DEVELOPMENT OF CANDIDATE VACCINE STRATEGIES AGAINST RIFT VALLEY FEVER VIRUS

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Rift Valley Fever virus (RVFV) is an arthropod-borne bunyavirus that causes a zoonotic disease associated with abortion storms, neonatal mortality in livestock and hemorrhagic fever with a high case/fatality ratio in humans. To date, vaccine developments against RVF have been based on inactivated or attenuated strains but their widespread use has been hampered due to deleterious effects or incomplete protection, justifying further studies to improve the existing vaccines or to develop others. To address this, DNA plasmid and alphavirus replicon vector (VEEV) expressing RVFV Gn glycoprotein were constructed and evaluated for their ability to induce protective immune responses in mice against RVFV. An experimental live-attenuated vaccine (MP12) and its inactivated counterpart (WIV MP12) were developed to serve as benchmarks for comparison. Test vaccine candidates efficiently expressed the RVFV glycoprotein *in vitro* and elicited anti-RVFV antibody responses in immunized mice, as determined by RVFV specific ELISA, IgG isotype ELISA, and virus neutralization. Interestingly, these vaccine strategies elicited cellular immune responses as determined by Gn specific ELISPOT. More importantly these vaccines not only protected immunized mice from virulent RVFV when challenged via intraperitoneal route, but also conferred protection when challenged via aerosol route. This work is of public health significance as it describes the development of safe and effective vaccine candidates that have the ability to protect both livestock and humans against possible routes of exposure to this zoonotic threat.

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PREFACE

I offer my sincere gratitude to Dr. Ted M. Ross, my advisor and thesis committee chairman for his constant support and encouragement during my graduate career. I would also like to thank the members of my thesis committee-Dr. Todd A. Reinhart, Dr. Velpandi Ayyavoo and Dr. Paul R. Kinchington for their helpful guidance and suggestions. My big thank you goes to the past and present members of Ross lab for their intellectual and technical support during my project.

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1.0 INTRODUCTION

Rift Valley fever (RVF) is an arthropod-borne viral zoonosis. The causative agent, Rift Valley fever virus (RVFV), was first discovered in the Rift Valley of Kenya in 1931 [1]. RVFV infections in livestock are characterized by an acute hepatitis, abortion, and high mortality rates, especially in new born or young animals. Human infection with RVFV typically leads to a mild flu-like febrile illness. However, approximately 2% of infected individuals have more severe complications, such as retinal degeneration, fatal hepatitis, severe encephalitis and hemorrhagic fever [2]. The ability of RVFV to cross geographic or national boundaries, coupled with the fact that RVFV replicates in a wide range of mosquito vectors, has raised concerns that the virus might spread further into non-endemic regions of the world. Before 1977, RVFV circulation was not detected beyond the Sub-Saharan countries. In addition, RVFV is a potential bioweapon agent [3]. However, since 1997, RVFV outbreaks have occurred in Egypt [4], Mauritania in 1987 and 1998 [5], Saudi Arabia and Yemen [6]. In 2006–2007, RVFV outbreaks were recorded in Kenya, Somalia and Tanzania that resulted in human infections and deaths [7]. Thus, the ability of RVFV to cause explosive ‘‘virgin soil’’ outbreaks in previously unaffected regions demonstrates the need for prophylactic measures for this significant veterinary and public health threat.

1.1 RIFT VALLEY FEVER VIRUS

1.1.1 RVFV structure and classification

The family *Bunyaviridae* is comprised of a large group of arthropod-borne viruses distributed in among five genera; *Bunyavirus, Phlebovirus, Nairovirus, Hantavirus* and *Tospovirus.* Most viruses in this family infect vertebrates and are transmitted by arthropods such as mosquitoes, ticks, and sandflies with the exception of the viruses belonging to genuse *Tospovirus* which harbors plant viruses. Viruses in the *Bunyaviridae* family were originally classified based on their antigenic relationships. The International Committee on the Taxonomy of Viruses has designated 47 species in the *Bunyavirus* genus, 9 species in the *Phelobovirus* genus, 7 species in the *Nairovirus* genus and 22 species in the *Hantavirus* genus [8]. Rift Valley fever Virus (RVFV) is a typical member of the genus *Phlebovirus.* The virions of the *Bunyaviridae* family are spherical, measuring 80 to 120 nm in diameter, and have a bilayered lipid envelope with three circular nucleocapsids (Fig. 1). The virus genome contains three single-stranded negative sense RNA segments each in its own nucleocapsid. Recent studies have demonstrated that the RVFV possesses an icosahedral symmetry [9], and the two surface glycoproteins Gn and Gc (type I transmembrane proteins) are arranged as heterodimers on the surface of the virion [10]. A unique feature of the members of the family *Bunyaviridae* is the lack of matrix protein [11]. Recently, it has been shown that the cytoplasmic tails of the surface glycoproteins interact directly with the nucleoproteins of the ribonucleoprotein (RNP) complex and may be important for viral genome packaging [12-15].

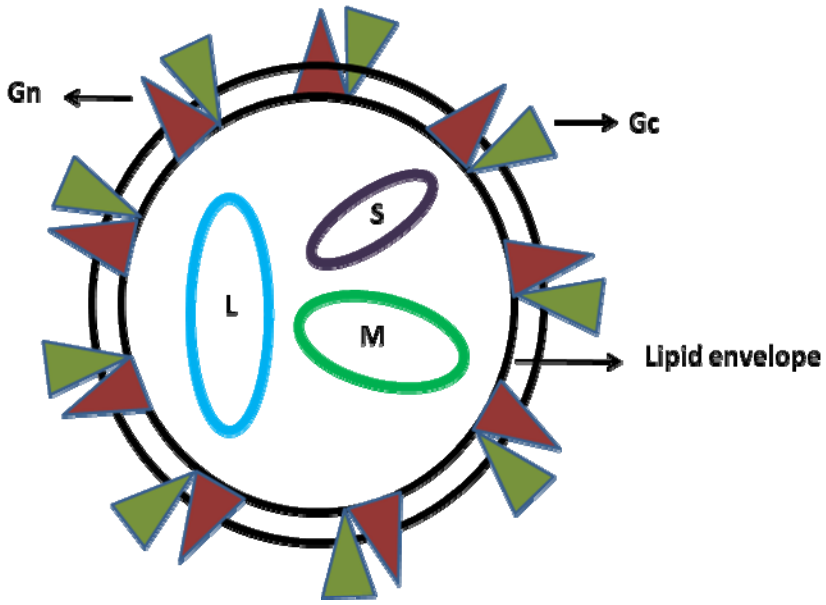


Figure 1. Schematic representation of Bunyavirus

RVFV is an enveloped virus with two surface glycoproteins Gn and Gc embedded in the lipid bilayer of envelope. L, M and S represent the large, medium and small RNA segments, respectively.

1.1.2 Coding strategy of RVFV genes

Virions contain a tripartite single-stranded RNA genome. The three segments are the large (L) segment (~6.4 kb) expressing virus RNA dependent RNA polymerase [11], medium (M) segment (~3.8 kb) encoding at least four proteins in a single open reading frame (ORF) out of which two are structural glycoproteins, Gn and Gc, and two are non-structural proteins, the 14kD NSm and a 78kD NSm+Gn fusion peptides [11, 16, 17]. The small (S) segment (~1.6 kb) encodes in an ambisense fashion the viral nucleoprotein (N) in the genomic oriteintation and the non-structural (NSs) protein in the anti-genomic orientation [11] (Fig.2).

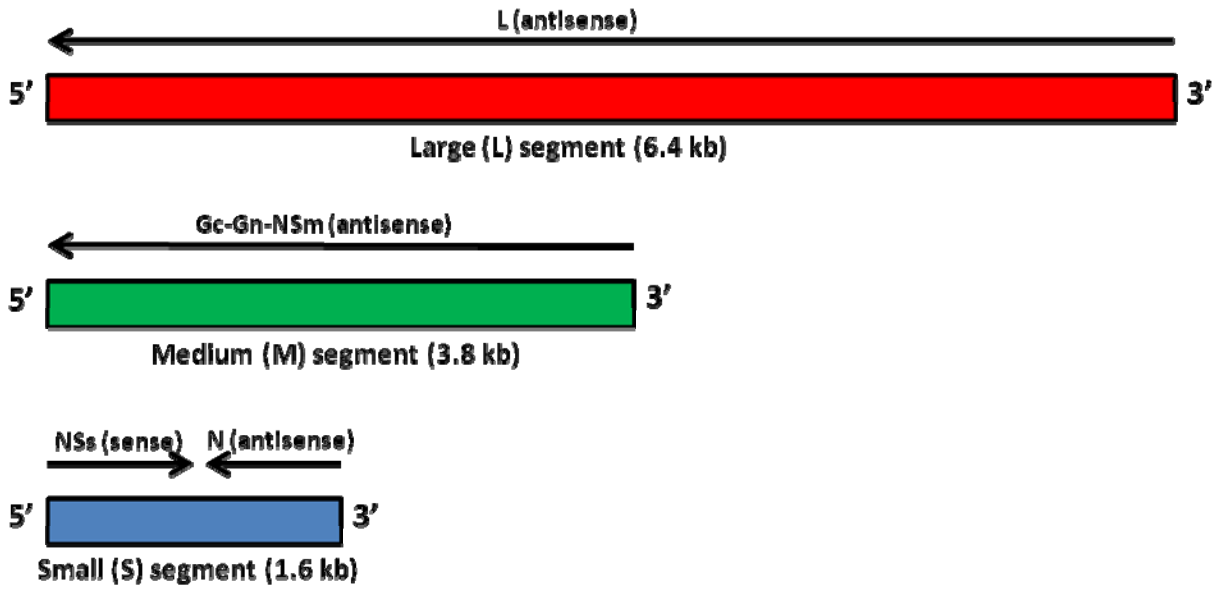


Figure 2. Schematic representation of RVFV genome

RVFV has a tripartite genome comprised of large (L), medium (M) and small (S) gene segments. The L segment encodes viral RNA polymerase, the M segment encodes viral structural glycoproteins (Gn, Gc) and and a nonstructural protein (NSm), and the S segment encodes non structural small protein (NSs) and a nucleocapsid protein (N)

Bunyaviruses replicate in the cytoplasm, and the progeny virions assemble by budding into the lumen of the Golgi apparatus [18]. The nucleocapsid (N) protein coats the genome segments to form ribonucleoproteins (RNPs). The two surface glycoproteins (Gn and Gc) are translated as polyproteins, which are later cleaved in the endoplasmic reticulum. A heterodimeric complex of the viral surface glycoproteins is required for targeting to the Golgi apparatus, since only Gn has the Golgi localization and retention signal [19].

RVFV genome is transcribed and replicated only when it is complexed with RNA polymerase and nucleocapsid protein, forming RNP complex. The structural glycoproteins encoded by the M segment ORF are initially translated as polyprotein precursors for the two mature structural proteins that are co- and post-translationally processed. The carboxy terminal

parts of NSm and Gn contain signal peptides that most likely play roles in the translocation of Gn and Gc into the endoplasmic reticulum (ER) followed by transport into the Golgi compartment for virus assembly. After budding into the Golgi, virions are transported to the cell surface within secretory vesicles and are released when these vesicles fuse with the plasma membrane of the cell.

1.1.3 Epidemiology and transmission

RVFV was originally characterized in 1931 and an association of RVFV epizootics/epidemics with heavy rainfall and high mosquito population was reported [1]. Since 1931, RVFV epidemics/epizootics have followed unusually heavy rainfall or in conjunction with construction of dams. Water plays an important role in the life of most blood feeding arthropods since they have aquatic immature larval stages. Therefore, the distribution of virus and associated outbreaks are linked to the presence of water. It has now become clear that the El Niño activity can lead to heavy precipitation in southern and eastern Africa and was responsible for outbreaks of RVFV in the horn of Africa during 1997-98 [20, 21]. RVFV epizootics are characterized by long inter-epizootic periods in a cyclical fashion. These cycles can vary from five to 15 years in areas experiencing rainfalls and change to 15 to 30 years in comparatively drier areas. Due to abundant rainfall in central and western Africa, RVFV outbreaks have a more continuous pattern, while in the comparatively drier northern Africa outbreaks are associated with irrigated lands.

Several arthropods can be experimentally infected with RVFV infection, but mosquitoes are important biological vectors for disease transmission [22-25]. RVFV has been isolated from greater than 30 different species of mosquitoes. Experimentally, North American mosquito

species belonging to genera *Aedes* and *Culex* are found to be highly competent for RVFV replication [26-28]. During inter-epizootic periods virus may be present in an endemic cycle between mosquitoes and livestock species and possibly gets amplified within the livestock and may then transmit to humans (Fig. 3). During the inter-epizootic period of RVFV infection in Kenya, the trans-ovarian transmission of RVFV in female mosquitoes (*Aedes lineatopennis*) was identified [29]. In dry to semiarid regions of Africa the survival of mosquitoes (*Aedes sp*) is dependent on drought-resistant eggs that remain viable for long dry seasons with below normal rainfall. Areas experiencing heavy rainfall where the water table is sufficiently raised promote virus activity with low level transmission to livestock with mosquitoes (*Aedes sp*). Shallow depressions or potholes (dambos) that are seasonally waterlogged offer an ideal environment for mosquito breeding, egg deposition and development of mosquito larvae [30].

During epizootic/epidemic RVFV cycles following heavy rainfall, there is emergence of large numbers of transovarially infected mosquitoes. These mosquitoes then infect the susceptible livestock species (cattle and sheep) that develop high-titer viremia and establish clinical infection leading to abortion storms and neonatal mortality. Humans acquire infection from percutaneous injury during handling aborted fetal materials or while performing necropsy/slaughter of infected animals [4, 31]. In addition, exposure to infectious aerosols in the laboratory and field have revealed the highly infectious nature of RVFV [32-36]. So far it has not been established if humans play an important role in the RVFV epizootic/epidemic cycles, but most likely act as dead end hosts.

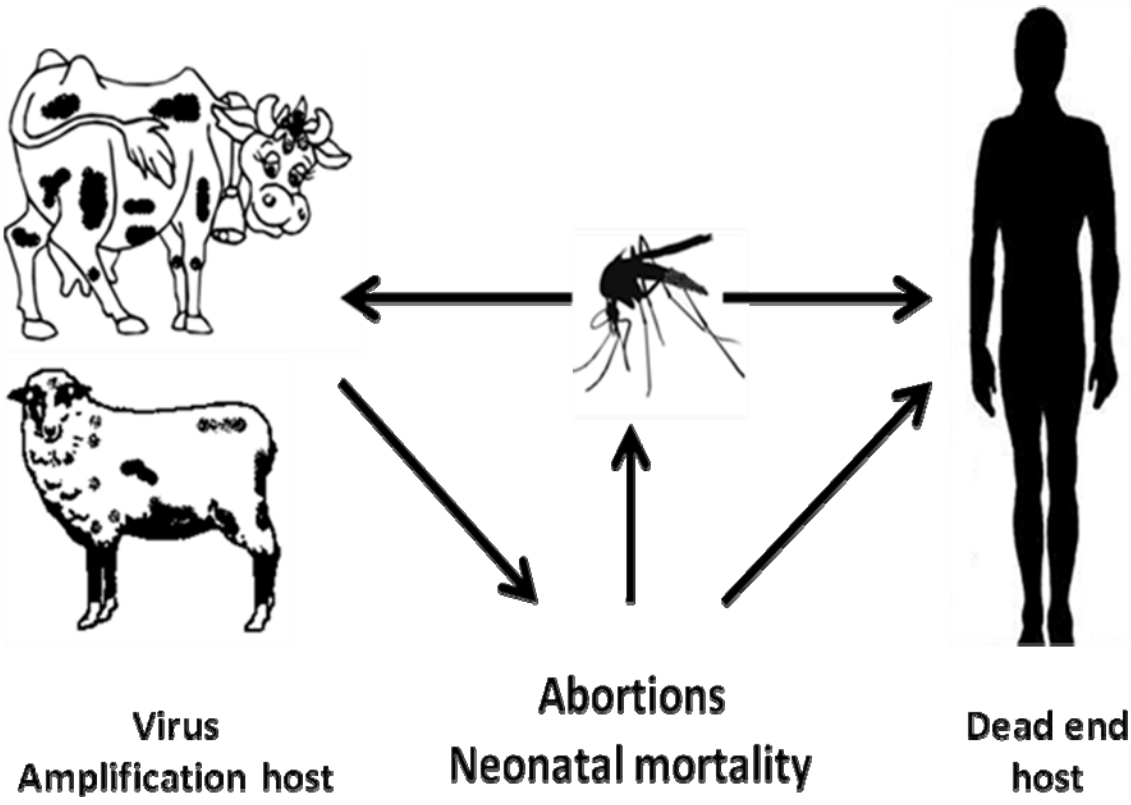


Figure 3. RVFV transmission cycle

Mosquitoes transmit virus to susceptible livestock hosts leading to abortions and neonatal mortality. Humans acquire infection either as a result of bites from infected mosquitoes, handling aborted fetal materials, infectious aerosol exposure or percutaneous injury during necropsy and slaughter procedures on infected animals.

1.1.4 Biology of RVFV infection

Although many animals are susceptible to RVFV infection, the virus causes disease primarily in sheep, goat, and cattle. Epizootics are often characterized by abortion storms that may occur at any stage of pregnancy. The incubation period may be as short as 12 hours in experimental infections, but usually lasts 24-36 hours or even longer in natural infections. Young animals, such as lambs, are highly susceptible to infection with 90-100% mortality. Once infected, lambs

develop a high grade fever (41^0C to 42^0C), exhibit abdominal pain and rapidly progress to death within 24 to 72 hours after the onset of the first clinical signs. Like lambs, newborn calves (<10 days age) also exhibit rapid progression to death once infected [30]. Adult sheep and cattle are somewhat resistant to disease and exhibit fever, loss of appetite, profuse salivation, nasal discharge, abdominal pain and bloody or fetid diarrhea [37]. In some cases, severe jaundice can develop with an overall low (10-30%) fatality rate in adult animals depending upon nutritional status [5].

In humans, RVFV infection is usually mild with a short incubation period of 4 to 6 days. Human infection is typically characterized by fever, myalgia, arthralgia, nausea, vomiting, and altered vision. However, in some cases, infection progresses to severe and sometimes fatal complications [2, 38, 39] such as retinitis, acute hepatitis, delayed onset encephalitis, and hemorrhagic fever that was observed in 1% of the cases in Egypt in 1977 [36]. Encephalitis is often associated with confusion and coma. A high incidence of retinal/ocular involvement was reported during the 2000 epidemic in the Arabian Peninsula [2]. The hemorrhagic syndrome characterized by coagulopathy, disseminated intravascular coagulation and multiple organ failure can develop in severely infected individuals and is the main cause of death with a 10-20% case fatality ratio [38]. Infected individuals have fever for two to four days and then exhibit jaundice, hemorrhages such as hematemesis, bloody stools (melena), hemorrhagic gingivitis, and petechial and purpuric cutaneous lesions. Hepatic necrosis has been one of the hallmark lesions found at autopsy. The meningoencephalitic syndrome is reported in some individuals and occurs one or two weeks after the febrile period.

1.2 LIVESTOCK AND PUBLIC HEALTH SIGNIFICANCE OF RVFV INFECTION

RVFV infection in livestock was first reported as an enzootic hepatitis with extensive necrosis [40]. RVFV was essentially known as a disease affecting domestic animals, before the Egyptian epidemic in 1977 [4]. It was implicated in producing high mortality rates in new-born animals and abortions in pregnant animals. Only a few fatal human cases were reported before 1977. During RVFV epizootic in South Africa in 1950-1951, 100,000 sheep died and 500,000 aborted [41]. In the successive outbreaks, RVFV caused great economic losses in livestock resulting from mortality of domesticated animals and restrictions in trade and export of animals several months after the end of outbreaks.

Human infections typically occur as a result of bites from infected mosquitoes or per-cutaneous or aerosol exposure during handling of aborted fetal materials or the slaughtering of diseased animals [42]. In most human cases, the disease is manifested as a self-limiting febrile illness, which progresses to more serious complications in 1-2% [42] of infected individuals with a hospitalized case fatality of 10-30% [43]. The Egyptian outbreak in 1977 was the first outbreak involving huge number of human cases with an estimated 200,000 cases resulting in 623 deaths from severe complications of disease [36]. Later in 1987, a large outbreak of RVFV infection in Mauritania and Senegal affected 89,000 individuals [5]. In the Arabian Peninsula in 2000, an estimated 2000 cases and 245 deaths were reported [6]. Recently, in 2006-2007 outbreaks in Kenya, Somalia and Tanzania resulted in estimated 1062 reported human cases and 315 deaths resulting from that outbreak [7]. The magnitude of RVF outbreaks in human and animal populations and the widespread vector population highlights the importance of developing preventive measures to meet the challenge in the face of an outbreak in non-endemic areas of the world.

An RVFV outbreak outside the endemic countries would cause serious public health and agricultural problems. One study evaluated the pathways for introduction of RVFV into United States (U.S.) [44]. RVFV can be introduced by the movement of infected travelers, animals and mosquitoes. An intentional release of RVFV in an act of bioterrorism is also a serious concern for national security [45]. RVFV is therefore classified as Category A biodefense agent by Centers for Disease Control (CDC) and United States Department of Agriculture (USDA). In the past, a number of laboratory acquired infections have occurred by the lack of adequate biosafety measures [35]. Accordingly, the handling of RVFV requires high containment facilities, including biosafety level (BSL) 4 laboratories or BSL 3 Agcriculture + laboratories in the U.S.

1.3 IMMUNE RESPONSE TO RVFV INFECTION

Most viral infections trigger both innate and adaptive immune responses in an infected host. Although little is known about the cell mediated immune response, it is a common feature among bunyavirus infections that the antibody mediated immune response plays an important role in protection. The viral nucleoprotein appears to be immunogenic, but antibodies are also raised against the envelope glycoproteins Gn and Gc, which carry neutralizing epitopes [46, 47]. It is known that neutralizing antibodies have a protective effect against a virulent RVFV challenge and passive transfer of RVFV immune serum is protective against lethal RVFV disease in animal models [35, 48]. The induction of a neutralizing antibody response is a good approach for the development of RVFV vaccine. A major role in virulence is played by the non-structural protein NSs [49, 50]. Researchers have now unearthed the mechanisms used by RVFV NSs protein to

counteract the host immune response [51, 52]. It appears that NSs protein although is dispensable for virus production; however it plays a major role acting as an IFN antagonist.

Although the correlates of immune protection for RVFV have not been elucidated, but there is strong evidence that neutralizing antibodies are a major contributor to protective anti-RVFV immune responses. Resolution of disease in animals that survive infection correlates with the generation of anti-RVFV antibody responses. In genomic analysis of the 33 RVFV strains collected from throughout Africa and Saudi Arabia from 1944 to 2000 revealed little viral diversity, with identity differences of only approximately 5% and 2% at the nucleotide and amino acid levels, respectively [53]. This could allow one efficacious vaccine construct to be employed throughout Africa, thereby potentially conferring protection against all RVF virus lineages.

1.4 RVFV VACCINES

No specific treatments are currently available to prevent RVFV infection. RVFV is sensitive to several antiviral agents and interferon treatment *in vitro.* Experimental administration of ribavirin and recombinant interferon alpha to RVFV infected rhesus macaques were as effective as prophylactic drugs, but the chemotherapeutic efficacy for the disease has not been demonstrated [54-56]. Passive antibody administration of serum or immune plasma may be effective, but impractical in an epizootic. The economic importance of disease in livestock industry and the highly pathogenic nature of the virus coupled with the absence of effective treatment against this zoonotic disease necessitate vaccine development to prevent the virus infection.

1.4.1 Live attenuated RVFV vaccines

A number of vaccines were developed to help control the spread of RVFV infection in livestock. One such vaccine, the Smithburn strain of RVFV, was developed by isolating the virus from mosquitoes in Uganda and serially passaging in mouse brains, has been developed as a live attenuated vaccine for veterinary use. [57]. Smithburn strain, created by alternative serial passage in mouse brains and embryonated chicken eggs was used for livestock vaccination for five years (1953-58) in South Africa [58]. Later, it was found that serial passages in mouse brain alone makes a better immunogen. Therefore, since 1958, the Smithburn strain passaged only in mouse brains has been used for immunization of animals in South Africa [58]. Modified live virus vaccine (MLVV) was produced in 1971 by amplification of Smithburn strain derived viruses for use in African countries which included Kenya and South Africa [58].

In 1985, Caplen and coworkers reported generation of a live attenuated vaccine (MP12) from the RVFV ZH548 strain for both human and animal use. The RVFV wild type strains ZH548 and ZH501 isolated from human patients in Egypt were subjected to serial passages in the presence of the chemical mutagen, 5-fluorouracil (5FU) [59]. RVFV ZH548 was passaged two times in suckling mice and once in FhRL cells prior to serial passage in the presence of 5FU. ZH501 on the other hand underwent serial passage in FhRL cells and subsequently was subjected to plaque cloning in MRC-5 cells in the presence of 5FU. ZH501 was found to keep the virulent phenotype in mice after 16 serial passages. However, ZH548 became attenuated in mice after 5 passages [59]. MP12 vaccine is currently in clinical trials and was derived from RVFV ZH548 virus, which was passaged 12 times in the presence of 5FU. MP12 is a temperature-sensitive

mutant virus with mutations in all three RNA segments (L, M, S) [60-62]. Although previous study revealed that mutations in all three genomic segments of MP12 contribute to its attenuation phenotype in mice, further investigation in a recent study revealed that mutations in medium (M) and large (L) segements of MP12 are primarily responsible for its attenuation in mice [60, 63]. MP12 vaccination in pregnant sheep in mid to late gestation (70-100 days) induces neutralizing antibody response without observing any fetal abnormality [64, 65]. Although newborn lambs from the immunized sheep did not exhibit neutralizing antibodies against RVFV. However, consuming colostrum from the MP12 vaccinated dams lead to rapid development of serum neutralizing antibody titers (≥ 1:80) in the newborn lambs against RVFV (1:80 and more) [66]. Furthermore, lambs from a few days old to 3-months of age were able to mount neutralizing antibodies against RVFV post-MP12 vaccination [67, 68]. In an experimental study MP12 was found to be safe and immunogenic in more than 100 human volunteers who received the vaccine if administered at an adequate dose [69].

Another vaccine candidate, clone 13 from the RVFV 74HB59 strain, can be used as live-attenuated vaccine. This virus was isolated in Central Africa from a patient infected with RVFV and it naturally lacks ~70% of an NSs ORF from RVFV S segment and is significantly attenuated [70]. Virus re-assortment experiments using clone 13 and RVFV strain ZH548 have revealed that the viruses that carry S segment from clone 13 had low virulence in mice, whereas viruses that harbor M and/or L segment from clone 13 were virulent in mice model. Further research using clone 13 revealed NSs as the virulence determining genes in mice [51]. Vaccination of pregnant sheep with clone 13 virus induced protective immune response without causing fetal defects or abortions [71]. A reassortant RVFV virus, R566 strain, that harbors a

clone 13 S segment and M and L segments from MP12, is also being developed as a veterinary vaccine candidate [39].

With the background knowledge about RVFV virulence determining gene, Bird *et al.* developed a mutant RVFV ZH501 virus using reverse genetics approach [72]. The mutant virus lacks NSm gene in the M segment and has green fluorescent protein (GFP) in place of NSs gene in the S segment. Inoculation of 1×10^3 PFU of this mutant virus (rRVF-ΔNSs:GFP-ΔNSm) resulted in the production of neutralizing antibodies 21 days post-inoculation. This vaccine was highly attenuated in rats. Challenge of vaccinated rats with virulent RVFV at 28th day post inoculation resulted in protection without development of detectable viremia [72].

The use of reverse genetics approaches to make viral vaccines against RVFV has the potential to go forward into clinical trials. One of the advantages of this approach is the differentiation of vaccinated from infected animals (DIVA) by inserting non-viral genes in place of some inherent viral genes (NSs or NSm). Vaccinated animals with such mutant viruses will not elicit antibody responses against the deleted viral genes, but they will elicit antibodies against the non-viral foreign genes.

1.4.2 Inactivated RVFV vaccines

In the continuing effort to develop safe and effective RVFV vaccines, Randall *et al*. first reported that vaccinating mice with a formalin-inactivated vaccine derived from RVFV Entebbe strain induced neutralizing antibody titers in mice [73]. The RVFV Entebbe strain was initially isolated from mosquitoes in Uganda and had subsequently undergone over 150 intraperitoneal (i.p.) or intravenous (i.v.) passages in mice. The formalin-inactivated vaccine prepared from

embryonated chicken eggs or chicken cell cultures was less immunogenic than the vaccine derived from primary rhesus macaque or African green monkey kidney cells [73, 74]. The new formalin-inactivated vaccine (NDBR 103) was manufactured by amplification of the mouse serum master seed (Entebbe strain, 184th passage) in primary monkey kidney cells [73]. Since 1977, more than 500 human volunteers have been vaccinated with NDBR 103 [75, 76]. A new lot of formalin-inactivated RVFV vaccine was manufactured by USAMRIID using a new master seed stock, which was prepared from two passages of the mouse serum master seed of the Entebbe strain in the diploid cells derived from fetal rhesus monkey lungs (FRhL-2) and named TSI-GSD 200 [75]. Further studies with this strain by Pittman *et al*. revealed that vaccination of human volunteers with three doses of TSI-GSD 200 resulted in the development of neutralizing antibody responses with a mean titer of 1:237 [77]. In addition, about 90% of the vaccinated individuals initially responded with antibody titers of 1:40 or more, whereas the remaining 10% of vaccinated individuals failed to achieve this titer and were considered non-responders [77]. This study further highlighted that a neutralizing antibody titer of 1:40 had a half life close to 250 days in the positive responders [77]. Presently, a neutralizing titer of ≥ 1:40 is recommended for at-risk individuals who might get exposed to RVFV [75]. In addition to the a series of primary vaccinations, a regular booster vaccination is considered necessary for maintaining the protective titers [78].

1.4.3 Limitations of the existing RVFV vaccines

Although live-attenuated and formalin-inactivated vaccines are immunogenic, their widespread use is limited due to safety issues. The MLVV based on the Smithburn strain, is used to

vaccinate livestock in Africa, but suffers from major limitations such as causing pathology, spontaneous abortions, and teratogenic effects in animals [79, 80]. Another MLVV based on ZH548 strain called MP12 vaccine was expected to be developed as a single vaccine for human and animal use induces abortions and teratogenicity in newborn lambs when pregnant sheep are vaccinated [81]. This indicates that the use of MP12 in pregnant animals might result in some loss of offspring and or birth of severely deformed lambs or calves. In addition to the adverse effects of the live-attenuated vaccines, there are considerable safety concerns including incomplete attenuation, reversion back to a virulent form during the vaccine manufacturing process. Furthermore, animals vaccinated with live-attenuated RVFV strains cannot be differentiated from naturally infected livestock, due to induction of similar antibody responses, which may preclude export of these animals to non-RVFV endemic areas.

Inactivated RVFV vaccine (TSI-GSD-200) elicits protective immunity in humans, however multiple booster vaccinations are required to achieve protective immunity, and perhaps most importantly, for many individuals, immunity rapidly wanes in the absence of follow-up booster vaccinations [77]. In addition, some local reactions such as swelling, pain and erythema were reported at the site of injection in individuals who received formalin-inactivated NDBR 103 and TSI-GSD 200 RVFV vaccines. In addition a single case of Guillain–Barré syndrome was also reported [76, 82].

Reverse-genetics based vaccine candidates lacking some of the viral genes like NSs or NSm might suffer from inability to replicate efficiently in immune competent animals or humans. The longevity of immunity induced by these mutant viruses has not been evaluated, but their inefficient replication could possibly lead to less robust long-term immunity.

1.4.4 New approaches in RVFV vaccine development

Given the limitation of existing RVFV vaccines, there is a need to explore alternative vaccine approaches for development of safe and effective global-use vaccines.

1.4.4.1 Vaccines based on recombinant proteins

Collet *et al*. first described the expression of RVFV envelope glycoproteins Gn and Gc in bacteria and vaccinia virus [83]. Vaccination of mice with these immunogens, especially the group that received vaccinia virus expressing RVFV glycoproteins, led to production of anti-RVFV antibody responses and most mice were protected from virulent RVFV challenge [83]. Subsequently Keegan and Collett used a bacterial expression system and identified amino acid sequences of the antigenic determinant present on Gn protein [46]. Later Dalrymple *et al*. did some pioneering work and mapped the protective determinants of RVFV glycoproteins in RVFV surface glycoprotein Gn by using a vaccinia virus based expression system [84]. Besselaar *et al*. followed this work and identified antigenic domains on both Gn and Gc that play important role in virus neutralization [85]. Schmaljohn and coworkers used baculovirus protein expression system to produce RVFV Gn and Gn and showed protective efficacy of these expressed proteins in protecting mice from virus challenge [48].

1.4.4.2 Virus like particle based RVFV vaccines

Another approach for vaccine development is to use virus-like particles (VLPs) that are formed when the structural (envelope and/or nucleocapsid) proteins self-assemble to replication-deficient particles [86]. RVFV VLP is composed of viral surface proteins Gn, Gc and nucleocapsid (N) protein [87, 88]. Näslund *et al*. reported the production of RVFV VLPs in mammalian cells by co-expressing viral structural genes along with a Renilla luciferase reporter minigenome [49]. These vaccines also elicited high titer neutralizing antibodies that protected immunized mice from virulent RVFV ZH548 challenge. Although RVFV VLPs has shown encouraging results however, several things need to be worked out such as lowering the significantly higher cost of production and selection of appropriate cell lines before they could be marketed.

1.4.4.3 DNA based vaccines

Vaccination with plasmid encoding antigens is another approach for inducing protective immunity against pathogens. The biggest advantage of using this vaccination strategy is that plasmids are extremely stable at wide temperature ranges. Therefore, a DNA-based vaccination strategy may be suitable for use in tropical areas, such as Africa, where access to refrigeration systems is sometimes difficult and where RVFV is endemic. Spik *et al*. showed that a series of four gene gun inoculations of DNA plasmids expressing RVFV Gn and Gc glycoproteins elicited neutralizing antibody titers (1:40 to 1:320). They also showed protection against virulent RVFV in mice vaccinated with the DNA plasmids [89]. A recent study using DNA plasmids in a similar

vaccination approach showed development of neutralizing titers ranging from 1:25 to 1:75. Although mice developed neutralizing titers, ~50% of vaccinated mice developed clinical symptoms post-challenge suggesting incomplete protection [90].

1.4.4.4 Vaccines based on Alphavirus replicon and other viral vectors

Alphavirus replicon vectors are single hit vectors capable of eliciting potent systemic and mucosal immune responses against a wide range of pathogens, including hemorrhagic fever viruses, such as Lassa and Ebola [91]. Gorchakov *et al*. used alphavirus as a vaccine vector for RVFV and showed protection in mice from wild-type RVFV challenge following immunization with a Venezuelan equine encephalitis virus (VEEV) replicon expressing RVFV Gn [92]. However, little to no protection was observed in mice vaccinated with Sindbis virus (SINV) replicon expressing Gn and Gc [92]. The study also highlighted the failure of efficient expression of RVFV Gn by SINV replicon. Furthermore, immunization of mice with chimeric VEEV expressing truncate Gn fused to the N-terminus of VEEV E2 protein protected mice from virulent RVFV challenge [92]. Another study using alphaviruses showed that vaccination of mice with SINV (AR86) or Girdwood-based replicons expressing Gn and Gc from RVFV M gene segment induced protective antibody titers and protected mice from subsequent virus challenge [93].

Another approach consisting of viral vectors for RVFV vaccine was based on using lumpy skin disease virus (LSDV), belonging to the *Poxviridae* family, for the expression of RVFV Gn and Gc proteins to protect sheep from RVFV as well as sheep poxvirus in South Africa [94]. These studies reflect the importance of viral vectors as promising candidates for

development of RVFV vaccines and can be furthered improved for both veterinary and human use against RVFV.

1.4.5 C3d as a molecular adjuvant

Although naked DNA is an efficient vaccination strategy, DNA immunization is not as efficient when used in large animal species. There are various ways to overcome this limitation, including codon optimization of gene expression and use of adjuvants. C3d, the final degradation product of the third component of the complement protein C3 is a molecular adjuvant in a number of preclinical vaccine studies [95-103]. The adjuvant potential of this molecule involves C3d binding to the complement receptor 2 (CR2) that is located on the surface of follicular dendritic cells (FDC), B cells, and T cells in many animal species [103]. Molecular adjuvant C3d stimulates antigen presentation by FDCs and helps to maintain immunological B cell memory. On the surface of B cells, C3d interacts with CR2 and associates with CD19 and TAPA. CD19 has a long intracellular tail that triggers a signaling cascade that results in cell activation and proliferation (Fig. 4). Furthermore, simultaneous C3d–CR2 ligation and surface immunoglobulin (sIg) by antigen, activates two signaling pathways that cross-talk and synergize to activate B cells, thereby leading to enhanced antibody secretion specifically directed to the fused antigen.

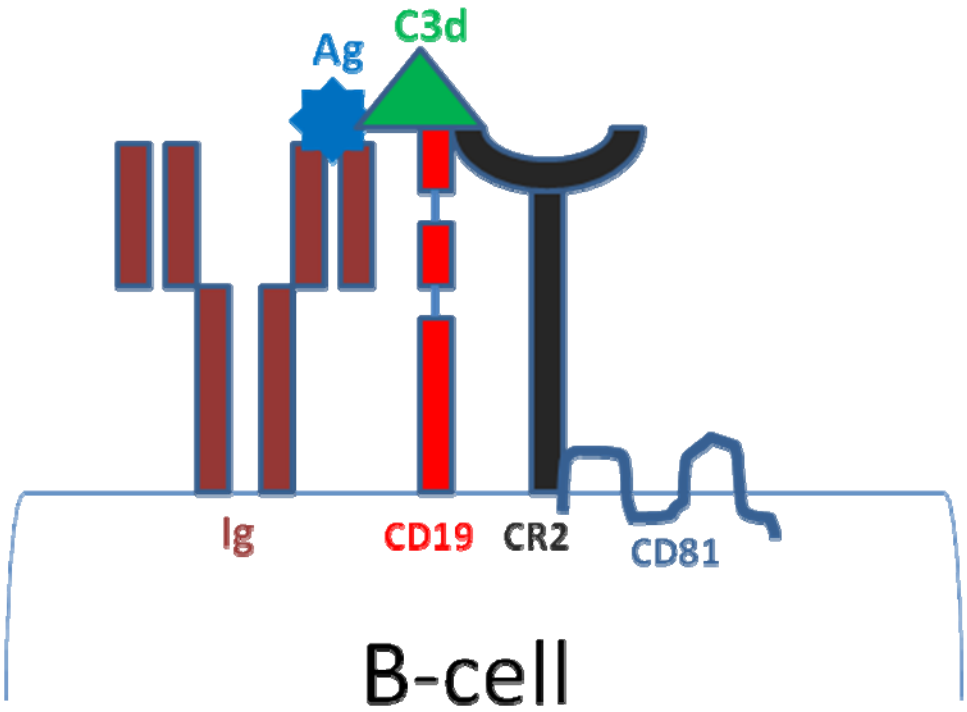


Figure 4. C3d: the molecular adjuvant

Invading microorganisms coated with C3d interact with B-cells through its surface immunoglobulin (sIg) and complement receptor (CR2). Co-ligation of these two receptors activate pathways that cross-talk and lead to activation of the B-cell thereby producing antigen specific immunoglobulins.

1.5 CONCLUSION

Perusal of the available literature reveals that the conventional live-attenuated and inactivated vaccines against RVFV, although successfully used in livestock in RVFV endemic areas have issues related with their safety and potency limits their widespread use. Live-attenuated vaccines cannot be used in pregnant animals due to the risk of abortions or birth of offspring with severe developmental defects. Alternatively, inactivated vaccines require multiple booster immunizations to achieve protective titers that increase their cost. Recent work with the advent

of alternative vaccination approaches holds some promise for further studies and future development. Among the new approaches, DNA and alphavirus replicons appear to be potential vaccine candidates that can be tested further. An ideal vaccine should not only protect from infection, but also prevent clinical symptoms and morbidity associated with infection. In this dissertation, I tested DNA and alphavirus replicons in homologous and heterologous vaccination approaches for their ability to protect against virus challenge and prevent clinical signs of RVFV infection in an animal model.

2.0 SPECIFIC AIMS

2.1 BACKGROUND AND SIGNIFICANCE

2.1.1 Background

Rift valley fever virus (RVFV) is an arthropod-borne Phlebovirus that causes periodic epizootics and epidemics in sub-Saharan countries of Africa and in Egypt. This viral zoonosis primarily infects livestock resulting in neonatal mortality and abortions. However, it has been implicated as the cause of hemorrhagic fever, encephalitis, retinitis and fatal hepatitis in humans. Though currently confined to Africa and the Arabian Peninsula, RVFV has the potential to be introduced into other countries by mosquito transmission or contact with infected tissues and aerosolized material. Currently, the inactivated and the experimental live attenuated RVFV vaccines for humans suffer from safety, potency and cost issues. Therefore, there is an urgent need for developing safe and effective vaccines that rapidly elicit protective immunity against RVFV infection.

2.1.2 Significance of the study

RVFV is a Class A bioterrorism agent with the potential to spread via mosquitoes or the aerosol route to cause disease in humans. Vaccination represents the most promising means of protecting humans against RVFV. DNA vaccines represent a novel means of expressing antigens in vivo, as it can induce both, humoral and cellular immune responses. Therefore, DNA vaccines encoding soluble RVFV glycoprotein linked to the multiple copies of C3d might show promise as a vaccine approach when administered in combination with alphavirus replicons expressing the soluble RVFV glycoprotein. This study not only facilitates the development of improved vaccines against RVFV, but also enhances our understanding of immune correlates that ultimately mediate protection against RVFV infection.

2.2 HYPOTHESIS AND SPECIFIC AIMS

The overall aim of this research project was to develop candidate vaccine strategies against Rift Valley fever virus using DNA and alphavirus replicons as vaccine delivery vectors. Preclinical studies with DNA and replicons have shown promise for further testing and optimization of these excellent vaccine vectors. Studies in the past using alternative vaccines against RVFV have provided encouraging results but were focused entirely on protection. The main focus of this comprehensive study was not only to test these vaccination strategies individually, but also in a heterologous prime-boost approach for their immunogenicity and protective efficacy against virulent RVFV challenge. In addition live-attenuated vaccine and inactivated RVFV vaccines were developed and used as strict benchmarks for comparison which was lacking in previous

studies. Although, protection is one aspect of evaluating vaccine efficacy, the present study was designed to evaluate DNA and alphavirus replicon-based vaccines in their ability to not only provide protection but also to prevent morbidity or clinical signs of infection.

DNA vaccines represent a novel and safe method of expressing antigens *in vivo* for the generation of humoral and cellular immune responses with low costs of production. Previous work has shown that C3d, the final degradation product of the third component of complement, can act as an adjuvant to selectively promote antibody responses to a foreign antigen. DNA vaccination with antigen-C3d fusion proteins resulted in enhanced specific antibody titers as well as accelerated affinity maturation. Alternatively, recombinant alphavirus vectors such as VEE replicons have excellent potential as vaccine vectors. The viruses from which these replicon vectors are derived can replicate in humans, but are not associated with any disease and are currently being used in other vaccine strategies to elicit protective immune responses in humans. **Therefore, the overall hypothesis of the study was that immunization with RVFV glycoprotein Gn on DNA and replicon vaccine platforms will elicit antigen-specific neutralizing antibody responses and confer some level of protection against virulent RVFV infection in mice. I addressed the hypothesis by the following specific aims:**

Aim 1. Construct and characterize candidate DNA and replicon vaccines in their ability to express RVFV glycoprotein Gn and develop benchmark live-attenuated and inactivated RVFV vaccines for comparison

Aim 2. Compare candidate DNA and replicon-based vaccine strategies in their ability to elicit anti-RVFV immune responses

Aim 3. Evaluate the ability of candidate vaccines in conferring protection against virulent RVFV challenge by parenteral routes

3.0 CONSTRUCTION AND VERIFICATION OF CANDIDATE DNA AND REPLICON VACCINES EXPRESSING RVFV GLYCOPROTEIN GN AND DEVELOPMENT OF BENCHMARK LIVE-ATTENUATED AND INACTIVATED RVFV VACCINES FOR COMPARISON

This chapter was modified with permission from:
Nitin Bhardwaj, Mark T. Heise, Ted M. Ross
Vaccination with DNA plasmids expressing Gn coupled to C3d or Alphavirus replicons expressing Gn protects mice against Rift Valley fever virus
PLoS Negl Trop Dis.2010 June 22. 4(6): e725

3.1 PREFACE

The study described in this aim was completed by Nitin Bhardwaj. The authors would like to thank Drs. Mike Parker and George Ludwig (USAMRIID) for providing anti-RVFV monoclonal and polyclonal antibodies, Dr. Robert Tesh (UTMB, Texas) for providing RVFV MP12 and Dr. Pierre Rollin (CDC, Atlanta, GA) for providing RVFV strain ZH501.

3.2 ABSTRACT

Rift Valley fever virus (RVFV) is an arthropod-borne virus associated with abortion storms, neonatal mortality in livestock and hemorrhagic fever or fatal encephalitis in a proportion of infected humans. Given the limitations of existing live-attenuated and inactivated vaccines, there is a need to explore alternative vaccination strategies with application for global use. To address this, two vaccination strategies were developed in this study based on DNA plasmid and alphavirus replicon. RVFV surface glycoprotein Gn was used as the vaccine antigen due to the presence of antigenic determinants and virus neutralizing epitopes on Gn. Well characterized DNA vaccine vector pTR600 was used to insert ectodomain of Gn behind the TPA leader sequence. C3d, a molecular adjuvant with the advantage of inducing antigen specific immune responses was used in conjunction with Gn to construct the DNA vaccine. Venezuelan Equine

Encephalitis virus (VEEV) based alphavirus replicon vaccine was developed by cloning Gn behind the highly efficient 26S subgenomic promoter on the RNA segment containing coding sequence for non-structural alphavirus proteins. Single cycle recombinant replicon particles expressing RVFV Gn were obtained by co-transfecting the RNA segment containing Gn with the helper RNA segment encoding VEEV structural genes into BHK21 cells. Both DNA plasmid and alphavirus replicon expressed Gn protein when tested by transfection followed by SDS-PAGE and western blot. Benchmark live-attenuated vaccine was developed by cultivation and titration of RVFV MP12 in Vero cells and the whole inactivated virus vaccine (WIV) was developed by inactivating pre-titrated MP12 virus with beta propiolactone.

3.3 INTRODUCTION

Since the current RVFV vaccines have several shortcomings preventing their widespread use, new RVFV vaccine strategies need to be considered. Genetic immunization with DNA vector based vaccines is an attractive alternative. A DNA vaccine incorporates genetic information of the suitable antigen(s) which are produced by the host cells and hence the antigen presentation resembles natural infections by intracellular parasites stimulating immune responses. In addition this strategy is cost-effective and avoids the need for expensive and laborious biosafety level manufacturing procedures. Studies from our group and others have demonstrated that the molecular adjuvant C3d can significantly enhance antibody responses against DNA vaccine delivered antigens and thus improves the overall immunization strategy [95-103]. Among various advantages of DNA vaccines, one of the most important is the ability to remain stable at

various temperatures which helps in easy storage and transportation to different parts of the world, especially in developing countries [104]. Alphavirus replicons present another promising vaccination approach against infectious diseases [91]. The natural targeting potential of replicons to lymph node cells helps mount an efficient humoral and cell mediated immune response against the antigen in question [105-107]. In contrast to the infection produced by live virus, where infection spreads from one cell to another, vaccination with replicons limits the gene expression to the cells initially infected with the replicon particles and coupled with their single cycle expression profile provides excellent safety. The 26S subgenomic promoter transcribes the gene of interest to high levels and subsequently helps in translation of multiple copies of antigenic protein. A number of studies have shown RVFV glycoprotein Gn harbors virus neutralizing epitopes and thus this became the antigen of choice for this study [46, 48, 83, 92]. In this aim, the construction and expression of DNA plasmid and alphavirus replicon expressing RVFV Gn glycoprotein is described. In order to have a fair comparison of our vaccine candidates, MP12 and WIV MP12, live and inactivated vaccines, respectively, were also developed to serve as benchmarks.

3.4 MATERIALS AND METHODS

3.4.1 Plasmid DNA

pTR600, a eukaryotic expression vector, has been described previously [100]. Briefly, the vector was constructed to contain the cytomegalovirus immediate-early promoter (CMV-IE) plus intron

A (IA) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription. The vector contains the Col E1 origin of replication for prokaryotic replication and the kanamycin resistance gene (Kan^r) for selection in antibiotic media. The gene sequence encoding for the RVFV, isolate ZH548 (Genbank DQ380206), Gn glycoprotein was used to PCR amplify a soluble form of Gn (Gn) without the transmembrane and cytoplasmic tail (Fig. 5). The Gn gene sequence was cloned into the pTR600 vaccine vector by using unique HindIII and BamHI restriction endonuclease sites. This Gn segment encoded a region from amino acids 131 to 557 (427 amino acids) and terminated in the sequence VAHCP. The vectors expressing Gn fused to three tandem repeats of the mouse homologue of C3d were cloned in frame and designated Gn-C3d, similar to constructs previously described [95]. Linkers composed of two repeats of four glycines and a serine [(G4S)2] were fused at the junctures of Gn and C3d and between each C3d repeat. Potential proteolytic cleavage sites between the junctions of Gn and the junction of C3d were mutated by ligating BamHI and BglII restriction endonuclease sites to mutate an Arg codon to a Gly codon [95]. The plasmids were amplified in *Escherichia coli* DH5a, purified by using endotoxin-free, anion-exchange resin columns (Qiagen, Valencia, CA, USA), and stored at -20^0C in distilled water. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined based on the optical density (O.D.) at wavelengths of 260 and 280 nm.

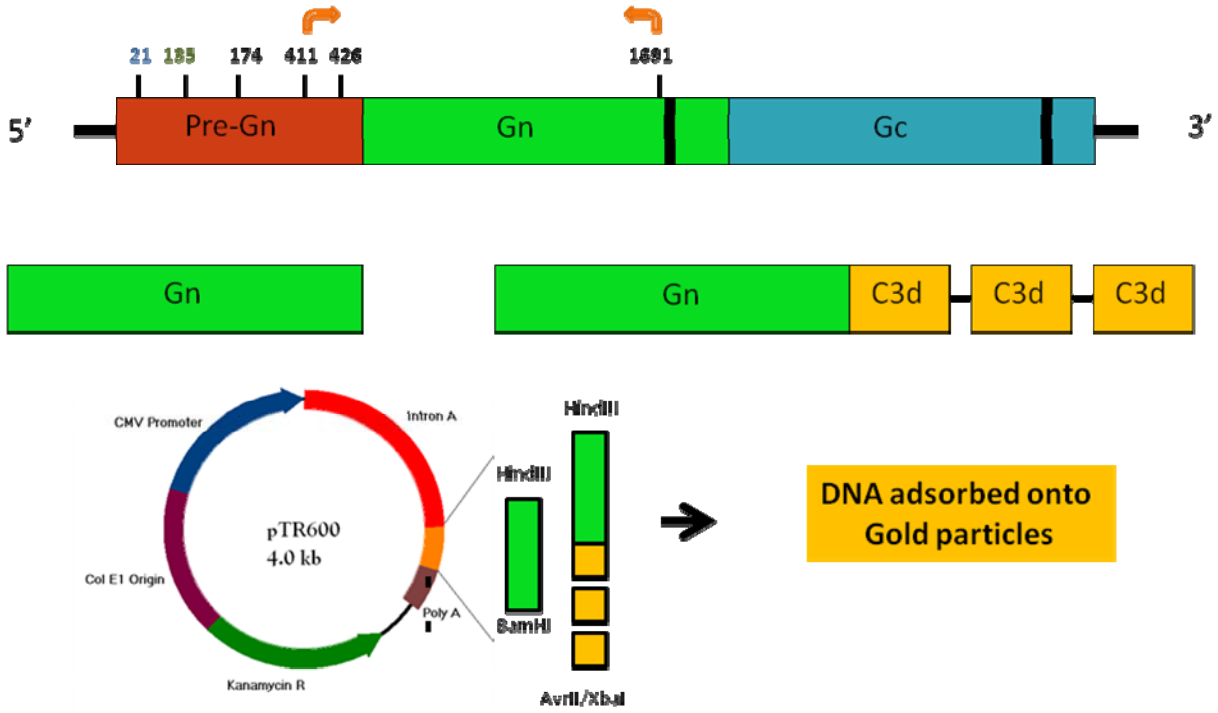


Figure 5. Schematic representation of DNA vaccine expression construct. See section 3.4.1 for more details

3.4.2 Replicons

A soluble form of RVFV Gn lacking the transmembrane and cytoplasmic tail (see above) was introduced behind the 26S subgenomic promoter of the VEE replicon plasmid pVR21 as outlined in Figure 6. VEE replicons expressing influenza hemagglutinin were used as negative controls. VEE replicon plasmids, as well as capsid and glycoprotein plasmids were linearized with NotI, replicon and helper transcripts were generated using mMessage mMachine T7 transcription kits (Ambion), and transcripts electroporated into BHK-21 cells to package replicon particles as described previously [108]. Following packaging, the replicons underwent two rounds of safety testing to ensure that no detectable replication competent virus was present [93, 108] at which point the replicons were concentrated by ultracentrifugation through a 20% sucrose cushion and

titered using polyclonal antiserum against the VEE nonstructural proteins. Expression of the truncated RVFV Gn protein from the replicon was confirmed by western blot with a Gn specific monoclonal antibody (RV5 3G2-1A) generously provided by Dr. George Ludwig, USAMRIID, Ft. Detrick, Frederick, MD, USA.

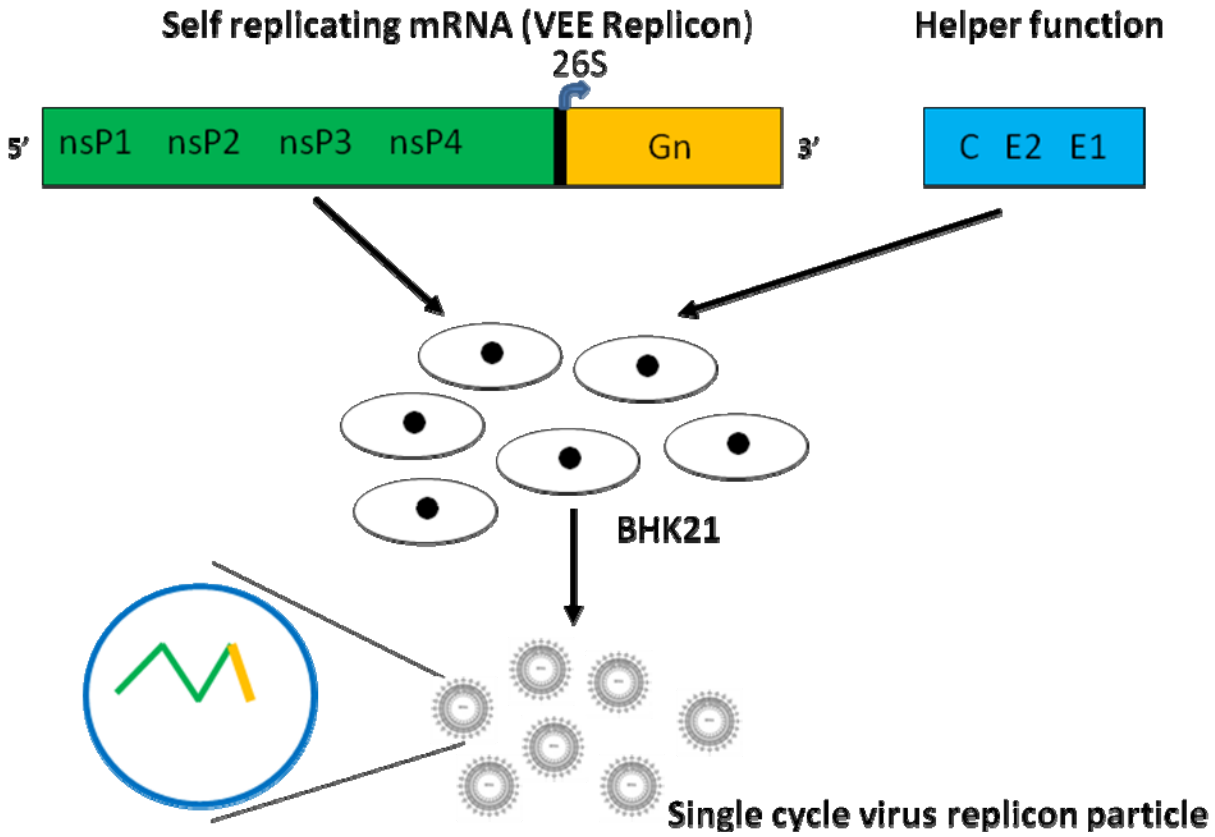


Figure 6. Schematic representation of VEEV replicon production. See section 3.4.2 for more details

3.4.3 In vitro expression of the vaccine plasmids

The human embryonic kidney cell line, 293T, was transfected (at $5x10^5$ cells/transfection) with 5µg of DNA by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA.) according to the manufacturer's guidelines. Supernatants were collected after 72 h and stored at -20^0C. Cell

lysates were collected in 500µl of 1% Triton X-100 buffer and stored at -20^{0}C. To detect specific proteins in the cell supernatant, it was diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA, USA) and loaded onto a 10% polyacrylamide–SDS gel. The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and incubated with a 1:5,000 dilution of anti-RVFV mouse sera (kindly provided by Drs. Mike Parker and George Ludwig, USAMRIID) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% skim milk powder. After an extensive washing, bound mouse antibodies were detected by using a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom).

3.4.4 Live attenuated and whole inactivated virus vaccines

The attenuated strain RVFV MP12 (MP12) (Kindly provided by Dr. Robert Tesh, UTMB) and ZH501 (kindly provided by Dr. Pierre Rollin was propagated and titrated using Vero cells. A pre-titrated RVFV MP12 was inactivated with 1% beta-propiolactone to a final concentration of 0.1% to make a whole virus inactivated preparation (WIV MP12). To ensure complete inactivation, an aliquot of inactivated virus was used to infect Vero cells and verify the lack of cytopathic effect.

3.5 RESULTS

3.5.1 Construction and expression of Gn and Gn-C3d from DNAvaccine vectors

Well described pTR600 plasmid was used to construct a DNA vaccine [100]. A 1281 bp gene segment encoding the ectodomain of RVFV Gn was successfully PCR amplified using high fidelity *Taq* polymerase under standard PCR conditions with Gn specific forward and reverse primers. The amplified gene segment that lacked TMD and CT regions of the glycoprotein was then successfully ligated with three copies of murine homologue of C3d. Restriction enzyme digestion of the plasmid containing unconjugated and conjugated Gn gene resulted in expected band sizes thus confirming the successful cloning.

A truncated, soluble form of Gn from the RVFV isolate ZH548 alone or fused to three copies of murine C3d (Gn-C3d) was efficiently secreted from cells transfected with DNA plasmid as determined by transient transfection and western blot analysis (Fig. 7). Anti-RVFV antibodies used for western blot revealed that RVFV Gn migrated at an expected 45kDa molecular weight and the C3d fusion with Gn increased the molecular weight to 135kDa. Not only did the conjugation with C3d help add the adjuvant effect to the DNA vaccine but perhaps it also helped in efficient secretion of the conjugated protein from the transfected cells. It however needs to be determined if C3d conjugation has any role in stability of the expressed protein.

3.5.2 Construction and expression of Gn from VEEV replicon vaccine

RVFV Gn ectodomain encoding gene (1281bp) was PCR amplified and cloned behind the VEEV 26S subgenomic promoter as described in Figure 6. Restriction enzyme analysis revealed the successful cloning of the gene into the VEEV vector. The new plasmid along with helper constructs was linearized and subjected to transcription as described in the materials and methods section before they were electroporated into BHK21 cells. This resulted in production of packaged replicons with a titer of 1x 10^9 infectious units (IU). Expression of the RVFV Gn protein from the replicon was confirmed by western blot with a Gn specific monoclonal antibody (Fig. 7). The replicons were then stored in screw cap vials at -80^0 C till further use.

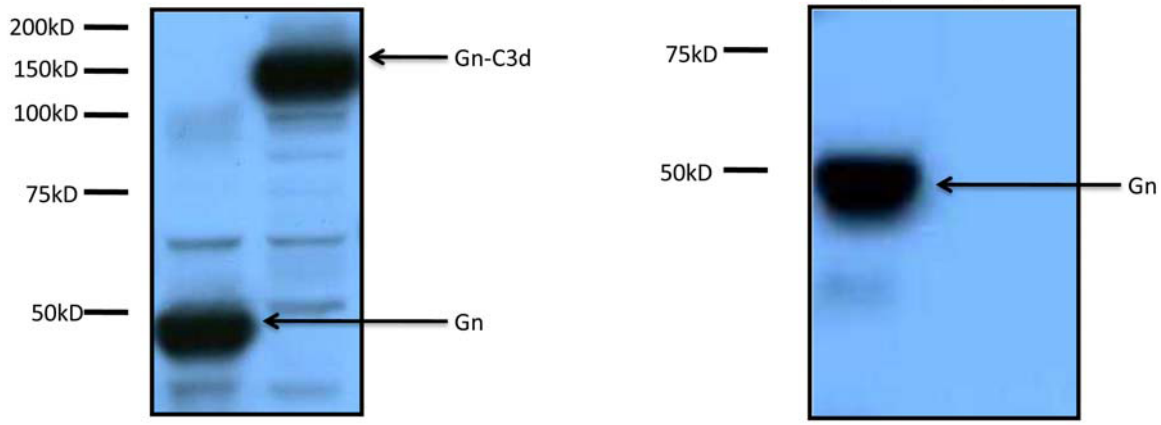


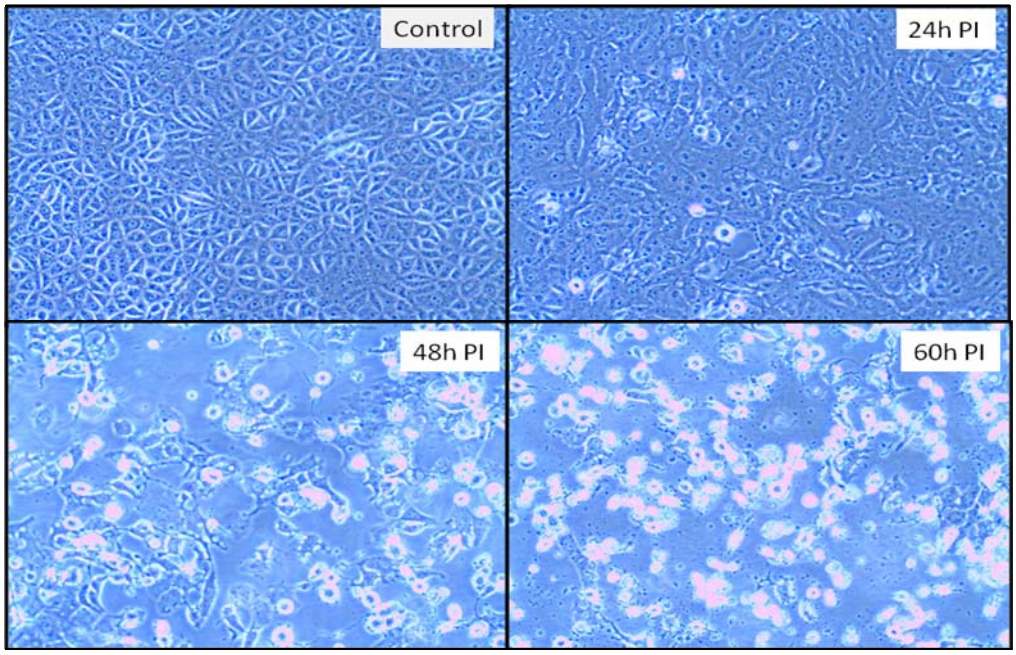
Figure 7. Expression of RVFV Gn from candidate DNA and replicon vaccines

(Left panel) Proteins expressed from 293T cells transiently transfected with plasmid DNAs were assessed by SDS-PAGE and Western blot. The membrane was probed with anti-RVFV polyclonal antibody. (Right Panel) Proteins expressed from BHK21 cells infected with packaged VEE replicons were assessed by SDS-PAGE and Western blot. The membrane was probed with anti-RVFV polyclonal sera.

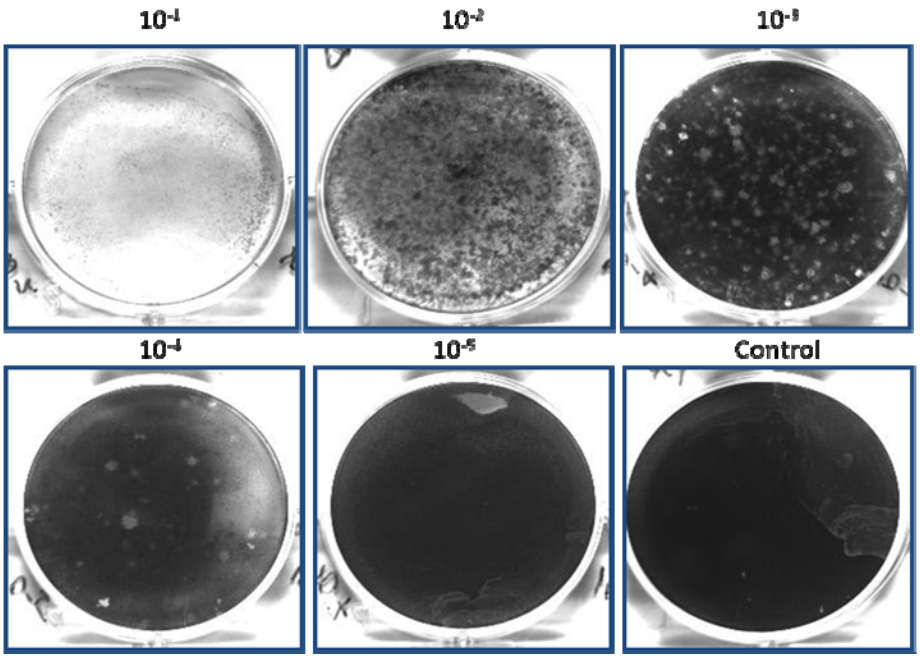
3.5.3 Development of live-attenuated and inactivated virus vaccines

3.5.3.1 Cultivation and titration of RVFV MP12

RVFV MP12 virus was grown in Vero cells and the first signs of cytopathic effect (CPE) were seen at 24h post infection (PI) and by 60h PI 90% of CPE was seen resulting in cell sheet disruption (Fig. 8A). Vero cells were plated in a 6-well plate ($4x10^5$cells per well) and virus titration was done using plaque assay in which various dilution of virus were incubated for 1 hr at 37^0C on Vero cells followed by 2x MEM mixed with SeaKem ME agarose and supplemented with HEPES, FBS, antibiotics and fungicide. The plates were kept at 37^0 in a 5% CO_2 environment for 4 days. Plaques were visualized by fixing cells with 10% formalin and staining with 1% crystal violet (Fig. 8B). Titrated virus was stored in 1.0 ml aliquots at -80^0C till further use.



A



B

Figure 8. Cultivation and Titration of MP12 in Vero cells

Panel A: CPE produced by MP12 at various time points and Panel B: Plaques produced by MP12 virus at different dilutions 4 days PI.

3.5.3.2 Development of whole inactivated RVFV vaccine (WIV)

Pretitrated MP12 Virus (10^5 pfu) was mixed with 1% betapropiolactone (BPL) to achieve a final concentration of 0.1%. The virus-BPL mixture was kept at 4^0 C for 24 h on a rocking platform to achieve complete inactivation. An aliquot of BLP inactivated (WIV MP12) virus was tested on Vero cells along with MP12 virus. The live-attenuated vaccine (MP12) produced characteristic cytopathic effect 72 h post infection whereas no cytopathic effect was observed with WIV MP12 vaccine (Fig. 9).

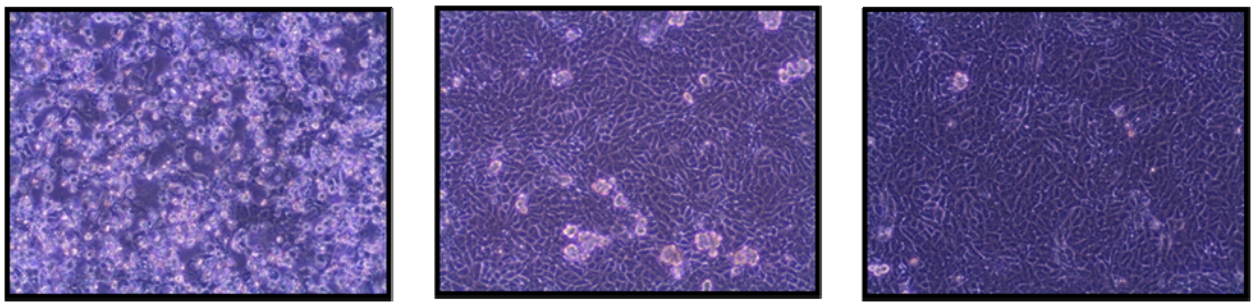


Figure 9. Evaluation of BPL inactivated RVFV on Vero cells

An aliquot of BPL-inactivated MP12 was added to a confluent monolayer of Vero cells and observed for presence or absence of CPE. RVFV MP12 virus was used as a positive control. MP12 virus produced CPE 72 h PI, however no CPE was observed with WIV MP12 and cell only control.

3.6 DISCUSSION

The goal of this study was to construct DNA and replicon-based vaccines and evaluate their ability to express RVFV Gn. The use of trunctated Gn as a vaccine antigen has been described previously [46, 48, 83, 92], therefore it became antigen of choice for the present study. The ability of molecular adjuvant C3d to present the tagged antigen by attaching to CR2 receptors on follicular dendritic cells (FDCs) in lymph nodes provides excellent oppurtunities for interaction of T and B lymphocytes with the antigen. This also helps in induction of high-titered antibody responses which are specific to antigen in question [109]. Within germinal centers, the role played by CR2 (CD21) present on B cells to generate antibody responses against protein antigens has been well characterized [110].

The use of alphavirus replicon as vaccine delivery vector against RVFV was first described by Gorchakov *et al*. [92]. They compared VEEV and Sindbis based vaccine vectors

expressing RVFV glycoproteins and tested their ability to elicit protective immunity against RVFV challenge in mice. The overall results from their study suggested that VEEV-based vector performance is superior to Sindbis based strategy. Later Heise and coworkers demonstrated efficient expression of RVFV glycoproteins using Sindbis replicons which lead to protective immunity against lethal RVFV challenge in mice [93]. The differences in results obtained from the two studies could be due to a different vector strain and or vaccination regimen. In this study, VEEV based replicons were used and the efficient production of RVFV Gn from packaged replicons corroborates the findings of Gorchakov *et al*. [92]. The most-promising finding from this study is the ability of replicon vectors to be used as potential vaccine platforms for veterinary/human vaccines. By introducing unique immunological tags in the packaged replicons, vaccination can be easily distinguished from natural infections.

RVFV vaccine studies in the past have focused on development of anti-RVFV immunity and or survival/protection data post-challenge. I believe that the use of appropriate benchmarks in parallel under the same experimental setting is essential to compare the true efficacy of the test vaccines in the. In order to achieve that I used live-attenuated virus (MP12) and developed an inactivated vaccine (WIV MP12) to serve as stringent benchmarks for comparison. Both live and inactivated vaccines are still under restricted use and account for a fair comparison of the vaccine candidates that I tested in the present study. Using both test vaccines and benchmarks in the same experimental setting not only help to ascertain the true potential of the test vaccines but also helps improve our understanding of anti-RVFV immunity.

4.0 COMPARISON OF CANDIDATE AND BENCHMARK VACCINES IN ELICITING ANTI-RVFV IMMUNE RESPONSES IN MICE

This chapter was modified with permission from:
Nitin Bhardwaj, Mark T. Heise, Ted M. Ross
Vaccination with DNA plasmids expressing Gn coupled to C3d or Alphavirus replicons expressing Gn protects mice against Rift Valley fever virus
PLoS Negl Trop Dis.2010 June 22. 4(6): e725

4.1 PREFACE

The study described in this aim was completed by Nitin Bhardwaj. The authors would like to thank Martha Collier, Nancy Davis, and Robert Johnston at UNC for assistance in production of VEE replicon particles.

4.2 ABSTRACT

I previous described the construction and expression of the candidate vaccines (section 3.0). To evaluate the potential of the DNA and replicon vectors as efficient vaccines, I conducted animal experiments using these vaccines which are described in this study. Both DNA and replicon vaccines were able to elicit anti-Gn antibody responses in immunized mice with a predominant bias towards Th2 immunity. The ligations of C3d to DNA vaccine resulted in an obvious boost in the elicited antibody levels. Most importantly DNA and replicon-based vaccination lead to development of high-titered neutralizing antibody responses which were comparable to those in the MP12 vaccine group. The replicon immunization emerged as an important strategy to elicit cell-mediated immune (CMI) responses. Although DNA alone did not contribute to the CMI, however priming with DNA and subsequent boosting did show improvement in IFN-γ mediated CMI. Epitope mapping of RVFV Gn ectodomain has also revealed presence of an immunodominant epitope (SYAHHRTLL). Heterologous DNA prime/replicon boost emerged as a balanced vaccination approach stimulating both humoral and CMI responses.

4.3 INTRODUCTION

Alternative vaccination strategies for RVFV prevention and control have shown some promise for further development in light of the limitations of live and inactivated vaccines. An ideal vaccine elicits both humoral and cell mediated immune responses, thus preventing establishment of infection or removing already established infection. Studies with DNA plasmid expressing the RVFV M gene has shown the potential of this strategy as a good vaccination approach [89, 94, 111]. The major advantages of DNA vaccines over other vaccine strategies have been well described in the past studies. However, their ability to induce protective immune responses in large animals often relies on other alternatives which help in enhancing and modulating the immune responses induced by the DNA plasmid vaccination approach. To address this, in this study DNA plasmid expressing RVFV Gn was tested for its ability to induce anti-RVFV humoral immunity and CMI when ligated with the molecular adjuvant C3d. I also used VEE replicon expressing the same glycoprotein either individually or in a heterologous DNA prime-replicon boost approach. Studies using alphavirus replicons as vaccine vectors for RVFV have been described in the past [92, 93]. However, no previous study has shown a direct comparison of DNA and alphavirus-based vectors, or an assessment of whether combining these vaccine strategies results in enhanced immunity or qualitative differences in the RVFV specific immune response. Furthermore, to date, RVFV vaccination studies have focused on antibody responses, and the ability of different vaccination strategies to elicit RVFV specific T cell responses has not been evaluated. Therefore, in this aim studies were conducted to directly compare DNA vaccines expressing either Gn or Gn-C3d to alphavirus vectors expressing Gn, evaluate whether combining these vaccines in a DNA prime/replicon boost strategy provided any advantage over

either vaccine on its own, and to assess the nature of the antibody and T cell response elicited by each of these vaccine strategies.

4.4 MATERIALS AND METHODS

4.4.1 Animals and immunizations

Six-to-eight week old female BALB/c mice (Harlan Sprague- Dawley, Indianapolis, IN, USA) were used for inoculations. Mice, housed with free access to food and water, were cared for under U.S. Department of Agriculture guidelines for laboratory animals. Mice were anesthetized with 0.03 to 0.04ml of a mixture of 5ml of ketamine HCl (100 mg/ml) and 1ml of xylazine (20 mg/ml). Gene gun immunizations were performed on shaved abdominal skin by using the hand-held Bio-Rad gene delivery system as described previously [100, 112-114]. For DNA immunizations, mice were immunized three times at three week intervals with 2mg of DNA per 0.5mg of 1-mm gold beads (Bio-Rad, Hercules, CA, USA) at a helium pressure setting of 400 lb/in^2. For replicon immunizations mice were given one dose at week 6 or three doses at weeks 0, 3, and 6 of $1x10^5$ infectious unit (IU) of replicons by foot pad route. Blood samples were collected at weeks 0, 2, 5, and 8 post-vaccination. A schematic of the vaccine regimen is listed in Table 1. Use of animals in this study was reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC).

Table 1. Vaccine groups and vaccination regimen

Immunogens	Immunization schedule			Route
	week 0	week 3	week 6	
Gn	Gn	Gn	Gn	GG
Gn-C3d	Gn-C3d	Gn-C3d	Gn-C3d	GG
Rep-Gn	Rep-Gn	Rep-Gn	Rep-Gn	FP
Gn-C3d/Rep-Gn	Gn-C3d	Gn-C3d	Rep-Gn	GG/FP
MP 12	MP 12	-	-	IP
WIV MP 12	WIV MP 12	WIV MP 12	WIV MP 12	IP
DNA control	DNA control	DNA control	DNA control	GG
Rep control	Rep control	Rep control	Rep control	FP
Naives	-	-	-	-

4.4.2 Immunological assays

Endpoint ELISA was performed on collected serum samples to assess the anti-Gn immunoglobulin G (IgG) response. Briefly, plates were coated with 100µl of inactivated RVFV MP12 overnight at 4^0C, blocked with 5% non-fat dry milk in PBS-T (1h) at 25^0C, and then extensively washed with PBS-T. Serial dilutions of mouse antisera were allowed to bind (1h) and the plates thoroughly washed with PBS-T. Subsequently, the primary antisera were detected by anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA, USA). The reaction was detected using tetramethybenzidine (TMB) substrate (Sigma, Saint Louis, MO,

USA) (1 h) at 25^0C. IgG isotypes were also assessed by ELISA as previously described [97, 100]. The secondary antibodies specific for IgG1, IgG2a, IgG2b, IgG3 (Southern Biotechnology, Birmingham, AL, USA) were used at varying concentrations determined by optimization.

4.4.3 Neutralizing antibody assays

Antibody-mediated neutralization of RVFV ZH501 was measured using plaque reduction and neutralization test (PRNT) [77]. Briefly, 100 plaque-forming units (PFU)/0.1 ml of RVFV ZH501 was mixed with serial two-fold dilutions of heat inactivated (60^0C for 30 min) serum samples in 96-well tissue culture plates. Virus-serum mixtures were incubated at 4^0C overnight and placed into duplicate 23-mm wells (0.1ml/well) containing confluent monolayers of Vero cells ($2x10^5$). Cells were incubated for 1h at 37^0C and 5% CO2 and overlaid with nutrient medium containing 0.8% agar, 5% fetal bovine serum, 200U penicillin/ml, and 200mg streptomycin/ml. The plates were incubated at 37^0C and 5% CO_2. After 4 days of incubation, cells were fixed with 10% formalin and stained with 1% crystal violet for visualization of plaques. The neutralizing antibody titer of a serum was considered positive at the highest initial serum dilution that inhibited >50% of the plaques as compared to the virus control titration. The whole experiment was conducted under strict BSL-3 conditions.

4.4.4 ELISPOT assays

The number of anti-Gn specific murine IFN-γ (mIFN-γ) secreting splenocytes was determined by enzyme-linked immunospot (ELISpot) assay (R & D Systems, Minneapolis, MN, USA). Briefly, pre-coated anti-mIFN-γ plates were incubated (25^0C for 1h) with RPMI (200µL) supplemented

with 10% fetal calf serum and then incubated with splenocytes ($5x10^5$/well) isolated from vaccinated mice. Cells were stimulated (48h) with peptides (15mers overlapping by 11 amino acids) representing the ectodomain of Gn glycoprotein. IL-2 was added to all wells (10 units/ml). Control wells were stimulated with PMA (+) (50 ng)/ionomycin (500 ng) or were mock stimulated (2). Plates were washed with PBS-T (3x) and were incubated (37^0C for 48h; 5% CO_2) with biotinylated anti-mIFN-γ and incubated (4^0C for 16h). The plates were washed and incubated (25^0C for 2h) with strepavidin conjugated to alkaline phosphatase. Following extensive washing, cytokine/antibody complexes were incubated (25^0C for 1h) with stable BCIP/NBT chromagen. The plates were rinsed with dH_2O and air-dried (25^0C for 2h). Spots were counted by an ImmunoSpot ELISpot reader (Cellular Technology Ltd., Cleveland, OH, USA).

4.4.5 Statistics

Differences in ELISA titers and virus neutralization titers between various vaccine groups were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. Statistical results are represented in the figure by * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$). Statistical analyses were done using GraphPad Prism software.

4.5 RESULTS

4.5.1 Anti-Gn total IgG responses

After 3 vaccinations, mice vaccinated with DNA expressing Gn elicited anti-Gn antibodies (1:180), however, the fusion of C3d to Gn enhanced the anti-Gn antibodies (1:1280), while mice vaccinated with replicons expressing Gn (Rep-Gn) had an average anti-Gn titer of 1:2560 (Fig. 10). There were no detectable antibodies following a single DNA vaccination (data not shown). In order to determine if Gn-C3d-DNA could prime and enhance antibody titers following a Rep-Gn boost, mice were vaccinated twice with Gn-C3d-DNA and then administered a single inoculation of replicon expressing Gn. These vaccinated mice had higher anti-Gn antibody titers (1:4160) compared to mice vaccinated with a single vaccination of alphavirus-replicon (1:280). Mice vaccinated the Gn-DNA only, did not elicit any detectable anti-Gn antibodies (Fig. 10). These antibody responses were comparable to mice immunized with live attenuated RVFV (MP12), but 1–2 $\log_{10}$ lower than mice vaccinated with three doses of whole-inactivated RVFV (WIV MP12).

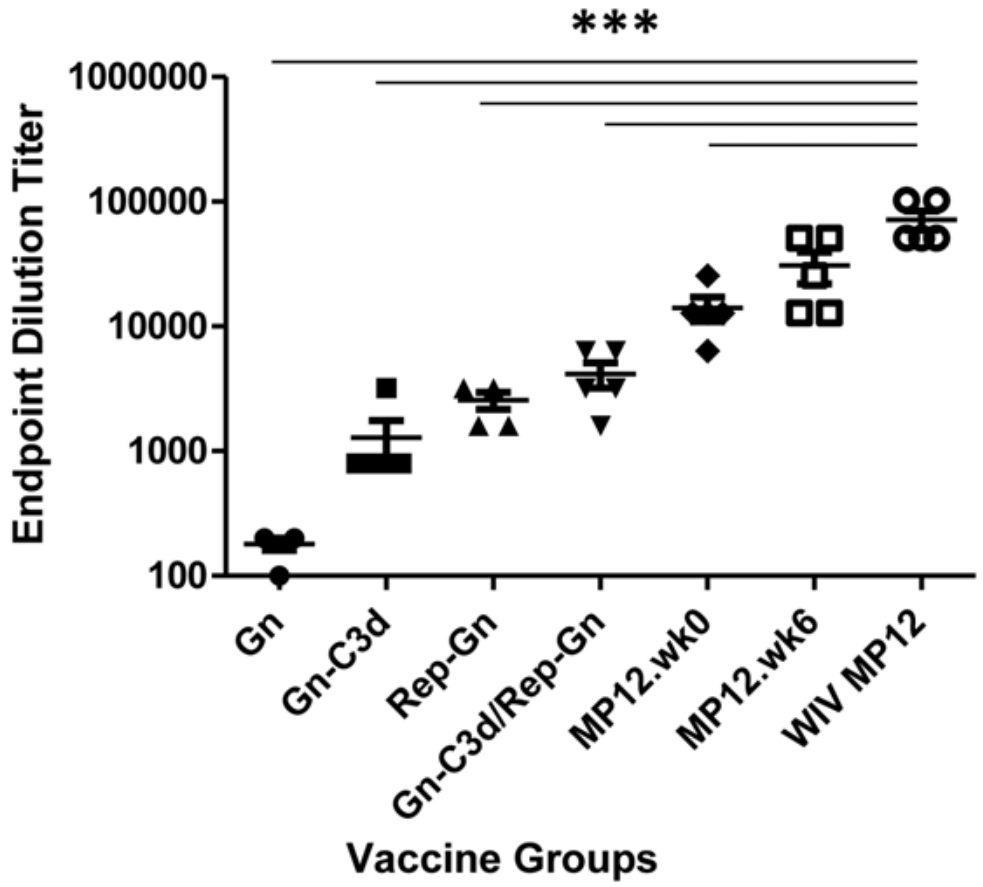


Figure 10. Indirect ELISA meauring RVFV specific IgG responses in mice immunized with indicated vaccines

All groups received primary and two booster immunizations (except MP12) spaced 3 weeks apart. Serum samples were collected two weeks after the last immunization (week 8 of the study), except for the group of mice vaccinated at week 0 with MP12. End point dilution titers were conducted by diluting the sera until the OD values reached the background levels. Each dot represents an individual mouse. Error bars denote the standard error within the samples with a measurable titer. Representative data from 1 of 2 experiments shown. A 1-way ANOVA with Tukey's multiple comparison test was used to determine the significance of the data between groups, which is denoted by asterisks; *** $p<0.001$ for both MP12 vaccine regimens compared to the other vaccine regimens.

MP12 infection elicited a mixed Th1 and Th2 response, whereas mice vaccinated with three doses of WIV MP12 had a Th2-restricted immune response (Fig. 11E and F). Mice vaccinated with Gn-C3d-DNA vaccines elicited predominately IgG1, suggesting a Th2 immune response

(Fig. 11B and D). In contrast, the replicons expressing Gn administered to mice three times elicited not only IgG1, but also IgG2a and IgG2b isotypes suggesting a mixed Th1/Th2 response similar to that elicited by the live attenuated MP12 vaccine (Fig. 11C). Interestingly, mice primed with Gn-C3d-DNA maintained an IgG1 isotype bias following a boost with Gn expressing replicons (Fig. 11D). These titers were specific to the Gn antigen, since controls (DNA plasmid with no insert and replicons expressing the influenza virus hemagglutinin) did not elicit anti-Gn antibodies (data not shown).

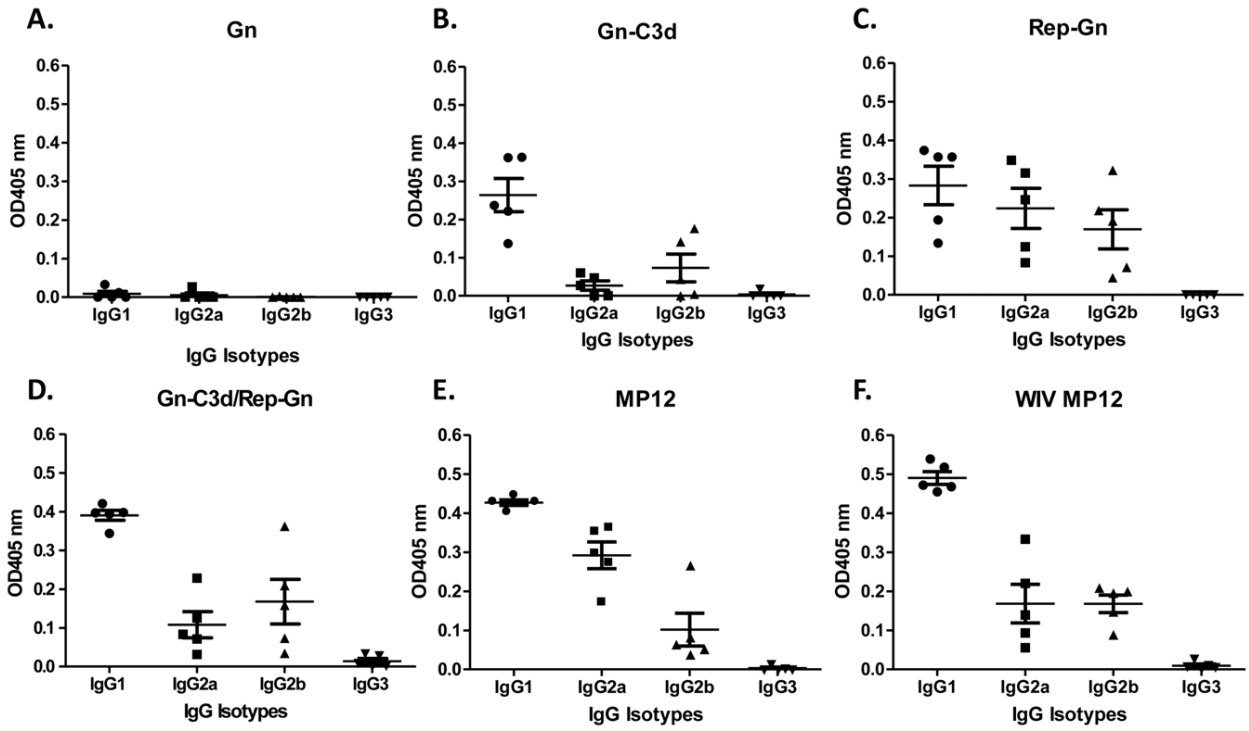


Figure 11. Isotype ELISA measuring RVFV-specific IgG isotype responses in sera of mice with indicated vaccine regimens 8 weeks post-vaccination

1:100 dilutions of serum samples from each vaccine group (A) to (F) were used and the results are represented in OD values. Each dot represents an individual mouse. Error bars denote the standard error within the samples with a measurable titer. Representative data from 1 of 2 experiments are shown.

4.5.2 Elicitation of antibodies that neutralize virus infection

At week 8 of the study, sera from mice vaccinated with Gn-C3d-DNA or Rep-Gn neutralized (PRNT50) RVFV ZH501, while priming mice with Gn-C3d-DNA and then boosting with Rep-Gn did not significantly enhance the neutralizing titers compared to Gn-C3d-DNA or Rep-Gn alone (Fig. 12). Mice vaccinated with the live attenuated MP12 vaccine strain had the highest neutralizing titers (average; 1:656–1:736) regardless if the mice were vaccinated at week 0 or week 6 of the study, and they were significantly higher than sera from mice vaccinated with Gn, Rep-Gn and WIV MP12 ($p<0.05$). In contrast, serum samples collected from Gn (1:22) vaccinated or WIV MP12 (1:8) had low virus neutralizing titers in spite of the fact that WIV MP12 elicited very high RVFV specific antibody levels as measured by ELISA (Fig 12).

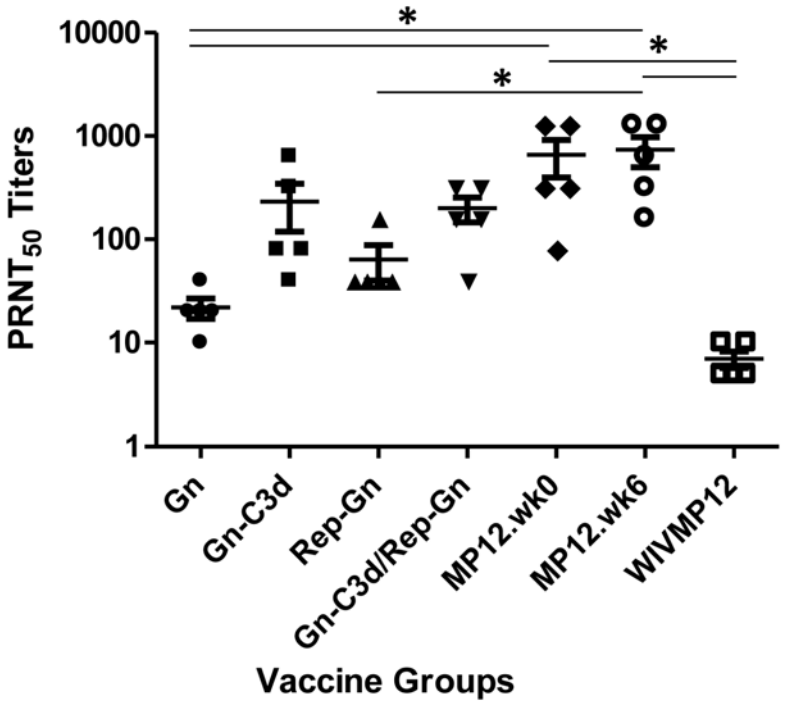


Figure 12. Neutralizing antibody responses of mice vaccinated with indicated vaccine regimens

$PRNT_{50}$ titers of week 8 sera from mice immunized with the indicated vaccines. Each dot represents an individual mouse. Error bars denote the standard error within the samples with a measurable titer. A 1-way

ANOVA with Tukey's multiple comparison test was used to determine the significance of the data between groups, which is denoted by asterisks; * $P<0.05$.

4.5.3 Elicitation of cellular immune responses

Mice vaccinated with DNA and replicon vaccines were challenged with MP12 virus two weeks after the last immunization and splenocytes were collected 6 days post-infection. Cells collected from mice vaccinated with Gn vaccines were stimulated in vitro with 8 overlapping pools of peptide (15mers overlapping by 11) specific for Gn. Mice vaccinated with Rep- Gn or Gn-C3d/Rep-Gn had responses to pools B and C (Table 2), representing a stretch of 111 amino acids starting at amino acid 53 in the Gn sequence. Only mice vaccinated with Gn-C3d/Rep-Gn had splenocyte responses to pool A. No responses were recorded from any mice to pools D-G. A few spots (10–12 spots) were detected following stimulation of splenocytes with an irrelevant peptide or left unstimulated following in vitro re-stimulation. Mice vaccinated with DNA vaccines did not elicit cellular responses (Table 2).

Table 2. Anti-Gn cell mediated immune responses of mice vaccinated with indicated vaccine regimens

Pools	A	B	C	D	E	F	G	H
Gn	0	0	0	0	0	0	0	0
Gn-C3d	0	0	0	0	0	0	0	0
Rep-Gn	0	541	415	0	0	0	0	0
Gn-C3d/Rep-Gn	77	301	253	0	0	0	0	0
Mock/Rep-Gn	0	171	124	0	0	0	0	0
Naive	0	0	0	0	0	0	0	0

A group of mice vaccinated with different vaccine regimens were challenged with MP12 virus at week 8 of the study and 6 days post-infection splenocytes were isolated and stimulated with overlapping RVFV Gn specific peptides (pools A to H). Each pool contained 13 overlapping peptides except pool H which contained 14. Responses are represented as average number of spots (SFU per million cells) from different vaccine groups.

The peptides in these pools B and C were further analyzed to determine the peptides responsible for eliciting these responses in replicon-vaccinated mice. Using a matrix format, 4 out of 10 pools (5 peptides/pool) were identified (peptide pools II, IV, VI, VII) (Table 3). From this analysis, four potential peptides (peptide # 18, 19, 36, 38) were identified as responsible for the vaccine elicited cellular responses. Two out of four peptides share a common amino acid sequence (SYAHHRTLL) predicted to be MHC class I restricted (www.immuneepitope.org) (Table 4). A unique peptide representing this region of Gn elicited similar mIFN-γ cellular immune response as compared to the four individual peptides as indicated in Figure 13.

Table 3. Peptide matrix with pool B and C peptides

Pools	I	II	III	IV	V
VI	14	18	22	26	30
VII	34	38	15	19	23
VIII	27	31	35	39	16
IX	20	24	28	32	36
X	17	21	25	29	33
	37				

Table 4. Immune epitope prediction for RVFV Gn sequence

Input Sequences

#	Name	Sequence
1	sequence 1	MAGIAMTVLPALAVFALAPVVFAEDFHLRNRPGKGHNYIDGMTQEDATCK PVTYAGACSSFDVLLEKGKFPLFQSYAHHRTLLEAVHDTIIAKADPPSCD LQSAHGNPCMKEKLVMKTHCPNDYQSAHYLNNDGKMASVKCPPKYGLTED CNFCRQMTGASLKKGSYPLQDLFCQSSEDDGSKLKTKMKGVCEVGVQAHK KCDGQLSTAHEVVPFAVFKNSKKVYLDKLDLKTEENLLPDSFVCFEHKGQ YKGTMDSGQTKRELKSFDISQCPKIGGHGSKKCTGDAAFCSAYECTAQYA NAYCSHANGSGIVQIQVSGVWKKPLCVGYERVVVKRELSAKPIQRVEPCT TCITKCEPHGLVVRSTGFKISSAVACASGVCVTGSQSPSTEITLKYPGIS QSSGGDIGVHMAHDDQSVSSKIVAHCP

Predictions (smm method) - Low IC50 values = good binders

Click on column headers to sort

Allele	Position	PepLength	Sequence	IC50 [nM]
H-2 Kd	1:75-83	9	SYAHHRTLL	19.1
H-2 Kd	1:37-45	9	NYIDGMTQE	131.6
H-2 Kd	1:298-306	9	QYANAYCSH	241.7
H-2 Kd	1:128-136	9	HYLNNDGKM	283.9
H-2 Kd	1:53-61	9	TYAGACSSF	289.7

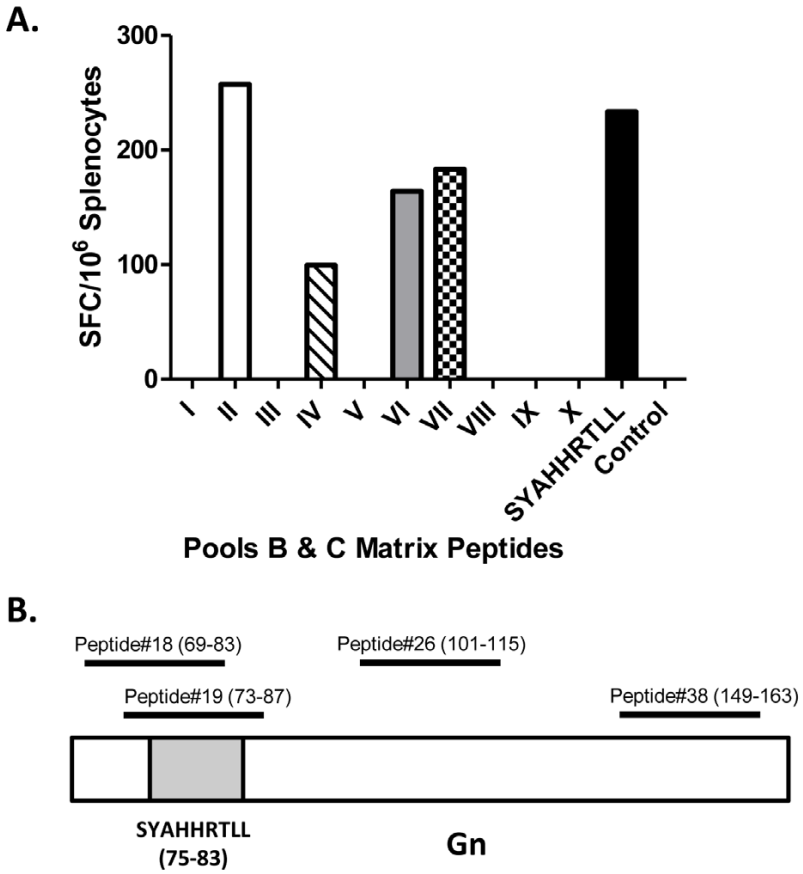


Figure 13. Identification of the peptide sequence eliciting cellular immune response in mice vaccinated with replicons

(A). Mice immunized with Rep-Gn vaccine were challenged with MP12 virus at week 8 of the study and 6 days post-infection splenocytes were isolated and stimulated with overlapping RVFV Gn specific peptides representing pools B and C and peptide SYAHHRTLL. Responses are represented as average number of spots (SFU per million cells). The highlighted peptides 18 and 19 share a common amino acid sequence SYAHHRTLL. Representative data from 1 of 2 experiments are shown. (B). Schematic alignment of identified peptides with Gn. Numbers in the parentheses represent amino acid positions of the individual peptide. The gray box indicates the region of Gn covered by the predicted CD8+ T cells epitope SYAHHRTLL.

4.6 DISCUSSION

One of the goals of an effective RVFV vaccine is to elicit protective neutralizing antibodies. In recent years, several RVFV vaccines strategies have been employed to elicit a potent neutralizing antibody responses [48, 49, 77, 89, 90, 92, 94], however, these vaccines did not always elicit high titer immune responses that protected against lethal challenge. Early RVFV vaccine studies focused on live-attenuated and inactivated virus strategies that induce long-lasting protection [68, 74, 77]. However, the induction of adverse reactions may likely limit the wide spread use of live-attenuated vaccines [79-81].

To overcome the limitations discussed above, this study describes development of two promising vaccine candidates based on DNA plasmid and alphavirus replicon vectors that express the virus envelope glycoprotein, Gn. Each vaccine was tested alone or in a DNA prime/replicon boost strategy formulation to elicit humoral and cell mediated immune responses. In order to enhance the antibody responses elicited by DNA vaccines, our laboratory has pioneered the use of the complement protein C3d as a molecular adjuvant [97, 100, 101]. Vaccination of mice with DNA or replicons administered individually or in a DNA prime/ replicon boost strategy elicited similar anti-Gn antibody titers (Fig. 10); however, different subclasses of IgG were elicited by each vaccine. The isotype of the polyclonal antibody in part determines the effector functions of the anti-Gn antibodies and identifies the T helper cell bias (required for antibody class switching). The predominant IgG isotype elicited by DNA vaccination was IgG1 indicating a Th2 bias. However, IgG1, IgG2a, and IgG2b were detected in both replicon vaccinated, as well as live MP12 immunized mice, indicating that both the replicon and the live attenuated vaccine elicit a mixed T helper response (Fig. 11). Even though both MP12 infection and the WIV vaccination elicited the highest anti-Gn titers, only the live MP12

infection elicited strong neutralizing antibody responses. Most importantly Gn-C3d- DNA and Gn-C3d-DNA/Rep-Gn vaccinated mice had statistically similar neutralizing titers as MP12 immunized mice (Fig. 12).

Mice vaccinated with replicons alone or in a DNA prime/replicon boost strategy, but not by DNA alone, had robust cellular responses directed at Gn. Cellular responses are critical for clearing virally infected cells in many systems. Although the elicitation of robust neutralizing antibodies are considered ideal for the development of an effective RVFV vaccine, induction of cellular responses by immunization may clear virally infected cells, reduce morbidity, and hasten recovery from infection. The replicon-based vaccines elicited cellular immune responses against the Gn protein, but Gn expressed from DNA plasmids did not, even though priming mice with DNA did not dampen the induction of cellular responses by the Gn-C3d/Rep-Gn in the DNA prime/replicon boost regimen (Table 2). Although nonspecific induction of T-cell responses against RVFV glycoproteins and nucleocapsid proteins have been previously reported [111], this is the first report to identify an MHC-I restricted immunodominant epitope (SYAHHRTLL) on the surface of Gn as predicted by multiple algorithm methods to detect the peptide sequence with lowest IC50 and hence better binding to MHC-I (Table 4) [www. immuneepitope.org].

5.0 EVALUATION OF THE ABILITY OF CANDIDATE AND BENCHMARK VACCINES TO PROTECT MICE FROM VIRULENT RVFV CHALLENGE BY INTRAPERITONEAL AND AEROSOL ROUTES

This chapter was modified with permission from:
Nitin Bhardwaj, Mark T. Heise, Ted M. Ross
Vaccination with DNA plasmids expressing Gn coupled to C3d or Alphavirus replicons expressing Gn protects mice against Rift Valley fever virus
PLoS Negl Trop Dis.2010 June 22. 4(6): e725

5.1 PREFACE

The study described in this aim was completed by Nitin Bhardwaj. The authors would like to Dr. Doug Reed from University of Pittsburgh for performing aerosol experiments.

5.2 ABSTRACT

RVFV has the ability to infect humans and animials by percutaneous and aerosol routes. In this study, I tested the ability of our vaccine candidates to protect against both intraperitoneal and aerosolized RVFV challenge in vaccinated mice. Both candidate vaccines were able to protect vaccinated mice against intraperitoneal challenge without the development of clinical illness. However, the groups that received DNA and replicon vaccines were partially protected following aerosol challenge with the same viral strain. The level of protection was similar to the group that received live-attenuated virus (MP12). Interestingly, DNA vaccine expressing Gn-C3d not only conferred complete protection in mice, but also prevented development of clinical signs post-aerosol challenge. This is the first report of any vaccine strategy that confers complete protection against aerosol RVFV challenge and warrants further investigation and development.

5.3 INTRODUCTION

Human infection with RVFV typically manifests itself as an acute self-limiting febrile illness with the exception of hepatitis, severe encephalitis, hemorrhagic fever and ocular sequelae in

complicated cases [2]. The primary route of RVFV transmission is by mosquitoes, however the virus can be transmitted via aerosol inhalation [32-36]. Humans can be infected by aerosols generated during the slaughtering procedure, by handling aborted fetuses, performing necropsies, and conducting laboratory procedures. The potential for aerosolization of RVFV and the high morbidity and mortality associated with infection, even at low doses, has led to RVFV being listed as a potential bioterrorism weapon. In addition, the U.S. National Institutes of Health has included RVFV in their list of Category A priority agents. (http://www.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/research/Pages/CatA.aspx). However, in light of this, only two studies have been performed using existing inactivated RVFV vaccines in protecting experimental animals from aerosolized RVFV infection [115, 116]. Incomplete protection after aerosol infection of mice vaccinated with inactivated RVFV vaccine was the outcome of both experimental studies.

Vaccine evaluation studies in the past (with the exception of the above two studies) have mainly focused on survival of vaccinated mice post-intraperitoneal virus challenge [48, 49, 72, 89, 90, 92, 94, 117]. An ideal vaccine should be able to protect from all potential routes of infection. In addition, an ideal vaccine will not only protect from viral infection, but also prevent development of clinical symptoms. In this study, we evaluated our candidate DNA and replicon based vaccines for the ability to confer protection, as well as the ability to prevent clinical symptoms using both intraperitoneal and aerosol routes for virulent virus challenge.

5.4 MATERIALS AND METHODS

5.4.1 Determination of RVFV ZH501 dose for intraperitoneal challenge route

Groups of naïve BALB/c mice were inoculated intraperitoneally (i.p.) with different concentrations (10^1 PFU to 10^5 PFU) of RVFV ZH501 virus. Post-infection, mice were housed in sealed negative-ventilation bio-containment units (Allentown Inc., Allentown, NJ, USA). All manipulations with infected mice and/or samples involving RVFV ZH501 were performed under strict BSL-3 enhanced conditions. The animals were examined twice daily for visual signs of morbidity or mortality.

5.4.2 RVFV ZH501 challenge of vaccinated mice by intraperitoneal route

At week 8 of the study, a challenge dose containing $1x10^3$ PFU of RVFV ZH501 was administered i.p to vaccinated or control mice. During challenge, mice were housed in sealed negative-ventilation bio-containment units (Allentown Inc., Allentown, NJ, USA). All manipulations with infected mice and/or samples involving RVFV ZH501 were performed under strict BSL-3 enhanced conditions. The animals were examined twice daily for visual signs of morbidity or mortality, using a lab-validated scoring system as previously described [37]. Mice were observed for clinical signs that ranged from lethargy, ruffled fur, and weight loss to neurological manifestations, such as hind-limb paralysis. Mice found in a moribund condition were euthanized.

5.4.3 Determination of RVFV ZH501 dose for aerosol challenge

Groups of naïve BALB/c mice were exposed to RVFV aerosols in whole-body exposure chambers housed within Class III biological safety cabinets maintained under negative pressure (-1 WC"), as previously described [118]. The animals were exposed inside a whole-body chamber which could contain up to four smaller stainless steel mesh restraint cages holding approximately 10 mice/cage or two guinea pigs/cage. The animal exposures were acute and lasted 30 min. A Collison nebulizer (BGI Inc., Waltham, MA) was used to generate the smaller (1 μm) particles. Exposure concentration, expressed in plaque-forming units (PFU)/ml, was determined by isokinetic sampling of the chamber with an all-glass impinger (AGI; Ace Glass, Vineland, NJ). DMEM medium with 3% sera w/v (Sigma, St. Louis, MO) was used to collect medium in the impinger. Post-challenge, mice were housed in sealed negative-ventilation bio-containment units (Allentown Inc., Allentown, NJ, USA). All manipulations with infected mice and/or samples involving RVFV ZH501 were performed under strict BSL-3 enhanced conditions. The animals were examined twice daily for visual signs of morbidity or mortality.

5.4.4 RVFV ZH501 challenge of vaccinated mice by aerosol route

At week 8 of the study, a challenge dose containing $1x10^6$ PFU of RVFV ZH501 was administered to vaccinated or control mice in whole-body exposure chambers as described above. Post-challenge, mice were housed in sealed negative-ventilation bio-containment units (Allentown Inc., Allentown, NJ, USA). All manipulations with infected mice and/or samples

involving RVFV ZH501 were performed under strict BSL-3 enhanced conditions. The animals were examined twice daily for visual signs of morbidity or mortality, using a lab validated scoring system as previously described [119]. Mice were observed for clinical signs that ranged from lethargy, ruffled fur, and weight loss to neurological manifestations, such as hind-limb paralysis or circling. We used a lab validated clinical scoring system as previously described [119]. Mice found in a moribund condition were euthanized.

5.4.5 Passive transfer of immune sera and RVFV challenge

Sera from vaccinated mice were diluted 1:10 in sterile PBS and 100µl of the diluted sera was injected (i.p.) into new, naïve BALB/ c mice. One hour following transfer, the mice were challenged (i.p.) with virulent RVFV ZH501 ($1x10^3$ PFU). Mice were observed daily for 8 days post-transfer for signs of morbidity and mortality.

5.4.6 IgA antibody ELISA

ELISA was performed on collected serum samples from vaccinated groups to assess the anti-Gn immunoglobulin A (IgA) response. Briefly, plates were coated with 100µl of inactivated RVFV MP12 overnight at 4^0C, blocked with 5% non-fat dry milk in PBS-T (1h) at 25^0C, and then extensively washed with PBS-T. Serial dilutions of mouse antisera were allowed to bind (1h) and the plates thoroughly washed with PBS-T. Subsequently, the primary antisera were detected by anti-mouse IgA conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA, USA). The reaction was detected using tetramethybenzidine (TMB) substrate (Sigma, Saint Louis, MO, USA) (1 h) at 25^0C.

5.5 RESULTS

5.5.1 RVFV ZH501 challenge dose for intraperitoneal infection

Groups of mice were infected with different concentrations of RVFV ZH501 by intraperitoneal route. All mice that received virus displayed loss in original body weight except mice that received the lowest concentration of challenge virus (10^1 PFU) (Fig. 14). The loss in original body weight started on day 3 post-infection which coincided with mortality in the groups that received 10^3 and 10^4 PFU of virulent ZH501 virus (Fig. 15). Two out of five mice survived in the group that received 10^1 PFU of virus however all mice succumbed to RVFV infection by day 5 in the rest of the groups (Fig. 15). From this pilot study the dose of 10^3 PFU was selected as the challenge dose for future use.

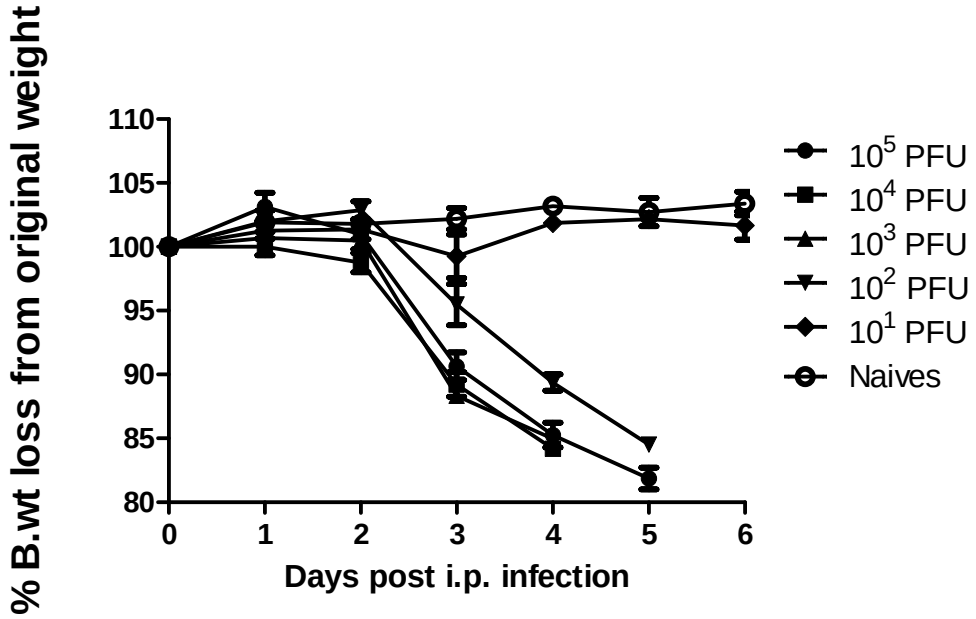


Figure 14. Weight loss curve of mice inoculated with indicated RVFV ZH501 dose by intraperitoneal route

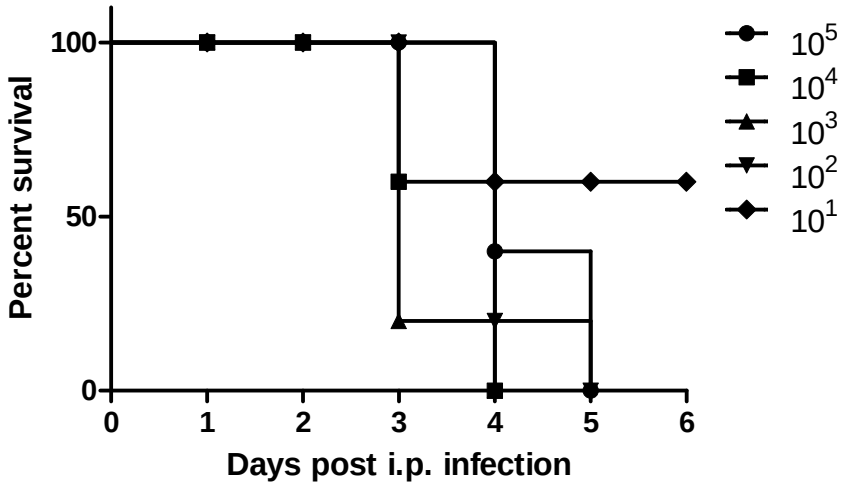


Figure 15. Survival of mice against intraperitoneal inoculation of indicated concentrations of RVFV ZH501

5.5.2 DNA and replicon vaccines protect mice against intraperitoneal virulent virus challenge

Two weeks after final vaccination, the mice were challenged with a lethal dose ($1x10^3$ PFU) of RVFV ZH501. All the mice vaccinated with an all Gn-C3d-DNA or Rep-Gn strategy or in a DNA prime/replicon boost strategy were protected from virulent virus challenge with no body weight loss or development of clinical signs (Fig. 16 and Fig. 17). Sixty percent of mice that received Gn without the molecular adjuvant C3d displayed ruffled fur and lethargy with one mouse succumbing to infection (Fig. 16D). As expected, all the mice immunized with MP12 and then challenged with RVFV ZH501 survived lethal challenge with no clinical signs of infection (Fig. 17C). However, mice vaccinated with WIV MP12 were not protected from challenge with all mice exhibiting reduced body weight (Fig. 17C), ruffled fur, lethargy, and all mice ultimately succumbing to infection (Fig. 16D).

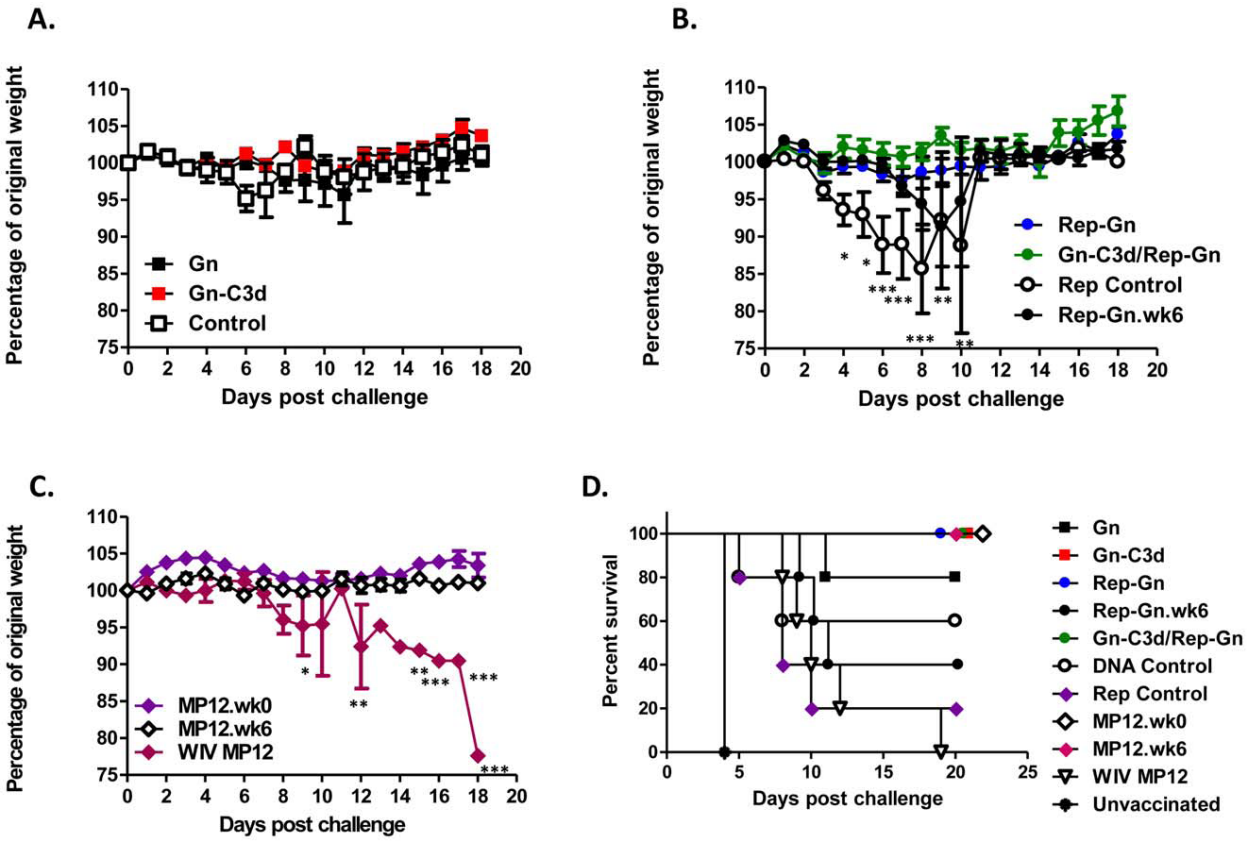
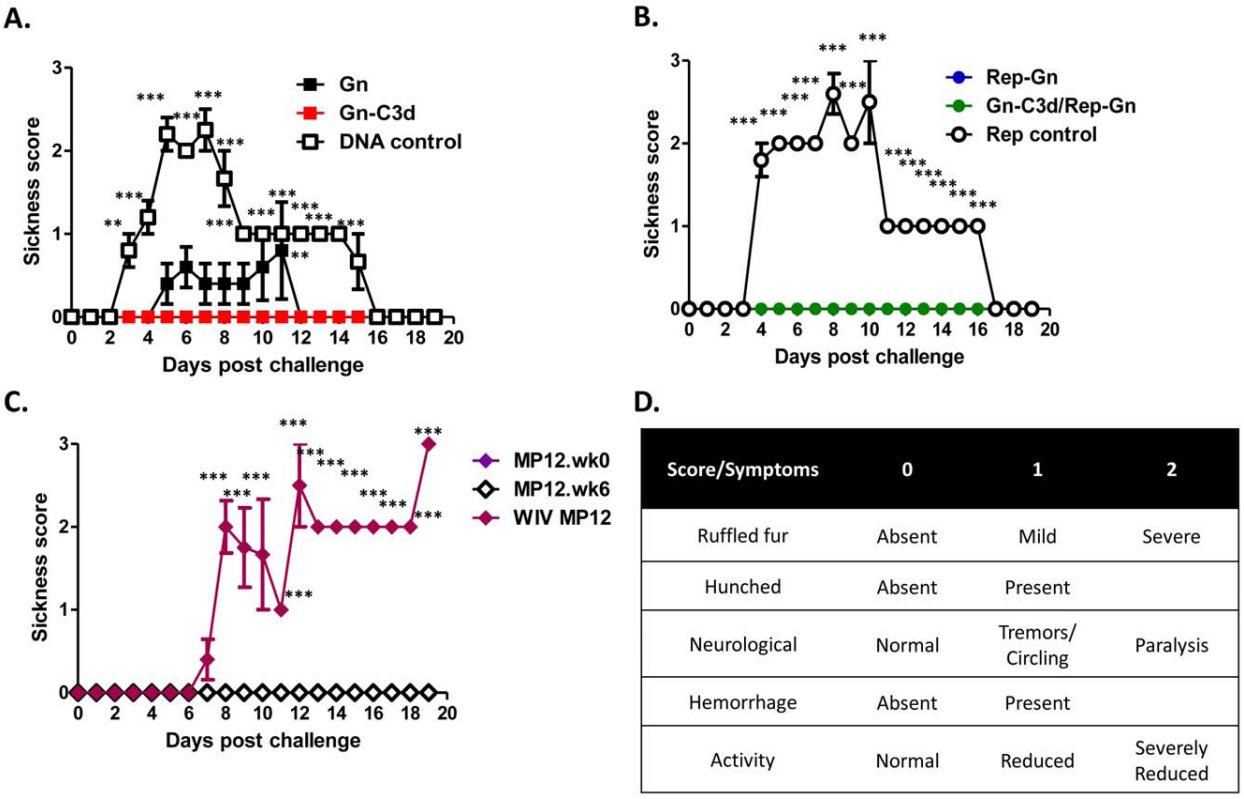


Figure 16. Weight loss curves and survival against virulent RVFV challenge of mice vaccinated with indicated vaccine regimens

Mice vaccinated with indicated vaccines or appropriate controls, DNA plasmid with no insert (DNA control) and replicon expressing influenza HA (replicon control) were challenged i.p. with 1000 PFU of RVFV ZH501 and monitored for loss in body weight (A) to (C) and mortality (D) daily post-challenge. Dead and moribund mice were included in the weight loss curves on the day of death, but not after. The daily weight of each mouse was compared to its weight on the day of challenge, and data are shown as the average percentage of initial weight for each cohort. Error bars represent the standard error for all samples available at that time point. A two-way ANOVA with Bonferroni's post tests was used to determine the significance of the body weight data between groups, which is denoted by asterisks; *P<0.05, **P<0.01, *** P<0.001. All vaccinated mice showed statistically significant protection (P<0.05, log rank test) compared to unvaccinated mice.

Unvaccinated naive mice had severe signs of infection and body weight loss which resulted in all mice succumbing to infection by day 4 post-challenge (Fig. 16D). Mice that received appropriate DNA and replicon controls displayed clinical signs of infection (Fig 17A and B) and mortality was also observed in the control groups.



Score/Symptoms	0	1	2
Ruffled fur	Absent	Mild	Severe
Hunched	Absent	Present	
Neurological	Normal	Tremors/ Circling	Paralysis
Hemorrhage	Absent	Present	
Activity	Normal	Reduced	Severely Reduced

Figure 17. Post-challenge sickness score in mice vaccinated with indicated vaccine regimens

Mice immunized with indicated vaccines or appropriate controls, DNA with no insert (DNA control) and replicon expressing influenza HA (replicon control) (A) to (C) were challenged with 1000 PFU of RVFV ZH501 and monitored for clinical signs associated with RVFV infection and mortality daily post-challenge. (D) Mice were evaluated daily and scored for individual symptoms. Ruffled fur (absent = 0, present = 1), activity (normal = 0, reduced = 1), hunched (absent = 0, present = 1). The final score was the addition of each individual score. The minimum score was 0 for healthy and 1–3 depending upon the severity. A two-

way ANOVA with Bonferroni's post tests was used to determine the significance of sickness score data between different groups, which is denoted by asterisks; **P<0.01, *** P<0.001.

5.5.3 Passive sera transfer protects mice from virus infection

Pooled antiserum from each vaccinated group was transferred (i.p.) into unimmunized mice, which were then challenged i.p. with a lethal dose of RVFV ZH501 (Table 5). Eighty percent of mice that received sera from MP12 immunized mice survived challenge. A similar outcome was observed in the Gn-C3d group where 80% of mice survived. Sera from mice primed with Gn-C3d-DNA and then boosted with Rep-Gn or immunized with Rep-Gn protected 40% (2/5) of mice, which was similar to the mice that received sera from Gn-DNA vaccinated mice. All the mice that received sera from WIV MP12 immunized mice or mice that received sera from control immunized mice (DNA control, Rep control, Naïve) succumbed to virulent RVFV ZH501 infection.

Table 5. Passive transfer of sera from vaccinated mice protects against virulent RVFV challenge

Vaccine groups	Survivors
Gn	3/5
Gn-C3d	4/5
Rep-Gn	2/5
Gn-C3d/Rep-Gn	2/5
MP12.wk0	4/5
MP12.wk6	5/5
WIV MP12	0/5
DNA control	0/5
Rep control	0/5
Naïve	0/5
Mock challenged	5/5

Five to six weeks old BALB/c mice were pre-treated with 100 µl of 1:10 diluted serum from the indicated vaccinated mice or naïve animals by intraperitoneal injection. One hour post-sera inoculation mice were infected with 1000 PFU of RVFV by intraperitoneal route and monitored for survival.

5.5.4 RVFV ZH501 challenge dose for aerosol infection

A starting dose of 10^6 PFU resulted in development of RVFV aerosols with a receiving end concentration of 1000 PFU thus suggesting a drop of 3 $\log_{10}$ in virus concentration during the aerosolization process whereas no plaques were detected in aerosolized virus sample with 10^5 PFU starting dose (data not shown). Groups of female BALB/c mice were infected with RVFV ZH501 strain by aerosol route using two different starting virus concentrations (10^5 and 10^6 PFU)

as described in the materials and methods section. Mice that received 1000 PFU as the aerosolized virus concentration developed clinical signs of infection and first mortality was observed on day 6 post-infection (Fig. 18). All mice in the 10^6 PFU group were dead by day 10 post-infection on the other hand, no mice from the 10^5 PFU group got sick or died of aerosolized RVFV infection (Fig. 18). A 10^6 PFU starting dose was employed in subsequent experiments as the challenge dose for aerosol RVFV infection.

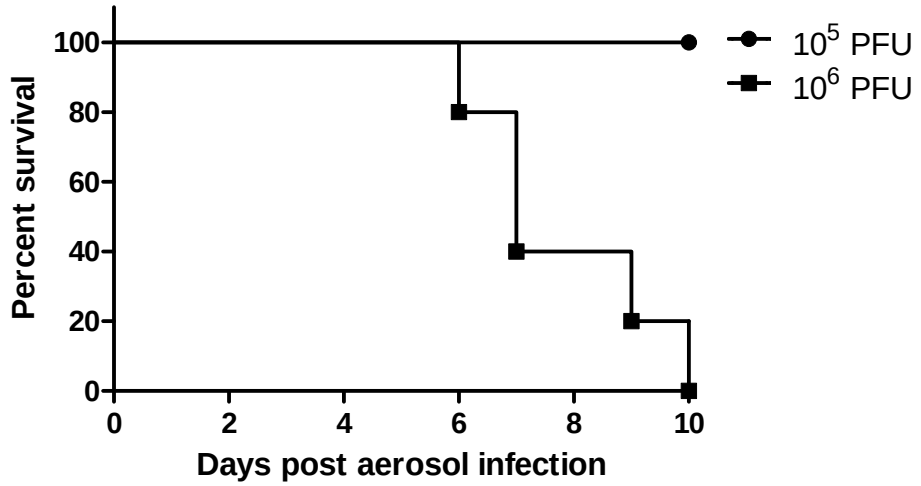


Figure 18. Survival of mice against aerosol inoculation of indicated concentrations of RVFV ZH501

5.5.5 Protective efficacy of DNA and replicon vaccines against RVFV aerosol challenge

The mice were challenged two weeks after final vaccination with $1x10^6$ PFU of RVFV ZH501. All the mice vaccinated with Gn-C3d-DNA were protected from virulent virus challenge with no body weight loss or development of clinical signs (Fig. 19A and C and Fig 20A). All mice that received Gn without the molecular adjuvant C3d displayed clinical signs of infection and 3 mice succumbing to infection (Fig. 20A and Fig 19C). Surprisingly only 2 out of 5 mice immunized

with MP12 and then challenged with RVFV ZH501 survived lethal challenge and all showed clinical signs of infection (Fig 20B and Fig. 19C). A similar result was observed with mice immunized with Rep-Gn alone or in prime boost fashion (Gn-C3d/Rep-Gn) where 3 out of 5 mice succumbed to infection (Fig. 19C). Unvaccinated naive mice had severe signs of infection and body weight loss which resulted in all mice succumbing to infection by day 7 post-challenge (Fig. 19A and C). Mice that received appropriate DNA and replicon controls displayed loss in body weight, clinical signs of infection, (Fig. 19 and Fig. 20) and mortality was also observed in the control groups (Fig. 19C).

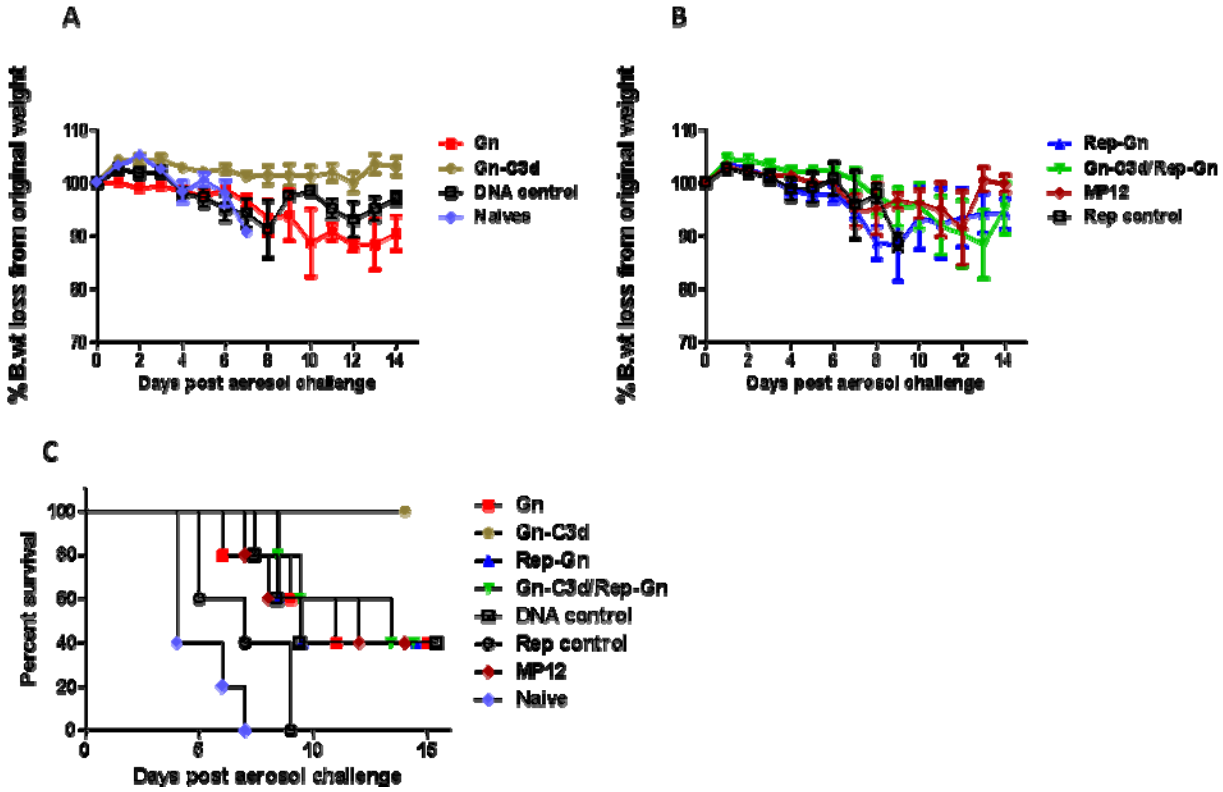


Figure 19. Weight loss curves and survival against virulent RVFV challenge of mice vaccinated with indicated vaccine regimens

Mice vaccinated with the indicated vaccines or appropriate controls, DNA with no insert (DNA control) and replicon expressing influenza HA (replicon control) were challenged with 1000 PFU of RVFV ZH501 by aerosol route and monitored for loss in body weight (A) and (B) and mortality (C) daily post-challenge.

Dead and moribund mice were included in the weight loss curves on the day of death, but not after. The daily weight of each mouse was compared to its weight on the day of challenge, and data are shown as the average percentage of initial weight for each cohort.

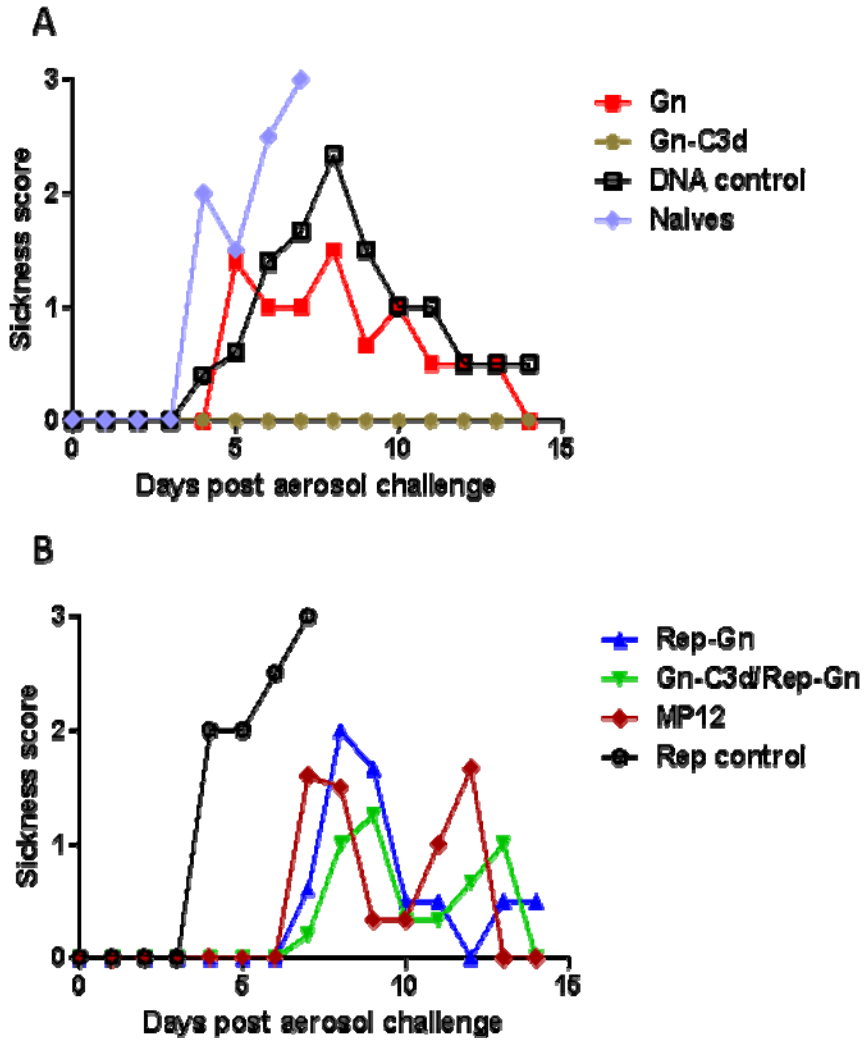


Figure 20. Post challenge sickness score in mice vaccinated with the indicated vaccines

Mice immunized with the indicated vaccines or appropriate controls were challenged with 1000 PFU of RVFV ZH501 by aerosol route and monitored for clinical signs associated with RVFV infection and mortality daily post-challenge. (D) Mice were evaluated daily and scored for individual symptoms. Ruffled fur (absent = 0, present = 1), activity (normal = 0, reduced = 1), hunched (absent = 0, present = 1), nervous symptoms/paralysis (absent = 0, present = 1). The final score was the addition of each individual score. The minimum score was 0 for healthy and 1–3 depending upon the severity.

5.5.6 DNA vaccination elicits serum IgA response

After 3 vaccinations, mice vaccinated with DNA plasmid expressing Gn by itself or in conjunction with C3d (Gn-C3d) (Fig 21). These titers were specific to the Gn antigen, since controls (DNA plasmid with no insert and replicons expressing the influenza virus hemagglutinin) did not elicit anti-Gn antibodies (data not shown).

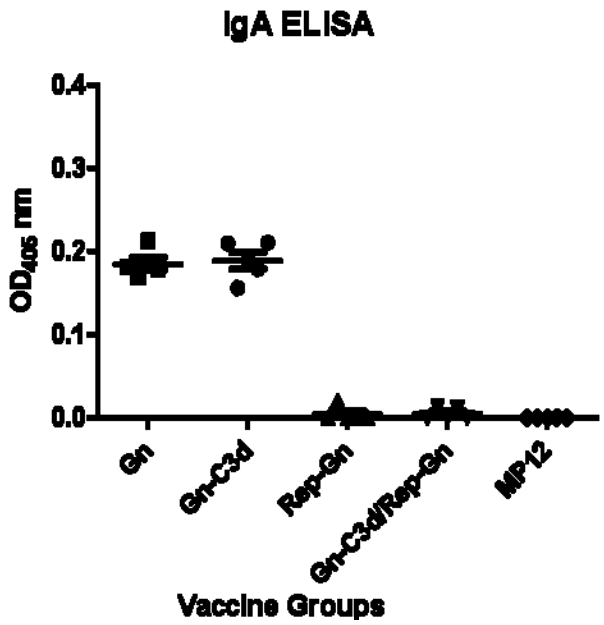


Figure 21. Indirect ELISA measuring RVFV specific IgA responses in mice immunized with indicated vaccines

All groups received primary and two booster immunizations (except MP12) spaced 3 weeks apart. Serum samples were collected two weeks after the last immunization (week 8 of the study). Error bars denote the standard error within the samples with a measurable titer.

5.6 DISCUSSION

In this study, we evaluated our candidate vaccines for the ability to confer protection, as well as the ability to prevent clinical signs after challenge by the intraperitoneal or aerosol routes. Few mice from DNA and replicon control groups survived virus infection similar to previous studies [89, 94]. However, all of the control mice displayed clinical signs of infection that were characterized by ruffled fur and lethargy. We observed a correlation between neutralizing antibody titers (Fig. 12) and development of clinical signs or mortality in the study involving intraperitoneal virus challenge. To further explore the ability of factors in the sera to protect mice from RVFV infection by the intraperitoneal route, passive transfer of serum from vaccinated mice to naïve mice demonstrated that humoral immune response play a major role in anti-RVFV immunity [48]. Not all mice that received passively administered serum were protected, which may be due to dilution of the neutralizing antibodies during preparation. Mice with a PRNT50 value of <1:10 succumbed to lethal infection and a PRNT50 value of ≥1:40 was sufficient to prevent clinical signs. This however, was not the case post-aerosol challenge in the vaccinated mice where neutralizing antibody titers did not correlate well with survival or development of clinical signs. This finding is in contrast to the experimental study performed by Anderson *et al*. [115]. They reported a correlation between the day 42 PRNT50 values after 3 vaccinations with survival. This could be explained by the fact that they performed experiments using rats and used formalin inactivated RVFV vaccine. However the authors did propose that serum neutralization antibody is not the only possible correlate of protection post-aerosol infection [115]. It should further be noted that a similar study performed in the past had strikingly different outcomes showing lack of protection in vaccinated mice after aerosol infection using same inactivated

RVFV vaccine [116]. This infers that the mechanism of protection after aerosol infection is not fully understood and demands further investigation. We evaluated pre-challenge sera from vaccinated mice for serum IgA levels and found that only DNA vaccination lead to development of any anti-RVFV serum IgA titers. Mice immunized with MP12 did not elicit IgA antibody reponse. This could be a general property of DNA vaccination or the route of immunization might also have some role in modulating IgA isotype. IgA is the most abundant immunoglobulin isotype produced in the body and is the second most dominant isotype in the circulation after IgG [120]. The function of serum IgA in development of systemic immune response has not been fully understood. One of its major roles might be to function as an inflammatory antibody through interactions with FcαR on immune effector cells [121]. I speculate that serum IgA plays an important role in modulating immunity to pathogens and might explain the survival of mice immunized with the Gn-C3d vaccine. The issue of survival from control DNA or mock vaccination is curious, but has been observed in previous publications. Spik *et al*. [89] also observed survival of a subset of mice following vaccination with DNA controls up to 31 days following challenge with Rift Valley fever virus. In addition, Bird *et al*. observed that sham mice did not succumb to lethal Rift Valley fever virus challenge, but they developed severe clinical signs of ruffled fur, hunched back, and lethargy [72]. In sum, the vaccination strategy employing Gn-C3d emerged as the promising strategy protecting animals from both i.p. and aerosol challenge without development of clinical signs and demands further test and evaluation in large animal species.

6.0 CONCLUSIONS AND FUTURE DIRECTIONS

One of the major thrust areas in the prevention of RVFV infection is to design safe and effective vaccines. In the beginning, research was focused on developing a live-virus based vaccine to prevent RVFV outbreak in livestock. These early vaccines although elicited long-lasting immunity after single dose vaccination, however it retained the virulent potential to cause abortions or fetal malformations in livestock species [79-81]. Inactivated whole virus-based vaccines were the next to get developed with the idea of inducing good immunity without worrying about the potential side effects as observed with its live counterpart [77]. Unfortunately, the inactivated RVFV vaccines developed so far have been shown to be less efficacious in inducing protective immunity than the live attenuated vaccines and require multiple booster vaccinations to obtain immunity [77]. Studies in the recent years reflect continuous efforts in the development of an effective vaccine strategy against this zoonotic pathogen [48, 49, 77, 89, 90, 92, 94]. Although preclinical research with the new vaccine strategies showed some promise, several factors including higher cost of production and questionable safety limits their potential use.

The recent outbreak of RVFV in Saudi Arabia and Yemen reflects the ability of this pathogen to create virgin soil epidemics [6, 38, 122]. In addition, high morbidity and mortality associated with RVFV infection poses a continuous threat of its malicious use by terrorist groups as a biological weapon [39, 44, 45, 123]. Therefore, there is a pressing need for developing

RVFV vaccines that optimally combine efficacy and safety for human and veterinary use. To address this issue, the present research study was undertaken, focused on development and testing of two promising RVFV vaccine candidates based on DNA and alphavirus replicon vectors that express the virus envelope glycoprotein, Gn. Since current diagnostic tests employ RVFV recombinant N protein based ELISA [124-126]. Therefore, an ideal RVFV vaccine, especially for livestock applications, would lack the RVFV N protein, which would allow differentiation between vaccinated and infected individuals. As a benchmark to compare the test vaccines, a live-attenuated (MP12) and whole inactivated virus (WIV MP12) vaccines were also developed in this study.

DNA vaccines have been licensed for veterinary use. However, they were found to be less effective in human clinical trials for other infectious diseases [127, 128]. In order to enhance the immunogenicity of DNA vaccines by enhancing the antibody responses directed at the antigen, I used a molecular adjuvant C3d which has shown positive results in previous studies [97, 100, 101]. Since the Gn glycoprotein is known to contain protective neutralizing epitopes [46, 48], my efforts were focused on characterizing whether fusion of the C3d molecule to Gn resulted in enhanced RVFV specific immunity. Each vaccine was tested alone or in a DNA prime/replicon boost strategy formulation to elicit protective immune responses against virulent RVFV infection in mice. The development of marker vaccines make it possible to differentiate infected from vaccinated animals [129]. To address issues associated with potential RVFV exposure in the real world scenario, both intraperitoneal (i.p.) and aerosol routes of virus challenge were employed in this study.

Mice vaccinated with Gn-C3d vaccine had high titer neutralizing antibodies compared to mice vaccinated with DNA expressing Gn alone. It remains to be determined whether this effect

is solely due to C3d's function as a molecular adjuvant or whether the fusion of C3d also enhances the secretion of Gn from the cell or the protein's stability in the extracellular environment. In addition to the DNA vaccine strategy, I also used a DNA prime/alphavirus replicon boost strategy to expand the repertoire of elicited immune responses. Previously Heise *et al*. used a Sindbis virus replicon vectors expressing the RVFV Gn and Gc glycoproteins, as well as the non-structural NSm protein to induce protective immune responses in mice against RVFV [93]. Studies in the past have mainly focused on survival of vaccinated mice post-challenge. However, an ideal vaccine should not only be able to protect from virus infection, but also prevent development of clinical symptoms. In this study, I evaluated candidate vaccines for the ability to confer protection, as well as ability to prevent clinical signs.

DNA or replicon vaccination individually or in a heterologous approach elicited identical total IgG antibody titers but different IgG isotypes (Fig. 10 and Fig. 11). DNA immunization resulted in a predominantly Th2 biased immune response indicated by predominant IgG1 isotype in prechallenge sera. Replicon and live attenuated vaccine (MP12) elicited a mixed helper T cell response indicated by the detection of IgG1, IgG2a, and IgG2b isotypes (Fig. 11). Among benchmark vaccines, only the live MP12 infection elicited strong neutralizing antibody responses, however despite of higher total anti-Gn IgG titers, WIV MP12 failed to elicit noticeable neutralizing antibody titers (Fig. 12). Most importantly, mice vaccinated with Gn-C3d and Rep-Gn had neutralizing antibody titers that were statistically identical to the titers of MP12 group (Fig. 12). The issue of survival from control DNA or mock vaccination is curious, but has been observed in previous publications. Spik *et al*. [89] also saw survival of a subset of mice following vaccination with DNA controls up to 31 days following challenge with Rift Valley fever virus. In addition, Bird *et al*. observed that sham mice did not succumb to lethal Rift Valley

fever virus challenge, but they developed severe clinical signs of ruffled fur, hunched back, and lethargy [72]. Another interesting observation was the results from passive sera transfer experiment suggesting the important role played by factors in serum in conferring protection against i.p. RVFV infection (Table 5). A correlation between neutralizing antibody titers and development of clinical signs/mortality were observed when vaccinated mice were challenged by i.p. route. This was reflected in the observation that mice with a PRNT50 value of <1:10 succumbed to RVFV infection and a PRNT50 value of ≥1:40 was sufficient to prevent clinical signs or body weight loss after i.p. challenge (Fig. 16 and Fig. 17)

On the other hand, vaccinated mice challenged by aerosol route had an interesting outcome where morbidity/mortality was found not to correlate with the PRNT50 values. Anderson *et al*. reported similar finding in their study where the inactivated vaccine that conferred almost complete protection by i.p. challenge failed to protect mice from aerosol virus challenge [116]. The group of mice immunized with Gn-C3d vaccine was the only group that displayed complete protection against aerosol challenge with virulent RVFV. Despite higher neutralizing antibody titers, the MP12 group had some mortality associated with aerosol challenge (Fig. 19). Although the complete function and role of serum IgA is not fully understood, but I speculate that serum IgA plays an important role in defense against pathogens and might explain this outcome since only DNA immunization produced detectable serum IgA levels (Fig. 21). Induction of serum IgA could be a general property of DNA-based vaccination and further experiments are required to corroborate these findings.

Elicitation of high-titered neutralizing antibody responses are considered one of the important features in vaccine development. However, an ideal vaccine would also induce cellular immunity. Cell-based immune responses would help clear virally infected cells thereby limiting

virus production and hasten recovery. In the present study, cellular immune responses directed towards RVFV Gn were observed only with Rep-Gn group. DNA-based immunizations did not induce any cellular immunity (Table 2). In addition this is the first report identifying a MHC-I restricted T cell epitope SYAHHRTLL in RVFV glycoprotein Gn (Table 4 and Fig. 13). This immunodominant epitope on the surface of Gn might play an important role in anti-RVFV immunity. However further studies are required before any such associations are made.

The study presented here demonstrates the efficacy of candidate vaccines against RVFV infection in mice. The results are encouraging and warrant further testing the efficacy of these vaccine candidates in livestock such as sheep and cattle.

6.1 SUMMARY OF THE FINDINGS

The present study describes the use of DNA and alphavirus replicon based vaccination approaches to elicit a protective immune response against RVFV. While both vaccines elicited high titer antibodies, DNA vaccination elicited high titer neutralizing antibodies, whereas the replicon vaccine elicited cellular immune responses. Both strategies alone or in combination elicited immune response that completely protected against not only mortality, but also illness against virus challenge. Further testing of the vaccine candidates resulted in DNA vaccination emerging as the single vaccine strategy conferring complete protection against aerosolized RVFV challenge. Even though the delivery vectors elicited some protection on their own, they did not prevent severe morbidity. These promising vaccines provide an alternative RVFV vaccine for livestock and humans.

6.2 PUBLIC HEALTH SIGNIFICANCE

Rift Valley fever virus (RVFV) is associated with abortion storms, neonatal mortality in livestock and hemorrhagic fever with a high case/fatality ratio in humans. In addition, it is a potential biowarfare agent which is a highly infectious via aerosol route. Several limitations prevent the widespread use of live and inactivated RVFV vaccines in livestock or humans. The present study demonstrates DNA expressing Gn-C3d and alphavirus replicons expressing Gn administered alone or in a DNA prime/replicon boost provide comparable protection as the live-attenuated MP12 vaccine. In addition, the vaccines prevented the development of clinical signs of infection. Gn-C3d emerged as the best vaccine vaccine candidate providing complete protection against both intraperitoneal and aerosol virus challenge. Encouraging results obtained from this study not only test the potential of DNA and replicons as efficient vaccines, but also improves our knowledge in vaccine design and immunity against this significant veterinary and public health threat.

6.3 FUTURE DIRECTIONS

The study presented in this dissertation demonstrates development of an improved and efficacious vaccination strategy against RVFV. Both DNA and replicon vectors have good potential to be used and licensed as common vaccines for both human and livestock. However,

further testing of these vaccines in non-human primates and large animal species such as sheep and cattle will be required and constitute the future direction for this project.

Recent research on developing prophylactic and medical interventions against RVFV has increased in the past few years. However we still lack information about correlates of protection against RVFV infection. Additional studies with knockout mice or mice immune deficient in a specific immune function would help address this and provide information about the host factors involved in anti-RVFV immunity. Results from this study highlight the role of serum factors in protecting mice against RVFV infection. However we still do not know if cell-mediated immunity plays any role in preventing infection.

How RVFV induces pathogenesis is one of the areas in RVFV research that still lacks a good understanding. We can specifically target certain genes/proteins to develop efficient treatment or control strategies against RVFV with the better understanding of the disease process and its effect on host immune system. Future studies looking at the pathogenesis and immune activation against RVFV infection would help design and test therapeutics and vaccines against this biological threat.

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