subgenomic 26S promoter. The genes encoding the VEEV structural proteins were replaced with heterologous gene of interest (GOI), such as the influenza virus hemagglutinin or the Lassa virus nucleocapsid gene (Pushko et al. 1997). After transfection into eukaryotic cells by electroporation, replicon RNAs directed the efficient, high-level synthesis of the influenza or Lassa virus protein of interest (Fig. 3.5). For packaging of replicon RNAs into vector particles, the VEEV capsid and glycoproteins are supplied in trans by expression of VEEV nucleocapsid and

glycoproteins from helper RNA(s) co-electroporated with the replicon (Fig. 3.5). A number of different helper constructs, expressing the VEEV structural proteins from a single or two separate helper RNAs, were derived from various attenuated VEEV strains (Pushko et al. 1997; Seregin et al. 2010). Regeneration of infectious virus was not detected when replicons were packaged using a bipartite helper system (Fig. 3.5) encoding the VEE capsid protein and glycoproteins on two separate RNAs (Pushko et al. 1997).

3.7 Applications of Alphavirus VLPVs (Replicon Particles)

Applications of alphavirus VLPVs, or replicon vector particles, involve development of human and veterinary vaccines against infectious diseases and immunotherapies for cancer, as well as adjuvants. The representative examples of these applications are described below and summarized in Table 3.1.

Table 3.1 Representative experimental alphavirus replicon vaccines for infectious diseases

Alphavirus replicon	Indication	Protective antigen	Testing in vivo	Notes	References
VEEV	Influenza	HA	Rodent		Pushko et al. (1997)
	Influenza	HA	Swine	Veterinary	Bosworth et al. (2010)
	BVDV	E2	Calves	Veterinary	Loy et al. (2013)
	Lassa	N, GPC	Rodent		Pushko et al. (1997, 2001)
	Machupo	GPC	Rodent		Carrion et al. (2012)
	Junin	GPC	Rodent		Seregin et al. (2010), Carrion et al. (2012)
	Ebola	NP, GP	Rodent, NHP		Geisbert et al. (2002), Pushko et al. (2000, 2001), Herbert et al. (2013)
	Marburg	NP, GP	Rodent, NHP		Hevey et al. (1998)
	Dengue	prME, E	NHP		White et al. (2013)
	HIV	gag	Rodent, human	Phase I clinical	Wecker et al. (2012), Carroll et al. (2011)
	CMV	gB, pp65/IE1	Human	Phase I clinical	Bernstein et al. (2009)
SINV	HCV	E2E1	Rodent		Zhu et al. (2013)
	CPV	VP2	Dog	Veterinary	Dahiya et al. (2011)
SFV	HIV	Gp160	NHP		Berglund et al. (1997)
	Influenza	NP, HA	Rodents		Berglund et al. (1999)
SAV[a]	ISAV	HE	Salmon	Veterinary	Wolf et al. (2013)

[a]*SAV* salmonid alphavirus, *ISAV* infectious salmon anemia virus

3.8 Alphavirus Replicons as Vaccines for Infectious Diseases

In the first study of VEEV replicon systems, subcutaneous immunization of BALB/c mice with VRP expressing either influenza HA or Lassa virus N gene (HA-VRP or N-VRP, respectively) induced antibody responses to each expressed protein. After two inoculations of HA-VRP, complete protection against intranasal challenge with influenza was observed (Pushko et al. 1997). Similarly, immunization of mice with SFV vectors encoding the influenza virus HA and nucleoprotein (NP) resulted in immune responses that were protective against challenge infection with influenza virus (Berglund et al. 1999).

Some of the applications of VEEV replicon vaccines involved vaccine development for viral hemorrhagic fevers. For example, one of the first VEEV replicon vaccines has been developed for Marburg filovirus (MBGV), for which there are no vaccines or treatments (Hevey et al. 1998). MGBV causes an acute hemorrhagic fever with a high mortality rate in people. VEEV RNA replicon was used to express genes for MBGV glycoprotein (GP), nucleoprotein (NP), VP40, VP35, VP30, or VP24. Guinea pigs were vaccinated with recombinant VEEV replicons packaged into VEEV-like particles and then experimentally infected with MBGV in a BSL4 laboratory. Survival and viremia in animals were evaluated. Results indicated that either GP or NP was protective antigen for MBGV, while VP35 afforded incomplete protection. As a more definitive test of replicon vaccine efficacy, nonhuman primates (cynomolgus macaques) were vaccinated with VEEV replicons expressing MBGV GP and/or NP. Three monkeys received packaged control replicons; these died 9 or 10 days after challenge, with typical MBGV disease. MBGV NP afforded incomplete protection, sufficient to prevent death but not disease in two of three macaques. However, three monkeys vaccinated with replicons which expressed MBGV GP, and three others vaccinated with both replicons that expressed GP or NP, remained aviremic and were completely protected from disease (Hevey et al. 1998).

RNA replicons derived from an attenuated strain of VEEV alphavirus have also been configured as candidate vaccines for another filovirus, Ebola hemorrhagic fever (Pushko et al. 2000). Similarly to other alphavirus replicons (Fig. 3.3), the Ebola virus (EBOV) nucleoprotein (NP) or glycoprotein (GP) gene was introduced into the VEEV RNA downstream from the 26S promoter in place of the VEEV structural protein genes. The resulting recombinant replicons expressing the NP or GP gene were packaged into VEEV replicon particles (NP-VRP and GP-VRP, respectively) using a bipartite helper system that provided the VEEV structural proteins in trans and prevented the regeneration of replication-competent VEEV during packaging (Fig. 3.5) (Pushko et al. 1997). The immunogenicity of NP-VRP and GP-VRP and their ability to protect against lethal Ebola infection were evaluated in BALB/c mice and in two strains of guinea pigs. The GP-VRP alone, or in combination with NP-VRP, protected both strains of guinea pigs and BALB/c mice, while immunization with NP-VRP alone protected BALB/c mice, but neither strain

of guinea pig (Pushko et al. 2000). Protection of nonhuman primates against EBOV challenge proved more challenging (Geisbert et al. 2002). Nevertheless, VEEV replicon particles were successfully evaluated as experimental vaccines against Sudan (SUDV) and EBOV filoviruses in nonhuman primates (Herbert et al. 2013). VRP vaccines were prepared that expressed the GP of either SUDV or EBOV. A single intramuscular vaccination of cynomolgus macaques with high dose of VRP expressing SUDV GP provided complete protection against intramuscular challenge with SUDV. Vaccination against SUDV and subsequent survival of SUDV challenge did not fully protect cynomolgus macaques against intramuscular EBOV back-challenge. However, a single simultaneous intramuscular vaccination with VRP expressing SUDV GP combined with VRP expressing EBOV GP did provide complete protection against intramuscular challenge with either SUDV or EBOV in cynomolgus macaques. Finally, intramuscular vaccination with VRP expressing SUDV GP completely protected cynomolgus macaques when challenged with aerosolized SUDV, although complete protection against aerosol challenge required two vaccinations with this vaccine (Herbert et al. 2013).

Vaccine development against dengue has also been reported by using VEEV replicons. VRP replicon particles expressing dengue virus E antigens as subviral particles [prME] and soluble E dimers [E85] successfully immunized and protected macaques against dengue virus (White et al. 2013). Anti-vector antibodies did not interfere with a booster immunization. Interestingly enough, compared to prME-expressing vectors, the E85 vectors induced neutralizing antibodies faster, to higher titers, and with improved protective efficacy. This study also mapped antigenic domains targeted by vaccination versus natural infection, revealing that, unlike prME-VRP and live virus, E85-VRP induced only serotype-specific antibodies, which predominantly targeted EDIII, suggesting a protective mechanism different from that induced by live virus and possibly live attenuated vaccines. A tetravalent E85-VRP dengue vaccine induced a simultaneous and protective response to all four serotypes after two doses given 6 weeks apart. Balanced responses and protection in macaques provided further support for exploring the immunogenicity and safety of this vaccine candidate in humans (White et al. 2013).

Other vaccines include vaccines against arenaviruses, such as Lassa, Junin, and other arenaviral hemorrhagic fevers. Arenaviruses cause severe disease in people. For example, Junin virus (JUNV) is the etiological agent of the potentially lethal, reemerging human disease, Argentine hemorrhagic fever. The replicon system was engineered from live investigational VEEV vaccine TC-83 that expressed glycoproteins (GPC) of JUNV (Seregin et al. 2010). Preclinical studies testing the immunogenicity and efficacy of TC83/JUNV GPC were performed in guinea pigs. A single dose of the TC-83 alphavirus-based vaccine expressing only GPC was immunogenic and provided partial protection, while a double dose of the same vaccine provided a complete protection against JUNV (Seregin et al. 2010).

A SINV virus vector was used to induce humoral and cellular responses against hepatitis C virus (HCV). The recombinant vector, pVaXJ-E1E2, expressing HCV glycoproteins E2 and E1, was constructed by inserting the E1E2 gene into the replicon pVaXJ, a DNA vector derived from Sindbis-like virus XJ-160 (Zhu

et al. 2013). The replication-defective replicon particles were produced by transfecting BHK-21 packaging cell line with pVaXJ-E1E2. Mice were vaccinated using a prime-boost regimen with SINV replicon particles combined with Freund's incomplete adjuvant via intramuscular injection, and HCV-specific IgG antibody levels and cellular immune responses were detected by IFA and IFN-γ ELISPOT, respectively (Zhu et al. 2013).

SFV replicon RNA vectors expressing the envelope protein gp160 of HIV-1IIIB were evaluated in cynomolgus macaques. Monkeys were immunized four times with recombinant SFV particles. Whereas two out of four monkeys showed T-cell-proliferative responses, only one monkey had demonstrable levels of antibodies to HIV-1 gp41 and gp120 as shown by enzyme-linked immunosorbent assay (ELISA) and Western blot. The vaccinated monkeys and four control animals were challenged with 10,000 MID100 (100 % minimum infectious doses) of cell-free monkey cell-grown SHIV-4 virus. Three out of four vaccinated monkeys had no demonstrable viral antigenemia and low viral load as opposed to one of the four naive control animals (Berglund et al. 1997).

Several experimental veterinary vaccines have also been developed from alphavirus replicons including replicons derived from VEEV and other alphaviruses. For example, replicon particles that expressed bovine viral diarrhea virus sub-genotype 1b E2 glycoprotein were generated (Loy et al. 2013). Expression was confirmed in vitro by using antibodies specific to E2 glycoprotein. Experimental replicon particle vaccine was generated in Vero cell culture and administered to BVDV-free calves in a prime-boost regimen at two dosage levels. Vaccination resulted in neutralizing antibody titers that cross-neutralized both type 1 and type 2 BVD genotypes after booster vaccination. Additionally, high-dose vaccine administration demonstrated protection from clinical disease and significantly reduced the degree of leukopenia caused by viral infection (Loy et al. 2013). Other experimental veterinary vaccines have also been developed from alphaviruses. An alphavirus-derived replicon particle vaccine expressing the H3N2 swine influenza virus hemagglutinin gene induced protective immunity against homologous influenza virus challenge (Bosworth et al. 2010). A replicon expression system based on the salmonid alphavirus (SAV) that encodes the infectious salmon anemia virus (ISAV) hemagglutinin-esterase (HE) was found to be an efficacious against infectious salmon anemia (Wolf et al. 2013). Following a single intramuscular immunization, Atlantic salmon (*Salmo salar*) were effectively protected against subsequent ISAV challenge. These results have shown that the alphavirus replicon approach may represent a novel immunization technology for the aquaculture industry (Wolf et al. 2013).

A SINV replicon-based DNA vaccine containing VP2 gene of canine parvovirus (CPV) was delivered by *E. coli* to elicit immune responses (Dahiya et al. 2011). The orally immunized dogs developed CPV-specific serum IgG and virus neutralizing antibody responses after vaccine administration. The cellular immune responses were analyzed using lymphocyte proliferation test and flow cytometry and indicated successful CPV-specific sensitization of both CD3+CD4+ and CD3+CD8+ lymphocytes. This research demonstrated that SINV replicon-based DNA vaccine

delivered by *E. coli* can be considered as a promising approach for vaccination of dogs against CPV (Dahiya et al. 2011).

3.9 Sequential Immunization with VLPVs

One of the advantages of alphavirus VLPVs, or replicon particle vectors, is that they do not induce significant self-immunity (Pushko et al. 1997). Even when anti-vector response was detected, this did not prevent booster vaccinations (White et al. 2013). This allowed efficient boosts with the same vectors, and even sequential immunizations with vectors expressing distinct immunogens. As described above, subcutaneous immunization of BALB/c mice with VEEV replicon vectors expressing either influenza HA or Lassa virus N gene (HA-VRP or N-VRP, respectively) induced antibody responses to each expressed protein. After two inoculations of HA-VRP vectors, complete protection against intranasal challenge with influenza was observed. Furthermore, sequential immunization of BALB/c mice with two inoculations of N-VRP prior to two inoculations of HA-VRP induced an immune response to both HA and N, which was essentially equivalent to immunization with either VRP construct alone. Protection against influenza challenge was not affected by previous N-VRP immunization (Pushko et al. 1997).

3.10 Multivalent and Bicistronic Replicon Vaccines

Vaccination against multiple pathogens can be achieved by blending several species of replicon particles, each species protecting against a single pathogen of interest. Alternatively, protection against multiple pathogens can be achieved by vaccination with replicon particles, which encapsidate multi-cistronic replicons co-expressing multiple protective antigens in a tandem fashion (Fig. 3.3b). An example of blended, tetravalent dengue vaccine derived from VEEV replicons has been described (White et al. 2013). Preparation of bivalent and bicistronic replicons and encapsidation of bicistronic replicons into VLPVs has resulted in bivalent replicon vaccines capable of protecting against two pathogens, Lassa and Ebola viruses. Experimental individual vaccines for Lassa virus and bivalent and bicistronic vaccines for Lassa and Ebola viruses were developed from an RNA replicon of attenuated VEEV. Recombinant replicons were incorporated into viruslike replicon particles. Expression of antigens was confirmed by immunofluorescence assay (Fig. 3.6). Guinea pigs vaccinated with particles expressing Lassa virus nucleoprotein or glycoprotein genes were protected from lethal challenge with Lassa virus (Pushko et al. 2001). Vaccination with particles expressing Ebola virus glycoprotein gene also protected the animals from lethal challenge with Ebola virus. In order to evaluate a single vaccine protecting against both Lassa and Ebola viruses, blended and bicistronic particles were prepared that expressed

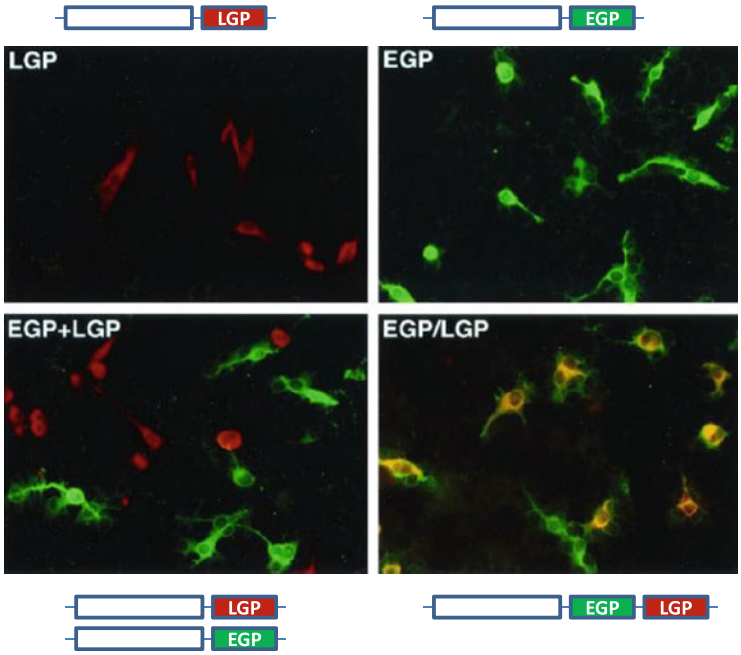

Fig. 3.6 Expression of Lassa and Ebola antigens from VEEV replicons in BHK-21 cells, by immunofluorescence (Pushko et al. 2001). *Top panel* depicts expression of individual Lassa (*left*) and Ebola (*right*) genes. *Bottom panel* shows expression of both Lassa and Ebola antigens from blended vaccine formulation (*left*) and from bicistronic replicon expressing both Lassa and Ebola antigens from the same replicon vector (*right*). *Yellow color* indicates co-localization of both antigens in the same cell

glycoprotein genes of both Ebola and Lassa viruses. Vaccination of guinea pigs with either bicistronic replicon particles or with a blended mixture of particles expressing Ebola and Lassa virus glycoprotein genes protected the animals against challenges with Ebola and Lassa viruses. The results showed that immune responses can be induced against multiple vaccine antigens co-expressed from an alphavirus replicon and suggested the possibility of engineering multivalent vaccines based upon alphavirus vectors for arenaviruses, filoviruses, and possibly other emerging pathogens (Pushko et al. 2001). Recently, bicistronic VEEV VLPVs were described that co-expressed Junin and Machupo arenavirus GPC genes (Carrion et al. 2012).

3.11 Clinical Trials

Clinical trials involving alphavirus replicon vaccines are underway. In order to develop cytomegalovirus (CMV) vaccine, a two-component alphavirus replicon particles expressing CMV gB or a pp65/IE1 fusion protein, previously shown to

induce robust antibody and cellular immune responses in mice, were evaluated in a randomized, double-blind phase 1 clinical trial in CMV seronegative individuals. Forty subjects received a low dose or high dose of vaccine or placebo by intramuscular or subcutaneous injection at weeks 0, 8, and 24 (Bernstein et al. 2009). The vaccine was well tolerated, with mild to moderate local reactogenicity, minimal systemic reactogenicity, and no clinically important changes in laboratory parameters. All vaccine recipients developed ex vivo, direct IFN-γ ELISPOT responses to CMV antigens and neutralizing antibodies. Polyfunctional CD4(+) and CD8(+) T-cell responses were detected by polychromatic flow cytometry. This alphavirus replicon particle vaccine was safe and induced neutralizing antibody and multifunctional T-cell responses against three CMV antigens that are important targets for protective immunity (Bernstein et al. 2009).

Another clinical trial was conducted to evaluate experimental HIV-1 vaccine. On the basis of promising preclinical data (Williamson et al. 2003), safety and immunogenicity of an alphavirus replicon HIV-1 subtype C *gag* vaccine, expressing a nonmyristoylated form of *gag*, were evaluated in two double-blind, randomized, placebo-controlled clinical trials in healthy HIV-1-uninfected adults (Wecker et al. 2012). Escalating doses of vaccine or placebo were administered subcutaneously to participants in the USA and Southern Africa. Although both trials were stopped prematurely due to various reasons, safety and immunogenicity were evaluated through assessments of reactogenicity, reports of adverse events, and assessment of replication-competent VEEV viremia. Immunogenicity was measured using the enzyme-linked immunosorbent assay (ELISA), chromium 51-release cytotoxic T lymphocyte (CTL), gamma interferon (IFN-γ) ELISPOT, and other assays. Vaccine was well tolerated and exhibited only modest local reactogenicity. There were five serious adverse events reported during the trials; however, none were considered related to the study vaccine. In contrast to the preclinical data (Williamson et al. 2003), immune responses in humans were limited. Only low levels of binding antibodies and T-cell responses were seen at the highest doses. This trial also highlighted the difficulties in the developing of HIV vaccine (Wecker et al. 2012).

3.12 Alphavirus Replicons as Vaccines Against Cancer

Cellular immunotherapy based on autologous dendritic cells (DCs) targeting antigens expressed by metastatic cancer has previously demonstrated clinical efficacy. However, the logistical and technical challenges in generating such individualized cell products require the development of alternatives to autologous DC-based cancer vaccines. Particularly attractive alternatives include delivery of antigen and/or activation signals to resident antigen-presenting cells, which can be achieved by alphaviral vectors expressing the antigen of interest and capable of infecting DCs.

The rationale for developing immunotherapeutic vectors and opportunities to enhance their effectiveness has been reviewed elsewhere including the use of alphaviruses (Osada et al. 2012). Alphavirus replicon vectors have been used in multiple studies to develop new immunotherapies against tumor-associated antigens (TAAs). Efforts to evaluate and discover TAAs as diagnostic and therapeutic markers for cancer have succeeded in identification of several TAAs. Many TAAs represent "self"-antigens and, as such, are subject to the constraints of immunologic tolerance. There are significant immunological barriers to eliciting antitumor immune responses to self-antigens. VEEV-derived alphavirus replicon vector system that has shown in vivo tropism to dendritic cells has been used to develop vaccines using expression of TAAs. For example, VEEV vectors have been shown to overcome the intrinsic tolerance to the "self"-TAA rat neu and elicited an effective antitumor immune response using VEEV replicon vector and a rationally designed target antigen in a rigorous rat mammary tumor model (Nelson et al. 2003). The VEEV vectored immunotherapy has shown the capacity to generate 50 % protection in tumor challenge experiments ($p = 0.004$). The establishment of immunologic memory was confirmed by both second tumor challenge and Winn assay ($p = 0.009$). Minor antibody responses were identified and supported the establishment of T helper type 1 (Th1) antitumor immune responses by isotype. Animals surviving in excess of 300 days with established effective antitumor immunity showed no signs of autoimmune phenomena. These experiments supported the establishment of T-lymphocyte-dependent, Th1-biased antitumor immune responses to a non-mutated "self"-TAA in an aggressive tumor model. Importantly, this tumor model is subject to the constraints of immunologic tolerance present in animals with normal developmental, temporal, and anatomical expression of a non-mutated TAA. These data supported the development and potential clinical application of VEEV replicon vectors along with the appropriately designed target antigens for antitumor immunotherapy (Nelson et al. 2003).

Prostate-specific membrane antigen (PSMA) is a transmembrane protein expressed in all types of prostatic tissue. PSMA may represent a promising diagnostic and possibly therapeutic TAA target (Slovin et al. 2013). PSMA-VRP was made and evaluated in phase I clinical trial for patients with castration-resistant metastatic prostate cancer (CRPC). Two cohorts of three patients with CRPC metastatic to bone were treated with up to five doses of either $0.9 \times 10(7)$ IU or $0.36 \times 10(8)$ IU of PSMA-VRP at weeks 1, 4, 7, 10, and 18, followed by an expansion cohort of six patients treated with $0.36 \times 10(8)$ IU of PSMA-VRP at weeks 1, 4, 7, 10, and 18 (Slovin et al. 2013). No toxicities were observed. In the first-dose cohort, no PSMA-specific cellular immune responses were seen but weak PSMA-specific signals were observed by ELISA. The remaining nine patients, which included the higher cohort and the extension cohort, had no PSMA-specific cellular responses. PSMA-VRP was well tolerated at both doses. While there did not appear to be clinical benefit nor robust immune signals at the two doses studied, neutralizing antibodies were produced by both cohorts suggesting that the vaccine dosing was not optimal (Slovin et al. 2013).

Another promising strategy of the use of alphavirus vectors for cancer therapy can be targeting and potentially eliminating of tumors by cytopathic effect exhibited by alphavirus vectors. For example, distribution of recombinant SFV particles (recSFV) and SFV naked viral RNA replicon was studied in tumor-free and 4T1 mammary tumor-bearing mice (Vasilevska et al. 2012). The predominant tumor targeting by recSFV was observed at a reduced dose, whereas the dose increase led to a broader virus distribution in mice (Vasilevska et al. 2012).

3.13 Alphavirus Replicons as Adjuvants

One of the most recent applications of alphavirus replicon vectors includes their use as novel adjuvants. VEEV replicon particles (VRP) have been used to augment humoral, cellular, and mucosal immune responses in mice. For the adjuvant purpose, replicons do not even need to express any transgene. For example, co-inoculation of VRP with no transgene (null VRPs) along with inactivated influenza virions resulted in a significant increase in antigen-specific systemic IgG and IgA antibodies, compared to antigen alone. Pretreatment of VRP with UV light diminished this adjuvant effect. These results demonstrate that alphavirus replicon particles possess intrinsic systemic and mucosal adjuvant activity and suggest that VRP RNA replication is the trigger for adjuvant activity (Thompson et al. 2006).

It was also demonstrated that VRP adjuvant induced an increased and balanced serum IgG subtype response to co-delivered antigen, with simultaneous induction of antigen-specific IgG1 and IgG2a antibodies (Thompson et al. 2008). VRP adjuvant also increased both systemic and mucosal antigen-specific CD8+ T-cell responses, as measured by an IFN-γ ELISPOT assay. Additionally, VRP further increased antigen-specific T-cell immunity in an additive fashion following co-delivery with the TLR ligand, CpG DNA (Thompson et al. 2008). VRP infection led to recruitment of CD8+ T cells into the mucosal compartment, possibly utilizing the mucosal homing receptor, as this integrin was upregulated on CD8+ T cells in the draining lymph node of VRP-infected animals, where VRP-infected dendritic cells reside. This newly recognized ability of VRP to mediate increased T-cell response towards co-delivered antigen provides the potential to both define the molecular basis of alphavirus-induced immunity and improve alphavirus-based vaccines (Thompson et al. 2008).

To assess the adjuvant activity of null VRP in the context of a licensed inactivated influenza virus vaccine, rhesus monkeys were immunized with either influenza Fluzone vaccine alone or with Fluzone vaccine mixed with null VRP and then challenged with a human seasonal influenza virus, A/Memphis/7/2001 (H1N1) (Carroll et al. 2011). Compared to Fluzone alone, Fluzone with null VRP-immunized animals had stronger influenza-specific CD4(+) T-cell responses (4.4-fold) with significantly higher levels of virus-specific IFN-γ (7.6-fold) and IL-2 (5.3-fold) producing CD4+ T cells. Fluzone/null VRP-immunized animals

also had significantly higher plasma anti-influenza IgG ($p < 0.0001$, 1.3 log) and IgA ($p < 0.05$, 1.2 log) levels. In fact, the mean plasma anti-influenza IgG titers after one Fluzone/null VRP immunization was 1.2 log greater ($p < 0.04$) than after two immunizations with Fluzone vaccine alone. After virus challenge, only Fluzone/null VRP-immunized monkeys had a significantly lower level of viral replication ($p < 0.001$) relative to the unimmunized control animals. Although little anti-influenza antibody was detected in the respiratory secretions after immunization, strong anamnestic anti-influenza IgG and IgA responses were present in secretions of the Fluzone/null VRP-immunized monkeys immediately after challenge. There were significant inverse correlations between influenza RNA levels in tracheal lavages and plasma anti-influenza HI and IgG anti-influenza antibody titers prior to challenge. These results demonstrate that null VRP dramatically improves both the immunogenicity and protection elicited by a licensed inactivated influenza vaccine (Carroll et al. 2011).

3.14 Advantages and Challenges for Alphavirus Replicon Vectors

Alphavirus replicon vectors are characterized by high-level expression of heterologous genes in cultured cells, little or no regeneration of plaque-forming virus particles, and the capability for sequential immunization to multiple pathogens in the same host. Induction of protective immunity against many pathogens and cancer-related indications has been demonstrated by using VEEV, SINV, and SFV replicon vectors. The vaccine products derived from attenuated VEEV viruses incorporate multiple redundant safety features (Pushko et al. 1997). Another feature of VEEV replicon vectors is the ability to target dendritic cells (DCs). Dendritic cells consist of heterogeneous phenotypic populations and have diverse immunostimulatory functions dependent on both lineage and functional phenotype. As exceptionally potent antigen-presenting cells, DCs represent excellent targets for generating effective antigen-specific immune responses. VRP replicon particle vectors derived from VEEV have been reported to transduce murine and human DC. The receptive DC subsets, the degree of restriction for this tropism, and the extent of conservation between rodents and humans have been extensively studied and characterized. By using fresh peripheral blood DCs, mononuclear cells, monocyte-derived macrophages, and monocyte-derived DCs, it has been demonstrated that VEEV vector has similar tropism for DCs between humans and rodents. It has been also observed that the VEEV target population represents a subset of immature myeloid DCs and that VRP-transduced immature DCs retain intact functional capacity, for example, the ability to resist the cytopathic effects of VRP transduction and the capacity to acquire the mature phenotype. These studies supported the demonstration of selective VRP tropism for human DCs and provide further insight into the biology of the VRP vector, its parent virus, and human DCs (Nishimoto et al. 2007).

Regarding the development of veterinary vaccines by using alphavirus vectors, one of the attributes of a good veterinary vaccine is the capability to permit differentiation of vaccinated vs. infected animals (DIVA). The DIVA concept relies on the principle that a vaccinated animal will have a different immune response than an animal that is infected with the wild-type pathogen and that this immune response is readily detectable by some immunoassay. Alphavirus vectors can be readily configured to allow DIVA, thus allowing the development of next-generation veterinary vaccines (Vander Veen et al. 2012).

3.15 Future Directions

The alphavirus replicon technology offers great potential for the next generation of human and veterinary vaccines (Pushko et al. 1997; Vander Veen et al. 2012). Both replicon particles and replicon DNA vaccines have demonstrated robust and balanced immune responses with subsequent protection against a variety of diseases that have implications for both human and animal health. Further improvements in both safety and the replicon vector design may significantly advance the field of replicon-based vaccines. Improvements of replicon packaging technologies in order to achieve better packaging efficacies and preventing recombination of replicons and helpers will be an important goal for further development of alphavirus vector development. In addition, replicons derived from other viruses can also be useful for many applications in vitro and in vivo, such as replicon derived from Kunjin flavivirus (Pijlman et al. 2006). The optimal vaccine regimens for prophylactic and therapeutic interventions may include a combination of distinct alphavirus vectors or combinations of alphavirus vectors with other vaccine-relevant immunogens.

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