DEVELOPMENT OF NOVEL VACCINATION STRATEGIES AGAINST EMERGING BUNYAVIRUSES

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ABSTRACT

DEVELOPMENTOF NOVEL VACCINATION STRATEGIES AGAINST EMERGING BUNYAVIRUSES

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Severe fever with thrombocytopenia syndrome virus (SFTSV) is a highly pathogenic emergent bunyavirus. First isolated in 2009 in China, SFTSV is now endemic to several east Asian countries where high case fatality ratios of 6-30% are reported. The primary tick vector of SFTSV, *Haemaphysalis longicornis*, has a large range and is a well reported invasive species throughout the world. This, in addition to SFTSV's ability to spread in the absence of its vector in nosocomial and veterinary settings, suggests SFTSV is well suited to cause widespread lethal outbreaks. Currently no vaccines or therapeutics against SFTSV exist, prompting health agencies to list SFTSV as a high priority pathogen. Here, we first develop a single dose recombinant vesicular stomatitis virus (rVSV) encoding the SFTSV glycoproteins Gn/Gc as a virus vectored vaccine. We demonstrate that this vaccine (rVSV-SFTSV) is safe in immunocompromised mice and not neuropathogenic when delivered intracerebrally. Additionally, this vaccine induces robust antibody responses that are protective from lethal challenge. Furthermore, we demonstrate that this vaccine elicits cross-protective responses against the closely related Heartland virus. We then developed an mRNA vaccine encoding SFTSV Gn/Gc and compared this platform with our rVSV-SFTSV vaccine in single dose, homologous prime-boost, and heterologous prime-boost regimens. We found that mRNA immunizations in single dose and homologous prime-boost regimens achieved the highest neutralizing antibody titers. Immunizations with rVSV-SFTSV also reached high antibody titers though they were the lowest titers of any immunization regimen, with

heterologous prime-boost having intermediate titers. When T-cell responses were analyzed, mRNA immunization achieved robust CD4+ and CD8+ responses in single dose and homologous prime-boost regimens. Heterologous vaccine regimens elicited similar responses to homologous mRNA strategies despite weak cellular activity after rVSV-SFTSV prime. Despite some differences in immunogenicity, all vaccines were protective from lethal SFTSV challenge. Overall, this work demonstrates the effectiveness of two vaccine platforms in their ability to elicit robust protective responses against SFTSV.

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CHAPTER 1: Introduction

1.1 – Emerging Bunyaviruses

As humans come into increasingly close contact with wild animals due to urban sprawl, the likelihood of encountering emergent viruses, newly discovered viruses with increasing incidence, increases concurrently[1]. In the last several decades there have been many new pathogenic viruses including Middle East respiratory syndrome virus, severe acute respiratory syndrome coronaviruses 1 and 2 (SARS-CoV-1 and SARS-CoV-2), and Nipah virus, among many others, have caused significant impact to humans. Indeed, many of the major epidemics and pandemics of the last century, including Ebola virus (EBOV), human immunodeficiency virus (HIV), and various coronaviruses (CoV), have been associated with zoonotic transmission from animal reservoirs[2-4]. Among the recent emergent viruses are the viruses in the *Bunyavirales* order which pose serious public health and economic threats[5].

1.1.1 Introduction to Emergent Bunyaviruses

The *Bunyavirales* order encompasses a large and diverse set of viruses found throughout the world (Figure 1.1). These viruses are generally characterized by having multisegmented, single stranded RNA genomes that are either ambisense or negative sense[6]. The genomes of these viruses are generally comprised of three segment (with the exception of the Arenaviruses, which have two segments and are a recent addition to the order *Bunyavirales*)[6,7]. Most viruses in this order are spread by arthropod vectors such as mosquitos, flies, or ticks; the two exceptions to this are the Hantaviruses, which are spread by aerosolization of rodent feces, and the Arenaviruses which cause chronic asymptomatic infections in their rodent hosts (due to their significant differences in virology, Arenaviruses are not discussed further)[6]. This order of viruses includes many

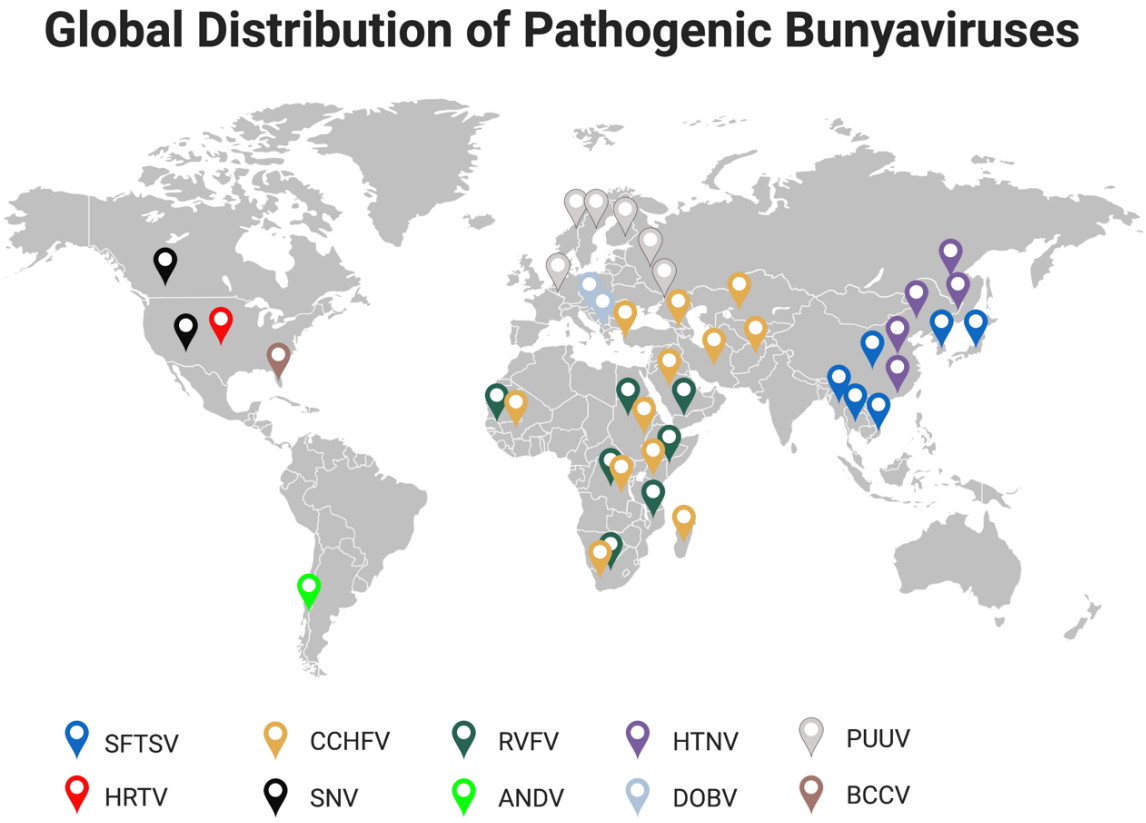

Figure 1.1 Global Distribution of Pathogenic Bunyaviruses The distribution of various pathogenic bunyaviruses discussed in the text are shown. RVFV and CCHFV share a similar distribution throughout much of Africa and reaching into the Arabian Peninsula. CCHF extends to western Asia and southeast Europe. SFTSV is endemic to much of east Asia while it's closely related counterpart, HRTV, is found in the midwest of the United States. Also shown are the locations of three New World hantaviruses, SNV, ANDV, and BCCV; and three Old World hantaviruses, HTNV, DOBV, and PUUV. Figure created in BioRender.com

important pathogens with high economic and health burdens associated with livestock and human disease.

Many bunyaviruses pose public health and economic hazards including Rift Valley fever virus (RVFV), Crimean-Congo hemorrhagic fever virus (CCHFV), and severe fever with thrombocytopenia virus (SFTSV). One of the first identified bunyaviruses was RVFV which was first isolated in the 1930's during a large outbreak that led to the death of many livestock animals and hemorrhagic fever in humans[8,9]. Since this outbreak, retrospective studies have identified suspected RVFV outbreaks dating as far back as 1912 and outbreaks continue to this day[8,9]. A member of the *Phenuiviridae* family, RVFV is spread by mosquitos. In mosquitos, it infects the midgut after a blood meal from an animal carrying the virus at a high enough viremia[6]. Once the virus replicates in the midgut it eventually travels to the salivary glands of the mosquito where, upon the next blood meal, it is injected into the skin of the bitten animal[6]. As is seen in many other bunyaviruses, RFVF can be transmitted transovarially and is seasonal/cyclical according to conditions favoring its vector[8]. RVFV is endemic to sub-Saharan Africa and the Arabian Peninsula where it represents a major economic and health hazard (Figure 1.1)[8,9]. The virus infects domestic ruminants causing high rates of mortality and abortions or fetal malformations in pregnant animals[8,9]. In humans, RVFV generally causes a self-limiting febrile illness but in some cases can cause severe disease including hemorrhagic fever, neurologic disorders, and blindness[8,9]. These symptoms, including hemorrhagic fever and neurologic disorders are common symptoms of many other bunyaviruses[6]. The potential for RVFV to cause large outbreaks and significant economic damage cannot be overstated. Since the discovery of RVFV, many more deadly bunyaviruses have been identified which often share similar disease manifestations and life cycles to those of RVFV.

Many other bunyaviruses represent public health threats. The hantaviruses include several species such as Sin Nombre virus (SNV) and Andes virus (ANDV) which are spread by aerosolized mouse feces (Figure 1.1)[6]. Human infection by hantaviruses often manifests itself through hemorrhagic fever with renal syndrome (HFRS) or hantavirus cardiopulmonary syndrome (HPS)[10]. HFRS is caused by old world hantaviruses, such as Hantaan virus (HTNV), Dobrava-Belgrade virus (DOBV), and Puumala virus (PUUV), with reported cases in China alone reaching as high as 100,000 cases a year[10]. New world hantaviruses, such as SNV, ANDV, and Black creek canal virus (BCCV), cause HPS which has a case fatality ratio of 40-50%[10]. Infection by new world hantaviruses is less common with 816 cases reported from 1993 to 2019 by the Centers of Disease Control[11].

The tick-borne virus CCHFV is another source of significant health and economic burden. Disease caused by CCHFV was originally described in 1944 and the virus was later identified in 1968[12,13]. CCHFV has a high case fatality rate of approximately 30% and shares a similar disease course and range to RVFV[13]. Like many bunyaviruses, the burden of disease for CCHFV remains understudied due to it primarily impacting rural farmworkers. More recently discovered bunyaviruses include SFTSV and heartland virus (HRTV), identified in 2009 and 2011 respectively[14-16]. HRTV was discovered in the Midwest of the United States and is transmitted by the lone-star tick[16]. Despite the prevalence of the HRTV tick in the Midwest, the disease remains uncommon with few recorded cases. Its Asian counterpart, SFTSV, on the other hand has become commonplace in East Asia and is considered an endemic seasonal disease (Figure 1.1)[17,18]. SFTSV has quickly become a major public health concern due to its high case fatality ratio, lack of treatment options, and the expanding range of its tick vector. A

common theme among the Bunyaviruses is the pervasiveness of high case fatality ratios and a lack of effective treatment and preventative therapies.

1.1.2 SFTSV Epidemiology

SFTSV first emerged in 2009 in the rural Hubei and Henan provinces of China[15]. An unknown illness with a case fatality ratio (CFR) of 30% was observed in patients presenting with fever, thrombocytopenia, leukopenia, and gastrointestinal symptoms[15]. After ruling out anaplasmosis and other suspected pathogens, a virus was isolated by culturing a variety of cell lines with patient serum[15]. Sequencing of viral RNA identified a novel Bunyavirus, SFTSV[15]. Since the initial report of SFTSV in China, several other countries including Japan, Vietnam, and South Korea, have reported CFRs ranging from 6-30%[17,19,20]. These numbers are likely overestimates of actual CFR due to SFTSV incidence in rural areas and the likelihood of unreported asymptomatic cases.

Clinical disease of SFTSV is characterized by fever, leukopenia, and gastrointestinal symptoms including nausea, vomiting, and diarrhea[21,22]. These initial symptoms generally occur 3-7 days after a tick bite or other exposure[21,22]. In general, patients with mild disease recover from these symptoms after approximately a week and see viral titers decrease over 2 weeks after initial disease onset[21]. In patients with severe disease, however, virus titers continue increasing and new symptoms manifest. These symptoms are far more serious including hemorrhagic manifestations such as disseminated intravascular coagulation and pulmonary and gastrointestinal bleeding, central nervous system (CNS) disorders such as tremors, convulsions, and comas, and acute respiratory distress[21,22]. Patients with severe illness often experience multiorgan distress which may be reversible in some patients, however, this often leads to multiorgan failure and death[21]. Infection by the closely related HRTV induces similar

disease manifestations[23]. Currently no approved therapeutics exist against SFTSV, thus, patients can only be treated for manifested symptoms, not the underlying disease.

Like other viruses in the *Phenuiviridae* family, SFTSV is a tick-borne disease. The primary vector of SFTSV is the Asian long-horned tick, *Haemophysalis longicornis*, though other tick species have been found to be positive for SFTSV[24]. Work with the closely related HRTV suggests that these viruses can be transmitted among ticks transstadially and transovarially[25,26]. Additionally, cofeeding of ticks allows uninfected ticks to acquire the virus, though this transmission method appears to be rare[26]. It remains unknown whether ticks, mammals, and/or birds are reservoir species for SFTSV and HRTV. Several serologic studies have found a wide range of animals to be sero-positive for SFTSV, including sheep, cattle, dogs, and chickens[27]. As a tick-borne disease, SFTSV incidence increases seasonally from early spring to late fall. Additionally, environmental predictors of tick populations including cattle density, temperature, rainfall, and forest coverage correlate with incidence of SFTSV[22]. Of particular concern is the expanding range of the invasive *H. longicornis* tick. Originally endemic to China, Japan, Korea, and eastern Russia, *H. longicornis* was introduced to Australia, and New Zealand in the 19th century and has been recently identified in eastern United States[28,29]. Spatial modeling of climates suitable for *H. longicornis* suggest a potential for the tick to greatly expand its range leading to ecologic, and economic damage[28]. Indeed, tick surveys in New Jersey have found all immature life stages of *H. longicornis* suggesting a viable population[30]. Currently, no ticks outside of eastern Asia have been found positive for SFTSV, yet the expanding presence of this vector threatens to increase SFTSV incidence around the world.

Though primarily tickborne, SFTSV has been shown to be infrequently transmitted by other means. Ferret studies have shown that SFTSV can be transmitted

in the absence of ticks when animals are co-housed or co-housed with a separator[31]. This transmission is thought to be due to contact with bodily fluids, as SFTSV was detected at high titers in ferrets' saliva, urine, and feces[31]. Indeed, several cases of nosocomial transmission have been reported in humans that have been exposed to blood from infected individuals[32]. Additionally, zoonotic transmission from cat bites have been reported in veterinary settings[33,34]. Due to the potential for severe disease, evidence of human-to-human transmission, the ever-increasing range of the SFTSV vector, and a lack of vaccines or therapeutics, SFTSV is classified as a Category C Priority Pathogen by the National Institutes of Health and is often included in lists of high priority pathogens by other health organizations[35,36].

1.1.3 SFTSV Biology

As with other members of the *Phenuiviridae* family of viruses, SFTSV has a tripartite RNA genome composed of a large (L), medium (M), and small (S) segment (Figure 1.2)[15]. Both the 6.3 kilobase-pair (kb) L and the 3.3 kb M segments are negative sense and encode the RNA dependent RNA polymerase (RdRp) and the glycoproteins (Gn/Gc) respectively[15]. The 1.7kb S segment is ambisense with the nucleocapsid (N) encoded in one direction and a non-structural protein (NSs) encoded in the opposite orientation[15]. Each RNA segment has terminal complimentary regions and thus each segment is predicted to form a noncovalently closed loop (Figure 1.2)[6]. The N protein encapsidates the viral genome and is essential for transcription and replication by the RdRp, which is found in complex with the viral RNA and N[6]. The glycoproteins Gn/Gc are found on the membrane of the virus and confer specificity and enables viral entry into cells[6,37]. The NSs protein is an interferon (IFN) antagonist[38]. Fully formed SFTSV virions are pleiomorphic and range from 80-100nm[15].

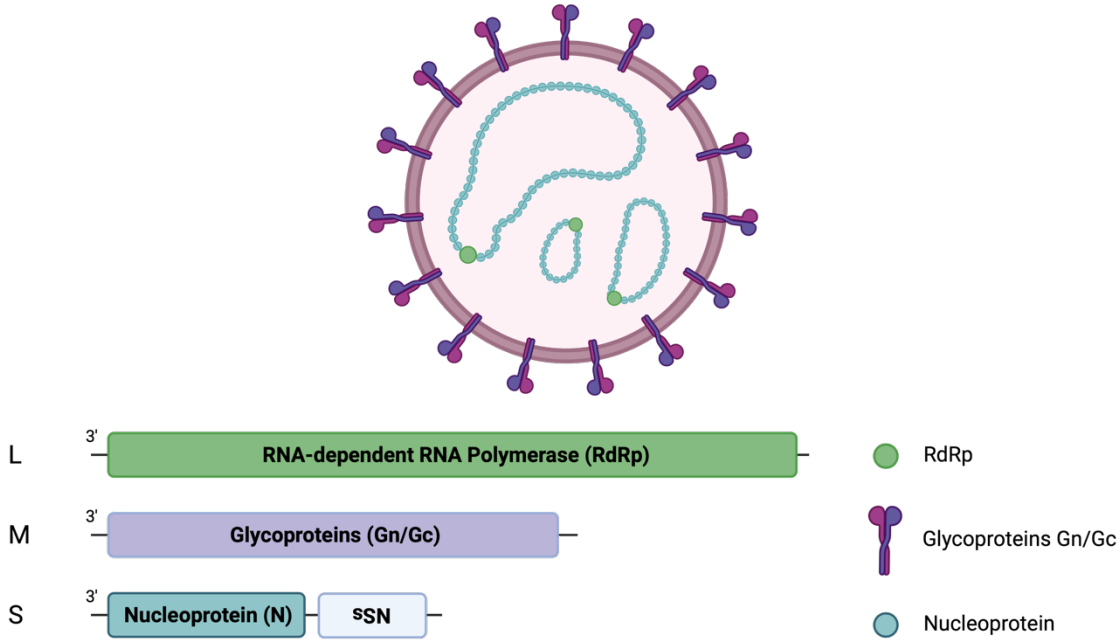

Figure 1.2 STSV Virion Structure and Genome Organization SFTSV is an enveloped virus with a pleiomorphic structure. Its surface is coated with the glycoprotein complex Gn/Gc. The virion carries the three segmented negative sense RNA genome. The large segment (L) encodes the polymerase, the medium segment (M) encodes the glycoproteins as a polyprotein, and the small segment (S) encodes the nucleoprotein and NSs. Unlike other segments, the S segment is ambisense. Created with BioRender.com

The infection cycle of SFTSV and other Bunyaviruses begins with interaction of the glycoproteins, Gn/Gc, and their receptor, which induces receptor-mediated endocytosis[6,39]. As endosomes acidify, Gn/Gc mediated fusion of viral and endosomal membranes occurs, releasing viral nucleoproteins into the cytoplasm where SFTSV and other bunyaviruses replicate[6,39]. The RdRp mediates viral transcription and replication within the cytoplasm, which is thought to be membrane associated. Replication and transcription are dependent on both the presence of RdRp and N[6]. The SFTSV glycoproteins Gn/Gc are produced as a single polyprotein which is then cleaved by a yet to be identified protease[6,39]. In cells, the uncleaved precursor of Gn/Gc is not observed, indicating that cleavage occurs co-translationally[6]. Once cleaved, Gn and Gc remain associated as dimers within the endoplasmic reticulum (ER) and golgi compartments due to the presence of ER and golgi localization signals[40]. Interestingly, Gn can exit the ER when expressed alone, but Gc cannot[6]. This indicates that oligomerization of the two glycoproteins is necessary to overcome the ER retention signal found in Gc[6]. The Gn/Gc complex is capable of budding and is sufficient for the formation of virus like particles[6]. Viral particles are made when Gn/Gc bud into the golgi with S, M, and L fragments. Once in the golgi the virus is transported out of the cell by vesicles.

SFTSV entry is mediated by Gn/Gc presenting a potential target for therapeutic and vaccine development. It is unclear which glycoprotein subunit is responsible for imparting specificity; both proteins are likely involved in binding. Multiple studies have failed to isolate an SFTSV receptor, and entry factors remain poorly characterized. Several studies have indicated that the lectin dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) is likely involved in SFTSV entry, as knockout cells showed decreased viral infection[37]. While DC-SIGN is involved in SFTSV entry it cannot be the sole receptor, as cell types lacking DC-SIGN can be infected by

SFTSV[37]. After receptor-mediated endocytosis, fusion is then mediated by the Gc subunit[37,41]. This has been shown to occur in late endosomes and is pH dependent with fusion occurring at a pH of approximately 5-5.5[42]. Proteins associated with viral entry are also poorly characterized, though studies show that UDP-glucose ceramide glucosyltranferase (UGCG) is a contributing factor. In UGCG-knockout cells, SFTSV virions are unable to fuse and fail to exit late endosomes, preventing viral replication[43]. It is known that antibodies against either Gn or Gc can neutralize virus[37,44,45]. This indicates that both subunits likely play a role in interacting with receptors. Neutralization of Gn/Gc thus presents a potential target in the development of vaccines, although further work is needed to identify key entry factors on the host cell.

Although the receptor for SFTSV is unknown, several groups have investigated the cellular and tissue tropism of the virus. In one study in humans, it was found that in fatal disease the primary target organs for SFTSV were the lymph nodes[46]. High levels of SFTSV were also detected by immunohistochemistry and real-time quantitative polymerase chain reaction (RT-qPCR) in the spleen and bone marrow[46]. The primary cell types infected by SFTSV were macrophages and class-switched B cells[46]. Mouse models of SFTSV have shown similar targets with virus detected in the spleen, lymph nodes, liver, and kidney of infected animals[47]. The targets of infection in mice appear to be primarily macrophages and B cells, resembling human infections[47]. Another similarity between infections in mice and humans is the high levels of virus detected in the lymph nodes in fatal disease[47]. Notably, a difference between infection in mice and humans is the absence of hemophagocytosis in the mouse model[47]. This symptom is characteristic of fatal cases in humans but not observed in mice. This is perhaps due to the lack of IFN signaling in the most commonly used SFTSV animal models.

1.1.4 Animal Models for SFTSV

The development of efficacious vaccines and therapeutics is dependent on the availability of good animal models to test potential therapies. Quality animal models must meet several criteria, among them: achieving similar disease course as in humans, reasonable cost of use, and availability of tools to assess immune responses. In general, all animal models currently employed to study of SFTSV are lacking in at least one of these areas (Table 1.1). The current animal models of SFTSV infection are aged ferrets, cats, and immunosuppressed or newborn mice and hamsters (Table 1.1).

One of the better models at recapitulating human disease is the SFTSV aged ferret model[31,48]. In a study with various mouse strains as well as aged and young ferrets it was found that aged ferrets (≥4 years old) succumbed to SFTSV by 8 days post challenge[48]. In contrast, young ferrets challenged equivalently exhibited no lethality and only minor clinical symptoms[48]. Aged ferrets showed a variety of clinical symptoms of disease that mimicked what is seen in human patients; this included fever, severe thrombocytopenia, decreased white blood cell count, and increases in both aspartate aminotransferase and alanine aminotransferase[48]. High viral titers were detected in the spleen, liver, and serum of aged animals[48]. Overall, this model captures clinical manifestations of SFTSV well, despite a lack of CNS symptoms and a lack of any mortality in young animals (despite a higher mortality in older humans, SFTSV can be fatal in younger individuals). Notably, an aged ferret model is unlikely to gain traction as a widely used animal model. Very few vendors offer ferrets ≥4 years old, and when they are available these animals carry a significant financial cost. Labs are also unlikely to purchase and maintain ferrets until they are old enough to manifest clinical symptoms, again due to the overwhelming cost associated with such an experiment. Finally, few tools are available for the analysis of immunological responses limiting the ability to

Animal	Strain/Age	Immunocompetence	Lethality	Immunologic tools	Accessibility/barriers to use
Mouse	WT Newborn	Incompletely developed immune system	Lethal	Readily available	Not a good model for vaccine studies
	WT Adult	Immunocompetent	Non-lethal		
	WT Aged	Immunocompetent	Non-lethal		
	IFNAR Ab	Temporarily immunocompromised	Lethal		Expense of IFNAR Ab
	Ifnar$^{-/-}$	Immunocompromised	Lethal		Lacks full immune capabilities useful for vaccine studies
	Stat1$^{-/-}$	Immunocompromised	Non-lethal		
	Stat2$^{-/-}$	Immunocompromised	Lethal		Lacks full immune capabilities useful for vaccine studies
Hamster	WT Newborn	Incompletely developed immune system	Non-lethal	Somewhat available	
	WT Adult	Immunocompetent	Non-lethal		
	Stat2$^{-/-}$	Immunocompromised	Lethal		Lacks full immune capabilities useful for vaccine studies
Ferret	≤2 Years Old	Immunocompetent	Non-lethal	Lacking	
	≥4 Years Old	Immunocompetent	Lethal		Not readily available; Expensive; Risk of bites and scratches; Known transmission of SFTSV in absence of tick
Cats	Russian blue, American shorthair	Immunocompetent	Lethal	Lacking	Expensive; Risk of bites and scratches; Known transmission of SFTSV in absence of tick; Ethical concerns
Non-Human Primates	Rhesus macaque	Immunocompetent	Non-lethal	Somewhat available	

Table 1.1 Current animal models of SFTSV

dissect responses elicited by potential vaccines. Taken together, ferrets recapitulate human SFTSV disease well, but are limited in their use for the development of treatment strategies. This model may however become useful in the end-stages of development of therapeutics.

Another animal that shows clinical symptoms mirroring what is seen in humans is the SFTSV cat model. It has been widely reported that cats (including domestic cats and in one case a cheetah) can be infected by SFTSV and manifest clinical symptoms[49,50]. A small study of 6 cats experimentally infected with SFTSV demonstrated a 66% fatality rate[50]. All animals showed severe thrombocytopenia and decreased white blood cell counts, surviving animals began recovery at approximately day 14 post infection[50]. Additionally, cats infected with SFTSV developed high fever, extensive gastrointestinal symptoms, and hepatic and renal damage consistent with what is seen in humans infected with SFTSV[50]. As previously discussed with the ferret model, an SFTSV cat model has many shortcomings such as cost and lack of tools for deep analysis of immune responses. Both ferrets and cats are also far more difficult to handle and pose a danger to researchers who must conduct these studies in animal biosafety level 3 facilities (ABSL3). Transmission of SFTSV through a cat bite has been documented and ferrets have been shown to transmit SFTSV to cohoused animals[33]. Both animal models have been shown to contain high titers of SFTSV in their bodily fluids[48,50]. Therefore, a bite or scratch from a challenge animal could prove fatal making these animal models potentially dangerous and unlikely to become commonplace.

The most common animal used as a model in research is the mouse. Extensive work has been done to identify mouse models that effectively recapitulate human SFTSV disease. The mouse model is ideal for research and development of novel vaccines and therapeutics due to the short lifespan, rapid reproduction, and ease of

handling, as well as their affordability and availability of extensive genetic and biological tools. Unsurprisingly, due to the ease of use of this animal model various groups have attempted to find mouse strains that recapitulate with reasonable accuracy human SFTSV disease. One study analyzed 12 immunocompetent mouse strains (including aged mice in these strains) and found that none of the tested immunocompetent mouse strains succumbed to lethal disease[47]. This study demonstrated that wild type C57/BL6 mice did have some weight loss upon challenge despite no lethality[47]. SFTSV has been shown to cause lethal disease in wild type mice only when mice are newborn[47]. This is likely due to newborn mice lacking a developed immune system capable of mounting a significant response to infection. As such, newborn mice are not useful in vaccination studies or for the analysis of pathologic responses.

Due to the lack of lethality of SFTSV in wild type mice, most groups rely on immunocompromised animals. The primary immunocompromised mouse model is the IFN alpha/beta receptor knockout (*Ifnar*$^{-/-}$) mouse[47,51,52]. Most studies developing novel SFTSV targeted therapies or vaccines have made use of *Ifnar*$^{-/-}$ mice as SFTSV causes lethal disease that closely mimics SFTSV symptoms in humans. The caveat of studies using *Ifnar* $^{-/-}$ mice in vaccine studies is that IFN signaling is critical in initiating immune responses that lead to immunity. Regardless, even without an IFN response many studies have been able to make strides in the development of SFTSV therapeutics. Other immunocompromised animals that have been identified to be susceptible to lethal SFTSV infection are mice and hamsters that are *Stat2*$^{-/-}$[53]. Like *Ifnar*$^{-/-}$ mice, the IFN signaling pathway is disrupted in *Stat2*$^{-/-}$ mice allowing them to succumb to SFTSV infection.

The most recent SFTSV animal model is the use of wild-type mice treated with an α-IFNAR monoclonal antibody (mAb)[54]. This group reported (and we later confirmed,

see chapter 3) that use of this α-IFNAR mAb strategy led to complete lethality in all mice given a sufficient challenge dose[54]. α-IFNAR mAb was administered at low doses on day -1 and day 2 post infection[54]. The use of this mAb treatment likely stifles the animal's immune response, allowing the virus to rapidly expand and cause tissue damage. When compared to *Ifnar* $^{-/-}$ mice, lethality in α-IFNAR mAb treated animals lagged and SFTSV in mAb treated animals did not reach titers as high as those in *Ifnar*$^{-/-}$ mice[54]. This is likely due to the inability of the mAb to control all IFN signaling resulting in weak immune responses that slightly dampen SFTSV mediated disease. For vaccine work this model represents a great improvement in available tools by allowing vaccine candidates to be tested in immune competent animals, thereby enabling a better understanding of the characteristics of the immune response elicited by vaccine candidates. Additionally, it allows for challenge studies with animals that can mount a full immune response with the caveat that this immune response is briefly dampened by the α-IFNAR mAb therapy.

1.2 – Vaccines

1.2.1 Vaccines Overview

Vaccines are the most effective public health intervention available and save an estimated 2-6 million lives each year from preventable diseases[55,56]. Vaccination is a powerful strategy to combat infectious diseases and has allowed for the elimination of rinderpest and smallpox as well as the near elimination of polio virus[56]. The primary function of vaccines is to expose an individual's immune system to immunogens from disease causing pathogens thereby eliciting protective immune responses[55]. An immunogen here is defined as a component of a pathogen against which an immune response can be directed, most commonly these are proteins, though sugars and lipids are also potential immunogens. Successful vaccines drive an immune response that will be recalled if the vaccinated individual ever encounters the immunogen again[55].

Immunization is safer than infection from the pathogen and once a sufficient portion of the population is immunized, herd immunity can be achieved[56]. Achieving high vaccine efficacy requires vaccination with appropriate immunogen using the ideal delivery method for the chosen immunogen and eliciting the correct type of immune response.

Vaccines vary widely on how an immunogen is delivered, each delivery strategy comes with benefits and drawbacks. Here, four classes of vaccines approved for human use will be discussed, these include live attenuated pathogens, inert vaccines, virus vectored vaccines, and nucleic acid vaccines, the most recently approved class of vaccines. Historically, most licensed vaccines have used live attenuated or inert vaccine platforms, however, these platforms present difficulties that underscore the need for new vaccine platforms[55]. Broadly, live attenuated vaccines contain a version of the live pathogen which has been weakened sufficiently to no longer cause significant disease in humans[55]. Inert vaccines deliver one or more non-replicating immunogens[55]. New vaccine technologies such as virus vectored and nucleic acid vaccines are becoming more common and hold the promise of improving upon the historical vaccine platforms. Virus vectored vaccines use live viruses that are non-pathogenic to humans to deliver an immunogen from another pathogen[55]. Finally, nucleic acid vaccines deliver mRNA or DNA (currently no DNA based vaccines are approved for human use) encoding an immunogen which is then produced by cells in the vaccinated individual[55].

The first vaccines developed were attenuated live vaccines[57]. These vaccines are generally produced by passaging pathogens many times through various animals or cell lines that are slightly permissive of pathogen replication or by using related pathogens that do not cause disease in humans[55,57]. For example, the oral polio virus vaccine was developed by passaging polio virus through rats and monkeys which are not infected efficiently[55,57]. Due to this passaging, the virus accumulated various

mutations that enabled it to adapt to growth in the animals where it was passaged. The adaptation of the virus to infect these animals in turn resulted in a virus that is less virulent in humans. Another example of an attenuated vaccine is the smallpox vaccine[55,57]. The original smallpox vaccine used by Edward Jenner used the cowpox virus, which is related to variola virus, the cause of smallpox[57]. Patients immunized with the cowpox virus would develop immunity that cross-protects them from smallpox[57]. Live attenuated vaccines continue to be widely used to this day, some examples of this vaccine type include the measles, mumps, and rubella vaccine, rotavirus vaccine, chickenpox vaccine, and the yellow fever vaccine[57].

There are many advantages and disadvantages to consider in using live attenuated vaccines. The primary advantages lie in the robust long-lived responses they generate[55,58]. Because they are live viruses the immune polarization (see section 1.2.3) is similar to what would be achieved by natural infection[58]. These vaccines thus elicit powerful responses optimal for the pathogen they target while largely eliminating the risk of severe disease[55,58]. The disadvantage of these vaccines, however, is the potential for reverted virulence[57]. Because the immunogens in these vaccines are replicating, there is the potential for the accumulation of mutations that revert the pathogen to a virulent strain[57]. The oral polio vaccine is an example of the risks associated with live attenuated vaccines. Every year a small number of children vaccinated with the oral polio vaccine develop polio disease due to the virus reverting to wild type[57,59]. An additional consideration that is needed for the use of live attenuated vaccines is the immune status of patients. While attenuated vaccines are generally safe for immunocompetent people, they can cause severe disease and even death in immunocompromised patients[60-62]. Additionally, these vaccines are not recommended for pregnant women as their immune systems are somewhat compromised, and they can pose a risk of crossing the placenta

and infecting the baby which is largely unable to respond to infectious pathogens[63]. Finally, the production of live attenuated vaccines can be complicated and time intensive. It frequently takes many repeated passages through animals or tissue culture cells to achieve a pathogen that is sufficiently attenuated[57]. Additionally, it is unclear what cells or animals will yield the best results in attenuating pathogens or even if the pathogen will be sufficiently attenuated[57]. Because of these drawbacks, live attenuated vaccines may not always be optimal and alternatives should be considered.

The next and perhaps largest class of vaccines are inert vaccines. These vaccines deliver an immunogen that is not capable of replicating and causing disease[55]. These vaccines range from delivering a single immunogen that is sufficient to provide protection up to delivery of a whole killed pathogen[55]. The immunogens delivered in these vaccines can vary widely, in some cases, recombinant proteins are delivered to target an immune response against a single protein that is essential for disease[55]. Vaccines of this type are widely used to target bacterial toxins[55]. In toxoid vaccines, an inactive version of the toxin is delivered to the patient creating immunity against the virulence agent as opposed to the pathogen itself[55]. Polysaccharides, complex sugars coating the exterior of many bacteria, can also be targeted with this vaccination type[55]. In vaccinations against viruses, viral-like particles, which lack nonstructural proteins, or whole killed virus may be used[55]. Some pathogens targeted by inert vaccines currently in use include, tetanus, diphtheria, shingles, hepatitis B, and human papilloma virus[55].

While inert vaccines are advantageous due to their safety in immunocompromised individuals, their disadvantage lies in their inability to drive a strong immune response alone[55]. Inert vaccines by nature of not replicating or being virulent are unable to elicit the signals associated with infections and responsible for initiating immune responses[55,64]. Because of this, inert vaccines are often delivered with

adjuvants, which are compounds able to stimulate immune responses[55,64]. Unfortunately, adjuvants may not always elicit immune responses that are ideal for controlling the targeted pathogen[58,64]. Inert vaccines are generally associated with immune responses that are not long-lived and thus may require booster immunizations.

A relatively new class of human vaccines are the virus vectored vaccines[55,65,66]. This type of vaccine typically uses viruses that are non-pathogenic to humans to deliver an immunogen[65,66]. While extensively studied, only recently have these vaccines become more widely adopted with the development and approval for human use of the vesicular stomatitis virus (VSV) vectored Ebola virus vaccine, and adenovirus vectored SARS-CoV-2 vaccines[66,67]. Virus vectored vaccines are advantageous in their ability to produce robust long-lasting immunity[55,65,66]. A major concern for viral vectored vaccines is the potential for platform immunity[66]. Delivery of immunogens through a virus elicits immune responses not only to the desired immunogen, but also to the proteins that comprise the viral vector. Therefore, vaccination with a VSV vector may render future immunizations with VSV platforms ineffective due to immune responses against the vector itself[66]. Additionally, insertion of immunogens into a non-pathogenic virus may alter that virus's pathogenicity[65,66]. Because of these characteristics of virus vectored vaccines, they often require more extensive testing to ensure their safety. Virus vectored vaccines using the VSV platform are discussed in detail in section 1.2.4.

The most recent class of vaccines approved for human use are the nucleic acid vaccines. Nucleic acid vaccines, both mRNA and DNA based, have been under development for decades[68,69]. These vaccines rely on the delivery of nucleic acid sequences encoding an immunogen. As an example, the two SARS-CoV-2 mRNA vaccines, which are the only currently approved nucleic acid vaccines in humans, encode the spike protein of SARS-CoV-2[70]. Cells that take up the mRNAs produce the

encoded immunogen, spike protein in this case, which leads to the induction of a strong immune response[69]. The advantages of nucleic acid vaccines are that once the platform is optimized and an immunogen is found to be protective, they are easy produce and manufacture is scalable[69]. Once an immunogen's nucleic acid sequence is known, producing needed mRNA or DNA sequences for vaccination is a relatively fast process. There are however some disadvantages to these vaccines, the primary challenge is often delivery[69]. Nucleic acids need to be delivered into cells; in DNA vaccines this often involves electroporation which can be painful to the patient[68]. Other concerns for DNA vaccines are the potential for integration into the vaccinated patients' chromosomal DNA[68]. Delivery of mRNA vaccines has been largely addressed (see section 1.2.5) but these vaccines require a strict cold chain to maintain effectiveness[69]. mRNA vaccines must be maintained at -80 °C and once thawed for use they cannot be frozen again due to degradation of the mRNA[69]. This can complicate delivery of vaccines to isolated rural populations where maintaining a cold chain may be unfeasible. It is likely that in the coming years more mRNA-based vaccines will become available due to their various advantages and the robust responses they achieve. Section 1.2.5 delves deeper into mRNA vaccines.

1.2.2 Characteristics of Immune Responses

Immune responses are generally characterized as either innate or adaptive immunity. The innate immune system is non-specific, meaning innate immune responses are not pathogen specific[71,72]. The innate immune system serves as a broadly acting response that can slow infections while the adaptive immune system is activated[71,72]. Innate immunity includes physical barriers, cellular populations, and various proteins[71]. Physical barriers include skin and mucous membranes which serve as a first line of defense preventing pathogens from entering the body[71]. The cellular

populations involved in innate immunity are extensive and indeed can include all nucleated cells[71,72]. The more commonly discussed cellular elements of the innate immune system include macrophages, dendritic cells, natural killer cells, basophils, eosinophils, and mast cells[71,72]. In brief, macrophages and dendritic cells are phagocytic cells considered to be professional antigen presenting cells (APCs) [72]. These cells phagocytose proteins and pathogens in the extracellular milieu and present peptides, short protein sequences (approximately 8-15 amino acids long) that are found within protein sequences in pathogens, to B- and T-cells[72]. Natural killer cells cause cytotoxicity to cells infected with pathogens[72]. Eosinophils, basophils and mast cells are associated with tissue damage, large extracellular pathogens, and allergenic responses[72]. All nucleated cells can be considered part of the innate immune system as these cells encode proteins for the detection of pathogens and the initiation of the adaptive immune response[71,72]. The protein components associated with innate immunity are vast and can be constitutively active or activated by certain danger signals elicited by infections or tissue damage[71-73]. Recognition of danger signals is done by pathogen recognition receptors (PRRs) which recognize pathogen associated molecular patterns (PAMPs)[72,73]. Many different PAMPs are recognized by the innate immune system including various species of mRNA (ex. double stranded or uncapped mRNA), cytoplasmic DNA, polysaccharides, and lipids[72,73]. Most PRRs are constitutively active and once activated they initiate a cascade of protein interactions resulting in altered transcription and the production of immune signaling proteins such as interferon (IFN), and various cytokines and chemokines[72,73]. The subset of cytokines produced is dependent upon the type of PAMPs, each PRR generally results in a slightly different cytokine profile which is able to shape the adaptive immune response[73]. The cytokines produced directly influence the type of immune response mounted, with detection of

RNA PAMPs leading to different responses than polysaccharide PAMPS. Importantly, the innate immune system is separate from the adaptive immune system but interactions between the two systems are essential to mounting and shaping adaptive responses[71].

The adaptive immune system is composed primarily of B- and T-cells which are activated when they encounter their cognate peptide (Note that B-cells can also recognize nucleic acids and sugars. T-cells have also been described to respond to lipids instead of peptides)[71,72,74-76]. Each B- and T-cell is specific to one specific epitope giving these cells very narrow specificities[71,72]. While innate immunity is important to initiate an immune response to vaccines, the adaptive immune response is typically examined when analyzing vaccine responses.

The adaptive immune system can be further broken down into humoral and cellular immunity[71,72]. Humoral immunity is associated with immunoglobulins (Ig), which are produced by B-cells[71,72]. Cellular immunity is associated with T-cells which are broken into two subsets CD4+ helper cells, and CD8+ cytotoxic cells[71,72]. Both B- and T-cells share a similarity in having a receptor that is highly specific[71,72]. In the case of B-cells, this receptor is the B-cell antigen receptor (BCR). The BCR genes encode the immunoglobulin proteins which are referred to as the BCR when anchored to B-cell membranes, and antibodies when secreted[71,72]. In T-cells, the T-cell receptor (TCR) is responsible for the detection of antigens presented by the major histocompatibility complex (MHC) [71,72]. Both these receptors are formed by somatic rearrangements of germline-encoded elements. This somatic rearrangement allows for the recognition of millions of epitopes with each having a unique specificity[72]. This vast repertoire of B- and T-cell specificities allows almost any encountered immunogen to be recognized and an immune response mounted.

T-cells can be further subdivided into two main subtypes which are defined by surface expression of CD4 or CD8. CD8+ T-cells are called cytotoxic T-cells and function primarily to kill cells infected with intracellular pathogens, though they also secrete cytokines thereby regulating immune responses[71,72]. CD4+ T-cells on the other hand act primarily by regulating the immune cellular and humoral response through cytokine secretion and are often referred to as helper T-cells (Th)[71,72]. There are many subpopulations of CD4+ T-cells that have been described, these populations are characterized by the cytokines they secrete and the types of pathogens that they help in controlling.

T-cells must encounter their cognate epitope to be activated. For T-cells to become activated they must form an interaction through the TCR and a peptide containing MHC[71,72,77]. Notably, the TCR is not activated upon interaction with its cognate peptide or MHC proteins alone; they must both be found in complex[71]. T-cell activation is broken down into three signals (Figure 1.3). The first signal occurs when the T-cell recognizes, through the TCR, its cognate peptide presented in an MHC (Figure 1.3). The initial activation of T-cells results in the CD4 or CD8 molecules then binding the MHC to stabilize the complex[72,77]. Signal two includes a variety of protein interactions that lead to further activation and clonal expansion of activated T-cells (Figure 1.3)[72,77]. In the case of CD4+ T-cells, CD28 activation by B7.1(CD80) or B7.2(CD86) found on the APC is critical in initiating proliferation[72,77]. To control T-cell expansion CTLA-4(CD152) is produced to compete for CD28 interaction, thereby slowing the expansion of T-cells[72,77]. CD8+ T-cells also rely on CD28 signaling though to a lesser extent, other costimulatory molecules which are used by CD8+ T-cells are CD70 and 4-1BB(CD137)[72,77]. To generate a strong response, pro-survival signals are given to T-cells at this stage through ICOS, 4-1BB, and OX40[72,77]. If co-stimulation does not occur

at this stage, the T-cells become inactive again without replicating, thereby preventing aberrant activation[72,77]. Once a T-cell has become active, it is then able to induce an immune attack the next time it encounters its antigen without the need for further co-stimulation. Signal three occurs once the activated T-cell encounters cytokines, this often occurs at the site of infection or tissue damage (Figure 1.3)[72,77]. These cytokines shape the activity of the T-cell by polarizing it to best response for the type of damage detected[72,77,78]. For further discussions on T-cell polarization see section 1.2.3.

As discussed above, T-cells are activated through interactions with MHC proteins which are divided into two main classes. Class I are present in all nucleated cells and present peptides from within the cell[71,72]. Class II presents internalized and proteolytically digested peptides within specialized APCs[71,72]. Since MHC class I cells present endogenous peptides (note, exogenous presentation can also occur for more information see cited source), they are particularly important for protection from intracellular pathogens[71,72,79]. Cells infected with intracellular pathogens present proteolytically processed peptides on MHC class I proteins[71,72,80]. Upon recognition of the MHC class I and it's cognate peptide, CD8+ T-cells are activated secrete perforin and granzyme which kills the infected cell and, in many circumstances, the infecting pathogen[71,72]. In contrast, MHC class II proteins are primarily expressed on "professional" APCs such as macrophages, dendritic cells, and B-cells[71,72,80]. During inflammation epithelial and endothelial cells can also express MHC class II[71]. CD4 T-cells recognize MHC class II-presented antigens. Once CD4+ T-cells are activated, the cytokines secreted by the APC or present at the site of infection or tissue damage impacts their maturation and the cytokines that will be produced by the T-cell. Both CD4+ and CD8+ T-cells can be categorized into subtypes that have different functions[78]. CD4+ T-cells in particular have various subpopulations characterized by their secreted

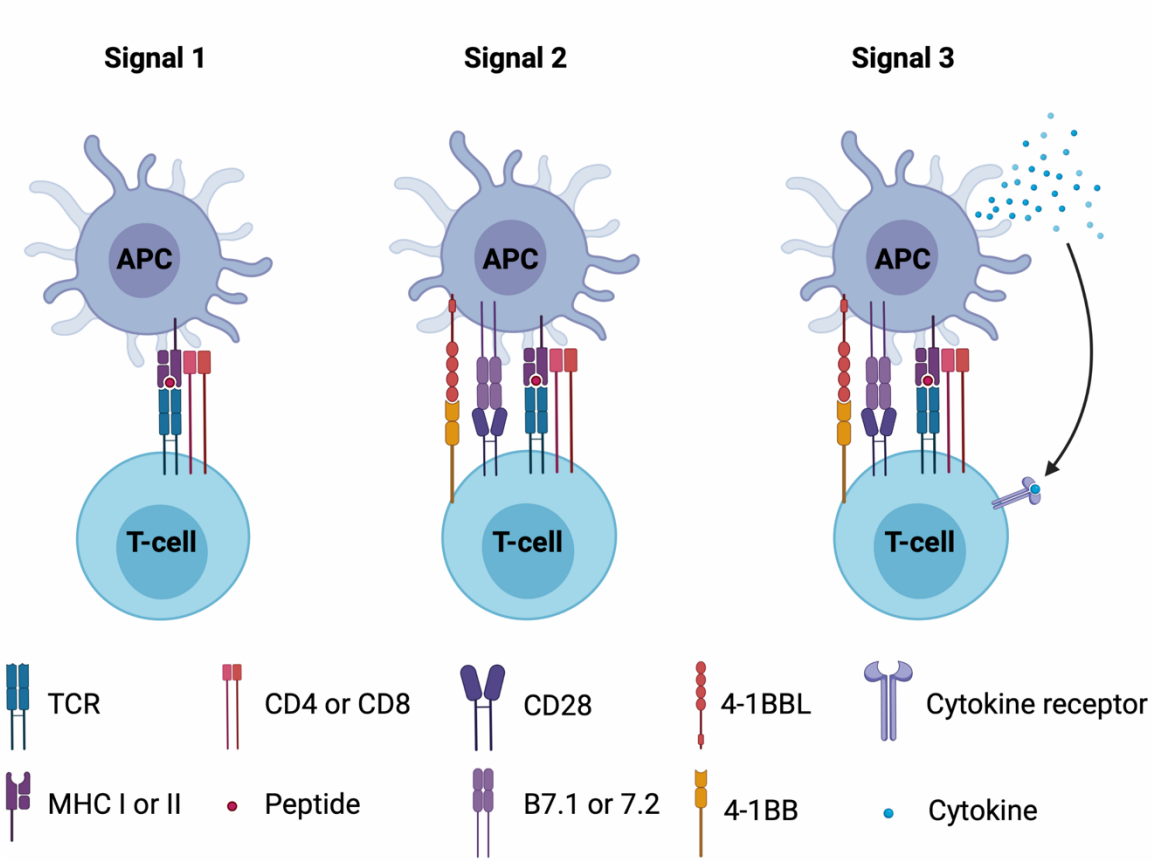

Figure 1.3 Signals 1, 2, and 3 in T-cell activation The first signal in activation of T-cells comes when the TCR interacts with an MHC molecule containing it's cognate peptide. This interaction is stabilized by CD4 or CD8. Co-stimulatory signal occurs inducing survival and proliferation. Many co-stimulatory proteins exist, shown here are CD28 and 4-1BB and their respective ligands. The presence of tissue damage or pathogen associated cytokines is signal three. The presence of these cytokines polarizes T-cells to have functions suited for the elimination of the pathogen they respond to. Created with BioRender.com

cytokine profiles[78]. Polarization into each subtype is largely dependent on the cytokines present upon initial activation of the T-cell. Once polarized, T-cells secrete cytokines which are important for instructing other aspects of the immune response[78]. One consequence of T-cell polarization is the induction of antibody class switching by B-cells. Further discussion into certain immune polarizations is described in the next section.

Production of antibodies is driven by B-cell activation. Several types, called isotypes, of antibodies may be produced by B-cells. These isotypes in mice are IgM, IgD, IgG (which is found in isotypes IgG1, IgG2a/c, IgG2b, and IgG3), IgA, and IgE[71,72,81]. Humans have similar antibody isotypes, though functions are not necessarily homologous between mouse and human antibodies of the same isotype. Some differences between human and mouse isotypes include human IgA antibodies having the subclasses IgA1 and IgA2, and IgG being split into IgG1, IgG2, IgG3, and IgG4 in humans (all further antibody discussion focuses on mouse antibodies)[81]. These different isotypes are structurally and functionally distinct, for example secreted IgM is pentameric and cannot cross into mucosa[72,81]. In contrast, IgA antibodies are found as monomers or dimers and are capable of crossing into mucosa when dimerized[72,81]. Naïve B-cells generally produce antibodies of isotypes IgM and IgD. Once activated isotype switching and somatic hypermutation of the variable region, the epitope sensing region of antibodies, occur. This allows for selection of higher affinity antibodies and production of antibodies in isotypes that have functions useful in the response against a pathogen[71,72,81]. The process by which a B-cell is activated and matures is the determinant of antibody isotype produced after class switching.

Activation of B-cells leads to class switching and production and secretion of antibodies. B-cell activation may occur in a T-cell dependent or independent manner. In T-cell dependent activation, a B-cell binds its cognate antigen by the BCR[71]. The antigen

is then internalized and degraded for presentation on MHC class II proteins[71]. In this way B-cells can act as antigen presenting cells which activate CD4+ T-cells. Activation of B-cells occurs in lymphoid organs in highly organized structures called germinal centers[72,82]. In these structures B-cells interact primarily with a specialized subset of CD4+ T-cells called follicular helper cells (Tfh)[82]. Upon recognition of the antigen by a Tfh cell's TCR, CD40 on the B-cell interacts with its ligand found on the T-cell and cytokines secreted by the T-cell lead to activation of the B-cell and rapid clonal expansion[71,72]. Cytokines secreted by the Tfhs induce and control isotype switching. Of particular importance for later discussions, the cytokines interleukin (IL)-4 and IFNγ are associated with the induction of IgG1 and IgG2c, respectively, in C57/BL6 mice[71,72,78,81]. Once B-cells are activated, a subset of cells differentiates into short-lived plasma cells which produce large amounts of antibodies before their eventual death some weeks after differentiation[83]. Another subset of cells undergoes affinity maturation, then differentiates into either memory B-cells or long-lived plasma cells[83]. Memory B-cells generally remain dormant until their cognate antigen is encountered again, allowing for rapid response and increased antibody production[83]. Long-lived plasma cells reside in the bone marrow and constantly secrete their antibody providing passive immunity from further infection by its cognate pathogen[83]. Long-lived plasma cells can survive and continue producing antibodies for decades.

Antibodies have several mechanisms of action during an infection. Most antibody isotypes can neutralize a pathogen preventing further infection or virulence (Note that neutralization is dependent not on antibody isotype but where on the immunogen the antibody binds to)[71,72,81]. In viruses for example, neutralization occurs when an antibody binds to the virus's glycoprotein. This prevents the virus from infecting a cell by either blocking recognition of the virus receptor or preventing conformational changes

necessary for viral fusion. Another function important during viral infections is antibody dependent cellular cytotoxicity (ADCC) where antibodies bind to antigens on the surface of infected cells and recruit natural killer cells or CD8+ T-cells to kill the infected cell[71,72,81,84]. Various antibody isotypes can activate the complement pathway leading to complement-mediated cytotoxicity[81,84]. Finally, another important function of some antibodies is the ability to drive opsonization[81,84]. Opsonization occurs when an antibody binds it's cognate antigen, the constant region of the antibody (Fc) is then bound by an Fc receptor in a phagocytic cell (such as a macrophage) inducing phagocytosis[81,84]. Taken together, the role of B-cells is to produce antibodies, the repertoire of secreted antibodies provides passive protection from pathogens and aids in directing other cellular responses.

1.2.3 Type 1 vs Type 2 Immunity

T-cell polarization can lead to different immune functions. Type 1 and type 2 immune responses are often discussed in vaccination due to their differing abilities to provide protection against various pathogen types. It is accepted that a type 1 response is important for clearance of intracellular pathogens and that type 2 immune responses are more suited in directing responses to inflammation caused by large extracellular pathogens and allergic responses (Figure 1.4)[78,85]. When developing vaccines, it is important to drive a response best suited to the targeted pathogen.

Type 1 immunity is important for clearance of intracellular pathogens. Cytokines that drive skewing towards type 1 immunity are the type 1 IFNs, IFNγ, and IL-12 (Figure 1.4)[78,85]. These cytokines are initially produced during innate immune responses in infected cells that have detected a PAMP from an infecting pathogen. CD4+ T-cells activated in the presence of IFNγ, and IL-12 cytokines become polarized to Th1 cells[78,85]. Once polarized, Th1 cells start secreting pro-inflammatory cytokines, including IFNγ, IL-

2, and tumor necrosis factor (TNF) (Figure 1.4)[78,85]. These cytokines upregulate antiviral and intracellular pathogen sensing pathways such as PRRs and lead to increased expression of MHC proteins in some cell types thereby allowing further detection of pathogens by T-cells[71,72]. Secreted cytokines also impact other immune cells such as dendritic cells by instructing them to increase phagocytosis[71,72]. Further effects are seen with the dilation and increased leakiness of blood vessels, allowing innate immune cells to access areas of inflammation more easily[78,85]. CD8+ T-cells are also polarized by IFNs and IL-12[71]. Type I CD8+ T-cells act in a similar manner as Th1 cells by secreting the pro-inflammatory cytokines IFNγ, IL-2, and TNF[71]. In addition to cytokine secretion CD8+ T-cells also kill infected cells by ADCC or releasing perforin and granzymes when interacting with MHC class I proteins presenting its cognate antigen[71,72]. Perforin forms pores in the target cell's plasma membrane allowing for the entry of granzyme which leads to cell death[72].

The skewing of an immune response by Th1 cells also has an impact on humoral immunity. As previously discussed, naïve B-cells express antibodies of the isotypes IgM and IgD. IgM antibodies offer high avidity with its pentameric structure but lacks high affinity which is achieved by somatic hypermutation after class switching. Tfh cells, which can be skewed to aTh1 or Th2 profile, interact with B-cells and the secreted IFNγ drives class switching to IgG2c (in C57/BL6 mice, in other mouse strains IgG2a may be induced by IFNγ) (Figure 1.4)[81,86]. IgG2c is important during intracellular infections by efficiently activating the complement pathway and having a high affinity for Fc receptors capable of mediating opsonization and ADCC[85]. Together, the various arms of the immune system regulated by type 1 immune polarization can effectively control many intracellular pathogens.

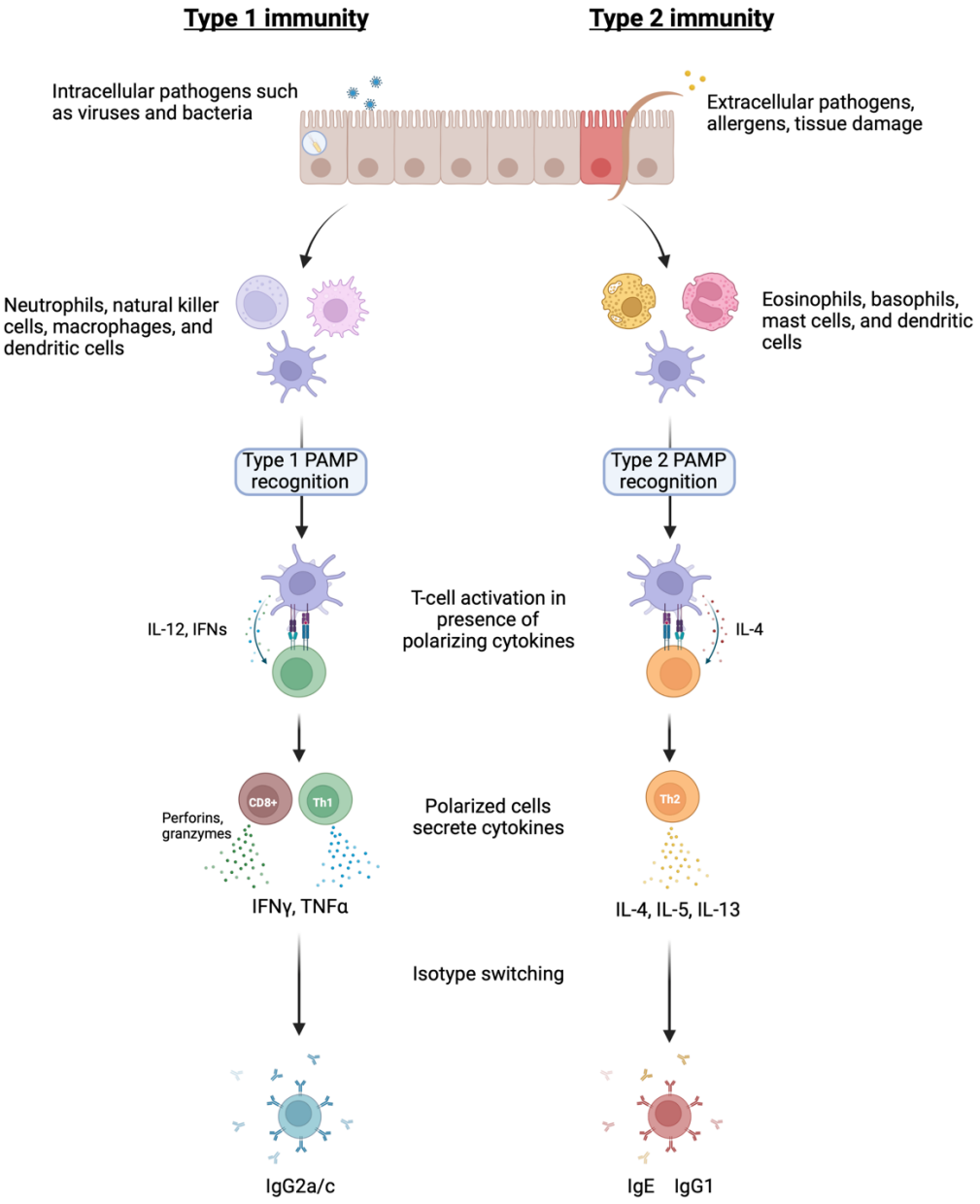

Figure 1.4 Type 1 vs Type 2 Immunity Type 1 immunity is associated with intracellular pathogens such as viruses and bacteria. Type 2 immune responses detect large extracellular pathogens and are associated with tissue damage as well as allergic responses. Different subsets of innate immune cells respond to these infections and recognize PAMPs from the infecting pathogen. This induces APCs to produce cytokines (signal 3) to polarize T-cells. Th1 polarization stimulates CD8+ T-cells and Th1 cells to produce IFN, TNF and other cytokines. Th2 cells produce IL-4, IL-5 and other cytokines. These cytokines when secreted by polarized Tfh cells lead to isotype switching, IgG2a/c in type 1 and IgE and IgG1 in type 2 immunity. Created with BioRender.com

In contrast to type 1 immunity, type 2 immunity is characterized by effective responses against extracellular pathogens and for causing allergies when overstimulated (Figure 1.4)[71,85]. The cytokine responsible for activating a type 2 response is IL-4, which is associated with tissue damage and repair[85]. Type 2 immunity polarized CD4+ T-cells are called Th2 and secrete a wide variety of anti-inflammatory cytokines such as IL-4, IL-5, IL-10, IL-13, and GM-CSF[85]. Th2 immunity is primarily characterized by activation and increases in numbers of eosinophils, basophils, and mast cells (Figure 1.4)[71,85]. Th2 responses lead to tissue infiltration by eosinophils and basophils as well as mast cell degranulation. In some situations, an overabundance of the Th2 cytokines is known to cause allergies and the airway inflammation seen in asthma. Polarization of CD8+ T-cells to a type 2 profile also occurs. In these cases, CD8+ cells can produce IL-4 and IL-5 and cytotoxicity of these cells by perforin and granzyme is decreased[71].

As in type 1 responses, Th2 polarized Tfhs interact with B-cells to induce class switching[78,85]. The primary isotype induced by B-cell interaction with Th2 cells is IgE (Figure 1.4)[78,85]. Class switching to IgE cannot occur without the presence of IL-4 or IL-13. Degranulation of mast cells is associated with cross-linking of IgE, a process associated with allergic reactions[71]. The presence of type 2 cytokines also drives some IgG antibodies, primarily IgG1 (Figure 1.4)[85]. IgG1 antibodies activate complement weakly and only bind inhibitory Fc receptors. Due to an inability to drive ADCC or activate complement, IgG1 is not suited for elimination of intracellular pathogens[85].

The polarization of the immune response is necessary for the effective clearance of pathogens. BALB/c mice, which favor a type 2 response, are particularly susceptible to infection by the intracellular parasite *Leishmania,* which is more effectively controlled by type 1 responses[87]. Incorrect immune polarization is well described to worsen

infections with respiratory viruses in humans and small animal models[88-90]. In these infections, patients tend to have increased mucus production which leads to worsened disease. The initial polarization of the immune response is thus clinically important in determining infection outcomes. This is particularly important in the design of vaccines as Th1 and Th2 cells counteract each other[72]. The presence of IFNγ is inhibitory of Th2 cells and conversely IL-4 is inhibitory of Th1s[72]. Therefore, initial polarization is likely to be reinforced and not shift over time. If a vaccine skews the response to the wrong polarization, it could not only impact the effectiveness of the vaccine but patient safety. This has been previously demonstrated in vaccines against respiratory syncytial virus, these vaccines drove a strong Th2 immune polarization[88-90]. This led to vaccine associated enhancement of disease making RSV infections worse. This highlights the importance of immune polarization in the development of vaccines and the need for vaccine platforms that are able to efficiently skew polarization to a desired profile.

1.2.4 Recombinant Vesicular Stomatitis Virus Vaccines

One of the more recently approved vaccine platforms, recombinant VSV (rVSV) presents a strong candidate in eliciting potent immune responses against viral glycoproteins. VSV is a member of the *Rhabdoviridae* family. It is a negative sense, single stranded RNA virus with a simple genomic structure comprised of 5 genes. Commonly seen as a livestock pathogen, VSV has low sero-prevalence in humans and causes no known disease to immunocompetent individuals[91,92]. Researchers have used VSV as a backbone to study glycoproteins of highly pathogenic viruses due to tractable genetic systems and VSVs ability to incorporate foreign glycoproteins into its virion[93-95]. Most rVSVs are designed with the removal of the wild-type glycoprotein (G) and addition of a foreign glycoprotein[95]. The use of rVSV as a vaccine platform against viruses has

several advantages such as the efficient elicitation of a type 1 immune response, and the ease of producing large amounts of vaccine virus.

Currently the Ebola virus vaccine is the only FDA approved rVSV vaccine[96]. In the 2016 outbreak of Ebola virus (EBOV) an rVSV-EBOV vaccine was deployed and shown to give strong protection in ring studies[97]. While the vaccine did have strong side effects including arthralgia, fatigue, and myalgia, this deployment successfully demonstrated the capabilities of rVSV vectored vaccines[97]. Despite the rVSV-EBOV vaccine being the only currently approved rVSV-based vaccine, researchers have developed and tested rVSV vaccines for a wide variety of viral diseases[94,98-102]. Current progress in the field has demonstrated that rVSV can elicit immune responses when expressing the glycoproteins of Lassa fever virus, Marburg virus, various species of Ebola virus, Zika virus, and more recently SARS-CoV-2[94,98-102]. Another benefit of rVSV vaccines is the ability to elicit rapid immunity. In non-human primate studies using an rVSV expressing Marburg virus glycoprotein (rVSV-MARV), it was demonstrated that protection upon vaccination can occur as soon as 3 days post-vaccination. Macaques vaccinated with rVSV-MARV had 100% survival when challenged 7- or 14-days post-immunization[103]. Impressively, even when challenged 3 days post-immunization 75% of animals were protected from lethal challenge[103]. Other studies with rVSV-MARV have shown that post-exposure vaccination in rhesus macaques is fully protective when administered 30 minutes post lethal challenge, and partially protective (83% of animals survived) when administered 24 hours after challenge[104,105]. The rapid protective responses elicited by rVSV vaccines make this platform powerful for the development of vaccines against laboratory pathogens where vaccination post-needle-stick could provide protection and useful in the context of controlling rapidly spreading epidemics and pandemics.

The immune response elicited by rVSV vaccines is favorable for immunization against viral pathogens. Infection with VSV elicits high levels of IFNs which drive a type 1 immune response as expected in a viral infection[95,102,106,107]. This skewing ensures that rVSV elicits the appropriate immune response for protection from viral diseases. Vaccinations with rVSV elicit both high levels of antibodies and T-cell responses[95,102,106,107]. Many studies have demonstrated that rVSV based vaccines are particularly capable of eliciting high levels of neutralizing antibodies which are often sufficient for protection in passive transfer models.

A major consideration in the use of vaccines to control epidemics and pandemics is the ability to deliver vaccines to rural and hard to reach areas, and the necessity for cold chains. The effective roll-out of the rVSV-EBOV vaccine in Africa demonstrated that rVSV vaccines can be delivered in rural areas. Additionally, research shows that rVSV can be lyophilized or dried and retain infectivity and immunogenicity[108]. This represents a mechanism to eliminate the cold chain in delivery of rVSV based vaccines.

Two often cited downsides of rVSV vaccination are the neuropathologic effects of VSV and the potential for platform immunity[66,109,110]. As a member of the *Rhabdoviridae* family, VSV is closely related to the highly neuropathic rabies virus. Due to this and the neuropathogenicity of VSV itself it is important to verify rVSV vaccines are safe. In wild type VSV, neuropathogenicity has been conclusively linked to the virus's glycoprotein[109,110]. Therefore, most rVSV based vaccines have removed the innate risk of pathogenicity from VSV with a deletion of the wild type VSV glycoprotein. However, it is then important to consider the potential neuropathic effects of the newly inserted glycoproteins. It has been shown that addition of certain viral glycoproteins into the VSV genome leads to gain of neuropathogenesis[98]. In the development of novel rVSV based

vaccines it is thus important to ensure that vaccine candidates do not cause neurologic symptoms.

The concern of platform immunity is also an important consideration. Upon vaccination with rVSV, immune responses develop not just to the foreign glycoprotein but to the other viral proteins such as nucleoprotein and polymerase[111]. T-cell immunity against these proteins has the potential to inhibit infection with following rVSV based vaccines thereby leading to a less potent immune response that may not be protective. Studies have shown that despite the potential for platform immunity, booster shots and subsequent heterologous vaccinations are still effective[94,112]. Overall, studies have demonstrated that vaccination against differing pathogens as well as booster doses of rVSV are effective at eliciting immune responses to the foreign glycoprotein and provide protection. These studies suggest that rVSVs are a powerful vaccine platform to deliver viral antigens but must be thoroughly verified for safety.

1.2.5 mRNA Vaccines

The most recently approved vaccine platform is the mRNA platform. In light of the SARS-CoV-2 pandemic (often referred to as coronavirus disease 2019, COVID19) the FDA granted emergency use authorization to two mRNA vaccines in 2020, both of which have since been given full FDA approval[113,114]. Despite only being recently approved, mRNA vaccines have been in development for decades[69,115]. These vaccines are safe, modular, and efficacious with an ability to induce high levels of antibodies. The mRNA vaccine platform also has downsides that have prevented earlier use, these include the instability of mRNA, considerations of delivery, and previous issues with overstimulation of the immune system[69,115,116]. After decades of research and innovation, many of these concerns have now been addressed to achieve a powerful vaccine platform.

mRNA vaccines mimic endogenous mRNA with a few modifications. Like endogenous mRNA, mRNA vaccines encode a molecule with a 5' cap, 5' untranslated region (UTR), open reading frame (ORF), 3' UTR, and a polyA tail[69,115]. In brief, the 5' cap is key in preventing recognition of the mRNA by cytosolic RNA sensors[69,115]. Without this cap, the mRNA may be detected as viral and initiate innate immune responses. The UTRs are important determinants of stability and half-life of mRNAs[69,115]. The polyA tail together with the 5' cap is critical for translation initialization and tail length is a determinant of half-life[117]. The ORF is critical as this section encodes the desired immunogen. To design an ORF, you must first select a protein that will be a suitable immunogen at driving protective immune responses. The coding sequence of the desired antigen must be known, due to advances in sequencing technologies, rapid sequencing of novel pathogens is possible allowing for fast development of mRNA vaccines. Modifications to the ORF are critical in optimizing immunogenicity of mRNA vaccines. Some pathogens use rarely used codons in their genomic sequence, modifying these to more commonly used codons is often done to increase efficiency of translation[115]. The use of modified nucleosides is also critical in mRNA vaccines. Unmodified nucleosides are markers of viral infections and elicit innate immune responses shutting down protein production, thus modified nucleosides are often included in mRNA vaccines[118,119]. Once delivered to a cell, mRNA vaccines function similarly to endogenous mRNA and lead to production of the coded immunogen and an eventual targeted immune response against the delivered immunogen.

RNA delivery and production are critical processes to consider when designing mRNA vaccines capable of generating effective immune responses. DNA plasmids are used as templates in the production of mRNA[69,115]. Plasmids encoding mRNAs allow for the rapid production of vaccines at scale with relative ease. The primary concern when

preparing mRNA vaccines is the presence of double stranded (ds)RNA, a production contaminant[120]. Many PRRs can detect the presence of dsRNA and initiate immune responses preventing translation of the encoded antigen[69]. Additionally, exogenous mRNA can also be detected by PRRs in endosomes, initiating antiviral pathways and thereby preventing effective immunization[121]. To address these issues, thorough dsRNA decontamination is done on synthesized mRNA by chromatographic methods prior to delivery[120]. To ensure intracellular delivery of mRNA, several strategies have been developed. The most widely used strategy is currently lipid nanoparticles (LNPs)[69,115]. LNPs resemble commonly used lab reagents such as Lipofectamines that are used to transfect cells *in vitro*. Clinically used LNPs are specially formulated to deliver mRNA into cells *in vivo* and contain four components: an ionizable cationic lipid, cholesterol, a lipid-linked polyethylene glycol (PEG), and phospholipids[69,115]. Briefly, ionizable cationic lipids are important in the production of LNPs as they promote assembly of the particle, their positive charge attracts negatively charged mRNA for inclusion in particles. Ionizable cationic lipids are preferable to non-ionizable cationic lipids as the latter are immunostimulatory[122,123]. Ionizable cationic lipids used are neutral at blood pH but positively charged in acidic environments such as endosomes allowing for fusion and delivery of mRNAs into the cytoplasm. Cholesterol helps stabilize the lipid structures. Lipid-linked PEG increases the half-life of LNP particles and regulates particle size. Finally, phospholipids are key for formation of a lipid bi-layer and modulate membrane fluidity which aids in fusion at endosomes. Together, these developments in mRNA production and delivery have made the mRNA platform viable for use in humans.

Vaccination with mRNA vaccines induce strong immune responses characterized by robust antibody responses[69,115]. Recent work has indicated that mRNA vaccines induce IFNs which skew immune responses towards type 1 immunity[124-126]. CD4+ T-cells

are stimulated to high levels and tend to be strongly Th1 polarized. Studies have shown that mRNA vaccines are powerful inducers of Tfhs which are critical in controlling B cell responses and inducing class switching[124-126]. Recent studies have shown that Tfh cell polarization can be dependent on the antigen delivered[124]. Some antigens drive a strong Th1 polarized response while other antigens can drive a mixed Th1/Th2 response[124,127]. mRNA vaccines are widely lauded for their ability to induce high antibody titers which remain stable for extended periods of time[128,129]. This is likely due to the strong induction of Tfh cells and long-lasting germinal centers[124]. Due to the propensity of some antigens to drive a mixed Th1/Th2 response, it has been noted that antibodies produced upon vaccination likewise can have a mixed IgG1/IgG2 phenotype[124,127]. This mixed phenotype has been posited as a potential benefit as IL-4 (a Th2 cytokine) is associated with increased B-cell survival[127]. Notably, mRNA studies have not noted a strong Th2 response and thus there have been no reports of vaccine-induced enhancement of infection as is common with some respiratory viruses[127]. The ability of mRNA to induce CD8+ T-cells remains unclear. In mice, it has been shown that powerful CD8+ responses are elicited upon vaccination with SARS-CoV-2 mRNA vaccines[125]. However, macaques immunized with mRNA vaccines against SARS-CoV-2 had no or poor induction of CD8+ immunity except when high dosages were used[130]. In humans, few studies have broken down T-cell responses into CD4+ and CD8+ subsets. Therefore, it remains largely unclear what CD8+ responses are elicited in humans, though early studies suggest that mRNA can in fact induce CD8+ T-cells. Overall, mRNA vaccines elicit powerful immune responses well suited to protect against viral infections.

Despite improved mRNA vaccine technologies, these vaccines are still not widely adopted and continue to be challenging in rural settings. The first approval of a novel vaccine platform is often the most difficult due to the need to meet a high bar of safety.

Due to the high efficacy of the COVID19 mRNA vaccines, it is likely that there will be an increased prevalence of mRNA vaccines in the coming years. Indeed, many mRNA vaccines to other pathogens including Zika virus, respiratory syncytial virus, and influenza are already in clinical trials[69,115,131-135].

Despite the widely understood efficacy and safety of mRNA vaccines by scientists, certain populations of the public remain skeptical of mRNA vaccines. Nucleic acid vaccines are misunderstood by the general public with a common misconception that mRNA can alter a person's genes. Further public education is thus required to highlight that mRNA is non-integrative and is incapable of genetic modification. Another currently problematic property of mRNA is its instability and requirement for storage at -80° C. Delivery to hard-to-reach areas is currently difficult or impossible due to the difficulty of maintaining this cold chain. Taken together, mRNA is a powerful vaccine platform that is safe and efficacious and will likely see increased use in the coming years.

1.2.6 Heterologous Vaccination Strategies

Heterologous vaccination, in the context of this work, refers to prime-boost vaccination strategies where the first and second dose vaccines utilize different platforms. Heterologous vaccinations have been studied for decades and the data consistently shows that they can have beneficial effects on the immune response. As previously described, different vaccine platforms have benefits and drawbacks in their abilities to elicit specific immune responses. Heterologous vaccination offers the potential for combining the benefits of each platform to attain a more well-rounded response. Some of the first heterologous vaccine studies focused on human immunodeficiency virus (HIV) vaccines where it was observed that immunization with recombinant proteins elicited potent antibody responses but weak cellular responses[136].

Conversely, immunization with recombinant vaccinia viruses expressing HIV proteins induced strong T-cell responses but low antibody titers[136]. These initial studies found that combining these two platforms in a prime-boost model induced both strong antibody and cellular immunity. The responses generated by heterologous immunization was stronger than that of homologous prime boosts indicating that heterologous vaccinations may be a useful vaccine regimen.

No currently approved vaccines have been designed for heterologous use, however, during the COVID19 pandemic several governments around the world have approved heterologous boosting with emergency use authorized vaccines[137]. Studies around the world using either adenovirus based or mRNA based SARS-CoV-2 vaccines have found that heterologous vaccination provides better protection and induces better immune responses. In a Swedish study comparing heterologous vaccination (adenovirus prime and mRNA boost) to adenovirus homologous vaccination, it was found that heterologous vaccination was 68% effective compared to 50% for homologous immunization[138]. These findings are corroborated in other studies that measured cellular and humoral responses in homologous and heterologous vaccinated individuals[139-141]. One such study from Hannover found that heterologous vaccination induced significantly higher neutralizing antibody titers and T-cell activity than homologous vaccination[139]. In mice, it has also been shown that heterologous vaccination (using adenovirus and self-amplifying RNA platforms) induced stronger humoral and cellular immune responses than homologous vaccination[142]. While most studies show that differences exist between heterologous immunization and homologous adenovirus-vectored regimens, these differences are less pronounced when comparing heterologous immunization and homologous immunization with mRNA[140,141]. Two studies in humans show that homologous mRNA regimens induce higher levels of antibodies than heterologous

immunization using adenovirus and mRNA platforms[140,141]. Conversely, cellular responses are significantly better after heterologous vaccination[140,141]. Overall, current data on SARS-CoV-2 heterologous mRNA vaccination suggests that immune responses are superior or identical to homologous vaccination.

Heterologous vaccination using the rVSV platform has been done in the context of HIV. These studies often combined a DNA vaccine platform prime with rVSV boosts. One such study demonstrated that immunization with a plasmid encoding simian HIV (SHIV) SIVgagp39 and IL-12 followed by a boost with an rVSV expressing env89.6P gp160 and SIVmac239 gag p55 had improved immunogenicity and efficacy upon challenge[143]. This was associated with preserved CD4+ cells in the peripheral blood, and increased antibody titers[143]. Heterologous vaccination represents a clearly understudied area with few pathogens having had experimental heterologous vaccines tested, immune responses remain not fully characterized, and few vaccine platform combinations have been tested.

1.3 – Current SFTSV Vaccine Candidates

Currently, no approved SFTSV vaccines exist. Several groups have developed experimental SFTSV vaccines and tested for efficacy and immunogenicity in *Ifnar*$^{-/-}$ mice or aged ferrets. A variety of vaccine platforms have been used to immunize animals, these include DNA, protein subunit, and viral vectored vaccines. Most vaccines target the SFTSV glycoprotein Gn/Gc, though some also target other viral proteins such as N and NSs. The currently tested vaccines and characteristics of their responses are discussed below.

1.3.1 DNA Vaccines

A DNA vaccine encoding SFTSV Gn, Gc, N, and NSs elicited weak immune responses[45]. In this study, Kang et al. immunized *Ifnar*$^{-/-}$ mice with a single plasmid

encoding several SFTSV proteins (Gn, Gc, N, and NSs)[45]. In this study, no antibody responses were detected in vaccinated mice and only slight, not statistically significant, increases were observed in T-cell activity. To elicit cellular responses, the authors added IL-12 to the vaccine plasmid[45]. In mice immunized with the IL-12 containing plasmid, antibodies were detected against SFTSV N, but no antibodies against Gn or Gc were detected[45]. This suggests that immunization with this DNA vaccine would not lead to antibody mediated neutralization of viruses, as Gn/Gc are the only viral proteins found on the virion surface. Addition of IL-12 led to increased cellular responses as the authors predicted[45]. The original plasmid protected only 40% of animals from lethal challenge with SFTSV, while the IL-12 containing vector was 100% protective[45]. The lack of high titer Gn/Gc antibodies indicates that protection was mediated by T-cells[45]. Overall, this vaccine platform did provide protective immunity, though it failed to stimulate antibody responses. Additionally, DNA vaccines are currently not approved for human use and are problematic due to complications in delivery methods.

Another group tested a different DNA vaccine consisting of 5 plasmids encoding SFTSV Gn, Gc, N, NSs, or RdRP[144]. This vaccine was initially tested in wild type BALB/c mice with two doses given 21 days apart, each dose was given with electroporation to induce more efficient take up of the plasmids. This vaccination protocol elicited potent cellular immunity and modest levels of neutralizing antibodies[144]. To test whether this DNA vaccine was protective, 3 doses were given to aged ferrets which were then challenged with a lethal dose of SFTSV. Immunized animals were fully protected and displayed strong cellular immunity particularly to the glycoproteins, Gn and Gc[144]. As with the mouse model, vaccination elicited modest levels of neutralization. Experiments using only plasmids encoding only SFTSV Gn and Gc demonstrated that these antigens are sufficient for protection from lethal challenge. Furthermore, serum transfer from mice

immunized against Gn and Gc to naïve mice was protective upon lethal challenge[144]. Immunization with a combination of plasmids excluding Gn and Gc showed that full protection occurred only when animals were immunized with all three plasmids containing N, NSs, and RdRP[144]. Importantly, passive transfer of sera from these animals to naïve ferrets did not impart protection. Several important conclusions can be made from the data shown in this paper, the first being that SFTSV Gn and Gc are sufficient to impart protection from lethal challenge. The second important finding of this paper addresses the correlates of protection in SFTSV infection. Protection upon challenge in mice that received passively transferred sera suggests that antibodies against SFTSV are sufficient to prevent lethal disease. This protection is even more impressive given the modest levels of neutralizing antibodies reached in vaccinated mice. Additionally, full protection achieved by immunizing animals against N, NSs, and RdRp suggests that cellular immunity may also be sufficient for protection, though this is not directly tested. Therefore, future vaccine development against SFTSV can focus primarily on antibody responses as this is sufficient for protection, but the presence of cellular immunity is likely to provide a more thorough response that could be beneficial. The downside of this vaccination platform is the requirement for electroporation which is often painful. Despite that drawback, this SFTSV vaccine is quite promising.

1.3.2 Protein Subunit Vaccines

Only one study to date has investigated the immunogenicity of protein subunit vaccines against SFTSV. This study fused SFTSV Gn or Gc to the Fc region of a human antibody and immunized mice with each of these proteins using the adjuvant Alum[45]. As expected from protein subunit vaccines, antibody titers in immunized animals reached impressive levels of approximately 3,500 for both protein vaccines[45]. Interestingly, neutralizing titers were higher in Gn immunized mice (mean titer = 929) than Gc

immunized mice (mean titer = 209) [45]. Total antibody titers were identical in both vaccines, yet Gn produced higher levels of neutralizing antibodies suggesting the Gn subunit may be a better target for vaccine development. Alternatively, this result may be due to expression of these proteins individually as opposed to in complex as it is naturally found in SFTSV infections. Expression of the proteins individually may lead to Gn neutralizing epitopes being maintained, while misfolding or a lack of Gc-Gn interactions leads to a loss of Gc neutralizing epitopes. When animals were challenged with a lethal dose of SFTSV, all of the Gc immunized mice succumbed, with deaths delayed 3 days compared to unimmunized mice, and half of the Gn immunized mice succumbed to infection[45]. On the surface, these results seem to conflict with results from other groups who have demonstrated that antibodies are sufficient for protection and antibody titers elicited by these protein vaccines are higher than any currently discussed vaccine has achieved. The explanation for this incongruency may be two-fold: protein subunit vaccines are known to be poor inducers of cellular immunity, therefore immunized mice likely did not have high levels of CD8+ T-cell activity. Additionally, Alum is well described to drive a type 2 skewed immune response[145]. Therefore, antibodies produced by this immunization are unlikely to drive opsonization, ADCC, or activation of complement pathways effective for controlling viral infections. Cellular responses are also unlikely to be optimized for responding to intracellular pathogens resulting in a lack of protection. Overall, protein subunit vaccination against SFTSV appears to be an ineffective platform.

1.3.3 Viral vectored vaccines

To date, three different studies have reported SFTSV immunization using viral vectors. The virus vectors that have been studied so far include vaccinia virus, rabies virus, and rVSV. The study with vaccinia virus used three different viruses for

immunizations in a two-dose regimen, each virus was an LC16m8 strain of vaccinia virus encoding either SFTSV N, Gn/Gc, or both N and Gn/Gc[146]. Immunization with either virus encoding Gn/Gc led to high levels of total IgG (approximate titer of 4,000) but incredibly low neutralizing antibody titers with serum at a dilution of 1:40 not neutralizing 50% of virus particles[146]. Despite low levels of antibodies these vaccines all were fully protective against lethal challenge. Interestingly, these investigators determined that pre-existing immunity to vaccinia virus inhibited protective responses. *Ifnar*$^{-/-}$ mice were first inoculated with the vaccinia lister strain, then vaccinated one month later with each of the three vaccines in a two-dose regimen. In this experiment, mice were only partially protected from lethal challenge indicating that pre-existing immunity to vaccinia virus negatively impacts immunization efficacy[146]. In this study, serum transfer only partially protected against SFTSV disease. This was despite a two-dose serum transfer and a low dose SFTSV challenge, further highlighting the weak neutralizing activity elicited by this vaccine. To assess CD8+ contributions to protection, immunized mice were depleted of their CD8+ T-cells through anti-CD8 antibodies. No change in lethality was observed which is interpreted by the authors as CD8+ T-cells being unnecessary for protection from SFTSV infection[146]. This interpretation however is confounded since the challenge was done in immunized mice which had previously been shown to have high IgG titers against SFTSV. The authors had previously shown that antibodies alone did provide partial protection in a passive transfer model. Additionally, vaccinia virus is known to induce robust type 1 immunity meaning antibodies present in these mice likely neutralized low amounts of virus and carried out other protective functions such as increased opsonization, activation of the complement pathway, and increased cytotoxicity by natural killer cells through ADCC[147,148]. A better model to assess CD8+ T-cell contribution to infection would be an adoptive transfer which would exclude all

antibody mediated immunity. Overall, the vaccinia virus SFTSV vaccine does induce protective immunity though neutralizing antibodies are of relatively low titer. Additionally, this vaccine platform may not be ideal as the authors demonstrated decreased efficacy when pre-existing immunity to vaccinia virus existed. This is likely a downside of many viral vectored vaccines, though this remains to be fully determined.

Another report describes SFTSV vaccine using an avirulent strain of rabies virus as a vaccine platform[149]. Rabies virus is a close relative of VSV, however unlike VSV, rabies virus infection is fatal in humans and highly neurotropic. A human vaccine using a rabies virus vaccine is unlikely to ever pass FDA approval due to fears of neurotropism, therefore the authors therefore present this as a veterinary vaccine designed for use in dogs and cats to immunize against both rabies virus and SFTSV[149]. The virus platform used does not remove the cognate glycoprotein as has been discussed with VSV platforms. Instead, SFTSV Gn (note Gc is not present) is added to the rabies genome after its cognate glycoprotein, thereby giving the virus an additional open reading frame[149]. Single dose immunization of BALB/c mice with this vaccine led to high titers of SFTSV neutralizing antibodies by 4 weeks after immunization[149]. These neutralizing titers notably had high variability in immunized animals, and titers decreased rapidly over the next 4 weeks. Immunization of mice with this vaccine was protective upon lethal challenge with rabies virus however protection is unknown for SFTSV as the authors used C57BL/6 mice which do not display lethal disease upon infection with SFTSV[149]. The only protection data displayed was a significant decrease in SFTSV viral load in the spleen 7 days after challenge in immunized animals relative to unimmunized mice[149]. This vaccine is interesting as it presents the possibility of controlling both SFTSV and rabies, however, it is unlikely to ever be used in humans and we currently lack information of its efficacy against SFTSV.

The final virus vectored SFTSV vaccine that has been evaluated in animals is an rVSV based vaccine. This vaccine removes the cognate VSV glycoprotein and inserts SFTSV Gn/Gc[112]. The authors demonstrate that insertion of SFTSV glycoprotein slows replication of rVSV relative to a virus with the wild-type glycoprotein[112]. Single dose immunization of *Ifnar*$^{-/-}$ mice with rVSV-SFTSV was completely protective against lethal challenge with all vaccine doses used (lowest dose given was $2x10^1$ focus forming units)[112]. Passive transfer of sera was partially protective with 60% of challenged mice surviving[112]. This group tested whether pre-existing immunity against VSV would prevent effective immunization[112]. Mice were first immunized with an rVSV containing a hantavirus glycoprotein, then vaccinated with rVSV-SFTSV, and finally the animals we challenged with a lethal dose of SFTSV. It was found that pre-existing immunity did not affect protection imparted by rVSV-SFTSV, as all challenged mice survived[112]. Finally, this group showed that immunization of animals with rVSV-SFTSV produced high titers of neutralizing antibodies against SFTSV as well as the closely related HRTV[112]. Overall, this study demonstrated the power and efficacy of rVSV-SFTSV vaccines suggesting that this is a powerful platform with good efficacy, although neuropathogenesis and protection from HRTV challenge were not assessed.

1.4 – Experimental Questions

The goals of this dissertation are to develop novel vaccine platforms against bunyaviruses, with a particular focus on SFTSV, and to assess the safety and immunogenicity of these vaccine platforms. As previously discussed, there are currently no approved vaccines or therapeutics against SFTSV in any country. Additionally, the vaccines that have been reported in the literature to date often have immunological short-comings, or the safety and immunogenicity are not fully characterized. To address this, we developed an rVSV-SFTSV and an mRNA SFTSV vaccine. Both platforms are

currently in use and approved for human use with a proven track record for other viral diseases. We then sought to answer the broad question, are these vaccines safe and protective, and do they stimulate a favorable immune response against SFTSV?

To begin addressing these questions, in chapter 2 we determine the safety and efficacy of a single dose rVSV-SFTSV vaccine. While this study is similar to the previously described study by Dong et al. which was published in the midst of our own study, we add further data reinforcing the safety and cross-reactivity of the vaccine[112]. We hypothesized that single dose immunization with rVSV-SFTSV would be fully protective in *Ifnar*$^{-/-}$ mice and safe when injected intracranially. This would address widespread concerns of safety for rVSV based vaccines, which can in some cases cause neuropathogenesis, that have not previously been addressed in other studies. Additionally, we hypothesized that immunization with rVSV-SFTSV would be fully protective upon challenge with the related HRTV. While Dong et al. found that rVSV-SFTSV vaccination induced cross-neutralizing antibodies, they did not assess survival upon lethal challenge with HRTV.

In chapter 3 we use wild type C57/BL6 mice to dissect the immune responses to rVSV-SFTSV and mRNA SFTSV vaccines in both single dose and prime-boost regimens, including a heterologous strategy. Detailed analysis of both the humoral and cellular adaptive immune responses were done to compare the different vaccination strategies. This study is novel in not only in its use of mRNA as a platform for immunization against SFTSV, but in the testing of heterologous vaccination strategies and the use of wild-type mice treated with anti-IFNAR antibodies for our challenge studies. We hypothesized that all immunization conditions would be protective, but that heterologous rVSV-SFTSV + mRNA SFTSV would induce the most potent cellular immune responses. These studies address important, unknown safety concerns for the

rVSV vaccine platform, and carefully analyze the immune responses elicited by the tested vaccine platforms, which have previously been unclear. Furthermore, we drive the field forward by developing an mRNA vaccine, representing a novel vaccine platform against SFTSV, and demonstrating the utility of an IFNAR antibody blockade mouse model for immunological and challenge studies. Ultimately, the goal of this study was to further our understanding of immune responses elicited by various vaccine strategies with the hope that this work may further the development of safe and efficacious human vaccines against SFTSV.

CHAPTER 2: Safety, Immunogenicity and Efficacy of a Recombinant Vesicular Stomatitis Virus Vectored Vaccine Against Severe Fever with Thrombocytopenia Syndrome Virus and Heartland Bandaviruses

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2.1 – Abstract

Severe fever with thrombocytopenia syndrome virus (SFTSV) is a recently emerged tickborne virus in east Asia with over 8,000 confirmed cases. With a high case fatality ratio, SFTSV has been designated a high priority pathogen by the WHO and the NIAID. Despite this, there are currently no approved therapies or vaccines to treat or prevent SFTS. Vesicular stomatitis virus (VSV) represents an FDA-approved vaccine platform that has been considered for numerous viruses due to its low sero-prevalence in humans, ease in genetic manipulation and promiscuity in incorporating foreign glycoproteins into its virions. In this study, we developed a recombinant VSV (rVSV) expressing the SFTSV glycoproteins Gn/Gc (rVSV-SFTSV) and assessed its safety, immunogenicity and efficacy in mice. We demonstrate that rVSV-SFTSV is safe when given to immunocompromised animals and is not neuropathogenic when injected intracranially into young immunocompetent mice. Immunization of *Ifnar*$^{-/-}$ mice with rVSV-SFTSV resulted in high levels of neutralizing antibodies and protection against lethal SFTSV challenge. Additionally, passive transfer of sera from immunized *Ifnar*$^{-/-}$ mice into naïve animals was protective when given pre- or post-exposure. Finally, we demonstrate that immunization with rVSV-SFTSV cross protects mice against challenge with the closely related Heartland virus despite low neutralizing titers to the virus. Taken together, these data suggest that rVSV-SFTSV is a promising vaccine candidate.

2.2 – Importance

Tick borne diseases are a growing threat to human health. Severe fever with thrombocytopenia syndrome (SFTS) and Heartland viruses are recently recognized, highly-pathogenic, tick-transmitted viruses. The fatality rates for individuals infected with SFTSV or HRTV are high and there are no therapeutics or vaccines available. The recent introduction of the tick vector for SFTSV (Haemaphysalis longicornis) to the

eastern half of the United States and Austrailia raises concerns for SFTSV outbreaks outside East Asia. Here we report the development of a potential vaccine for SFTSV and HRTV based on the viral vector platform that has been successfully used for an Ebola vaccine. We demonstrate that the rVSV-SFTSV protects from lethal SFTSV or HRTV challenge when given as a single dose. We evaluated possible pathogenic effects of the vaccine and show that it is safe in immune compromised animlas and when introduced into the central nervous system.

2.3 – Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tickborne disease caused by the SFTS virus (SFTSV, recently renamed *Dabie bandavirus,* formerly known as *Huaiyangshan Banyangvirus*). First identified in 2011 in China, SFTSV is a novel bunyavirus that can cause fever, thrombocytopenia, and leukocytopenia in infected individuals [14,15]. Subsequent reports later showed that SFTSV had been causing human disease since 2009 with a high case fatality ratio of approximately 30% [14,15]. Retrospective studies have found SFTSV to be endemic to, and causing disease in, South Korea, Japan, and Vietnam [17,19,150]. Recent literature indicates the case fatality ratios range from 6-20% depending on the country studied, and disease progression is characterized by hemorrhagic tendency with fatal cases exhibiting multi-organ failure [151]. In 2009 a novel related bunyavirus named Heartland virus (HRTV) was discovered in Missouri exhibiting a similar disease progression and transmission cycle to SFTSV [16].

The recently discovered SFTSV and HRTV are both bandaviruses in the order *Bunyavirales* and family *Phenuiviridae*. These viruses have a trisegmented, single-stranded RNA genome encoding 4 proteins. The S segment is ambisense and encodes the nucleoprotein (N) in the negative sense and a nonstructural protein (NSs) in positive

sense [15]. The L and M segments are negative sense and encode the RNA-dependent RNA polymerase (RdRp) and envelope glycoproteins, respectively. The glycoprotein is translated as a polyprotein which is proteolytically cleaved into two subunits, Gn/Gc [6,15]. The Gn/Gc complex recognizes its receptor or binding factor and entry is mediated by the fusion peptide within Gc [37,41,152]. While studies have failed to isolate a receptor for SFTSV and HRTV Gn/Gc, some molecules have been identified as important binding or entry factors, including DC-SIGN and UGCG [37,43,152]. Antibodies against both Gn and Gc have been shown to inhibit viral entry into cells [37,44,153].

The primary vector for SFTSV is the tick *Haemaphysalis longicornis* found throughout eastern Asia [154,155]. However, SFTSV has been found in other tick genera as well including *Ixodes* and *Amblyoma* suggesting that numerous tick species might transmit this pathogen [156,157]**.** Due to transmission resulting from contact with ticks, SFTSV generally infects rural farmworkers. In recent years, the geographic distribution of *H. longicornis* has expanded and now includes Australia, New Zealand, and the United States, presenting further opportunities for SFTSV to spread [28,158-160]. Although likely more rare, other transmission routes have been shown to be possible for SFTSV. Ferret studies have shown that SFTSV can be transmitted in the absence of ticks between co-housed ferrets or ferrets co-housed with a separator [31]. The detection of SFTSV in ferret saliva, feces, and urine suggests that these fluids are a likely route of SFTSV transmission in the absence of ticks [31]. Indeed, one report indicates that SFTSV can spread between humans in a nosocomial setting through contact with patient blood [32]. Another case report showed likely zoonotic transmission to a human through the bite of an infected cat [34]. Due to the high potential of SFTSV to cause deadly outbreaks, it has been categorized as a high priority pathogen for the development of vaccines and

therapeutics by both the World Health Organization (WHO), and the National Institute of Allergy and Infectious Diseases (NIAID) [35,36].

Currently no approved therapeutics or vaccines exist for use against SFTSV. This is in part due to lack of adequate animal models. Wild-type mice do not develop severe disease upon challenge thus alternatives must be used [47,161,162]. Animals that succumb to SFTSV infection include cats, aged ferrets, *Stat2*$^{-/-}$ hamsters, and *Ifnar*$^{-/-}$ mice [47,48,50,52,53]. Despite these difficulties, several groups have designed and tested SFTSV vaccines, primarily using *Ifnar*$^{-/-}$ mice. Tested vaccine platforms include DNA, virus-vectored, and attenuated recombinant SFTSV vaccines [112,144,146,149,163]. These vaccines vary in their effectiveness and come with drawbacks. Here, we focus on developing and characterizing a recombinant vesicular stomatitis virus (rVSV) vaccine.

The livestock pathogen vesicular stomatitis virus (VSV) is generally non-pathogenic to humans and of low sero-prevalence [91,92]. Additionally, VSV is a powerful vaccine platform with genetically tractable models and a promiscuity to incorporate foreign glycoproteins in the virion [93]. An often cited detriment of rVSV vaccines is the propensity for VSV to be neurotropic. It is, however, known that neuropathogenicity is conferred by the tropism of the viral glycoprotein [109,110]. Currently, the rVSV vaccine platform is approved for use against Ebola virus (EBOV) and has been successfully distributed in Africa during recent EBOV outbreaks [96,97]. Due to the proven nature of the rVSV platform, we made an rVSV-SFTSV virus containing the SFTSV Gn/Gc glycoprotein in place of the cognate VSV glycoprotein VSV-G.

It has been previously reported by another group that rVSV-SFTSV confers protective immunity to *Ifnar*$^{-/-}$ mice [112]. To go beyond what has been previously shown, we demonstrate that our rVSV-SFTSV is non-neurotropic and safe in immunocompromised animals. We also show that a single administration of vaccine

virus is sufficient to induce protection against SFTSV challenge. Additionally, rVSV-SFTSV vaccination induces high levels of antibodies in wild-type animals suggesting it can effectively be used in immune competent animals. Both therapeutic and prophylactic passive transfer of sera from immunized animals leads to protection upon challenge of unvaccinated animals suggesting antibodies correlate with protection against SFTSV. Finally, we demonstrate that our rVSV-SFTSV vaccine is cross-protective upon lethal HRTV challenge.

2.4 – Results

2.4.1 rVSV-SFTSV is attenuated in Ifnar$^{-/-}$ mice and exhibits a favorable safety profile

rVSV-SFTSV was launched in HEK293T cells as previously described[43]. Expression of SFTSV Gn and Gc was confirmed by Western blot of cell lysates prepared from VeroE6 cells infected with rVSV-SFTSV (Fig. S2.1A). Before performing in vivo studies, we first determined if rVSV-SFTSV was attenuated in cell culture relative to parental VSV. To evaluate growth kinetics, VeroE6 cells were inoculated at a multiplicity of infection of 0.01 with rVSV-SFTSV or VSV. Cell supernatants were sampled every 24 hours and infectious virus was titrated by plaque assay. At 24 hours post infection, infectious titers of VSV were nearly 90-fold higher than titers of rVSV-SFTSV (Fig. S2.1B). Both viruses achieved similar maximum titers by 72 hours post infection. rVSV-SFTSV caused cytopathic effect in VeroE6 cells as evidenced by cell rounding and detachment, as well as the formation of plaques on VeroE6 cell monolayers by 48 hours post infection (Fig. S2.1C). However, the plaques created by rVSV-SFTSV were significantly smaller than those created by parental VSV (Fig. S2.1D). Taken together, these results suggest that rVSV-SFTSV is attenuated in cell culture and replicates with slower kinetics than parental VSV.

One safety concern with rVSV vaccines is the potential for neurotropism [164,165]. VSV-G, the cognate glycoprotein of VSV, is sufficient to catalyze viral entry into neurons, and VSV can replicate efficiently in neuron-like cells both *in vitro* and *in vivo* [166,167]. In addition, other rhabdoviruses such as rabies virus are known human pathogens that cause lethal neurotropic infections [168]. It is currently unclear whether neurons can be infected by SFTSV or viruses harboring SFTSV glycoproteins. Neurologic symptoms have been observed in human SFTS cases, but it remains unclear whether the SFTSV glycoproteins can initiate entry into neurons *in vivo* [169]. To test the neuropathogenic potential of rVSV-SFTSV, we injected escalating doses of rVSV-SFTSV or VSV Indiana strain intracranially into the right cerebral hemisphere of 4-week-old C57BL/6 mice and observed the mice for 14 days. All groups of mice lost 2-5% body weight the day following intracranial injection independent of inoculum composition (Fig. 2.1A). Weight loss in mice injected with rVSV-SFTSV was reversed by 4 days post-infection (dpi). In contrast, weight loss was more severe in mice challenged with VSV and survivors exhibited slower recovery. None of the mice injected with rVSV-SFTSV met humane endpoints during the experiment (Fig. 2.1B). In contrast, lethal disease was seen in mice injected with VSV that trended with inoculum dose. Neurologic effects were quantified by using a neurologic sign scoring scale that ranged from 0 (no neurologic signs) to 4 (severe neurologic signs). No neurologic signs were observed in mice inoculated with rVSV-SFTSV or vehicle. In contrast, a range of neurologic manifestations were observed in most mice injected with VSV (Fig. 2.1C). All the observed neurologic effects occurred between 2-7 dpi (data not shown).

The rVSV-EBOV vaccine received FDA approval despite severe pathogenicity in *Ifnar*$^{-/-}$ and *Stat1*$^{-/-}$ immunocompromised mice [164,165]. To evaluate the pathogenicity of rVSV-SFTSV, we challenged groups of *Ifnar*$^{-/-}$ mice with escalating doses of rVSV-

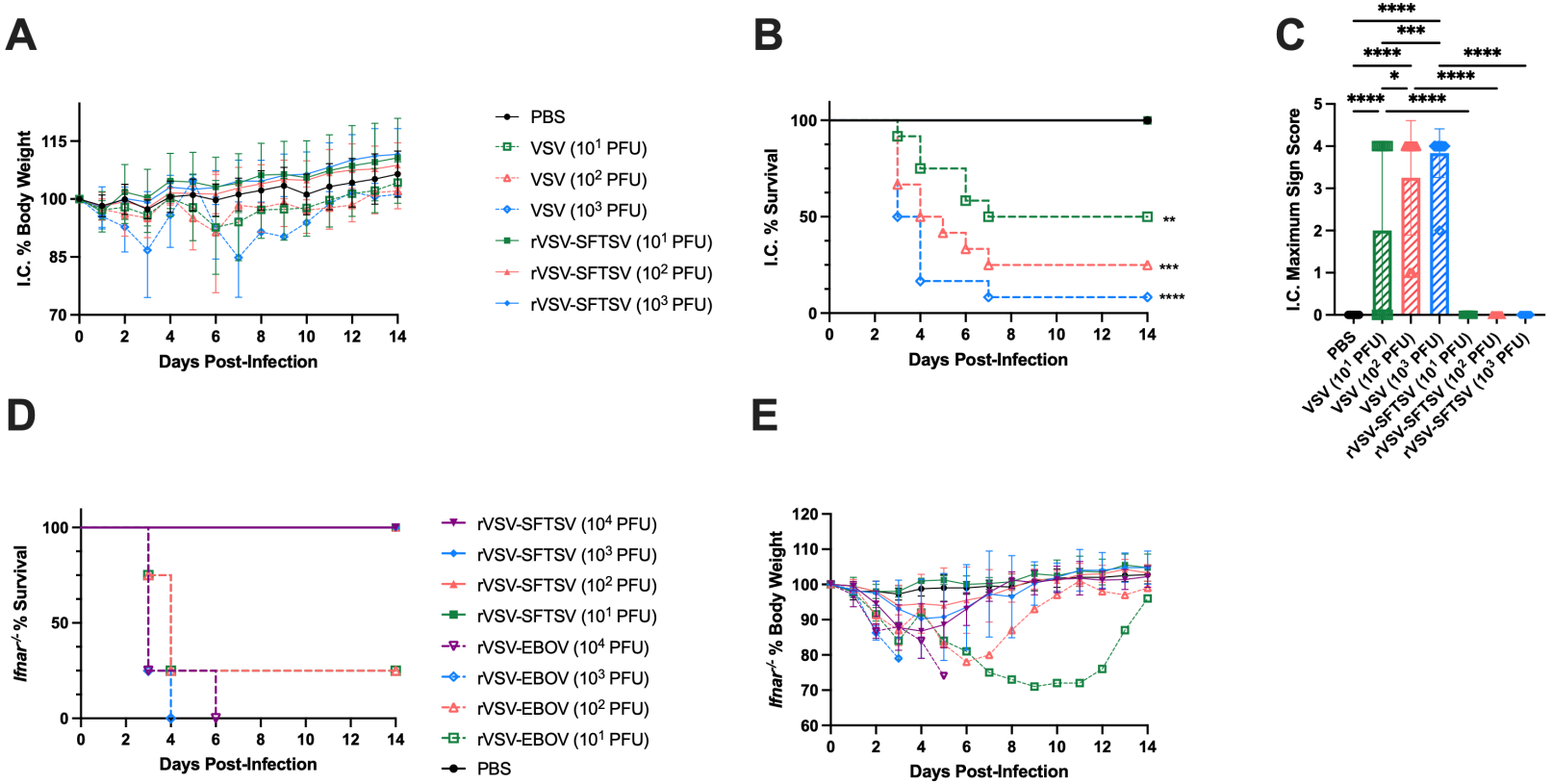

Figure 2.1 rVSV-SFTSV has a favorable safety profile compared to rVSV-EBOV and parental VSV. (A) Weight change, (B) survival proportions, (C) and maximal neurologic disease severity score in C57BL/6 mice challenged intracranially (IC) with 10^1, 10^2, or 10^3 PFU of parental VSV or rVSV-SFTSV into the right cerebral hemisphere (Mantel-Cox test and ordinary one-way ANOVA; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001) (D) Survival proportions and (E) weight loss of *Ifnar*$^{-/-}$ mice challenged intraperitoneally with PBS or 10^1, 10^2, 10^3, or 10^4 PFU of either rVSV-SFTSV or rVSV-EBOV. Weight changes were reported as percentages of body weight measured immediately pre-challenge. (Mantel-Cox test; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001)

SFTSV or rVSV-EBOV. All mice injected with rVSV-SFTSV were alive 14 dpi (Fig. 2.1D). In contrast, at least 50% of mice infected with rVSV-EBOV met humane endpoints by 4 dpi, and all mice challenged with at least 10^3 PFU succumbed by 6 dpi. All groups of mice challenged with rVSV-EBOV exhibited weight loss beginning 2 dpi which progressed with time (Fig. 2.1E). The surviving mice challenged with rVSV-EBOV lost at least 20% body weight prior to their recovery. Mice challenged with rVSV-SFTSV also began losing weight by 2 dpi, but the weight loss was less severe compared to rVSV-EBOV groups. Of the groups of mice challenged with rVSV-SFTSV, only the group infected with 10^4 PFU lost more than 10% body weight. Recovery from weight loss began between 4-5 dpi for all mice challenged with rVSV-SFTSV. Collectively, these experiments showed that rVSV-SFTSV was not neuropathogenic and demonstrated a significantly more favorable safety profile than the currently approved rVSV-EBOV vaccine in immunocompromised mice.

2.4.2 Single vaccination with rVSV-SFTSV induces high levels of neutralizing antibodies

To functionally characterize humoral responses to rVSV-SFTSV vaccination we assessed antibody neutralization potential by focus reduction neutralization titer of 50% ($FRNT_{50}$) in several animal models. To assess whether the lethal *Ifnar*$^{-/-}$ mouse model could mount an antibody response, mice were immunized intraperitoneally (IP) with 10^2, 10^3, or 10^4 plaque forming units (PFU) of rVSV-SFTSV. At 21 dpi sera were collected and analyzed for $FRNT_{50}$. Approximately half of the animals vaccinated with 10^2 PFU failed to seroconvert (Fig. 2.2A). Increasing the vaccination dose increased rates of seroconversion and neutralization titers (Fig. 2.2A). These titers are promising given thay previous work on influenza and SARS-CoV-2 suggest that neutralizing titers of 40-80 are sufficient for protection [170,171].

The high levels of neutralizing antibodies achieved with vaccination of *Ifnar*$^{-/-}$ mice was somewhat surprising as interferons (IFN)s are important drivers of immune responses. To determine whether mice deficient in both type I and type II IFN receptors also elicit high levels of neutralizing antibodies, we immunized AG129 (IFN-α/β and γ receptor-deficient) mice with 10^1-10^4 PFU of rVSV-SFTSV. Notably, 2 of 4 mice immunized with 10^4 PFU rVSV-SFTSV succumbed to viral infection (Fig. 2.2B). Mice receiving 10 PFU rVSV-SFTSV failed to generate a neutralizing antibody response (Fig. 2.2C). Animals receiving higher doses had mean neutralizing titers ranging from 60 to 240 with increasing dosage (Fig. 2.2C). These results demonstrate that rVSV-SFTSV elicits humoral responses even in highly immunocompromised animals lacking type I and II IFN responses.

It is well documented that VSV infection is highly sensitive to IFN responses [172,173]. To determine whether rVSV-SFTSV can induce a neutralizing antibody response in immunocompetent mice, we immunized C57BL/6 mice with 10^4, 10^5, or 10^6 PFU of rVSV-SFTSV. Dosages were increased relative to *Ifnar*$^{-/-}$ mice to account for IFN responses interfering with rVSV-SFTSV replication and thus reducing the humoral immune response in the immune competent mice. In contrast to what was seen with the immune deficient mice, no weight loss was observed in the C57BL/6 mice at any vaccine dose (data not shown). Additionally, despite the increased dosages, neutralizing titers were far lower than those observed in *Ifnar*$^{-/-}$ mice suggesting that rVSV-SFTSV is sensitive to IFN, consistent with previous reports (Fig. 2.2D). A dosage dependent increase in $FRNT_{50}$ titers was observed with mice receiving 10^6 PFU rVSV-SFTSV achieving a mean titer of 113 (Fig. 2.2D). Notably, all mice immunized with 10^6 PFU rVSV-SFTSV sero-converted (Fig. 2.2D). These data indicate that despite VSV's

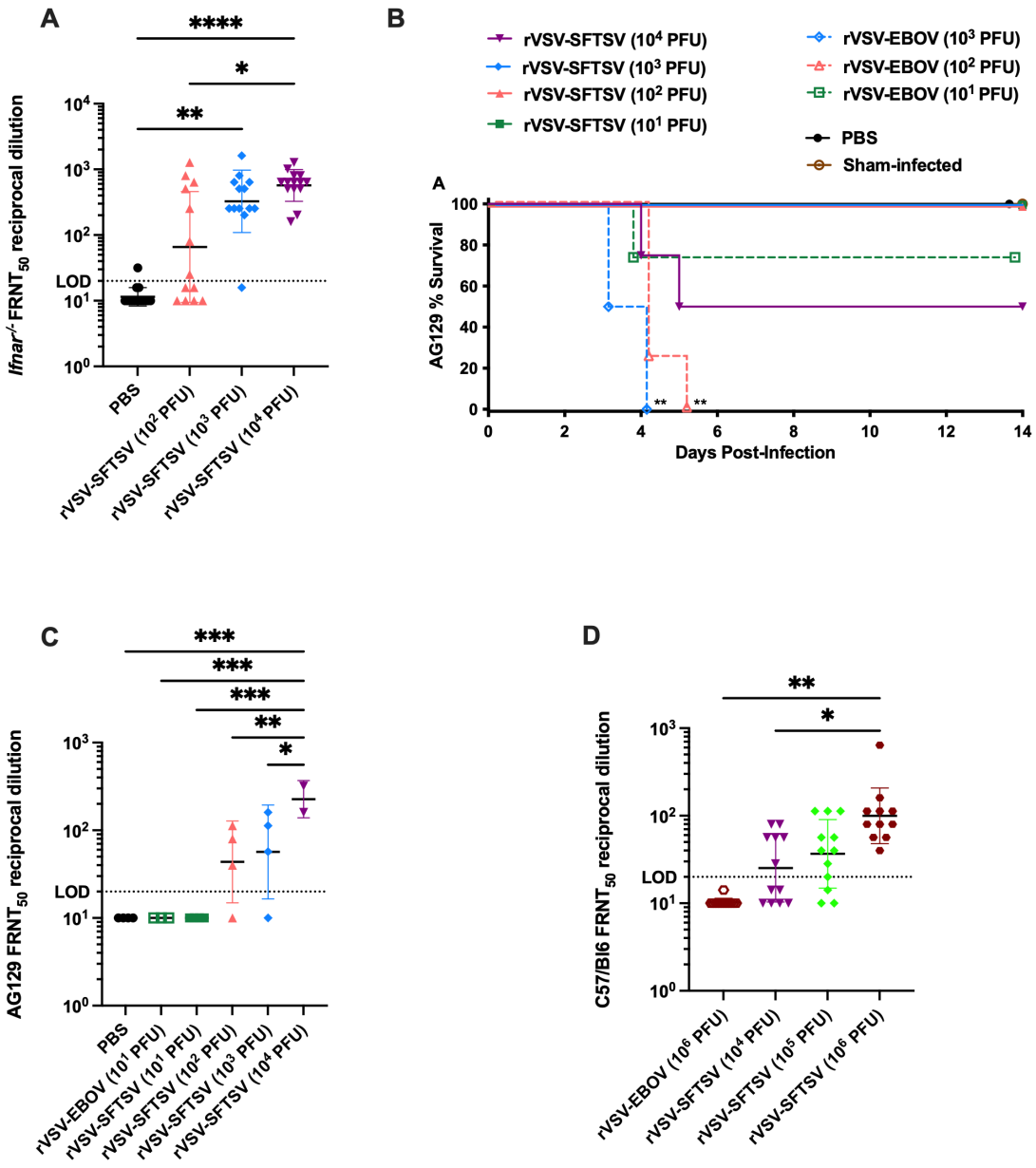

Figure 2.2 rVSV-SFTSV induces neutralizing antibodies across different mouse strains (A) *Ifnar*$^{-/-}$ mice were immunized with PBS, 10^2, 10^3, or 10^4 PFU rVSV-SFTSV. Serum neutralizing antibodies were quantified by measuring 50% decrease in pseudovirus foci, the reciprocal endpoint dilution is shown (Ordinary one-way ANOVA; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001). (B, C) AG129 mice were vaccinated with varying doses of rVSV-SFTSV and monitored for survival (B) and had serum collected 21 days post vaccination and $FRNT_{50}$ was assessed (C) (Mantel-Cox test and ordinary one-way ANOVA; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001). (D) Wild-type C57/Bl6 mice were immunized with rVSV-SFTSV and had serum neutralization titers determined at 21 days post treatment (Ordinary one-way ANOVA; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001).

sensitivity to IFN, rVSV-SFTSV induces responses in immunocompetent animals that reach neutralizing titers predicted to be protective.

2.4.3 rVSV-SFTSV protects Ifnar$^{-/-}$ mice from lethal SFTSV challenge and reduces viral titers in tissues

Because of the high neutralizing antibody titers measured in *Ifnar*$^{-/-}$ mice vaccinated with rVSV-SFTSV, we hypothesized that the vaccine would protect these mice against lethal SFTSV challenge. To test this hypothesis, vaccinated mice were challenged subcutaneously with 10 PFU of SFTSV 23 days post-vaccination (2 days after blood collection for neutralizing antibody titration). A single group of unvaccinated mice received 8 days of 100 mg/kg/day of favipiravir therapy following SFTSV challenge as a positive control for protection [174]. As expected, all mice vaccinated with PBS succumbed by 8 dpi (Fig. 2.3A). In contrast, only 60% of mice vaccinated with 10^2 PFU, and all *Ifnar*$^{-/-}$ mice vaccinated with at least 10^3 PFU, survived the lethal SFTSV challenge. Mice vaccinated with at least 10^3 PFU were protected from weight loss following SFTSV challenge while rapid weight loss was observed in PBS-vaccinated mice beginning by 3 dpi (Fig. 2.3B). Mild weight loss occurred post-challenge in mice that received 10^2 PFU of vaccine, but this trend was driven primarily by the three individuals that succumbed to disease. Vaccination-associated weight loss was also observed in this experiment and was consistent in magnitude to that shown previously (Fig. 2.1E).

To assess the effect rVSV-SFTSV vaccination has on SFTSV viremia and tissue viral loads, groups of 4 mice were vaccinated and challenged in, parallel following the timeline described above. These subsets of mice were sacrificed 5 days following SFTSV challenge and serum, liver, spleen, and kidney were collected for SFTSV quantification by endpoint titration on Vero E6 cells. All groups of vaccinated mice had

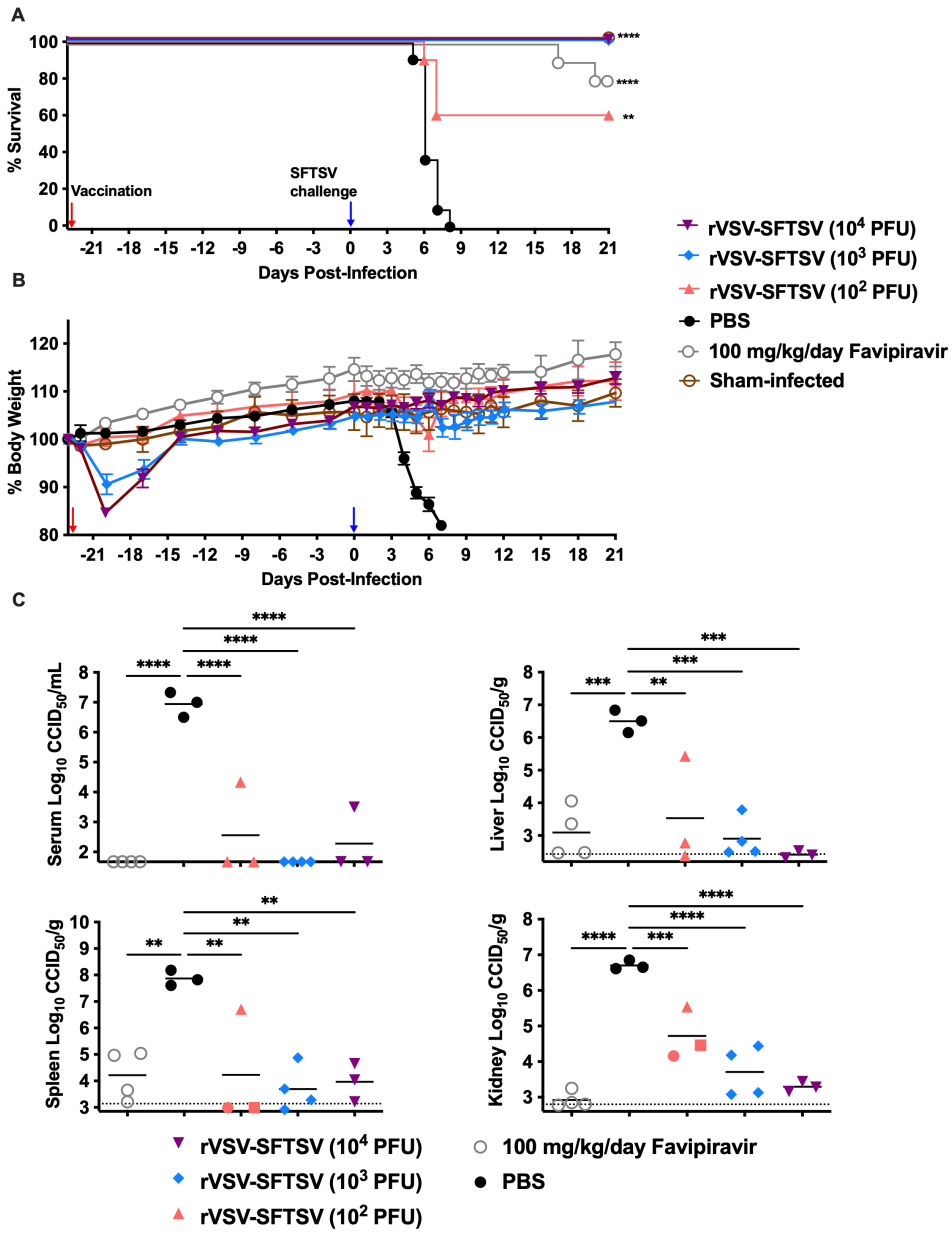

Figure 2.3 Vaccination with rVSV-SFTSV protects *Ifnar*$^{-/-}$ mice from lethal SFTSV challenge. (A) Survival proportions and (B) percent weight change in *Ifnar*$^{-/-}$ mice challenged subcutaneously with 10 PFU SFTSV (blue arrow) 23 days after intraperitoneal vaccination with PBS, or 10^2, 10^3, or 10^4 PFU rVSV-SFTSV (red arrow). Weight change is reported as percentage change in body weight relative to starting weight prior to vaccination. One group of mice received favipiravir daily for eight days following SFTSV challenge to serve as a positive control for protection. (Mantel-Cox test; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001). (C) SFTSV titers in serum liver, spleen, and kidney of mice five days post-challenge in mice subjected to the same vaccination schedule as those in (A) and (B). Horizontal dotted lines indicate the limit of detection of the assay (Ordinary one-way ANOVA; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001).

significantly reduced SFTSV serum and tissue viral titers (Fig. 2.3C). In the liver and kidney, there was a trend towards dose-dependence with mice vaccinated with the highest dose of rVSV-SFTSV having the lowest viral titers. Favipiravir treatment also reduced SFTSV titers compared to mice vaccinated with PBS. These data demonstrate that rVSV-SFTSV does not provide sterilizing immunity to SFTSV challenge but rather reduces replication in the vaccinated animals.

2.4.4 Passive serum transfer imparts protective immunity to naïve mice

Although our data demonstrate that rVSV-SFTSV vaccination induces neutralizing antibody response and protects against lethal SFTSV infection, it remains unclear if antibody-mediated immunity is sufficient to confer SFTSV protection. To assess whether antibodies alone impart protection against lethal SFTSV infection, we performed passive serum transfer. 60 µl or 20 µl of sera from rVSV-SFTSV-immunized or negative control *Ifnar*$^{-/-}$ mice were administered to naïve *Ifnar*$^{-/-}$ mice either prophylactically (2 days prior to challenge) or therapeutically (2 days post challenge). The pooled sera used for passive transfer had an approximate $FRNT_{50}$ titer of 450, while the $FRNT_{50}$ titer for negative control sera was below the limit of detection. Approximately 33% of *Ifnar*$^{-/-}$ mice receiving 60 µl of immune sera prophylactically were protected against lethal SFTSV challenge (Fig. 2.4A). When 60 µl of immune sera was administered therapeutically, 62% of mice survived (Fig. 2.4A). Animals given 20 µl of immune sera were not protected from challenge regardless of when sera were administered (Fig. 2.4A). All mice in the positive control group receiving 10^3 PFU rVSV-SFTSV 7 days prior to challenge survived (Fig. 2.4A).

Animal weights measured daily during the study positively correlated with the survival data (Fig. 2.4B). The most dramatic weight loss after the SFTSV challenge occurred in the group treated with the non-immune sera and the groups treated with the

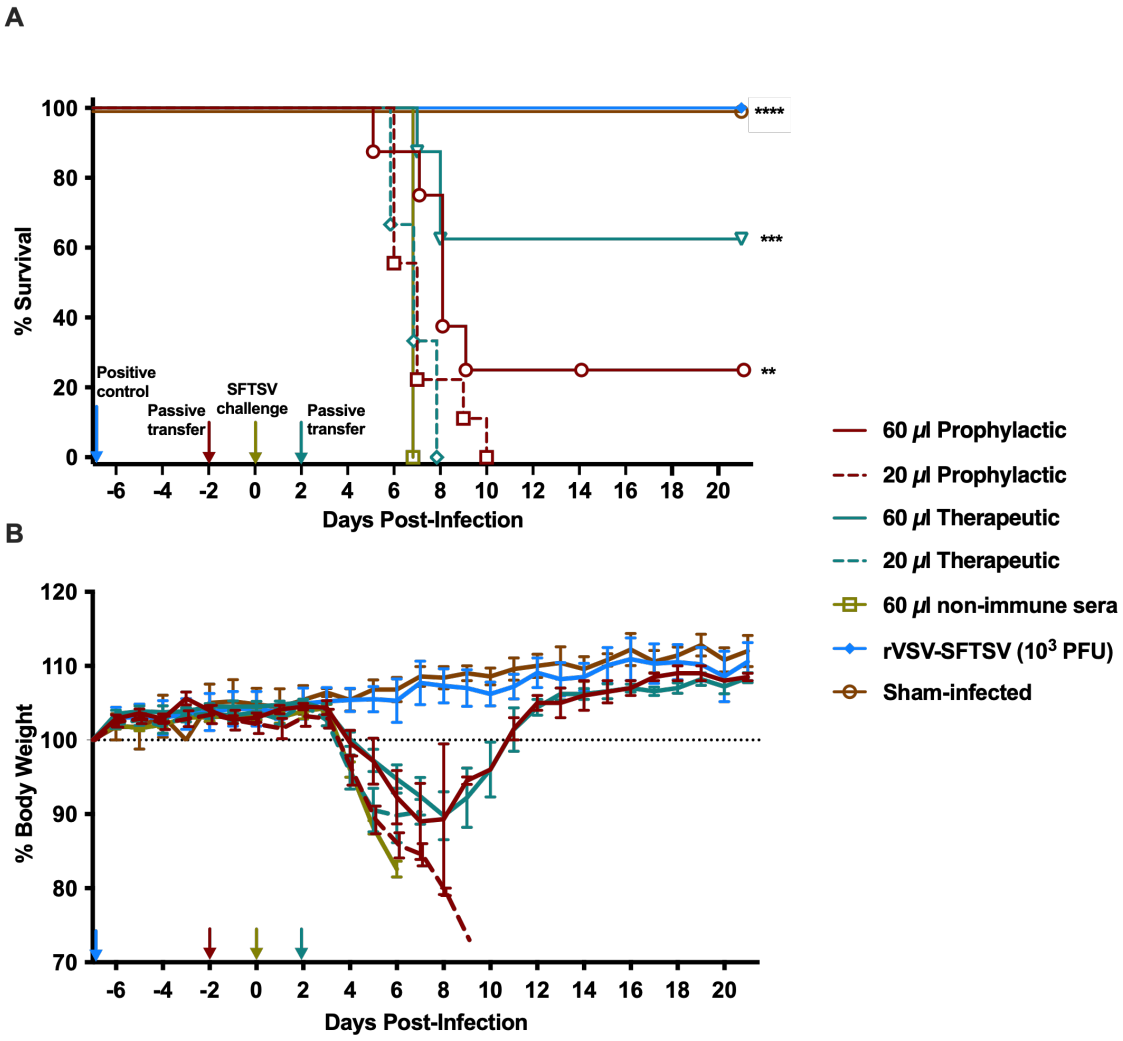

Figure 2.4 Passive transfer of sera from immunized mice protects naïve mice against SFTSV challenge. Survival (A) and weight loss (B) curves are shown from naïve animals receiving immune sera either 2 days prior to or 2 days post challenge with 10 PFU of SFTSV. Mice immunized with 10^3 PFU of the rVSV-SFTSV 7 days prior to challenge served as the positive control. Blue arrow, immunization with rVSV-SFTSV 7 days prior to challenge; Red arrow, passive transfer 2 days prior to challenge; Yellow arrow, SFTSV challenge; Teal arrow, passive transfer 2 days post SFTSV challenge. (Mantel-Cox test; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001).

lower quantities of immune sera. All surviving mice treated with the 60 µl dose of immune sera recovered fully from the infection after losing approximately 10% body weight (Fig. 2.4B). The mice vaccinated with 10^3 PFU rVSV-SFTSV did not experience any weight loss due to the vaccine virus or upon SFTSV infection (Fig. 2.4B).

2.4.5 rVSV-SFTSV vaccination cross protects against lethal HRTV challenge

Next, we evaluated whether vaccination with rVSV-SFTSV confers cross protection against HRTV challenge. In AG129 mice, a dose of 10^4 PFU rVSV-SFTSV was partially lethal (Fig. 2.2B). Thus, we modified immunization dosages to 10^2, $10^{2.5}$, and 10^3 PFU rVSV-SFTSV. Mice were then challenged with a lethal dose of a mouse-adapted HRTV (MA-HRTV) 21 days post vaccination. A group of unvaccinated mice were treated with 100 mg/kg/day favipiravir for 8 days following MA-HRTV challenge. Eighty percent of the mice that received the two highest doses of $10^{2.5}$ or 10^3 PFU rVSV-SFTSV survived the challenge, with 60% of the mice vaccinated with the lowest dose (10^2 PFU) also surviving (Fig. 2.5A). As expected, all PBS-vaccinated mice succumbed to MA-HRTV disease by 8 dpi and all the favipiravir-treated animals were protected (Fig. 2.5A). Most of the infected mice experienced considerable weight loss beginning 4 to 6 days post MA-HRTV challenge (Fig. 2.5B). Surviving mice fully recovered and had weight gain comparable to favipiravir-treated animals.

Subsets of 4 mice per experimental group were sacrificed on day 5 post MA-HRTV challenge for collection of blood, liver, and spleen tissue for measurement of viral loads by endpoint titration using an infectious cell culture assay. Mice immunized with rVSV-SFTSV had significantly reduced MA-HRTV titers comparable to the favipiravir positive control (Fig. 2.5C). In parallel, serum from vaccinated animals was analyzed for $FRNT_{50}$ to determine whether cross neutralization of HRTV occurred. Mice vaccinated with 10^2 or 10^3 PFU rVSV-SFTSV had moderate SFTSV neutralization titers of 40 and

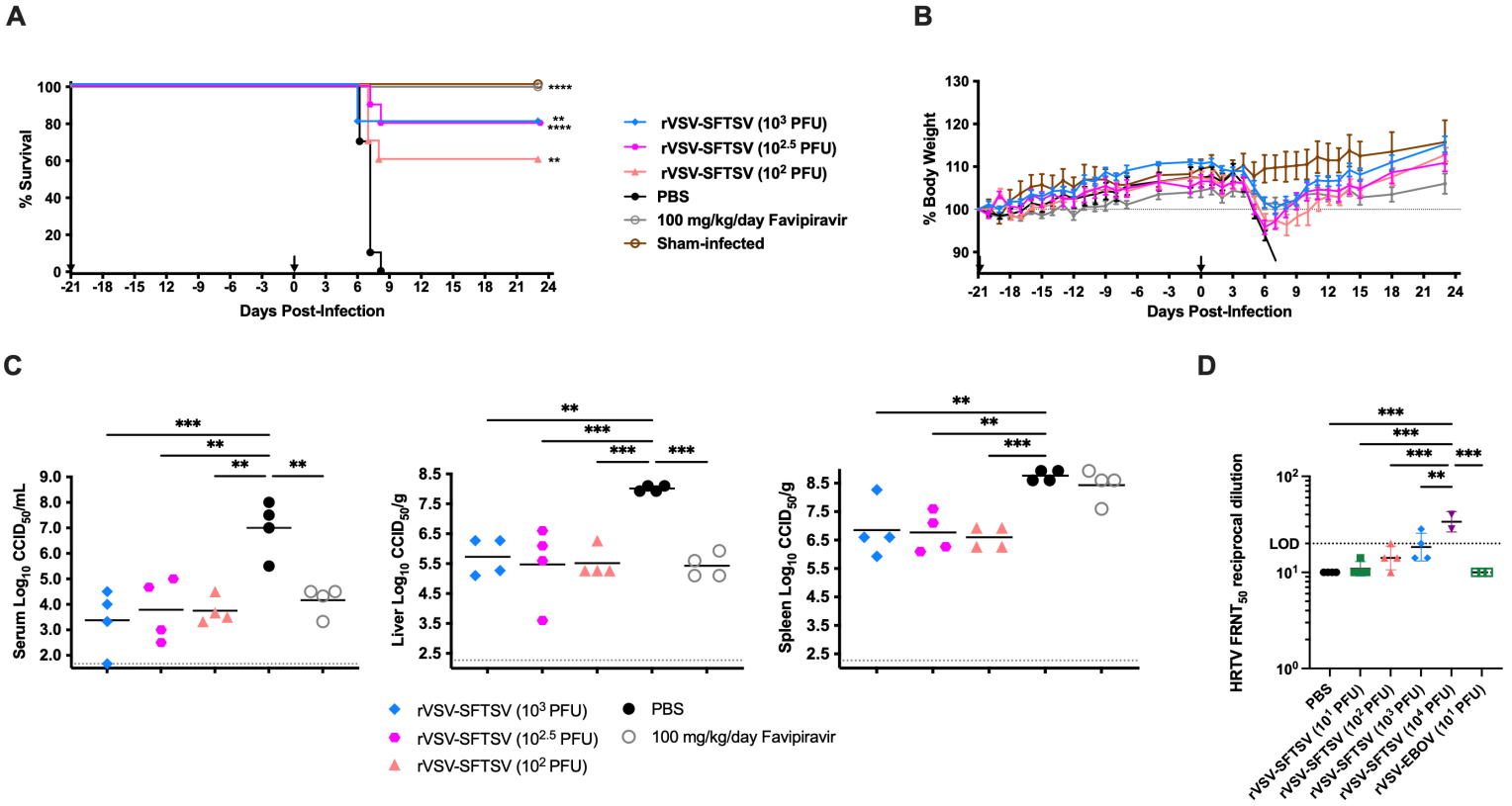

Figure 2.5 rVSV-SFTSV vaccination cross-protects animals against MA-HRTV challenge. AG129 mice were IP immunized with escalating doses of rVSV-SFTSV then challenged with MA-HRTV 21 days post immunization. (A) Survival and (B) weight loss curves are shown from immunization until completion of experiment. Black arrows indicate vaccination and challenge times at -21 and 0 days respectively (Mantel-Cox test; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001). (C) Four animals in each vaccination group were sacrificed 5 days post challenge to assess serum, liver, and spleen virus titers (Ordinary one-way ANOVA; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001) (D) Sera was collected from subsets of animals 21 days post immunization and prior to HRTV challenge. Sera was analyzed for neutralizing antibodies against HRTV using a pseudotyped virus with the HRTV Gn/Gc glycoprotein (Ordinary one-way ANOVA; *, P<0.0332; **, P<0.0021; ***, P<0.0002; ****, P<0.0001).

60, respectively, with one mouse in each group failing to seroconvert (Fig. 2.2C). Mice receiving 10^4 PFU immunization doses reached higher neutralization titers against SFTSV but 2 of 4 animals succumbed the vaccine virus (Fig. 2.2B, C). Interestingly, cross neutralization of HRTV was only observed in sera from 2 of 4 mice receiving 10^3 PFU immunizations and the surviving 10^4 PFU immunized animals from the safety and immunogenicity study (Fig. 2.5D). In mice immunized with 10^3 PFU rVSV-SFTSV, neutralizing titers against HRTV were at or just above the limit of detection indicating weak cross reactivity (Fig. 2.5D). Sera from mice receiving lower immunization doses did not have neutralization activity despite partial protection from MA-HRTV challenge (Fig. 2.5A, C, D). Lack of cross neutralization titers suggests that the protective effect in the context of survival and reduced viral loads may be due to cell-mediated immunity or other non-neutralizing antibody functions.

2.5 – Discussion

SFTSV is an emerging public health threat in southeast Asia with case fatality rates ranging from 4 to 30%. This high variability in case fatality rates may reflect access to health care, the genetic background of infected populations, and the virulence of the infecting SFTSV strain [175-177]. Given that no therapeutics or vaccines are available to curtail or prevent an outbreak of SFTS and that the virus is transmitted by multiple tick species with expanding geographic ranges, the threat SFTSV poses to public health is significant [157,178]. This has caused the WHO to list SFTSV in its prioritized pathogen research blueprint and the NIAID to include it as a category C priority pathogen [35,36]. In response to the potential threat from SFTSV, many SFTSV vaccines are being developed using a variety of different technologies including protein subunit, DNA, and recombinant viral platforms [45,112,144,146,163,179].

Ifnar $^{-/-}$ mice are susceptible to lethal SFTSV challenge and have therefore been used to evaluate an array of SFTSV vaccines that use DNA, protein subunit, and recombinant viral technologies [45,112,146,163]. A DNA vaccine encoding the ectodomains of Gn and Gc and a chimeric N-NSs fusion protein on a single plasmid only protected 40% of mice from lethal challenge after three doses of vaccine [45]. However, a similar vector that contained an additional open reading frame encoding IL-12 was fully protective using an identical vaccination schedule [45]. Interestingly, neither regimen induced neutralizing antibodies. This same group also developed a protein subunit vaccine by fusing Gn and Gc to the Fc region of human immunoglobulin heavy chain to create Gn-Fc and Gc-Fc, respectively [45]. Vaccination of mice with either Gn-Fc or Gc-Fc in alum induced neutralizing antibody titers greater than 1:100 in a $FRNT_{50}$ assay. This vaccination regimen, however, achieved only 50% and 0% protection from lethal SFTSV challenge with Gn-Fc or Gc-Fc, respectively [45]. Recombinant vaccinia virus and VSV technologies have also been explored. LC16m8 strain recombinant vaccinia virus vaccines encoding SFTSV N alone, SFTSV Gn/Gc alone, or both N and Gn/Gc in combination fully protected against lethal SFTSV challenge [146]. These vaccines however failed to induce $FRNT_{50}$ neutralizing titers greater than 1:40, and not surprisingly, the vaccine encoding N alone failed to induce detectable neutralizing activity. Of the vaccine platforms evaluated thus far in *Ifnar* $^{-/-}$ mice, recombinant VSVs induced the highest neutralizing titers. In our study, mean $FRNT_{50}$ neutralizing titers ranging from 282 to 642 were induced by a single dose of rVSV-SFTSV HB29 strain that ranged from 10^2 - 10^4 PFU. A similar rVSV encoding the SFTSV AH12 strain glycoprotein sequence elicited a neutralizing titer of 682 following vaccination with a single dose of 2×10^4 PFU [112].

Aged ferrets have also been used as a lethal SFTSV model to study the efficacy of both DNA vaccines and live-attenuated vaccines harboring mutations in NSs [144,163].

Representative vaccines from these platforms that fully protected ferrets from lethal challenge included a combination of two plasmids that encoded SFTSV Gn and Gc, a combination of five plasmids that encode SFTSV Gn, Gc, RdRp, N, and NSs, a recombinant SFTSV HB29 strain virus encoding the P102A substitution in NSs, and a recombinant SFTSV HB29 strain virus encoding an NSs containing a truncated carboxy-terminus of 12 amino acids. Both DNA vaccine platforms are given in a three dose regimen and elicit modest levels of neutralizing antibodies. Passive transfers from animals vaccinated against SFTSV Gn/Gc were protective suggesting neutralizing antibodies are a correlate of protection [144]. It remains unclear what contribution cellular responses make to protection from SFTSV challenge. Unfortunately, the age-related changes that render ferrets susceptible to lethal SFTSV challenge are unknown. This makes it difficult to directly compare results obtained from aged ferrets to other SFTSV animal models.

The only currently FDA-approved rVSV vaccine, rVSV-EBOV, is highly pathogenic and lethal in *Ifnar* $^{-/-}$ mice. In contrast, rVSV-SFTSV only caused mild-to-moderate weight loss at doses that elicited protective immunity. Unlike the parental VSV vector, rVSV-SFTSV did not cause neurologic disease when injected intracranially into 4-week-old C57BL/6 mice, suggesting that this vaccine strain is not neurotropic. Despite these promising results, it is possible that rVSV-SFTSV may be too attenuated in animals containing a functional IFN system. This possibility is supported by the lower neutralizing antibody titers measured in vaccinated C57BL/6 mice compared to those in *Ifnar* $^{-/-}$ mice. However, neutralizing serum levels in vaccinated C57BL/6 mice were higher than titers that are typically expected to protect against other viruses such as influenza [170]. This uncertainty highlights the need for immune-competent animal models to evaluate the efficacy of SFTSV vaccines and therapeutics.

Neutralizing antibodies elicited in the context of a functional humoral response are thought to be a correlate of protection against lethal SFTS in humans [180]. As such, passive transfer of sera from vaccinated animals has been previously shown to be protective when used prophylactically in an *Ifnar* $^{-/-}$ mouse model of lethal SFTSV [112]. Extending these findings, our results demonstrated that serum from mice vaccinated with rVSV-SFTSV could protect against lethal SFTSV challenge when used either prophylactically or therapeutically suggesting the protective capacity of Gn/Gc-targeted antibodies against SFTSV. In support of the role for antibodies in protection against SFTSV disease, a monoclonal antibody recognizing Gn protects *Ifnar*$^{-/-}$ mice from lethal challenge [181]. The efficacy of neutralizing sera administered therapeutically has implications for monoclonal antibody or antibody cocktail management of patients with SFTS.

In addition to direct neutralization by elicited antibodies, our data also suggest other mechanisms for protection against SFTSV pathogenesis. In our passive transfer studies, following redistribution of transferred sera from the peritoneal cavity to the circulation and subsequent dilution within the host's blood, it is likely that the neutralizing titer of the recipient's sera would be below the limit of detection of our $FRNT_{50}$ assay. Still, the dose of antibodies contained within 60 µL of donor sera was sufficient to protect mice under both prophylactic and therapeutic conditions. Surprisingly, we observed better protection when sera were transferred therapeutically (2 dpi) compared to prophylactically (-2 dpi). While the mechanisms underlying this observation are unknown, it is possible that uncharacterized variables such as the pharmacokinetics and bioavailability of the sera components responsible for protection at sites of virus replication may be responsible. In addition, our results suggest that passive transfer intervention may be beneficial in areas endemic for SFTSV within high-risk populations

for severe SFTS that have reported recent tick bites but are yet to show symptoms. Further studies using appropriate animal models should be conducted to better evaluate the temporal relationship of passive transfer therapies with both infection (by experimental inoculation or tick bite) and clinical disease onset.

Protection against lethal challenge in the absence of high neutralizing antibody titers has been observed in SFTSV vaccines using recombinant vaccinia virus and DNA technologies [45,146]. These observations suggest that other mechanisms in addition to neutralizing antibodies may also contribute to protection against SFTS. In this study, we demonstrated that vaccination with rVSV-SFTSV either 7 or 21 days prior to SFTSV challenge was fully protective (Figures 3A and 4A). Protection at 7 days, a time at which humoral responses are not fully developed, suggests that mechanisms other than antibodies can be protective. Since the T cell immune response peaks at approximately 7 days post-vaccination, it is possible that these cells also contribute to the protection elicited by the vaccine [182,183]. Additionally, we noted protection against SFTSV lethality in some of the rVSV immunized mice where the neutralizing titer was below the limit of detection. rVSVs are known to stimulate robust T_H1 immune responses in vaccinated animals and humans [102,106,107]. The data presented here suggest that rVSV-SFTSV may also confer protection by inducing type 1 immunity and CD8+ T cells. It is also possible that NK cells or other components of the innate immune system are important mediators of this early protective effect of the rVSV vaccine. More studies will be required to elucidate the potential role of T cells following vaccination with rVSV-SFTSV or infection by authentic SFTSV.

HRTV is an emerging bandavirus closely related to SFTSV that can cause lethal disease [184,185]. Previous studies have shown that sera raised by vaccination against SFTSV glycoproteins can cross-neutralize viruses harboring HRTV glycoproteins [112]. In

addition, this same study showed that a rVSV-HRTV protects *Ifnar* $^{-/-}$ mice against lethal SFTSV infection. The present study is the first to report that rVSV-SFTSV protects *Ifnar* $^{-/-}$ mice against a lethal HRTV challenge. Despite protection against lethal disease, sera from rVSV-SFTSV-vaccinated mice neutralized viruses pseudotyped with HRTV glycoproteins much less efficiently than viruses pseudotyped with SFTSV glycoproteins. Of the four serum samples collected from AG129 mice vaccinated with 10^2 PFU of rVSV-SFTSV, only one had a detectable neutralizing antibody titer (reciprocal dilution of 20) against VSV pseudotyped with HRTV glycoproteins. Despite this, 60% of AG129 mice that received this dose of vaccine survived MA-HRTV challenge. These data suggest that other systems stimulated by rVSV-SFTSV vaccination, such as a T_H1 response, could have contributed to the protection against HRTV. Additionally, alternative effector functions of antibodies beyond neutralization may contribute to the protective effect described here. Further studies are needed to elucidate which immune system components are responsible for cross-protection as the results of these studies could inform future vaccine development for these recently emerged bandaviruses.

2.6 – Acknowledgements

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2.7 – Author Contributions

P.B. and B.B.G. conceived and supervised the study. P.H., J.B.W., T.B.M., B.R., G.L.R., K.M.B. and D.J.B. performed the experiments. P.B., B.B.G., P.H., J.B.W., T.B.M., analyzed the data. P.H., J.B.W., T.B.M wrote the manuscript.

2.8 – Materials and Methods

2.8.1 Cells, Viruses, and Mice

ATCC verified and mycoplasma free 293T and Vero E6 cells were maintained in DMEM (Corning, #10-013-CV) containing 10% FBS (Corning, #35-010-CV), and 2mM L-glutamine (Corning, #25-005-Cl). Cells were passaged every 2-3 days.

Recombinant viruses harboring an additional open reading frame encoding EGFP (refered to as VSV throughout this paper) in genomic position 5, or encoding heterologous viral glycoproteins in genomic position 4 (rVSV-SFTSV and rVSV-EBOV) were launched and described previously [43,186]. rVSV-SFTSV and rVSV-EBOV also contain an additional open reading frame in position five encoding mCherry. All recombinant viruses were grown in Vero E6 cells by infecting a confluent T-175 flask at an MOI of 0.3-0.5. Virus was collected at 48-72 hours post infection with the addition of Hepes buffer pH7.4 to 25mM. Media was clarified by centrifuging at 6000 times gravity for 5 minutes at 4 ^{o}C twice. Virus was then frozen at -80 ^{o}C until used for ultracentrifugation. Virus was concentrated by ultracentrifuging virus-containing media through a 20% sucrose gradient at 26,000rpm for 2 hours at 4 ^{o}C using SW-32 tubes in a Beckman Coulter Optima XPN-80 ultracentrifuge. After removal of the sucrose and media, pelleted virus was placed on ice with 500 µl hepes buffered saline overnight. The next day virus pellets were resuspended and frozen at -80 ^{o}C. Viral titer was determined by plaque assays on Vero E6 cells with a 1.25% Avicel RC-591 NF (DuPont, #RC591-NFBA500) overlay and then stained with 1% crystal violet. Due to the size difference of plaques created by VSV and rVSV-SFTSV, plates were processed at 36 or 72 hours post infection, respectively unless otherwise stated.

SFTSV, strain HB29, was obtained from Dr. Robert Tesh (WRCEVA; World reference Center for Emerging Viruses and Arboviruses at the University of Texas

Medical Branch, Galveston, TX). The virus stock (5.6 x 10^6 PFU/ml; 1 passage in Vero E6 cells) used was from a clarified cell culture lysate preparation. Virus stock was diluted in sterile minimal essential medium (MEM) and inoculated by subcutaneous injection of 0.2 ml containing approximately 10 PFU.

The mouse-adapted HRTV (MA-HRTV) strain used was derived from the MO-4 strain obtained from Dr. Robert Tesh (WRCEVA). The MA-HRTV stock (4.7 x 10^8 50% cell culture infectious dose ($CCID_{50}$/ml); 1 passage in Vero E6 cells, 5 passages in AG129 mice) used was prepared from clarified liver homogenate. The virus stock was diluted in sterile MEM and inoculated bilaterally in two IP injections of 0.1 ml each for a total inoculation of 40 $CCID_{50}$.

C57BL/6 mice were ordered from Jackson Labs (Bar Harbor, ME). AG129 and *Ifnar*$^{-/-}$ mice were obtained from breeding colonies at Utah State University. 4 week old C57BL/6 mice were used for intracranial challenge experiments. 8 week old C57BL/6 mice or *Ifnar*$^{-/-}$ mice on the C57BL/6 background were used for all other experiments. All mouse experiments were done using equal numbers of male and female mice. All mice were given approximately 7 days to acclimate to their cages and vivarium prior to each experiment. Mice were weighed immediately prior to all vaccination and infection procedures. All mice were anesthetized using 1% isoflurane in air delivered by vaporizer (Northern Vaporisers, Skipton, UK) to the anesthesia chamber. Injection sites were first prepared by cleaning with a 70% ethanol pad. Intracranial injection experiments and some vaccination experiments without authentic SFTSV challenge were performed under animal biosafety level (ABSL) 2 conditions at the University of Pennsylvania. All other vaccination experiments that included authentic SFTSV challenged were performed in BSL3 conditions at Utah State University.

All animals were treated ethically using complying with guidelines set by the USDA and Utah State University Institutional Animal Care and Use Committee and the University of Pennsylvania Laboratory Animal Resources guidelines.

2.8.2 Western Blot

Vero E6 cells were mock infected or infected with rVSV-SFTSV. At 24 hours post infection cells were lysed, Laemmli buffer was added and samples were denatured at 95°C for 5 minutes. Samples were run on a 4-15% Biorad gel (Bio-Rad, #5671084). Protein was transferred to a PVDF membrane (Millipore, #IPVH00010) and stained with anti-SFTSV Gn (ProSci, #6647) or Gc (ProSci, #6651) polyclonal antibodies followed with a secondary HRP conjugated antibody (GE Healthcare, #NA934V). Between staining's, membrane was stripped for an hour at room temp using restore western blot stripping buffer (Thermo Scientific,#21059). Western blots were developed using SuperSignal west pico PLUS chemiluminescent substrate (Thermo Scientific, #34580) and read on an GE Healthcare Amersham 600 imager (Piscataway, NJ).

2.8.3 rVSV-SFTSV replication kinetics assay

Vero E6 cells were infected at a multiplicity of infection of 0.01 with rVSV-SFTSV or VSV diluted in DMEM + 10% FBS for 2 hours. The inoculum was then removed and cells were gently washed three times with PBS. Cells were then covered in fresh complete growth medium and incubated at 37 ^{o}C. Every 24 hours, 5% of the growth medium was removed and replaced with an equal volume of fresh growth medium. Samples were clarified by centrifugation, transferred to fresh tubes, and frozen at -80 ^{o}C until they were titrated by plaque assay as previously described.

2.8.4 Measurement of plaque area

Wells were imaged individually using a GelDoc XR+ with Image Lab Software (Bio-Rad Laboratories, Hercules, CA) with Coomasie Blue settings. Images were

analyzed using FIJI (ImageJ). First, images were thresholded using a pixel intensity cutoff of 216. Thresholded images were then converted to binary masks. Regions of interest were automatically drawn around plaques using the “Analyze Particles” command. Regions of interest that contained two or more plaques were discarded and redrawn using the “polygon ROI” tool such that regions of interest only included a single plaque. The area of each region of interest was then measured using the “Measure” tool.

2.8.5 Immunization

Vaccines were diluted to the desired concentrations with sterile PBS just prior to vaccination by IP injection. All immunizations were done with a 200 µl inoculum. Favipiravir, the positive control for the rVSV-SFTSV vaccine efficacy study, was kindly provided by the Toyama Chemical Co., Ltd. (Toyama, Japan) and prepared in a meglumine solution for administration by IP injection.

2.8.6 Intracranial infection and neurologic sign scoring

To evaluate neuropathogenesis, 4-week-old C57BL/6 mice were injected intracranially into the right cerebral hemisphere using a 1ml Hamilton syringe with Repeating Syringe Dispenser (Hamilton Company, Reno, NV). Inocula contained 0, 10^1, 10^2, or 10^3 PFU of rVSV-SFTSV or rVSV-EGFP and were diluted to a total injection volume of 10 µl with PBS. Mice we monitored during anesthesia recovery until they were ambulatory. Mice were weighed daily and were observed for neurologic signs. Neurologic signs were assigned a severity score ranging from 0-4. Mice scored “0” showed no signs of illness and were bright, alert, and responsive when handled. Mice scored a “1” showed mild signs of illness without clear signs of neurologic illness including body hunching, depressed activity, or mild grimace. Mice assigned a “1” had normal ambulation and responded normally to being handled. Mice assigned a “2” had clinical signs consistent with mild encephalitis including hyperexcitability or altered gait

that did not impair linear locomotion and used all four limbs. Mice assigned a “3” had more severe neurologic signs which included paraparesis of one or two limbs, mild head tilt, and altered gait that did impair linear locomotion (such as spinning). Mice assigned a “4” had severe neurologic signs that were inconsistent with life including complete pelvic limb paraplegia, ataxia, or tremors/seizures. Mice scored with a “4” were humanely euthanized with CO_2.

2.8.7 Blood collection

Mice were isofluorane anesthetized and blood was collected through the submandibular route using Goldenrod lancets 5mm (Medipoint, Mineola, NY). Blood was maintained on ice after collection. Serum was separated from blood by centrifugation at 8,000 RPM for 30 minutes at 4 ^{o}C in an Eppendorf 5424R centrifuge (Eppendorf, Enfield, CT). Serum was heat inactivated by incubating at 56 ^{o}C for 30 minutes. While running neutralization assays, serum was stored at 4 ^{o}C, for long term storage serum was frozen at -80 ^{o}C.

2.8.8 Pseudovirus neutralization assay

Production of VSV pseudotype with SFTSV Gn/Gc: 293T cells plated 24 hours previously at 2 X 10^{7} cells per T-175 flask were transfected using Lipofectamine 2000 (Invitrogen, #11668-019) using manufacturers protocol. Briefly, tubes each containing 1.75ml optimem (Gibco, #31985-070) were made. In one tube 100ul of Lipofectamine 2000 reagent was added and gently mixed. In the other, 45ug of pCAG-SFTSV Gn/Gc expression plasmid was added, tubes were allowed to sit for 5 minutes at room temperature. Lipofectamine and DNA containing tubes of optimum were combined and gently mixed, after 20 minutes incubating at room temperature. Solution was added to flask of 293T cells, after 4 hours cells were fed with fresh media. Thirty hours after transfection, the SFTSV Gn/Gc expressing cells were infected for 2-4 hours with VSV-G

pseudotyped VSVΔG-mNeon at an MOI of ~1-3 (Generated by deleting the cognate VSV-G and linking mNeon to the n-terminus of P. Virus was launched as previously described [43]). After infection, the cells were washed twice with FBS-free media to remove unbound virus. Media containing the VSVΔG-mNeon SFTSV Gn/Gc pseudotypes was harvested 30 hours after infection and clarified by centrifugation twice at 6000g then aliquoted and stored at -80 °C until used for antibody neutralization analysis.

Antibody neutralization assay using VSVΔG-mNeon SFTSV Gn/Gc: Vero E6 cells were seeded in 100 µl at $2x10^4$ cells/well in a 96 well collagen coated plate. The next day, 2-fold serially diluted serum samples were mixed with VSVΔG-mNeon SFTSV Gn/Gc pseudotype virus (100-200 focus forming units/well) and incubated for 1hr at 37 °C. Also included in this mixture to neutralize any potential VSV-G carryover virus was 1E9F9, a mouse anti-VSV Indiana G, at a concentration of 600 ng/ml. The antibody-virus mixture was then used to replace the media on VeroE6 cells. 16 hours post infection, the cells were washed and fixed with 4% paraformaldehyde before visualization on an S6 FluoroSpot Analyzer (CTL, Shaker Heights OH). Individual infected foci were enumerated and the values compared to control wells without serum. The focus reduction neutralization titer 50% ($FRNT_{50}$) was measured as the greatest serum dilution at which focus count was reduced by at least 50% relative to control cells that were infected with pseudotype virus in the absence of mouse serum. $FRNT_{50}$ titers for each sample were measured in two to three technical replicates performed on separate days.

2.8.9 Virus titer determination

Virus titers were assayed using an infectious cell culture assay as previously described[187]. Briefly, a specific volume of tissue homogenate or serum was serially diluted and added to triplicate wells of Vero E6 (African green monkey kidney) cell

monolayers in 96-well microtiter plates. The viral cytopathic effect (CPE) was determined 11 days after plating and the 50% endpoints calculated as described[188]. The lower limits of detection were 1.67 $\log_{10}$ $CCID_{50}$/ml serum and 2.43-3.14 $\log_{10}$ $CCID_{50}$/g tissue. In samples presenting with virus below the limits of detection, a value representative of the limit of detection was assigned for statistical analysis.

2.8.10 Passive transfer

The immune sera from mice vaccinated with rVSV-SFTSV (approximate $FRNT_{50}$ of 453), non-immune sera, and recombinant vaccine rVSV-SFTSV (7.12×10^7 PFU/ml) were diluted with sterile PBS so that the volume of each treatment was 100 µl. Sera was delivered by IP injection 2 days prior to or post challenge with SFTSV. Mice receiving the rVSV-SFTSV vaccine were immunized 7 days prior to challenge. Monitoring of mouse weight began at 7 days prior to challenge and continued 21 days post SFTSV challenge.

2.8.11 Statistical and Data Analysis

The Mantel-Cox log-rank test was used for analysis of Kaplan-Meier survival curves. A one-way analysis of variance (ANOVA) with the Dunnett's post test to correct for multiple comparisons was used to assess differences in virus titers. A one-way ANOVA with Tukey's multiple comparisons post-hoc test was used to assess $FRNT_{50}$ titers and maximum neurologic sign scores. Two-way ANOVA with Tukeys multiple comparisons test was used for replication kinetics. Unpaired student's t-test with unequal variance was used to assess differences in plaque area. All statistical evaluations were done using Prism 9 (GraphPad Software, La Jolla, CA).

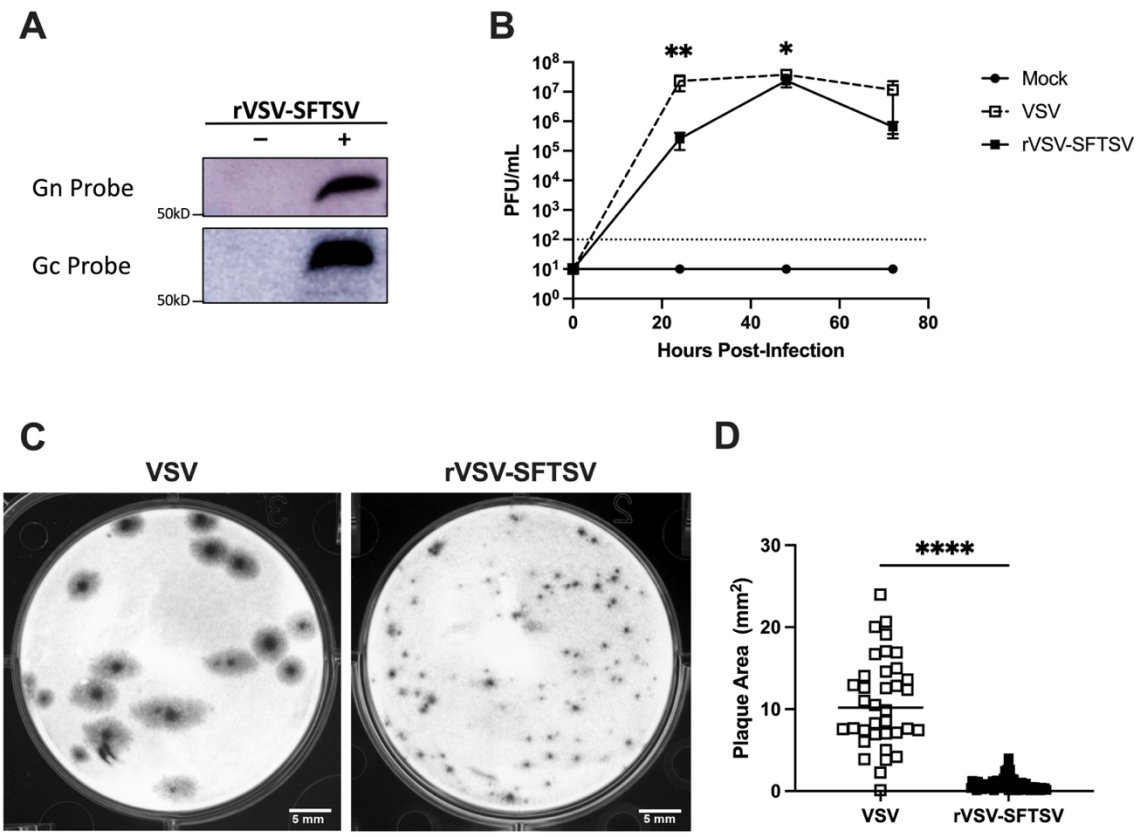

Figure 2.S1 rVSV-SFTSV expresses SFTSV glycoproteins and is attenuated *in vitro*. (A) Expression of SFTSV Gn and Gc by cells infected by rVSV-SFTSV. (B) Growth kinetics of rVSV-SFTSV and VSV in Vero E6 cells infected at a multiplicity of infection of 0.01(Two-way ANOVA; *, P<0.0332, **, P < 0.0021). Images (C) and surface area (D) of plaques created by VSV and rVSV-SFTSV on Vero E6 cell monolayers 48 hours post infection. (Unpaired t-test with unequal variance; ****, P < 0.0001)

CHAPTER 3: Vaccination With mRNA and rVSV Platforms Induce Potent Protective Immune Responses Against SFTSV in Homologous and Heterologous Regimens

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3.1 – Abstract

Severe fever with thrombocytopenia syndrome virus (SFTSV) is a highly pathogenic emergent bunyavirus first isolated in China in 2009. Since its discovery, SFTSV has been shown to have a high case fatality rate and wide distribution throughout eastern Asia where it is endemic in China, Japan, South Korea, and Vietnam. Currently, no therapeutics or vaccines are available for the treatment or prevention of SFTSV infection. Due to its pathogenicity, expanding range of its tick vector, and the ability for person-to-person transmission, SFTSV is a high-priority pathogen for the development of vaccines and therapeutics. Here, we develop a novel lipid nanoparticle (LNP)-encapsulated nucleoside-modified mRNA-based vaccine targeting the SFTSV glycoproteins, Gn and Gc. We compare this vaccine to the previously described recombinant VSV SFTSV (rVSV-SFTSV) vaccine in single dose, homologous, and heterologous prime-boost regimens. We conducted our immunizations utilizing a newly described mouse model with an α-IFNAR antibody blockade allowing us to determine vaccine immunogenicity in a non-immunocompromised animal model. We show that all vaccine regimens used are protective from lethal challenge and elicit strong long-lasting antibody responses. Furthermore, strong cellular immunity is elicited by mRNA-LNP immunizations and by heterologous immunization with an rVSV-SFTSV prime and mRNA-LNP boost. Cellular responses have a robust functional type 1 immune polarization characterized by high levels of IFNγ, TNFα, and IL-2. Immunization with mRNA led to a mixed type 1/type 2 immune response, as determined by antibody isotypes IgG1 and IgG2c, despite no detectable levels of the type 2 cytokines IL-4 and IL-5 in spleenocytes. In comparing homologous immunization to heterologous strategies, we found that homologous immunization leads to stronger antibody responses while heterologous immunization drives a slightly stronger cellular response. Taken together,

the vaccine platforms described here represent strong vaccine candidates for further development.

3.2 – Introduction

Severe fever with thrombocytopenia syndrome virus (SFTSV, also known as *Dabie bandavirus*, and formerly *Huaiyangshan banyangvirus*) is an emergent tickborne bunyavirus. First identified in China in a 2011 study, SFTSV has since been identified and is considered endemic to several southeast Asian countries including Japan, South Korea, Vietnam, and most recently Taiwan[14,15,17,19,150,189]. Infection with SFTSV results in a variety of disease symptoms including leukopenia, thrombocytopenia, fever, and the presence of gastrointestinal symptoms[14,15,151]. In more severe cases hemorrhage and multiorgan dysfunction can occur with patients generally succumbing to multiorgan failure in fatal cases[151]. The initial discovery of SFTSV reported a case fatality ratio (CFR) of 30%, more recent studies have found CFRs ranging from 6-20%[14,15,151]. The wide distribution of SFTSV coincides with the range of its primary vector, the tick *Haemophysalis longicornis*[154,155]. Other tick genera such as *Ixodes* and *Amblyoma* have also been found positive for SFTSV though these are more uncommon[156,157]. The geographic distribution of *H. longicornis* has been greatly expanding and now includes Australia, New Zealand, and the United States presenting the opportunity for further spread of SFTSV[28,158-160]. Additionally, though less common, SFTSV has been found to be able to spread without its tick vector. Ferret studies show that the virus is can be transmitted to co-housed animals in the absence of ticks, presumably through contact with blood, feces, urine, or saliva which contain high viral loads[31]. Transmission from a cat bite to a human, and human-to-human nosocomial transmissions have also been reported[32,34]. Taken together, the high CFR and potential for rapid spread of SFTSV along with a lack of approved therapeutics and vaccines have led to SFTSV being

categorized as a high priority pathogen by both the National Institute of Allergy and Infectious Diseases (NIAID) and the World Health Organization (WHO)[35,36].

SFTSV is in the order *Bunyavirales* and the family *Phenuiviridae*. Like other viruses in this family, SFTSV is a tri-segmented, single-stranded RNA virus with a negative and ambisense genome. The three segments of the virus are named by their sizes with a small (S), medium (M), and large (L) segment of which S is ambisense with M and L being negative sense[15]. The S segment encodes the nucleoprotein (N) and a non-structural protein (NSs) which antagonizes interferon (IFN) signaling[15,38]. The L segment encodes the RNA-dependent RNA polymerase (RdRp) which is essential along with N for virus replication. The M segment encodes the envelope glycoprotein which is translated as a polyprotein and then proteolytically cleaved into its two subunits Gn and Gc[15]. The Gn/Gc complex coats the exterior of the SFTSV virion and mediates receptor binding and entry through the fusion peptide found in the Gc subunit[37,41,152]. The receptors and entry factors for SFTSV remain poorly defined. It has been shown that DC-SIGN may be a potential receptor with studies showing that cells refractive to SFTSV infection can be made susceptible with the expression of DC-SIGN[37,152]. Additionally, UGCG has been shown to be an entry factor with SFTSV virions becoming unable to exit endosomes in UGCG knockout cells[43]. Studies have indicated that antibodies directed against Gn or Gc are able to inhibit viral entry into cells making the Gn/Gc complex the primary target for vaccine development[44,45,153].

Currently there are no approved vaccines against SFTSV. In part, the lack of vaccines and therapeutics can be attributed to the limitations of animal models. Infection with SFTSV does not cause severe disease in wild-type mice so other mouse genotypes or animals must be used for SFTSV vaccine development[47,161,162]. Currently the only immunocompetent animal models with lethal SFTSV disease are cats and aged ferrets,

the use of these animals for immunologic studies is limited due to lack of tools for the analysis of immune responses and difficulty in handling[48,50]. This leaves small animal models such as *Stat2*$^{-/-}$ hamsters, and *Ifnar*$^{-/-}$ mice which have been the primary animal models used for vaccine development against SFTSV[47,53,161]. More recently, one study has shown that using anti-IFNAR (α-IFNAR) antibodies in wild-type mice makes them susceptible to lethal SFTSV challenge[54]. Previous vaccine development against SFTSV has included platforms such as DNA, virus-vectored, and attenuated recombinant SFTSV vaccines[45,112,144,146,163]. Here we focus on the development of a novel mRNA-LNP based SFTSV vaccine and a heterologous prime-boost strategy with a recombinant vesicular stomatitis virus (rVSV) vaccine we have previously described[190].

Vesicular stomatitis virus (VSV) is a livestock pathogen that has been considered as a vaccine platform for a wide variety of pathogens[91,94,97]. The primary draw of VSV as a vaccine is the low sero-prevalence in humans suggesting most individuals would not have a pre-existing immune response against the vaccine platform[92]. Many groups have incorporated foreign glycoproteins into the VSV backbone due to the genetically tractable models available and the inherent ability of VSV to incorporate foreign viruses glycoproteins into virions[93,94,97]. Currently, an Ebola virus (EBOV) vaccine using an rVSV platform is approved for use by the United States Food and Drug Administration (FDA) and this vaccine has been successfully distributed to combat recent EBOV outbreaks in Africa[96,97]. As a virus platform, rVSV-based vaccines also elicit strong type 1 immune responses which are ideal for the elimination of intracellular pathogens such as viruses[85,102,106,107]. A shortcoming of many vaccine platforms is the skewing of immune responses to a type 2 response which is more suited to attack extracellular pathogens[85]. Here, we use an rVSV-SFTSV vaccine previously shown to be safe and effective in mice[112,190].

The most recently FDA-approved vaccine platform utilizes mRNA. mRNA vaccines use an in vitro transcribed mRNA strand, often including various modified nucleosides, to elicit production of an antigen in the vaccinated individual[69]. This platform is powerful, allowing the rapid design and manufacturing of vaccines against emerging pathogens along with the benefits of no platform immunity and the potential to easily modulate the immunogenicity of the delivered mRNA[69]. The biggest detriment of the mRNA platform is the need for storage at -80°C. Recent vaccination campaigns against SARS-CoV-2 have demonstrated the effectiveness of mRNA vaccines in controlling viral infections[191]. Indeed, mRNA vaccines induce primarily a type 1 immune response with high titers of antibodies and CD4+ T-cells[124-126]. Recent studies suggest the immunogen used can impact antibody skewing to a mixed type 1/type 2 response and the ability to elicit strong CD8+ T-cell responses is confounded by the strong responses induced in mice but no responses in macaques[124,127,130]. This platform remains represents a potential strong candidate for future vaccine development but remains unstudied in its use against SFTSV. Here we designed an mRNA vaccine targeting the SFTSV Gn/Gc glycoproteins to define its immunogenicity and efficacy.

In light of the SARS-CoV-2 pandemic, heterologous vaccination strategies have come to the forefront of discussion in the scientific community. The draw of heterologous vaccination strategies is the potential to combine the best aspects of different vaccine platforms to get a superior response. In our study, we combined an rVSV platform known to drive strong type 1 immune responses, and then boosted with an mRNA vaccine to achieve high antibody titers[69,107,125]. Recent reports of heterologous vaccination using an adenovirus platform followed by mRNA and vice versa have indicated that an mRNA boost does increase antibody levels further than a heterologous virus boost[140-142,192]. In regard to cellular immunity, current data indicates heterologous

vaccination induces similar or better responses compared to homologous vaccination[140-142,192]. Currently, detailed analysis of immunologic phenotypes upon heterologous vaccination remains largely undefined and attempts to analyze these responses are limited to SARS-CoV-2 vaccines[142]. Here we utilize a novel mouse model using wild type mice and α-IFNAR antibody blockade to develop a novel mRNA vaccine against SFTSV and compare it to the previously described rVSV-SFTSV vaccine in single dose as well as prime-boost studies. Additionally, we consider a heterologous prime boost strategy and show that all vaccines tested elicit powerful antibody responses. Immunization with homologous mRNA and heterologous vaccination yield comparable antibody and type 1 cytokine responses in T-cells with heterologous immunization being slightly superior in eliciting CD4+ responses and degranulation of CD8+ T-cells. Furthermore, all immunization strategies protect mice from lethal SFTSV challenge. Taken together, the mRNA platform is a strong candidate for development of a human SFTSV vaccine and heterologous prime-boost regimens may induce superior immune responses.

3.3 – Results

3.3.1 IFNAR antibody blockade recapitulates lethal infection in WT mice

Previous studies using various viruses have shown that using an α-IFNAR monoclonal antibody blockade can lead wild type mice to succumb to infections that are generally non-lethal[193]. Indeed, a previous study using a different strain of SFTSV (KH1 strain) showed that wild type C57BL/6 mice succumbed to SFTSV infection with similar, but delayed, symptoms and disease manifestations as seen in the more commonly used *Ifnar*$^{-/-}$ mouse model[54]. Since the *Ifnar*$^{-/-}$ model cannot mount a comprehensive immune response, we optimized an α-IFNAR blockade model with SFTSV strain HB29 to fully characterize our candidate vaccines in immunocompetent animals and then challenge them with lethal SFTSV doses. Animals were given two doses of the α-IFNAR

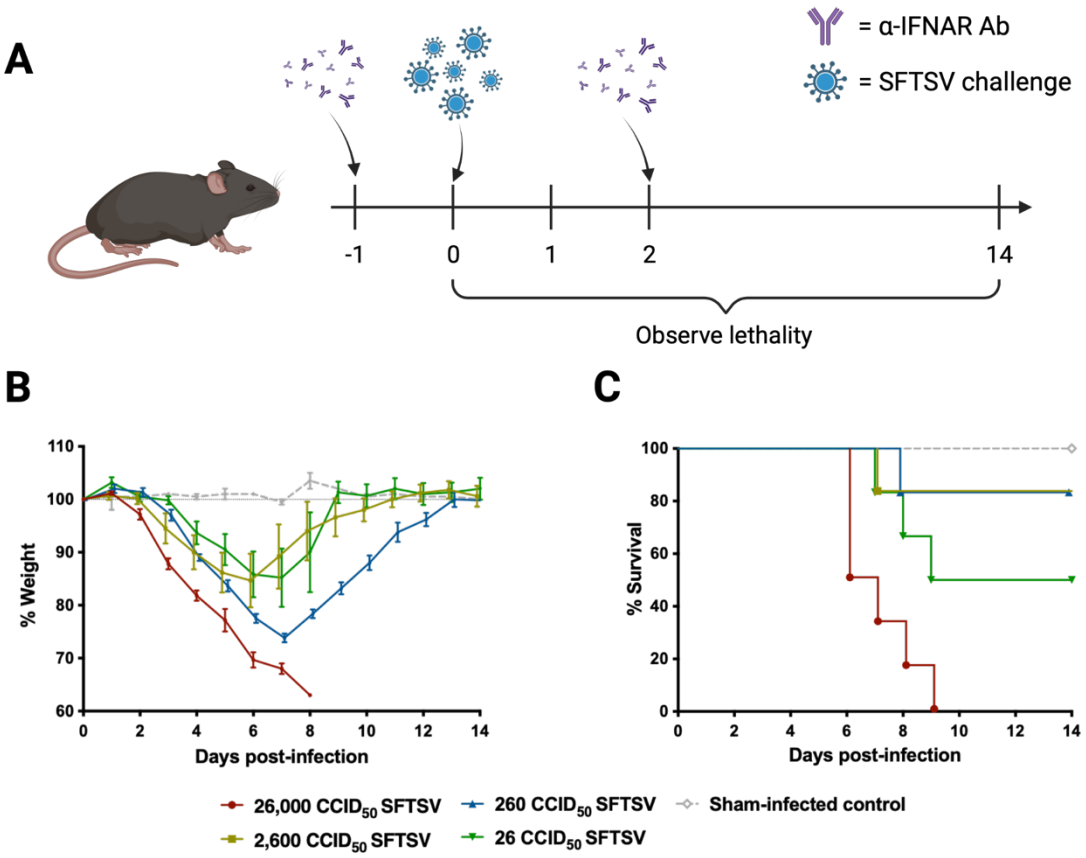

Figure 3.1 Determination of SFTSV LD_{50} in α-IFNAR blockade (A) Scheme for LD_{50} studies. (B) Weight loss and survival (C) in mice challenged with 26, 260, 2,600, or 26,000 $CCID_{50}$ of SFTSV following α-IFNAR blockade. Panel A created using BioRender.com

monoclonal antibody, one dose on day -1 from challenge and one dose on day 2 post infection (Figure 3.1 A). Several challenge doses were used ranging from 26 to 26,000 cell culture infectious dose 50% ($CCID_{50}$) to determine the median lethal dose (LD_{50}). Challenge with SFTSV caused severe weight loss with animals beginning to succumb at 6 days post infection (dpi) (Figure 3.1 B, C). By day 9, all animals challenged with 26,000 $CCID_{50}$ succumbed. (Figure 3.1 C). Interestingly, the lowest challenge dose (26 $CCID_{50}$) achieved the second highest mortality with 50% of animals succumbing to infection (Figure 3.1 C). From these data, the LD_{50} was determined to be 18,000 $CCID_{50}$. Based on these results, a challenge dose of 26,000 $CCID_{50}$ was selected for future viral challenges.

3.3.2 Prime-boost vaccination strategies induce powerful antibody responses against SFTSV

To assess the efficacy and immunogenicity of an SFTSV mRNA vaccine relative to the previously described rVSV-SFTSV, we immunized wild type C57BL/6 mice in either single dose or prime-boost regimens (Figure 3.2 A)[190]. Single dose vaccine conditions included SFTSV Gn/Gc mRNA (RS), rVSV-SFTSV (VS), and as negative controls, firefly luciferase mRNA (RL), and rVSV-EBOV (VE). Prime boost regimens included mRNA homologous regimens RS+RS and the negative control RL+RL, viral homologous regimen VS+VS, the heterologous regimens VS+RS, and the negative control VE+RL (Figure 3.2 A). A heterologous vaccination strategy using an mRNA prime and rVSV boost was not considered due to rVSV-SFTSV containing SFTSV Gn/Gc on the virion surface which would likely be neutralized by antibodies elicited by an mRNA vaccination thereby preventing any significant boost. Singly vaccinated animals were euthanized at 7 days post immunization for T-cell analysis and at 14 days for antibody assays (Figure 3.2 A). For boosted animals, a boost dose was administered at

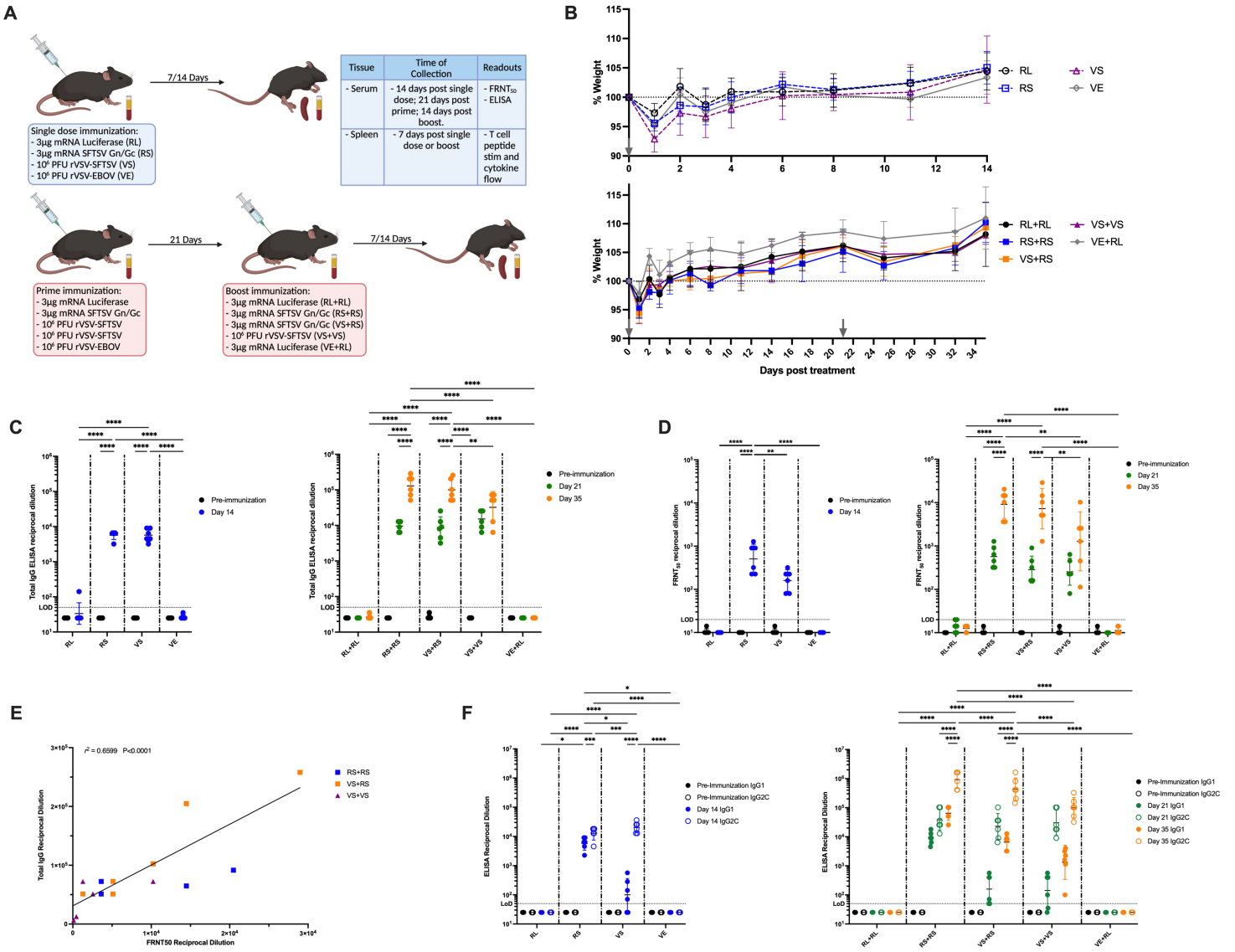
A
7/14 Days
Single dose immunization:
- 3µg mRNA Luciferase (RL)
- 3µg mRNA SFTSV Gn/Gc (RS)
- 10⁶ PFU rVSV-SFTSV (VS)
- 10⁶ PFU rVSV-EBOV (VE)
Tissue | Time of Collection | Readouts
- Serum | - 14 days post single dose; 21 days post prime; 14 days post boost. | - FRNT50 - ELISA
- Spleen | - 7 days post single dose or boost | - T cell peptide stim and cytokine flow
21 Days
7/14 Days
Prime immunization:
- 3µg mRNA Luciferase
- 3µg mRNA SFTSV Gn/Gc
- 10⁶ PFU rVSV-SFTSV
- 10⁶ PFU rVSV-SFTSV
- 10⁶ PFU rVSV-EBOV
Boost immunization:
- 3µg mRNA Luciferase (RL+RL)
- 3µg mRNA SFTSV Gn/Gc (RS+RS)
- 3µg mRNA SFTSV Gn/Gc (VS+RS)
- 10⁶ PFU rVSV-SFTSV (VS+VS)
- 3µg mRNA Luciferase (VE+RL)
B
% Weight
RL
RS
VS
VE
RL+RL
RS+RS
VS+RS
VS+VS
VE+RL
Days post treatment
C
Total IgG ELISA reciprocal dilution
LOD
Pre-immunization
Day 14
Day 21
Day 35
D
FRNT50 reciprocal dilution
E
r² = 0.6599 P<0.0001
Total IgG Reciprocal Dilution
FRNT50 Reciprocal Dilution
F
ELISA Reciprocal Dilution
LoD
Pre-Immunization IgG1
Pre-Immunization IgG2C
Day 14 IgG1
Day 14 IgG2C
Day 21 IgG1
Day 21 IgG2C
Day 35 IgG1
Day 35 IgG2C

Figure 3.2 Immunization with mRNA and rVSV-SFTSV platforms induce potent humoral responses (A) Study schematic. (B) Weight loss in mice immunized in single dose (top panel) or prime-boost regimens (bottom panel). Grey arrows indicate dates of immunization. (C) Analysis of neutralizing antibodies by $FRNT_{50}$ in single dose (left panel) and prime-boost regimens (right panel). (D) Total IgG titers as determined by ELISAS in single dose (left panel) and prime-boost regimens (right panel). (E) Total IgG and $FRNT_{50}$ titers plotted against each other from mice receiving prime-boost immunizations at day 35. Simple linear regression r^2=0.6599. (F) Analysis of mouse sera in single dose (left panel) and prime-boost regimens (right panel) for antibody serotypes IgG1 and IgG2c by ELISA. Each panel includes data from 5-6 mice per group with equally split sexes. Limit of detection (LoD) of 20 and 50 for $FRNT_{50}$s and ELISAs respectively are represented by a dashed line. Two-way ANOVA with tukey's multiple comparison test used in panels C, D, E, and F (p= *<0.0332, **<0.0021, ***<0.0002, ****<0.0001). Panel A created using BioRender.com.

21 days post prime, and animals were euthanized at day 28 post initial immunization for T-cell analysis and day 35 for antibody assays (Figure 3.2 A). Immunization led to no weight loss regardless of vaccination regimen (Figure 3.2 B). All mice showed a slight drop in weight the day after immunization likely from handling stress. All mice recovered by 2 days post immunization (Figure 3.2 B).

Using an SFTSV Gn/Gc ectodomain enzyme-linked immunosorbent assay (ELISA), we then determined total IgG titers achieved by each vaccination strategy. In single dose vaccinated mice, the total IgG endpoint titers reached were identical in both RS and VS immunized mice (Figure 3.2 C). In sera from 21 days post immunization, mice primed with RS and VS continued to have comparable endpoint titers though these titers are approximately 2-fold higher than on day 14 suggesting continued antibody class switching (Figure 3.2 C). After boost, homologous RS+RS and heterologous VS+RS vaccinated mice had similar endpoint titers with geometric means of approximately 129,000 and 100,000 respectively (Figure 3.2 C). Homologous VS+VS immunized mice had the lowest titers at approximately 32,000 (Figure 3.2 C). Fold changes in total IgG titers upon boost had a similar 15- and 14-fold titer increase upon

boost between RS+RS and VS+RS groups respectively. Mice immunized with the VS+VS regimen had a 3-fold increase in total IgG titers upon boost.

We next sought to characterize the neutralization ability of generated antibodies. At 14 days post vaccination all single dose vaccinated mice had sero-converted, the geometric mean titers reached were 500 and 170 for RS and VS respectively (Figure 3.2 D). Prime-boost vaccination regimens induced powerful neutralizing antibody titers from all groups. At 21 days post vaccination serum titers were slightly increased from what was observed at day 14 in singly vaccinated mice (Figure 3.2 D). Upon boost all mice had increased neutralizing antibody titers (Figure 3.2 D). Homologous RS+RS immunized mice had higher titers than all other groups with a mean neutralizing titer of 9,000, though this was not significantly higher than heterologous VS+RS immunized mice with a mean titer of 7,240 (Figure 3.2 D). Homologous VS+VS vaccinated mice had the lowest mean neutralizing titers (1,280) and the greatest variability in neutralizing titers (Figure 3.2 D). Interestingly, heterologous VS+RS immunization had a 31-fold increase in neutralizing titers upon boost, higher than the 23 and 11-fold increases seen in RS+RS and VS+VS regimens respectively. These data suggest that homologous mRNA and heterologous immunizations yield the best neutralizing responses though all conditions tested, both as a single dose and as a prime-boost, induce neutralizing antibody levels theorized to be sufficient for protection. Analysis of $FRNT_{50}$ titers relative to total IgG titers indicate a positive correlation (Figure 3.2 E). Taken together, these data show that all vaccination strategies elicit strong antibody responses. As expected, homologous RS+RS had the highest responses, however, heterologous boost induced the greatest increase in neutralizing antibody titers.

3.3.3 Homologous mRNA and heterologous immunizations stimulate powerful type I CD4+ and CD8+ responses

To assess differences in T-cell responses from each vaccination strategy, mouse spleens were harvested at 7 days post single dose vaccination or 7 days post boost (day 21 from experiment initiation). Spleenocytes were harvested and stimulated overnight with either a peptide pool covering SFTSV Gn or SFTSV Gc. Cells were then analyzed by flow cytometry for the type 1 cytokines interferon γ (IFNγ), tumor necrosis factor α (TNFα), or interleukin 2 (IL-2) as well as for degranulation markers CD107a and Granzyme B (GranzB). Flow data was analyzed flowing the gating strategy shown in figure 3.3 A. In single dose immunization, only RS vaccinated animals showed strong CD8+ T-cell activation with both the Gn and Gc peptide pools (Figure 3.3 B). Mice receiving a single dose of VS also showed increases in type 1 cytokines above negative controls RL and VE, though cytokine positivity was seen only in cells stimulated with the Gc peptide pool (Figure 3.3 B). Stimulated CD8+ cells in both RS and VS mice showed a large increase in INFγ+ cells and modest increases in all other cytokines including polyfunctional cells positive for multiple cytokines (Figure 3.3 B).

In CD4+ T-cells, single dose immunization only induced a detectable response in animals immunized with RS (Figure 3.3 D). CD4+ T-cell activation in RS immunized mice occurred with both the Gn and Gc peptide pools, interestingly, in higher activation of CD4+ T-cells occurred with the Gn peptide pool as opposed to CD8+ T-cells which were more potently activated by the Gc pool. (Figure 3.3 B, D). Activated CD4+ T-cells showed increases in all cytokines tested and particularly large increases in IFNγ and IL-2 (Figure 3.3 D). Taken together this data indicates that in single dose immunizations, RS induces much more potent cellular immune responses than VS.

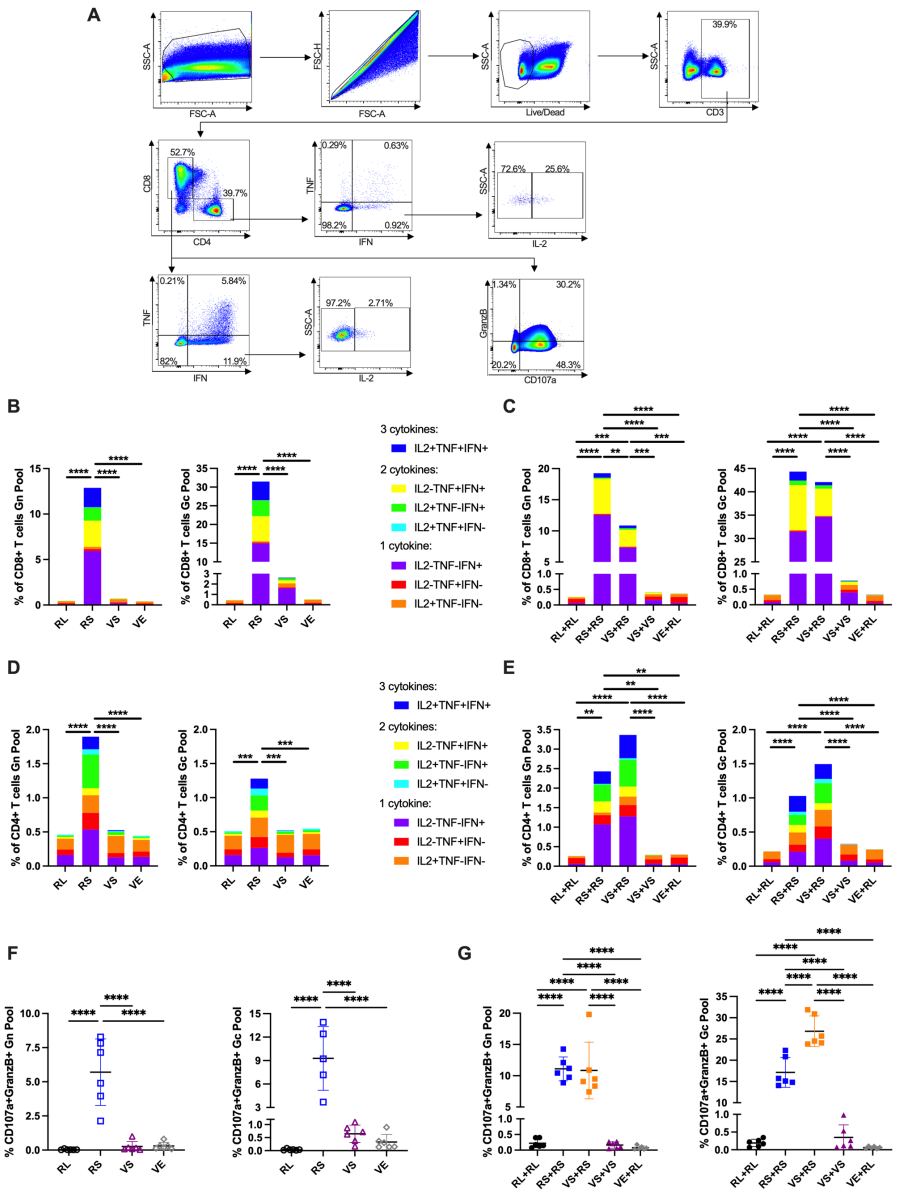

Figure 3.3 Single dose RS and prime-boost regimens RS+RS and VS+RS induce potent cellular responses (A) General gating strategy used for analysis of flow cytometry data from spleenocytes stimulated overnight with SFTSV Gn or Gc peptide pools 7 days after immunization. Cytokine profiling of CD8+ T-cells in single dose immunized animals (B) and prime-boost immunized animals (C). Throughout figure, Gn peptide pool stimulated cells are shown on the left and Gc peptide pool stimulated cells are shown on the right. CD4+ T-cell cytokine profiling in single dose immunized animals (D) and prime-boost immunized animals (E). Analysis of degranulation in CD8+ T-cells by CD107a and Granzyme B are shown in single dose immunized animals (F) and prime-boost immunized animals (G). Each panel includes data from 5-6 mice per group with equally split sexes. Ordinary one-way ANOVAs with tukeys multiple comparisons test was used in panels B-G (p= *<0.0332, **<0.0021, ***<0.0002, ****<0.0001). In panels B-E analysis was run on total cytokine positive cell percentage, not based on specific cytokine profiles.

In prime-boost studies, CD8+ T-cell responses in RS+RS and VS+RS were relatively comparable and were far superior to a homologous VS+VS strategy. Animals immunized with RS+RS and VS+RS both had potent CD8+ immune responses when stimulated with Gn and Gc peptide pools (Figure 3.3 C). Responses to Gn peptides were of smaller magnitude to Gc stimulation consistent with observations of single dose immunized mice. Interestingly, RS+RS and VS+RS achieved a similar response with stimulation by the Gc peptide pool, with approximately 42% of cells being cytokine positive; In contrast, VS+RS mice had a significantly lower response compared to RS+RS when stimulated with the Gn peptide pool (Figure 3.3 C). As seen in mice receiving a single vaccine dose, cytokine positivity is primarily attributed to the robust production of IFNγ and a large population that is IFNγ+TNFα+IL2- (Figure 3.3 C). Animals receiving a homologous VS+VS immunization regimen did not achieve a detectable CD8+ T-cell response with Gn peptide stimulation and only achieved a mild response over negative controls when stimulated with Gc peptides (Figure 3.3 C).

In contrast to CD8+ T-cells, cytokine profiling of CD4+ T-cells revealed a stronger response in heterologous VS+RS immunization than homologous RS+RS treated mice, though this was not statistically significant (Figure 3.3 E). CD4+ responses were detected with both Gn and Gc peptide pools and were stronger in cells stimulated with Gn peptides, consistent with single dose immunization data (Figure 3.3 D, E). Higher induction of CD4+ T-cells in heterologous immunized mice is surprising due to no detection of CD4+ T-cell responses with either peptide pool in mice receiving a single dose VS immunization (Figure 3.3 D, E). Perhaps differing vaccination strategies lead to different tissue homing for activated T-cells explaining the weak T-cell responses seen in VS and VS+VS immunized mice. CD4+ responses had a cytokine profile featuring high levels of both IFNγ and IL-2 as well as a large population of triple positive cells producing

IFNγ, TNFα, and IL-2 (Figure 3.3 E). All trends shown here are consistent with analysis of spleenocytes 14 days post immunization (Figure 3.S1) and when cells are stimulated for only 6 hours (data not shown), though magnitudes of responses are decreased.

To determine CD8+ T-cell functionality we also assessed positivity to the cytotoxic marker Granzyme B (GranzB) and the degranulation marker CD107a. In animals receiving a single dose immunization only RS induced CD107a+GranzB+ cells when stimulated with both the Gn and Gc peptide pool (Figure 3.3 F). Animals receiving the VS vaccine appeared to have a very slight but statistically insignificant increase in CD107a+GranzB+ CD8+ T-cells stimulated with the Gc peptide pool (Figure 3.3 F). In Animals receiving a boost, RS+RS and VS+RS mice induced equivalent levels of CD107a+GranzB+ CD8+ T-cells when stimulated with Gn peptides (Figure 3.3 G). When stimulated with Gc peptides, VS+RS induced significantly higher levels of CD107a+GranzB+ CD8+ T-cells than the homologous RS+RS strategy (Figure 3.3 G). For mice immunized with VS+VS no significant induction of CD107a+GranzB+ CD8+ T-cells occurred (Figure 3.3 G). Taken together the T-cell analysis presented here suggests that in single dose immunizations, RS is superior. However, when a boost is given heterologous VS+RS vaccination generally performs equivalently or better than homologous RS+RS.

3.3.4 Antibody isotype analysis indicate mRNA induces some type 2 associated immunity despite lack of type 2 cytokines in stimulated T-cells

To further determine whether immunization strategies skewed responses to type 1 of type 2 immune profiles, we performed SFTSV Gn/Gc ELISAs to quantify IgG1 vs IgG2c levels. In C57BL/6 mice IgG2c is associated with a type 1 immune response with IFNγ driving class switching to IgG2c[81,194]. In contrast, IgG1 is associated with a type 2 immune response and isotype switch to IgG1 is driven by IL-4[81,194]. In single dose

immunizations, VS drives primarily an IgG2c response with only low levels of IgG1 as expected from a virus known to drive a type 1 response (Figure 3.2 F). Surprisingly, immunization with RS led to approximately equal levels of IgG1 and IgG2c indicating a balanced type 1 and type 2 response (Figure 3.2 F). Despite a mixed response RS and VS immunized animals had similar titers of IgG2c (Figure 3.2 F). Upon boost, VS+VS immunized animals had increased levels of both IgG1 and IgG2c though IgG2c titers remained approximately 2 logs higher than IgG1 (Figure 3.2 F). Mice receiving the RS+RS regimen had a slight boost to IgG1 though isotype IgG2c showed the biggest boost in titer (Figure 3.2 F). Despite this small boost in IgG1 RS+RS mice had the highest mean IgG1 titer of any group by approximately 1 log (Figure 3.2 F). Finally, heterologous VS+RS vaccination had both IgG1 and IgG2c responses boosted by approximately 1.5 logs. Heterologous VS+RS immunization had an intermediate polarization compared to homologous RS+RS and VS+VS regimens (Figure 3.2 F).

To further determine immune polarization to type 1 or type 2 responses we assessed peptide stimulated T-cells for the presence of the type 2 associated cytokines IL-4 and IL-5 at 7 days post immunization. Confusingly, despite the presence of the IL-4 driven IgG1 antibody isotype in some vaccination groups no IL-4+ CD4+ T-cells were detected in any vaccination regimen (Figure 3.4 B, C). Similarly, another type 2 cytokine IL-5 was not detected in any immunization regimen (Figure 3.4 D, E). Type 2 cytokines continued to not be detected when spleenocytes were analyzed 14 days post immunization (Figure 3.S2). Perhaps different tissues, such as the lymph nodes, may contain IL-4+ CD4+ T-cells or timing for the detection of these cytokines is crucial in our vaccination strategies.

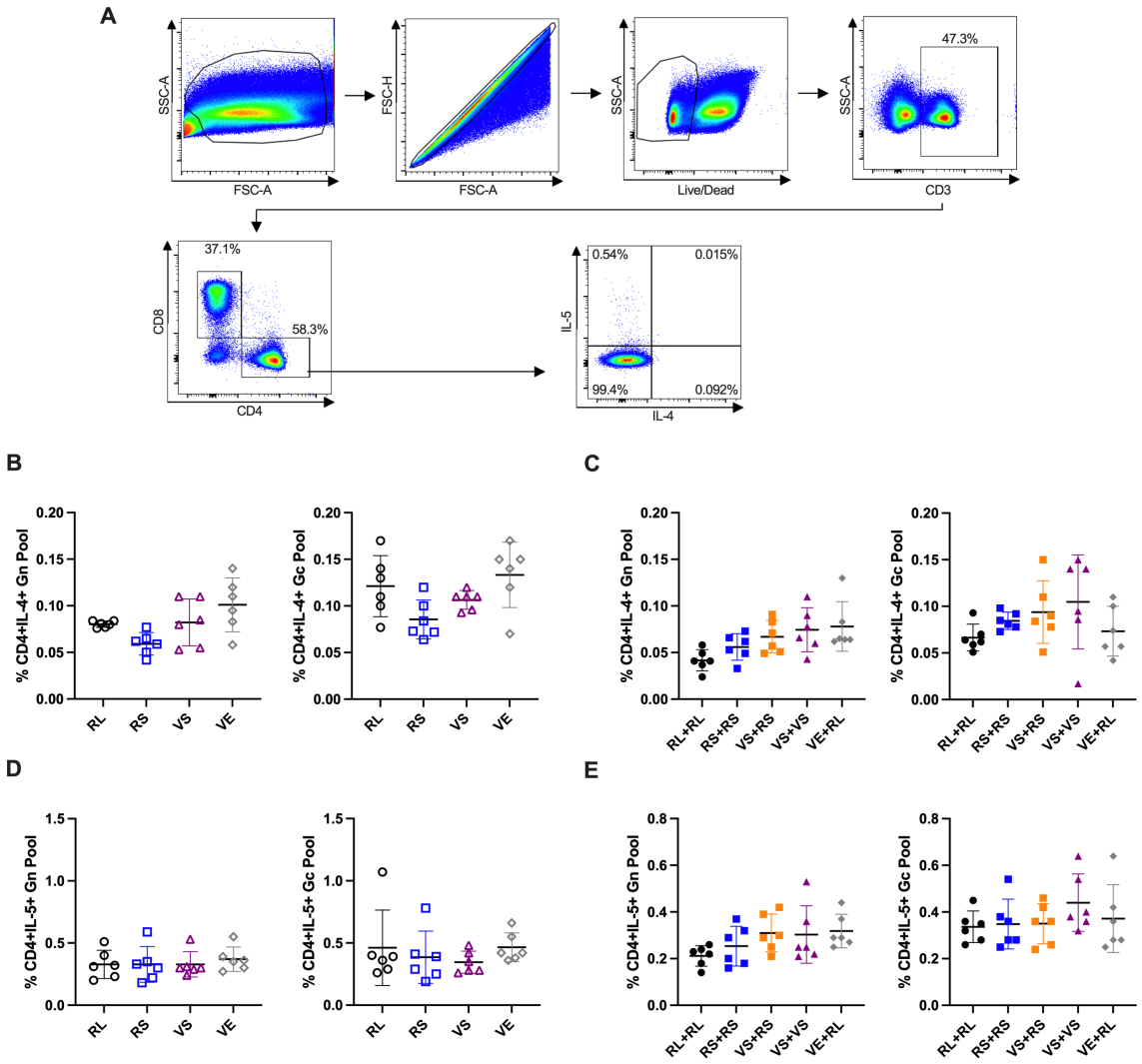

Figure 3.4 Immunization with mRNA and rVSV platforms do not induce type 2 cytokines IL-4 and IL-5 (A) General gating strategy used for analysis of flow cytometry data from spleenocytes stimulated overnight with SFTSV Gn or Gc peptide pools 7 days after immunization. IL-4 cytokine profiling of CD4+ T-cells in single dose immunized animals (B) and prime-boost immunized animals (C). Throughout figure, Gn peptide pool stimulated cells are shown on the left and Gc peptide pool stimulated cells are shown on the right. CD4+ T-cell IL-5 cytokine profiling in single dose immunized animals (D) and prime-boost immunized animals (E). Each panel includes data from 6 mice per group with equally split sexes. Ordinary one-way ANOVAs with tukeys multiple comparisons test was used in panels B-E, no significant comparisons were found.

3.3.5 Prime-boost vaccination regimens are fully protective from SFTSV challenge

After characterizing the immune responses elicited by our various vaccination strategies, we next sought to determine whether these vaccines would provide protection from challenge with a lethal dose of SFTSV. Date of challenge was set as day 0, initial immunization occurred on days -35 for animals receiving two vaccine doses, and day -14 for animals receiving a single dose as well as animals receiving a boost. On day -1 and day 2 mice had α-IFNAR antibodies administered IP.

Animals receiving a single dose of eithers RS or VS lost no weight upon challenge and were protected by immunization (Figure 3.5 A, B). A single mouse receiving VS immunization succumbed on day 1 post challenge as a result of injury during the virus challenge (Figure 3.5 B). Negative control immunized mice receiving RL or VE had severe weight loss after challenge and high lethality in RL immunized mice (Figure 3.5 A, B). Surprisingly, VE immunized mice appeared to be protected from lethal challenge with 90% of mice surviving despite severe weight loss (Figure 3.5 A, B). In prime-boost regimens, mice immunized with RS+RS, VS+RS, and VS+VS all were fully protected from lethal challenge and exhibited no weight loss (Figure 3.5 A, B). As with single dose immunized mice, RL+RL and VE+RL mice had severe weight loss and RL+RL mice succumbed by day 6 post-challenge with a 30% survival rate (Figure 3.5 A, B). Animals receiving a VE prime again appeared to be partially protected with an 80% survival rate (Figure 3.5 A, B). Decreased lethality relative to what was seen in Figure 3.1 is hypothesized to be partially attributed to mice being older and more acclimated to the animal facilities, due to a 35 day immunization protocol, as well as long lasting antiviral responses elicited by initial VE immunization.

Two days prior to SFTSV challenge, sera were taken from immunized mice for neutralizing antibody analysis. As previously reported, both single dose and prime-boost

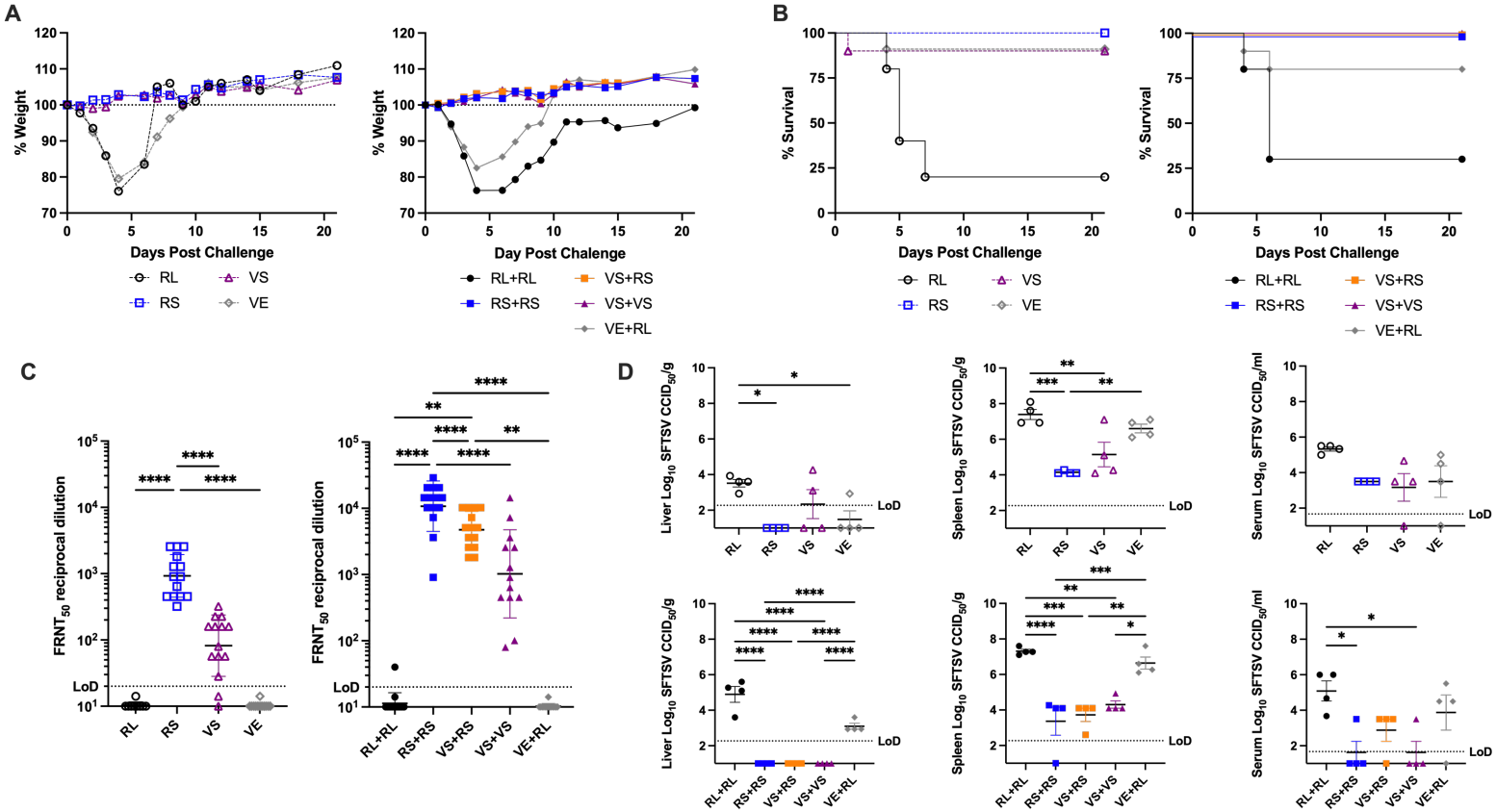

Figure 3.5 All immunization conditions are protective from lethal SFTSV challenge C57/Bl6 mice were immunized according to previously described vaccination regimens. Mice received α-IFNAR antibody blockades on day -1 and 2 post challenge with challenge (day 0) occurring 14 days after the last received immunization dose. Weight loss (A) and survival (B) are shown for both single dose and prime-boosted animals. (C) Serum was collected from all animals 2 days prior to challenge for neutralizing antibody titer determination. (D) SFTSV titers in liver, spleen, and serum were determined in a subset of mice sacrificed at 4 days post SFTSV challenge. Ordinary one-way ANOVAs with tukeys multiple comparisons test was used in panels C and D (p= *<0.0332, **<0.0021, ***<0.0002, ****<0.0001).

immunized animals had high titers of neutralizing antibodies (Figure 3.5 C). In VS immunization group, two animals failed to sero-convert, yet both of these animals survived lethal challenge implying non-neutralizing antibodies or cellular responses mediated protection from lethal challenge (Figure 3.5 C).

Lethal infections of SFTSV in humans are characterized by high viremia. To determine the ability of our vaccine candidates to control SFTSV infection, 4 mice from each immunization group were sacrificed at 4 days post challenge to determine viral titers in the liver, spleen, and serum. In single dose immunized animals both RS and VS immunization decreased tissue SFTSV titers equivalently (Figure 3.5 D). Similarly, all three prime-boost regimens, RS+RS, VS+RS, and VS+VS, induced significant decreases in SFTSV titers in the liver and spleen (Figure 3.5 D). Significant decreases in titers in the serum were only observed in RS+RS and VS+VS immunized mice, though VS+RS immunization did decrease serum titers relative to RL+RL (Figure 3.5 D). Immunization with VE also led to significant decreases in SFTSV titers in liver and spleen when given in single dose (Figure 3.5 D). In prime-boost studies, VE+RL mice also had decreased SFTSV titers though these decreases were not significant and were of lower magnitude than in single dose animals (Figure 3.5 D).

3.3.6 High levels of antibody are maintained for months after vaccination

An important factor in considering the efficacy of vaccines is the longevity of the immune response. To determine whether our vaccine regimens could produce a long-lived immune response, animals were immunized as described in figure 2A and maintained with sera collections occurring approximately every one to two months. Serum analysis by $FRNT_{50}$ and ELISA showed that immunized mice had similar titers as previously described at days 21 and 35 post immunization (Figure 3.2 C, 3.6 A, B). Over time, animals receiving a single dose VS immunization had the lowest titers of all groups

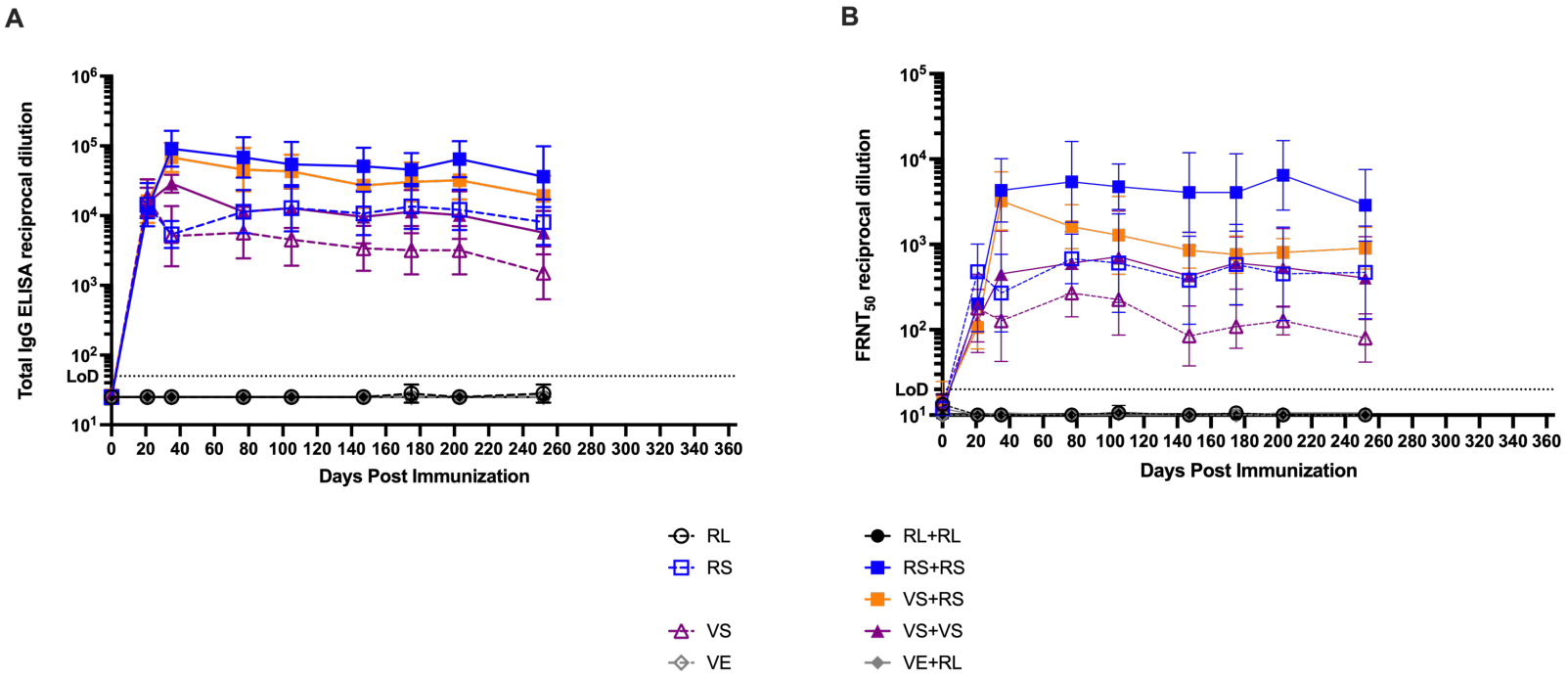

Figure 3.6 All immunization conditions elicit long lived antibody responses (A) Analysis of neutralizing antibodies by $FRNT_{50}$ in immunized mice over extended period. (B) Total IgG titers as determined by ELISA in immunized mice. Each panel represents data from 5-6 mice per group with equal sex distribution. Limit of detection (LoD) of 20 and 50 for $FRNT_{50}$s and ELISAs respectively are represented by a dashed line.

which stabilized at approximately 100 and 2500 for $FRNT_{50}$ and total IgG respectively (Figure 3.6 A, B). Animals receiving a single dose RS immunization and the prime-boost VS+VS regimen reached and maintained identical titers for both neutralizing antibodies and total IgG (Figure 3.6 A, B). For animals receiving the RS+RS or the heterologous VS+RS immunization regimens, titers peaked at day 35 at similar levels but VS+RS animals had decreasing neutralizing titers while RS+RS animals maintained neutralizing antibody titers of approximately 6,000 for 200 days before seeing decreasing titers (Figure 3.6 A). For VS+RS receiving animals neutralizing titers began to plateau at approximately 180 days post initial vaccination with neutralizing titers of 1100 (Figure 3.6 A). Interestingly, total IgG for both RS+RS and VS+RS had similar titers and a slight downward trend over time despite VS+RS having a more substantial decrease in neutralizing titers over the same period (Figure 3.6 A, B). Taken together, all vaccines induce long lived antibody responses that plateau in the long term with neutralizing antibody titers that are likely protective.

3.4 – Discussion

SFTSV is a recently emerged pathogen with a high case fatality ratio[14,15]. The expanding range of the vector responsible for transmitting SFTSV and the ability of the virus to spread in nosocomial and veterinary settings highlights the need for development of targeted vaccines and therapeutics to this pathogen[28,32,34,158]. In light of these factors, several health organizations including the NIAID and WHO have listed SFTSV as a priority pathogen for the development of therapeutics[35,36]. This has led to several vaccine platforms such as protein subunit, DNA, and recombinant virus platforms to be explored[45,112,144,146,149,190]. Here we added the mRNA platform as a potential candidate for an SFTSV vaccine. Previous work has shown that mRNA is able to drive high antibody titers and strong T-cell activity[124-126]. Furthermore, we characterize

and compare both rVSV and mRNA immunizations in single dose and prime-boost regimens including a heterologous model with an rVSV prime and mRNA boost (the reverse regimen was not considered due to the likelihood of mRNA induced antibodies neutralizing the rVSV vaccine boost). Heterologous vaccination has recently come to the forefront of vaccine research due to the approval for heterologous “mix-and-match” use of the SARS-CoV-2 vaccines around the world[192]. This vaccine regimen offers the potential to combine the strongest characteristics of each vaccine platform used to impart the strongest possible immune response. Our data suggest that heterologous immunization against SFTSV induces potent immune responses.

Most vaccine studies have thus far used *Ifnar*$^{-/-}$ mice as a challenge model to evaluate vaccine efficacy[45,112,144,146,149,190]. While these models have been sufficient and yielded data suggestive of vaccine efficacy, the model is not suitable for all studies due to its immunocompromised status. IFN signaling is essential for innate immune functions and for the development of adaptive immune responses. Following a previous study that indicated an antibody blockade against IFNAR made wild-type mice susceptible to lethal SFTSV infection, we were able to utilize a novel model wherein we immunized immunocompetent mice for characterization of immune responses[54]. Utilizing an α-IFNAR blockade, these mice could then be made susceptible to SFTSV infection for vaccine efficacy studies. Currently the only other immunocompetent animals available that exhibit lethal disease upon challenge with SFTSV are cats and ferrets[48,50]. While both animal models are superior to the aα-IFNAR blockade in the sense of not needing any immunomodulation, they have many draw backs. Both cats and ferrets are more expensive to purchase and maintain, and both animals have increased likelihoods of biting or scratching the researcher potentially leading to a transmission event in the absence of a tick as previously described[31,34]. Ferrets only manifest lethal disease when

aged >4 years, the reason for this age dependence remains unknown though it is likely due to the natural weakening of immune responses as animals age[48]. Finally, there is a lack of available reagents and tools for the analysis of immune responses in both cats and ferrets, making detailed immunological phenotyping as presented in this work not possible.

Currently developed and tested vaccines demonstrate inferior immune responses than what was measured in our homologous mRNA and heterologous rVSV+mRNA strategies. Current vaccine research against SFTSV includes a DNA vaccine encoding the SFTSV Gn and Gc ectodomains along with an N-NSs fusion protein[45]. In a three-dose immunization of *Ifnar*$^{-/-}$ mice, this vaccine imparted 40% protection from lethal challenge[45]. When the authors added IL-12 to the vaccine plasmid, to aid in cellular responses, full protection was observed[45]. Neither vaccination condition induced any detectable neutralizing antibodies[45]. This group then analyzed responses to recombinant protein vaccines by immunizing mice with Gn or Gc fusions to an Fc region with the adjuvant Alum[45]. These immunizations led to a neutralizing antibody response with titers ranging from 1:100 to 1:1000, interestingly this resulted in only 50% or 0% protection in mice immunized with Gn or Gc respectively[45]. Another strategy used was a vaccinia virus platform to deliver SFTSV Gn/Gc[146]. In this vaccine, high levels of total IgG are reached but neutralization is severely lacking with vaccinated mouse sera unable to neutralize 50% of virus at a 1:40 dilution[146]. In contrast to these and other studies, we achieve higher total IgG and neutralizing titers. Additionally, we demonstrate that these titers are maintained for almost a year after initial immunization. We and others have previously shown, by passive transfer studies, that antibodies are a correlate of protection against SFTSV[112,144,146,190]. The high levels of antibodies maintained over time

thus suggest these vaccination regimens are likely to provide long lived immunity from SFTSV infection.

T-cell responses and their contributions to protection from SFTSV remain largely unexplored. One study using vaccinia virus immunizations has shown that CD8+ T-cell depletion does not impact survival upon lethal challenge[146]. This data, however, is confounding as depletion was done in mice that were immunized and thus had SFTSV directed antibody responses[146]. Despite weak neutralizing titers, total IgG titers were high and could mediate protection through opsonization, complement activation, and natural killer cell mediated antibody dependent cellular cytotoxicity[146]. Indeed, CD8+ cell depletion did not affect survival in mice immunized with a vaccinia strain encoding SFTSV Gn/Gc but did have an impact in mice receiving just SFTSV N as an antigen[146]. Since antibodies cannot bind to N which is only found within cells or virions, this suggests that excluding antibody mediated protection T-cells do in fact contribute to protection[146]. Other groups using DNA vaccine platforms have also demonstrated that animals are protected when immunized with internal SFTSV proteins which do not induce neutralizing antibodies[144]. These studies suggest that T-cells may be important in the control and clearance of SFTSV upon infection. The cellular responses induced by other SFTSV vaccines are poorly characterized. Here we showed that mRNA vaccination as a single dose or in a prime-boost models potently induces polyfunctional CD4+ and CD8+ T-cells. CD8+ responses in heterologous vaccinated animals is comparable to homologous mRNA immunization though heterologous vaccinated animals exhibit higher degranulation of Granzyme B in cells stimulated with the SFTSV Gc peptide pool. Interestingly heterologous immunization consistently activates more CD4+ T-cells than homologous mRNA vaccination, though these differences are not statistically significant. Surprisingly, immunization with rVSV-SFTSV in a single dose or

prime-boost regimen induced weak or no T-cell responses. This is perhaps due to VSV being considerably attenuated by switching the cognate glycoprotein to SFTSV Gn/Gc which is not abundant on the cell surface where VSV buds[112,190,195]. This attenuation likely results in weak viral replication which is further controlled by VSV's sensitivity to IFNs. Taken together, heterologous and homologous mRNA immunizations displayed the strongest T-cell responses which other studies suggest may be an important factor in protection from SFTSV infections.

In designing vaccines, it is important to consider the polarization of immune responses towards a type 1 or 2 response[85]. Previous studies have shown that improper immune polarization may have deleterious impacts on the immunized patient[90]. This is best demonstrated in respiratory syncytial virus studies that determined the tested vaccines drove a type 2 response resulting in vaccine associated enhancement of disease[90]. Viral diseases and other intracellular pathogens are best controlled by type 1 responses characterized by cytokines such as type I IFNs, IFNγ, TNFα, and IL-2 along with antibody isotypes IgG2a/c (depending on mouse strain) [85]. In contrast, type 2 responses are suited for large extracellular pathogens and produce IL-4, IL-5, and IL-13 along with antibody isotypes IgE and IgG1[85]. In antibody analysis of type 1 and 2 responses we found that mRNA immunization led to a mixed polarization with approximately equal titers of IgG1 and IgG2c (Figure 3.2 F). Upon boost with mRNA these animals saw increased titers in both isotypes. In contrast, immunization with the rVSV platform led to a primarily IgG2c response. Heterologous immunization was a mixed phenotype with high levels of IgG2c and intermediate IgG1 titers. Interestingly, cellular analysis indicated a strong type 1 phenotype in mRNA immunized animals (for single and prime-boost regimens) and heterologous immunized mice with no IL-4 and IL-5 being detected. Perhaps this can be explained by mRNA vaccines being potent

activators of T follicular helper cells, which have been previously shown to be driven to a mixed polarization despite strong type 1 polarization in other CD4+ T-cells[124,127]. Further characterization of T-cell subsets and lymphoid T-cells is necessary to ascertain the reasons for a mixed polarization in antibody analysis versus a strong type 1 skewing in analyzed T-cells.

Despite differences in immune profiling, all tested conditions were protective upon lethal challenge. Interestingly our negative control mice immunized with VE and VE+RL were also partially protected from lethal challenge with SFTSV. This is presumably from activation and long-lasting effects of interferon stimulated genes upon immunization with VE. Similar observations of long-lasting non-specific vaccine responses have been reported after vaccination with the live attenuated polio virus vaccine and the live attenuated pertussis vaccine[196-198]. Other studies have implied that long-lasting non-specific immunity may be due to long-lived IFN responses, likely due to upregulation of IFN stimulated genes, inhibiting subsequent infection[199]. Infections with VSV induce strong IFN responses which are known to activate pathways that make an animal refractory to subsequent infection. This has previously not been described in other studies using similar rVSV negative controls, likely due to the use of *Ifnar*$^{-/-}$ mice which cannot initiate the innate immune responses our wild type mice can. Despite this protection in a negative control, we can still conclude that our VS immunizations induce specific protective responses not due to innate immunity as no weight loss was seen in VS or VS+VS immunized animals, in contrast VE and VE+RL immunized animals did survive challenge but suffered severe weight loss. Additionally, a temporal effect can be observed in tissue SFTSV titers in animals receiving a single dose VE immunization as opposed to VE+RL animals. Challenge of VE immunized animals occurred 14 days post immunization, in these animals SFTSV tissue titer was similar to titers in mice receiving

RS or VS immunizations. In prime-boost VE+RL animals, challenge occurred 35 days after the last RE dose was delivered. In these animals SFTSV tissue titers were decreased relative to our RL+RL negative control but not as low as animals receiving RS+RS, VS+RS, or VS+VS. This is consistent with the hypothesis of IFN mediated protection as we would expect waning protection over time.

Overall, the data presented highlight the potential for both mRNA and rVSV based vaccines against SFTSV. In homologous immunization, the mRNA platform greatly outperforms rVSV in immunologic stimulation. However, immune characterization cannot currently be directly correlated to protection. Due to this, extensive work is required to determine whether the differences seen in immunologic responses will translate to clinical differences. Additionally, other considerations must be considered when choosing a vaccine platform. An mRNA platform suffers from necessitating a cold chain which is difficult to maintain in isolated rural areas. Meanwhile, the rVSV-platform is well proven in isolated rural areas as demonstrated by the effective rollout of the rVSV based EBOV vaccine[97]. As has been suggested by other studies, heterologous immunization induces a somewhat superior or equivalent cellular response to homologous mRNA vaccination, and a somewhat weaker or equivalent humoral response[140-142,192]. These data are supported by human and animal studies of SARS-CoV-2[140-142,192]. Approval of vaccines designed to be delivered in heterologous strategies faces approval hurdles due to the need to demonstrate safety for two platforms. However, as we have recently seen in the SARS-CoV-2 pandemic, the use of heterologous immunizations has great potential for use in emergency situations where availability of different vaccine platforms may be lacking. Currently there are no approved SFTSV vaccines, in this study we demonstrate two viable platforms for potential development and three highly efficacious prime-boost regimens.

3.5 – Acknowledgements

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3.6 – Author Contributions

P.B., B.B.G., N.P., S.E.H. conceived and supervised the study. T.B.M., J.B.W., K.A.L., G.L.R., K.M.B., P.H., J.T.O. performed the experiments. N.P. and D.W. designed and made antigen-encoded mRNAs. Y.K.T. formulated mRNA to LNPs. P.B., B.B.G., T.B.M., J.B.W. analyzed the data. T.B.M., J.B.W. wrote the manuscript.

3.7 – Conflict of interest

N.P. and D.W. are named on a patent describing the use of modified mRNA in lipid nanoparticles as a vaccine platform. They have disclosed those interests fully to the University of Pennsylvania, and have in place an approved plan for managing any potential conflicts arising from licensing of these patents. XY are employees of Acuitas Therapeutics.

3.8 – Materials and Methods

3.8.1 Ethics Statement

All animals were treated ethically complying with guidelines set by the USDA and Utah State University Institutional Animal Care and Use Committee and the University of Pennsylvania Laboratory Animal Resources guidelines. Animals were humanely euthanized once approved endpoints were reached.

3.8.2 Cells, Viruses, and Mice

ATCC verified and mycoplasma free 293T and Vero E6 cells were maintained in DMEM containing 10% cosmic calf fortified serum (HyClone, #SH30087.03), 2mM L-

glutamine (Corning, #25-005-Cl), and 1mM Sodium Pyruvate (Gibco, #11360-070). Cells were passaged every 2-3 days and maintained for no more than 20 passages.

Recombinant viruses encoding heterologous viral glycoproteins in genomic position 4 (rVSV-SFTSV and rVSV-EBOV) were launched and described previously. rVSV-SFTSV and rVSV-EBOV also contain an additional open reading frame in position five encoding mCherry. All recombinant viruses were grown in Vero E6 cells by infecting a confluent T-175 flask at an MOI of 0.3-0.5. Virus was collected at 48-72 hours post infection with the addition of Hepes buffer pH7.4 to 25mM. Media was clarified by centrifuging at 6000 times gravity for 5 minutes at 4 ^{o}C twice. Virus was then frozen at -80 ^{o}C until used for ultracentrifugation. Virus was concentrated by ultracentrifuging virus-containing media through a 20% sucrose gradient at 115,500 times gravity for 2 hours at 4 ^{o}C using SW-32 tubes in a Beckman Coulter Optima XPN-80 ultracentrifuge. After removal of the sucrose and media, pelleted virus was placed on ice with 500µl hepes buffered saline overnight. The next day virus pellets were resuspended and frozen at -80 ^{o}C. Viral titer was determined by plaque assays on Vero E6 cells with a 1.25% Avicel RC-591 NF (DuPont, #RC591-NFBA500) overlay and then stained with 1% crystal violet.

SFTSV, strain HB29, was obtained from Dr. Robert Tesh (WRCEVA; World reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch, Galveston, TX). The virus stock (5.6 x 10^6 PFU/ml; 1 passage in Vero E6 cells) used was from a clarified cell culture lysate preparation. Virus stock was diluted in sterile minimal essential medium (MEM) and inoculated by intraperitoneal injection of 0.2 ml containing approximately 26, 260, 2,600, 26,000 $CCID_{50}$ for LD_{50} studies, and 26,000 $CCID_{50}$ in challenge studies. For challenge experiments, animals were given 1 mg of α-IFNAR1 monoclonal antibody clone MAR1-5A3 (Leinco Technologies, St. Louis,

MO, #I-1188) by intraperitoneal injection on days -1 and 0.5 mg of α-IFNAR1 monoclonal antibody on day 2 post challenge.

C57BL/6 mice were ordered from Jackson Labs (Bar Harbor, ME). All mouse experiments were done using equal numbers of male and female mice. All mice were given approximately 7 days to acclimate to their cages and vivarium prior to each experiment. Mice were weighed immediately prior to all vaccination and infection procedures. Mice receiving intradermal injections at the University of Pennsylvania were anesthetized using 1% isoflurane in air delivered by vaporizer (Northern Vaporisers, Skipton, UK) to the anesthesia chamber. Mice at Utah State University were anesthetized for intradermal injections using a bell jar and open-drop method. Injection sites were first prepared by cleaning with a 70% ethanol pad. Vaccination experiments without authentic SFTSV challenge were performed under animal biosafety level (ABSL) 2 conditions at the University of Pennsylvania. All other vaccination experiments that included authentic SFTSV challenge were performed in ABSL3 conditions at Utah State University.

3.8.3 Production of mRNA-LNP Vaccines

The codon-optimized Gn/Gc glycoprotein gene from SFTSV and firefly luciferase were synthesized (Genscript) and cloned into an mRNA production plasmid. A T7-driven in vitro transcription reaction (Megascript, Ambion) using linearized plasmid template was performed to generate mRNA with 101 nucleotide long poly(A) tail. One-methylpseudouridine (m1Ψ)-5'-triphosphate (TriLink) instead of UTP was used to generate modified nucleoside-containing mRNA. RNAs were capped using the m7G capping kit with 2'-O-methyltransferase (ScriptCap, CellScript) to obtain cap1 as described[200]. Cellulose-based purification of mRNAs was performed as described[201]. All mRNAs were then tested on an agarose gel before storing at -20°C. The cellulose-

purified m1Ψ-containing mRNAs were encapsulated in LNPs using a self-assembly process as previously described wherein an ethanolic lipid mixture of ionizable cationic lipid, phosphatidylcholine, cholesterol and polyethylene glycol-lipid was rapidly mixed with an aqueous solution containing mRNA at acidic pH[202]. The RNA-loaded particles were characterized and subsequently stored at -80°C at a concentration of 1 mg/ml.

3.8.4 Enzyme linked immunosorbent assay (ELISA)

Production of SFTSV Gn and Gc ectodomains: The SFTSV Gn ectodomain (amino acids 20-452) and Gc ectodomain (amino acids 562-996) were cloned from pCAG-SFTSV Gn/Gc and put into the pHLsec expression vector which adds a secretion signal and c-terminal His tag to inserted sequence. FreeStyle 293-F cells grown in FreeStyle 293 Expression Media (Gibco, #12338018) were co-transfected using 293Fectin (Gibco, #12347019) with pHLSec-SFTSV Gn and pHLSec-SFTSV Gc plasmids encoding ectodomains of SFTSV Gn or Gc. Supernatants were collected 4 days post-transfection and purified by nickel-nitrilotriacetic acid resin (Qiagen, #30210) according to the manufacturers protocol. Eluted protein was concentrated and buffer exchanged into PBS using an Amicon Ultra-15 Centrifugal Filter with a 30 kDa MWCO (Millipore, #UFC903024) and frozen at -80°C.

ELISA: Immulon 2HB (Thermo Scientific, #3455) plates were coated with 0.75μg/ml of purified SFTSV Gn/Gc ectodomains in sodium carbonate at 4ºC overnight. The next day ELISA plates were washed with phosphate buffered saline containing 0.1% Tween-20 (PBS-T) and blocked for an hour at room temperature with 3% milk in PBS-T. Mouse sera was diluted in 1% milk in PBS-T and serialy diluted 2-fold with an initial dilution of 1:50, 1:800, or 1:3200. Plates were incubated with diluted mouse sera for 2 hours at room temperature. Secondary HRP conjugated antibodies for total IgG (GE Healthcare, #NA931), IgG1 (Cell Signaling Technologies, #96714S), and IgG2c (Cell

Signaling Technologies, #56970S) were diluted in 1% PBS-T at 1:5000, 1:6000, or 1:4000 respectively and staining was done at room temperature for an hour. Prior to experiments IgG1 and IgG2c antibodies were normalized against known concentrations of their target antigens so comparisons between isotypes could be made. SureBlue TMB 1 component substrate (KPL, #52-00-01) was then added to plates and quenched after 5 minutes with 250mM HCl. Absorbance at 450nm was immediately read on a SpectraMax 190 microplate reader (Molecular Devices, San Jose, CA). Endpoints were determined as OD values twice as high as the background on a negative control run on each plate. Samples lacking absorbance at our lowest dilution of 1:50 were assigned a titer of 25 signifying titers below the limit of detection.

3.8.5 Immunizations

Vaccines were diluted to the desired concentrations with sterile PBS just prior to vaccination by IP injection for rVSV based vaccines and intradermal injections for mRNA vaccines. Viral vaccines were done with a 200µl inoculum and mRNA vaccines were done with a 50µl inoculum.

3.8.6 Blood collection

Mice were isofluorane anesthetized and blood was collected through the submandibular route using Goldenrod lancets 5mm (Medipoint, Mineola, NY). Blood was maintained on ice after collection. Serum was separated from blood by centrifugation at 8,000 RPM for 30 minutes at 4 ^{o}C in an Eppendorf 5424R centrifuge (Eppendorf, Enfield, CT). Serum was heat inactivated by incubating at 56 ^{o}C for 30 minutes. While running neutralization assays and ELISAs, serum was stored at 4 ^{o}C, for long term storage serum was frozen at -80 ^{o}C.

3.8.7 Pseudovirus neutralization assay

Production of VSV pseudotype with SFTSV Gn/Gc: 293T cells plated 24 hours previously at 2 X 10^7 cells per T-175 flask were transfected using Lipofectamine 2000 (Invitrogen, #11668-019) using manufacturers protocol. Briefly, tubes each containing 1.75ml optimem (Gibco, #31985-070) were made. In one tube 100ul of Lipofectamine 2000 reagent was added and gently mixed. In the other, 45ug of pCAG-SFTSV Gn/Gc expression plasmid was added, tubes were allowed to sit for 5 minutes at room temperature. Lipofectamine and DNA containing tubes of optimum were combined and gently mixed, after 20 minutes incubating at room temperature. Solution was added to flask of 293T cells, after 4 hours cells were fed with fresh media. Thirty hours after transfection, the SFTSV Gn/Gc expressing cells were infected for 2-4 hours with VSV-G pseudotyped VSVΔG-mNeon at an MOI of ~1-3 (Generated by deleting the cognate VSV-G and linking mNeon to the n-terminus of P. Virus was launched as previously described [43]). After infection, the cells were washed twice with FBS-free media to remove unbound virus. Media containing the VSVΔG-mNeon SFTSV Gn/Gc pseudotypes was harvested 30 hours after infection and clarified by centrifugation twice at 6000g then aliquoted and stored at -80 °C until used for antibody neutralization analysis.

Antibody neutralization assay using VSVΔG-mNeon SFTSV Gn/Gc: Vero E6 cells were seeded in 100 µl at 2x10^4 cells/well in a 96 well collagen coated plate. The next day, 2-fold serially diluted serum samples were mixed with VSVΔG-mNeon SFTSV Gn/Gc pseudotype virus (100-200 focus forming units/well) and incubated for 1hr at 37 °C. Also included in this mixture to neutralize any potential VSV-G carryover virus was 1E9F9, a mouse anti-VSV Indiana G, at a concentration of 600 ng/ml. The antibody-virus mixture was then used to replace the media on VeroE6 cells. 16 hours post infection, the

cells were washed and fixed with 4% paraformaldehyde before visualization on an S6 FluoroSpot Analyzer (CTL, Shaker Heights OH). Individual infected foci were enumerated and the values compared to control wells without serum. The focus reduction neutralization titer 50% ($FRNT_{50}$) was measured as the greatest serum dilution at which focus count was reduced by at least 50% relative to control cells that were infected with pseudotype virus in the absence of mouse serum. $FRNT_{50}$ titers for each sample were measured in two to three technical replicates performed on separate days.

3.8.8 Flow Cytometry

Mouse spleens were harvested and placed in RPMI (Gibco, # 11875-085) with 10% FBS (Corning, #35-010-CV). Spleens were smashed between two slides to make single cells suspension. Cells were filtered through a 70um cell strainer (Biologix, Cat # 15-1070). ACK lysis buffer (Quality Biological, Cat # 118-156-101) was used to lyse red blood cells. Remaining cells were then resuspended in RPMI containing 10% FBS, glutamine (Corning, #25-005-Cl), and β-mercaptoethanol (Sigma-Aldrich, #M7522). Peptide pools containing SFTSV Gn or SFTSV Gc peptides (GenScript, Piscataway, NJ) were added to cells at 1.5ug/ml for an hour at 37 C in the presence of α-CD28 antibody (BD, #553295). Golgi plug (BD, #555029) and golgi stop (BD, #554724) were then added to cells and incubated overnight at 37 C. If staining for CD107a PE-Cy7 (BD, #560647), antibody was added along with golgi plug/golgi stop and stained overnight. Extracellular staining was then done in FACS buffer for 30 minutes at 4 C with L/D Aqua (Invitrogen, #L34957), α-CD8 Pacific Blue (Biolegend, #100725), and α-CD4 PerCP/Cy5.5 (Biolegend, #100434). Cells were fixed using BD fix/perm solution (Cat #554722) then stained intracellularly with α-IL-2 BV711(Biolegend, #503837), α-TNFa PE-Cy7(BD, #557644), α-IFNg AF-700(BD, Ca#557998), α-CD3 APC-Cy7 (BD, #557596), α-Granzyme B (BD, #560213). Cells were analyzed on a BD LSRII flow

cytometer with high-througput system using FACSDIVA software (BD Biosciences). Flow data was then analyzed and prepared for presentation with FloJo software (FloJo LLC).

3.8.9 Serum and Tissue Virus Titers

Virus titers were assayed using an infectious cell culture assay as previously described[187]. Briefly, a specific volume of tissue homogenate or serum was serially diluted and added to quadruplicate wells of Vero E6 (African green monkey kidney) cell monolayers in 96-well microtiter plates. The viral cytopathic effect (CPE) was determined 10 days after plating and the 50% endpoints were calculated as described[188]. The assay lower limits of detection were 1.67 $\log_{10}$ $CCID_{50}$/ml serum and 2.27 $\log_{10}$ $CCID_{50}$/g tissue.

3.8.10 Statistical and Data Analysis

All serological assays were analyzed with two-way analysis of variance (ANOVA) with tukey's multiple comparisons test. T-cell assays were analyzed with ordinary one-way ANOVAs with tukey's multiple comparisons test. All statistical evaluations were completed on Prism 9 (GraphPad Software, La Jolla, CA).

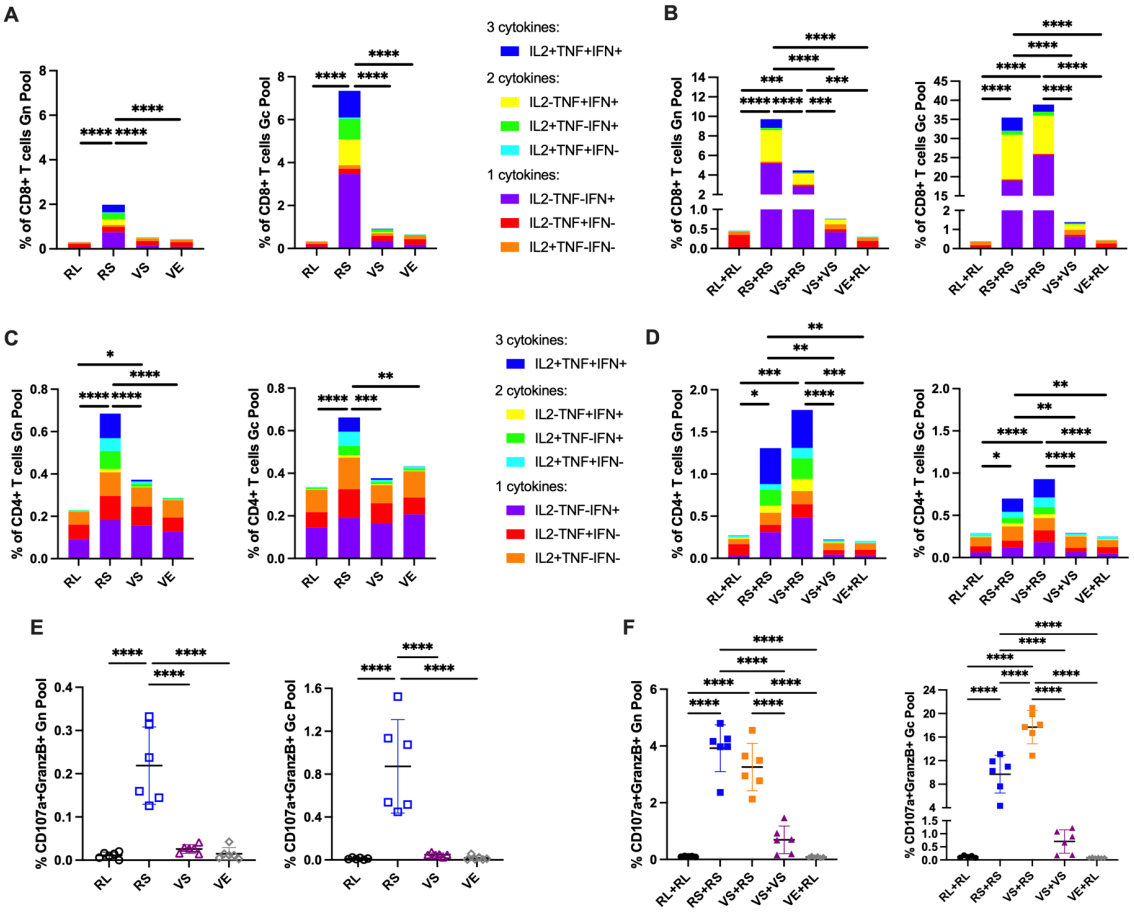

Figure 3.S1 Single dose RS and prime-boost regimens RS+RS and VS+RS continue having strong cellular responses 14 days after immunization Gating strategies like those used in Figure 3.3 were used for analysis of flow cytometry data from spleenocytes stimulated overnight with SFTSV Gn or Gc peptide pools 14 days after immunization. Cytokine profiling of CD8+ T-cells in single dose immunized animals (A) and prime-boost immunized animals (B). Throughout figure, Gn peptide pool stimulated cells are shown on the left and Gc peptide pool stimulated cells are shown on the right. CD4+ T-cell cytokine profiling in single dose immunized animals (C) and prime-boost immunized animals (D). Analysis of degranulation in CD8+ T-cells by CD107a and Granzyme B are shown in single dose immunized animals (E) and prime-boost immunized animals (F). Each panel includes data from 6 mice per group with equally split sexes. Ordinary one-way ANOVAs with tukeys multiple comparisons test was used in panels A-F (p= *<0.0332, **<0.0021, ***<0.0002, ****<0.0001). In panels A-D analysis was run on total cytokine positive cell percentage, not based on specific cytokine profiles.

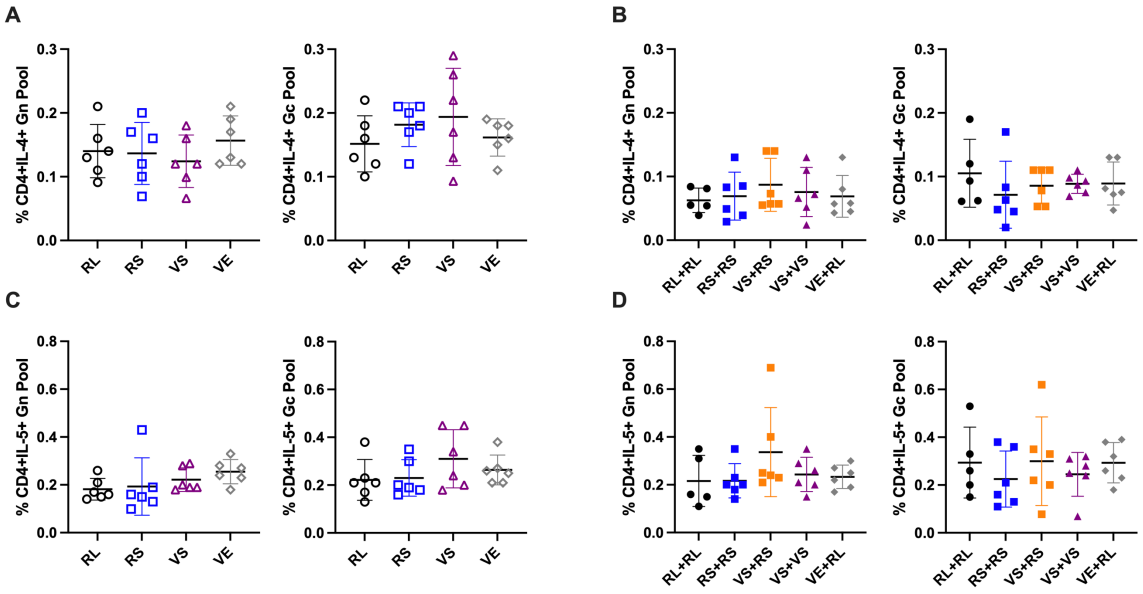

Figure 3.S2 No immunization conditions are positive for the type 2 cytokines IL-4 and IL-5 at 14 days post immunization. Gating strategies like those used in Figure 3.4 were used for analysis of flow cytometry data from spleenocytes stimulated overnight with SFTSV Gn or Gc peptide pools 14 days after immunization. IL-4 cytokine profiling of CD4+ T-cells in single dose immunized animals (A) and prime-boost immunized animals (B). Throughout figure, Gn peptide pool stimulated cells are shown on the left and Gc peptide pool stimulated cells are shown on the right. CD4+ T-cell IL-5 cytokine profiling in single dose immunized animals (C) and prime-boost immunized animals (D). Each panel includes data from 5-6 mice per group with equally split sexes. Ordinary one-way ANOVAs with tukeys multiple comparisons test was used in panels A-D, no significant comparisons were found.

CHAPTER 4: Discussion

4.1 – Summary of findings

Severe fever with thrombocytopenia syndrome virus (SFTSV) is an emerging bunyavirus with a high case fatality ratio, posing considerable risk of causing large, deadly outbreaks. Currently, no therapeutics or vaccines are approved for treatment or prevention of SFTSV infection. The goal of this dissertation was to explore recombinant vesicular stomatitis virus (rVSV) and mRNA vaccine platforms against SFTSV and to determine the safety, immunogenicity, and efficacy of these exploratory vaccines in small animal models.

In chapter 2, we assessed an rVSV-SFTSV vaccine in a single dose model. A common concern with rVSV based vaccines is neuropathogenesis, which is commonly observed in wild type VSV and sometimes reported in rVSVs[98,109,110]. To address this safety concern, we performed intracerebral injections into 4-week old wild type mice. None of the mice infected with rVSV-SFTSV developed any signs of neuropathy or succumbed to infection. To further demonstrate safety, we showed that immunization of the immunocompromised *Ifnar*$^{-/-}$ mouse strain was not lethal. This finding indicates that an rVSV-SFTSV vaccine may be safe in immunocompromised humans. Immunogenicity and challenge experiments showed that the rVSV-SFTSV vaccine induced high levels of neutralizing antibodies and was protective from lethal SFTSV challenge. Antibodies appear to be a correlate of protection from SFTSV infection as passive transfer of sera from immunized mice to naïve mice was protective upon lethal challenge with SFTSV. We then evaluated the ability of rVSV-SFTSV immunization to cross protect from lethal challenge with a mouse adapted strain of the closely related Heartland virus (HRTV). Despite a lack of detectable neutralizing antibodies, we found that rVSV-SFTSV

immunization induced partial protection from lethal HRTV challenge. Taken together, this work demonstrated the safety and efficacy of rVSV-SFTSV as a vaccine against SFTSV.

In chapter 3, we developed a novel mRNA based SFTSV vaccine and compared it to our previously described rVSV-SFTSV vaccine both in single dose, and prime-boost regimens. To assess the immunogenicity of our vaccines in an immunocompetent animal model, we first used an α-IFNAR antibody blockade to make wild-type mice susceptible to lethal SFTSV challenge. Once a working immunocompetent animal model was developed, we began assessing the immunogenicity of our vaccine platforms. In single dose immunizations we found that mRNA vaccines induced similar titers of total IgG, and higher levels of neutralizing antibodies against the SFTSV glycoproteins Gn/Gc than immunizations with rVSV-SFTSV. Interestingly, rVSV-SFTSV immunization induced only weak T-cell responses while mRNA robustly activated both CD8+ and CD4+ T-cells. Despite the weaker immune response observed in rVSV-SFTSV immunized animals relative to mRNA immunization, both groups were protected from lethal challenge with SFTSV, showing no weight loss upon challenge.

In prime-boost studies, trends were consistent with those observed in single dose immunizations. Animals receiving homologous mRNA vaccinations demonstrated higher total IgG and neutralizing antibody titers than homologous rVSV-SFTSV immunized animals. Similarly, homologous mRNA immunized animals had potent activation of CD4+ and CD8+ T-cells while homologous rVSV-SFTSV receiving mice had mild cellular responses. In a lethal SFTSV challenge model, both prime-boost regimens were equally effective in protecting animals from death and preventing weight loss. Further analysis of immune polarization showed that cellular responses from mRNA and rVSV-SFTSV vaccines in both single dose and prime-boost models were skewed towards a type 1 cytokine profile with no detectable levels of type 2 cytokines. Interestingly, antibody

isotype analysis indicated that rVSV-SFTSV vaccines drove a strong type 1 response (with high titers of primarily IgG2c), while mRNA immunization drove a mixed type 1/2 response with high titers of IgG1 and IgG2c. When homologous immunizations were compared to a heterologous (rVSV-SFTSV prime, mRNA boost) strategy, we found that total IgG and neutralizing antibody titers were similar to homologous mRNA immunization. T-cell response analysis showed heterologous immunization had similar cytokine profile compared to homologous mRNA strategies. As with all other conditions, no type 2 cytokines were detected in heterologous immunized animals; though, the type 2 associated antibody isotype IgG1 was detected at intermediate levels between those seen with homologous mRNA and rVSV-SFTSV immunizations. Heterologous immunization was protective from lethal challenge with no weight loss in challenged mice. Ultimately, our data suggests that all tested vaccine platforms and strategies are safe and immunogenic.

4.2 – General discussion and future directions

4.2.1 rVSV-SFTSV vaccine

Our studies in chapter 2 and 3 demonstrate that rVSV-SFTSV is protective but does not induce cellular and humoral responses as robust as seen with mRNA immunization. This finding was surprising as rVSV based vaccines have frequently been shown to induce high levels of antibodies and good cellular responses[102,106,107]. While good antibody responses were achieved, T-cell activity was limited in our studies. The cause of this limited activity is likely due to rVSV-SFTSV being overly attenuated. We and other groups have shown that while rVSV-SFTSV reaches similar titers to wild-type VSV, it lags about 36 hours behind wild-type VSV[112,190]. The slower growth of rVSV-SFTSV relative to wild-type VSV is clearly seen when comparing the much smaller plaque sizes of rVSV-SFTSV relative to wild-type VSV[112,190]. This slowed growth

combined with VSV's sensitivity to IFN likely result in the rapid control of rVSV-SFTSV in immunized animals preventing robust activation of T-cells. Several potential methods are available to address the over-attenuation of rVSV-SFTSV.

It is well described that VSV buds at the plasma membrane, with budding mediated by the matrix protein[6]. In contrast, SFTSV buds into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and Golgi, with SFTSV Gn/Gc demonstrating strong Golgi localization signals[6,195]. This mislocalization of the glycoprotein and the VSV proteins to different cellular locations is the likely cause of rVSV-SFTSV attenuation. Our vaccine strain rVSV-SFTSV likely relies on SFTSV Gn/Gc leaking from the Golgi to the plasma membrane, VSV components mislocalizing to the Golgi and exiting the cell similarly to SFTSV, or a combination of these. The localization signals maintaining SFTSV Gn/Gc in the ER/Golgi have not yet been fully mapped; However, preliminary data from our lab and others, suggest that a coat protein complex I (COPI) binding motif (RxxKxx) in the c-terminus of Gc may contribute to the observed ERGIC/Golgi localization[195]. A single amino acid mutation (K-3A) to the COPI motif has been shown to increase Gn/Gc localization to the plasma membrane thereby allowing us to correct viral component mislocalization[195]. Indeed, preliminary work from our lab shows that VSV pseudotype virus production with K-3A mutant Gn/Gc leads to much higher virus titers than wild type SFTSV Gn/Gc. Future work is needed to characterize whether an rVSV-SFTSV K-3A mutant would induce superior immunogenicity when used as a vaccine.

An alternative solution to solve attenuation of rVSV-SFTSV is the manipulation of the VSV genome. Wild type VSV has its genome organized from the 3' end with the nucleoprotein (N) encoded first, followed by the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), and finally the large RdRP (L). This organization is important

as the RdRp transcribes mRNAs sequentially from the 3' end with a probability of dislodging from the template strand after each mRNA is produced[6,203]. This leads to a higher quantity of N mRNA being produced than P, and more P than M and so on[204]. Altering the gene order can alter the relative levels of protein produced[204]. Therefore, changing the gene order for rVSV-SFTSV from it's current N-P-M-Gn/Gc-L to Gn/Gc-N-P-M-L may lead to increased production of SFTSV Gn/Gc, resulting in higher titers and a less attenuated rVSV-SFTSV. Some studies have shown that genome alterations like those described above can attenuate VSV, while other genetic modifications, such as the addition of a fluorescent protein open reading frame, have resulted in minimal virus attenuation[205,206]. If attenuation is observed, it may be possible to further manipulate protein levels and restore virulence with 2A self-cleaving peptides. These peptides allow 2 proteins to be encoded by one mRNA by inducing ribosomal skipping at the 2A sequence, which results in the release of the nascent protein. Once ribosomal translation begins again, a second protein is produced[207]. Development of next generation VSV platforms featuring altered genome organizations will require extensive testing to assess effects on virulence. Next generation rVSVs may also be useful in attenuating rVSVs that are too virulent. For example, the rVSV-EBOV vaccine virus that is currently FDA approved for use in humans is lethal in *Ifnar*$^{-/-}$ mice and is known to cause severe side effects in humans[97,165]. A next generation rVSV-EBOV vaccine could potentially offer improved immunogenicity and safety. Importantly, development of an rVSV-SFTSV K-3A or next generation rVSV-SFTSV platforms will require further evaluation of vaccine safety. It is likely that as we attempt to increase virulence to improve immunogenicity, we will in turn negatively impact safety, an ideal vaccine candidate must balance safety and immunogenicity.

Two alternatives to increase immunogenicity without altering our current rVSV-SFTSV platform include increasing immunization dosage, and the use of adjuvants. Immunizations in chapters 2 and 3 were done using 10^6 PFU inoculums. This is a high dose of virus considering that the only rVSV vaccine currently approved for human use has been found to have optimal immunogenicity at $2x10^7$ PFU[208,209]. Using too high of a dose may lead to inflammation and serious side effects. Furthermore, increasing inoculum titer leads to a reliance on production of more virus, which takes longer to grow and requires more resources, thereby making production inefficient. Adjuvants offer a viable alternative to overcome issues surrounding dosing by stimulating the immune system and thus amplifying immune responses upon vaccination. Unfortunately, most adjuvants in use today drive a type 2 immune response, which is not well suited to respond viral infections[210]. Type 1 response driving adjuvants have been developed in research settings but are not yet approved for human use[211,212]. Further work is required to determine whether novel adjuvants may improve rVSV-SFTSV immunogenicity and to achieve approval of type 1 skewing adjuvants in humans.

A final consideration of rVSV based vaccines is their ability to elicit rapid non-specific responses. In our *Ifnar*$^{-/-}$ studies, mice immunized with a single dose of rVSV-SFTSV 7 days prior to lethal challenge were fully protected. Other studies have demonstrated that immunization with rVSVs expressing Marburg glycoproteins provided rapid protection from challenge when administered 3-7 days prior to challenge and even when administered post-exposure[103-105]. One potential use for this property of rVSV vaccines is in post-exposure prophylaxis. Laboratory needle sticks are a relatively common source of infection, using an rVSV vaccine post-exposure may be protective in some circumstances. Another interesting application of rVSV based vaccines was seen in our α-IFNAR studies where we observed that rVSV-EBOV immunized animals were

protected from lethal challenge in single dose, and when receiving mRNA luciferase boosts. In contrast, vaccination with mRNA luciferase alone did not elicit protective responses. Non-specific rapid protection has been observed in several vaccines such as the live attenuated polio vaccine and the pertussis vaccine[196-199]. Similarly to what has been observed in our own work, studies suggest this non-specific protection may last over a month. Due to kinetics and functional characteristics of the adaptive immune response, this protection cannot be mediated by B- and T-cells and is thus likely due to upregulation of innate immune factors such as IFN stimulated genes (ISGs). ISGs are known to have antiviral functions that act broadly and rapidly. A potential use of this broad non-specific protection elicited by rVSVs is in remote and economically disadvantaged areas where it is difficult to follow up with patients and/or maintain a cold chain. Immunization with rVSV based vaccines (which can be lyophilized and maintain infectivity) could potentially protect patients from infection against the immunogen delivered and provide non-specific protection against other common pathogens[108]. Taken together, rVSV is a powerful vaccine platform that may be effectively used against many pathogens, and can be modified to provide the best possible responses.

4.2.2 mRNA SFTSV vaccine

Using the mRNA vaccine platform, we developed a novel SFTSV vaccine candidate and demonstrated strong immunogenicity. In both single dose and prime-boost regimens, mRNA SFTSV vaccination induced powerful cellular and humoral responses. Interestingly, we observed mRNA vaccines generated powerful cellular type 1 immunity characterized by the type 1 cytokines IFN, TNF, and IL-2. We did not detect CD4+ T-cells positive for the type 2 cytokines IL-4 and IL-5. Despite the lack of type 2 cytokines, mRNA immunization led to a mixed antibody response with both IgG2c (type 1 isotype), and IgG1 (type 2 isotype, IL-4 is required for class switch to IgG1). Other

studies have reported similar findings of mixed antibody responses and largely type 1 skewed CD4+ responses[124,127]. These studies found that while CD4+ T-cells remain type 1 skewed, a subset of CD4+ cells called follicular helper T-cells (Tfh) shows mixed type 1/2 polarization[124,127]. This explains the discrepancy we observed in the skewing of immune responses in antibodies and secreted cytokines. These same studies also suggest that different antigens encoded by mRNA vaccines may impact the immune polarization with some antigens skewing responses to type 1 while other antigens favor a mixed response[124,127]. Further work is required to dissect the responses incurred by our mRNA SFTSV vaccine to various cellular subsets.

mRNA vaccine technologies allow for the delivery of many potential immunogens. In our studies we used SFTSV Gn/Gc, but other SFTSV targets may be used alone or in conjunction to Gn/Gc. Protein subunit vaccine studies have demonstrated that neutralizing antibodies may be made to either subunit of SFTSV Gn/Gc, which highlights the possibility of mRNA vaccines encoding only Gn or Gc[45]. Immunization with individual Gn/Gc subunits may drive immune responses to target certain epitopes of SFTSV Gn/Gc more potently. Epitope mapping of SFTSV Gn/Gc would also be beneficial in order to establish which epitopes may drive strong T-cell responses allowing for targeted vaccine development. Targeting non-surface SFTSV proteins, such as the nucleoprotein (N), is also an interesting proposition. With non-structural targets, cellular responses such as CD8+ T-cells would be critical. We have demonstrated that our mRNA vaccines drive strong CD8+ responses in mice though other studies suggests this may not necessarily translate to other animal models[124,127,130]. It is important to consider the impact that different immunogens may have on the polarization of the immune response as previous work has demonstrated that changes in mRNA vaccine immunogens may alter immune polarization.

Nevertheless, exploring different mRNA immunogens against SFTSV warrants further work.

4.2.3 Heterologous vaccines

Historically, heterologous vaccination strategies in humans have been avoided; However, since the COVID19 pandemic, heterologous immunizations have received renewed attention and emergency authorization has been granted to “mix-and-match” COVID19 vaccines[192]. Few studies have done in depth analysis of immunologic responses to heterologous immunizations[140-142,192]. Preliminary analyses show that heterologous immunization induces similar to slightly inferior levels of antibodies as homologous mRNA immunization, and equivalent to slightly superior cellular responses[140-142,192]. These results are largely similar to what we have shown, though our studies dive into more immunologic detail. We also compare rVSV+mRNA immunization while other studies have studied an adenovirus prime in the context of COVID19.

Our studies demonstrated that heterologous immunization induces similar immunogenicity to homologous mRNA immunization despite an attenuated rVSV prime. Antibody responses in heterologous immunization demonstrated the highest fold increase in neutralizing antibody titers. Increasing the virulence of the rVSV-SFTSV prime may lead to higher antibody titers after prime and potentially superior titers to homologous mRNA after boost. Similarly, T-cell responses with a single dose of rVSV-SFTSV were lacking in comparison to responses in mice receiving a single dose of mRNA SFTSV; However, upon boost, mice receiving heterologous immunizations had slightly superior T-cell responses in CD4+ T-cells and CD8+ T-cell granzyme B degranulation. These results are particularly interesting due to the relative lack of T-cell stimulation observed upon immunization with rVSV-SFTSV. These results imply that rVSV-SFTSV did prime T-cells to mount a robust response despite our results. Further

characterization of the response to rVSV-SFTSV is necessary to determine whether certain cellular subsets, organs, or response timing can explain the discrepancies observed between the prime and boost immunizations. Taken together, increasing the immunogenicity of the first dose may lead to heterologous immunization eliciting superior antibody and T-cell responses.

Heterologous immunization demonstrated robust T-cell activation, however, T-cell contributions to protection from SFTSV currently remain unknown. Previous studies have suggested that T-cells may contribute to protection from lethal challenge with SFTSV while other studies have suggested CD8+ T-cells are not necessary[45,54,146]. Currently, no studies have been done to directly determine the contribution of T-cells. Further work is thus required in the form of adoptive transfers to conclusively assess T-cell contributions to protection from lethal SFTSV challenge. Further studies such as these could be used to determine whether CD4+, CD8+ T-cells populations, both, or neither are sufficient to impart protection from SFTSV. Currently, antibodies are the only known correlate of protection for SFTSV, therefore, determining the impact of T-cells would further our understanding of the correlates of protection for SFTSV infection and inform future vaccine development.

Attaining approval for two vaccine platforms simultaneously presents one of the primary obstacles to heterologous vaccines platforms. Vaccine approval requires that all reagents in the vaccine exhibit favorable safety profiles and meet good manufacturing practices. Having multiple platforms in a vaccine regimen would complicate safety trials and impose more work on pharmaceutical companies than using a single platform. As vaccine development is already relatively unprofitable for pharmaceutical companies, they are unlikely to push for vaccine strategies that may impose further financial barriers for approval unless governments impose incentives. The current COVID19 pandemic,

however, demonstrates that governments may embrace heterologous immunizations in times of vaccine shortages or emergency situations. Additionally, if evidence appears for mRNA vaccination inducing superior responses to other platforms it is reasonable that boosters may be given to individuals previously immunized with different vaccine platforms. However, an enduring difficulty of mRNA vaccines is the necessity for cold chains. The delivery of these vaccines to rural and hard to reach areas remains difficult and further work is necessary to address cold chain requirements of mRNA vaccines.

4.2.4 Animal models & cross protection

A concession that must be made in developing vaccines is that animal models do not accurately represent human immune responses. Nevertheless, it is necessary to select a model that has a similar disease progression and immune system for vaccine trials to occur. Most SFTSV work currently relies on the *Ifnar*$^{-/-}$ mouse which is immunosuppressed; therefore, these mice are not representative of the immune competent humans we would like to protect with vaccinations. The novel α-IFNAR blockade model attempts to address these shortcomings by using mice that have functional immune systems and are only temporarily immunosuppressed to sensitize animals to lethal disease. Further work is necessary to validate other candidate SFTSV vaccine platforms and assess whether immunogenicity of these vaccines is altered in animals with fully competent immune systems. Additionally, validation of the α-IFNAR blockade mouse model with other related bunyaviruses such as HRTV is required to assess its suitability for research of other pathogenic bunyaviruses lacking adequate mouse models.

We have demonstrated that rVSV-SFTSV immunization was cross-protective from lethal challenge with HRTV. Neutralizing antibody data demonstrated only low titers of cross-neutralizing antibodies, suggesting protection was mediated by cellular

responses or other non-neutralizing antibody functions. This is contradictory to previously published data where SFTSV/HRTV cross-neutralization was observed[112]. In this previous study, a different strain of SFTSV Gn/Gc was delivered as an immunogen than what was used in our studies, suggesting that cross-neutralization and maybe cross-protection may be determined to some extent by the immunogen strain[112]. Further work is necessary to assess SFTSV strain impacts upon cross-protection from HRTV or other pathogenic bunyaviruses. In preliminary experiments we observed cross-neutralization of the Puumala (PUUV) hantavirus upon immunization with rVSV-SFTSV. This suggests that conserved epitopes may exist that could be targets for the development of vaccines targeting highly divergent bunyaviruses. Further work is necessary to determine whether such epitopes can be targeted and what vaccine platforms and immunogens may effectively drive an immune response towards these epitopes.

4.3 – Closing remarks

Overall, this work addresses the lack of SFTSV targeting vaccines by analyzing the safety and immunogenicity of two vaccine platforms. Additionally, we explored novel animal models for use in SFTSV research, and used a heterologous vaccine regimen that proved to be effective at inducing robust immune responses. The two vaccine platforms we explored have favorable immunogenicity and represent strong candidates for the development of anti-SFTSV vaccines for human use. As evidenced by the recent COVID19 pandemic, it is necessary to have easily modified vaccine platforms with well described safety and immunogenicity to facilitate the rapid response to global disease threats. Further work is necessary with both vaccine platforms to address safety, immunogenicity, and delivery concerns; however, these platforms are strong options for use against future emergent pathogens.

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