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DEVELOPMENT OF REPLICATION-COMPETENT VSV-BASED VACCINES FOR PROTECTION AGAINST HANTAVIRUSES

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DEVELOPMENT OF REPLICATION-COMPETENT VSV-BASED VACCINES FOR PROTECTION AGAINST HANTAVIRUS INFECTION

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Abstract:

This study looks at the effects of the position and numbers of foreign immunogens in the vesicular stomatitis virus (VSV) genome on vector attenuation and vaccine efficacy. To generate the recombinant vaccine vectors, a VSV Indiana strain was modified by removing the native VSV glycoprotein and replacing it with one or two foreign glycoproteins; one derived from the filovirus Zaire ebolavirus (ZEBOVgp) and the other from the hantavirus Andes virus (ANDVgpc). Two monovalent (rVSVΔG-ANDVgpc and rVSVΔG-ZEBOVgp) and two bivalent (rVSVΔG-ANDVgpc-ZEBOVgp and rVSVΔG-ZEBOVgp-ANDVgpc recombinant vectors were compared. All vectors were attenuated in cell culture and in immunocompetent hamsters when compared to rVSV wildtype (rVSVwt). rVSVΔG-ZEBOVgp and other unrelated monovalent rVSV vectors did show pathogenicity in an immunocompromised mouse model making this model unsuitable for vaccine efficacy testing. rVSV vaccine vector efficacy testing was explored in the established lethal ANDV hamster model. In addition, two routes of immunization, intraperitoneal and intranasal, were compared. The two bivalent vectors both mediated full protection when administered even 3 days prior to ANDV challenge independent of the route of immunization. The monovalent rVSVΔG-ANDVgpc was similarly protective but intranasal immunization showed reduced efficacy when administered close to challenge. The bivalent rVSVΔG-ZEBOVgp-ANDVgpc was most potent in post-exposure treatment, followed by the monovalent rVSVΔG-ZEBOVgp. The monovalent rVSVΔG-ANDVgpc was least potent in post-exposure treatment. Intraperitoneal immunization was superior over the intranasal route in post-exposure treatment. Our data indicates the benefit of bivalent rVSV vaccine vectors based on the rVSVΔG-ZEBOVgp backbone with the second immunogen expressed in the downstream position in peri-exposure application.

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PRELUDE

In this dissertation, ongoing work toward developing replication-competent viral-based vaccines against a selected hantavirus is described.

In Chapter 1, an introduction to hantaviruses, their biogeography and life cycle, and the zoonotic human diseases that they cause is provided. This is followed by an introduction to Vesicular Stomatitis Virus (VSV) which is commonly used as an experimental vaccine platform. The chapter concludes with an overview of the core problem or "scientific question" addressed as part of the work that is presented in subsequent chapters of the thesis.

In Chapter 2, an expanded discussion on the advantages and disadvantages, benefits and limitations of employing a rVSV platform for vaccine development against deadly negative single-strand RNA (–ssRNA) viruses is provided. This chapter is based on work completed in 2009-2015 and recently published (October, 2015). Specifically, the efficacy of rVSV-based vaccines in distinct animal models is considered. This background information and work presented in Chapter 2 forms the basis for the model used for the core research effort presented in Chapter 3.

In Chapter 3, a series of experiments in the Syrian Golden hamster (*Mesocricetus auratus*) model system demonstrates pre- and post-exposure protection using rVSV-based vaccines against the human pathogenic hantavirus, Andes virus. Data demonstrate significant post-exposure protection against hantavirus with these rVSV-based vaccines compared to previously reported vaccine constructs.

In the final chapter (Chapter 4), future directions for design and development of replication-competent rVSV-based vaccines are discussed. The approaches and lines of experimentation described extend beyond the scope of this dissertation project and forward this work to a point where these rVSV-based vaccines may be used in other animal model systems thereby testing the utility of these constructs. Implementation of replication-competent vaccines (rVSV-based or otherwise) as standard medical practice remains controversial. However, the emergence of, and inability to predict and control devastating outbreaks of highly pathogenic viruses may result in the future testing and use of this class of vaccines in humans.

Cumulatively, the body of work presented in this thesis contributes to a larger body of literature on novel approaches to vaccine development and the focus area of developing replication-competent vaccines for emergency use. It further demonstrates how vaccine platforms such as rVSV-based vectors can be tailored to elicit specific responses while decreasing side effects often seen with traditional vaccines.

CHAPTER 1 INTRODUCTION

Hantaviruses and a Rhabdovirus Reverse Genetic System

Hantaviruses: Introduction

Hantaviruses are a group of zoonotic pathogens that are part of the virus family *Bunyaviridae*. Hantaviruses present as a single-stranded segmented RNA genome of approximately 12.1 kb divided into three segments: a large segment (L), a medium segment (M), and a small segment (S) of RNA lengths 6.5 kb, 3.6-3.7 kb, and, 1.6-2 kb, respectively (Schonrich et al. 2008). The hantavirus virion presents as an enveloped pleomorphic pseudo-spherical particle with a diameter ranging from 80-120 nm (Schonrich et al. 2008). Interestingly, each of the three RNA segments comprising the virion are packaged in a separate nucleocapsid. The three nucleocapsids are bound within the virus particle and surrounded by a common cell-derived lipid bilayer featuring the viral glycoprotein spike complex (Plyusnin et al. 1996; Vaheri et al. 2013).

The hantaviruses differ from the rest of the *Bunyaviridae* family in that they are the only group that are transmitted by a rodent vector. It has been proposed that hantavirus transmission can occur through the inhalation of dried rodent excreta (Colleen et al. 2010). In contrast, the other genera of bunyaviruses are all arthropod-borne (Plyusnin et al. 1996; Liu et al. 2012). Hantavirus genotypes are commonly grouped together based on their biogeography. Those from Europe and Asia are known as the “Old World” hantaviruses whereas those found in North America and South America are known as ”New World” hantaviruses (Figure 1-1). All hantaviruses are non-pathogenic in their natural hosts and indeed many do not cause disease in humans. However, several hantaviruses of both “Old World” and “New World” lineages can cause severe disease in

humans. (Hantaviruses that cause disease in humans are classified as Biosafety Level 3 pathogens because of the threat of severe disease without the availability of therapeutic treatments or preventative vaccines).

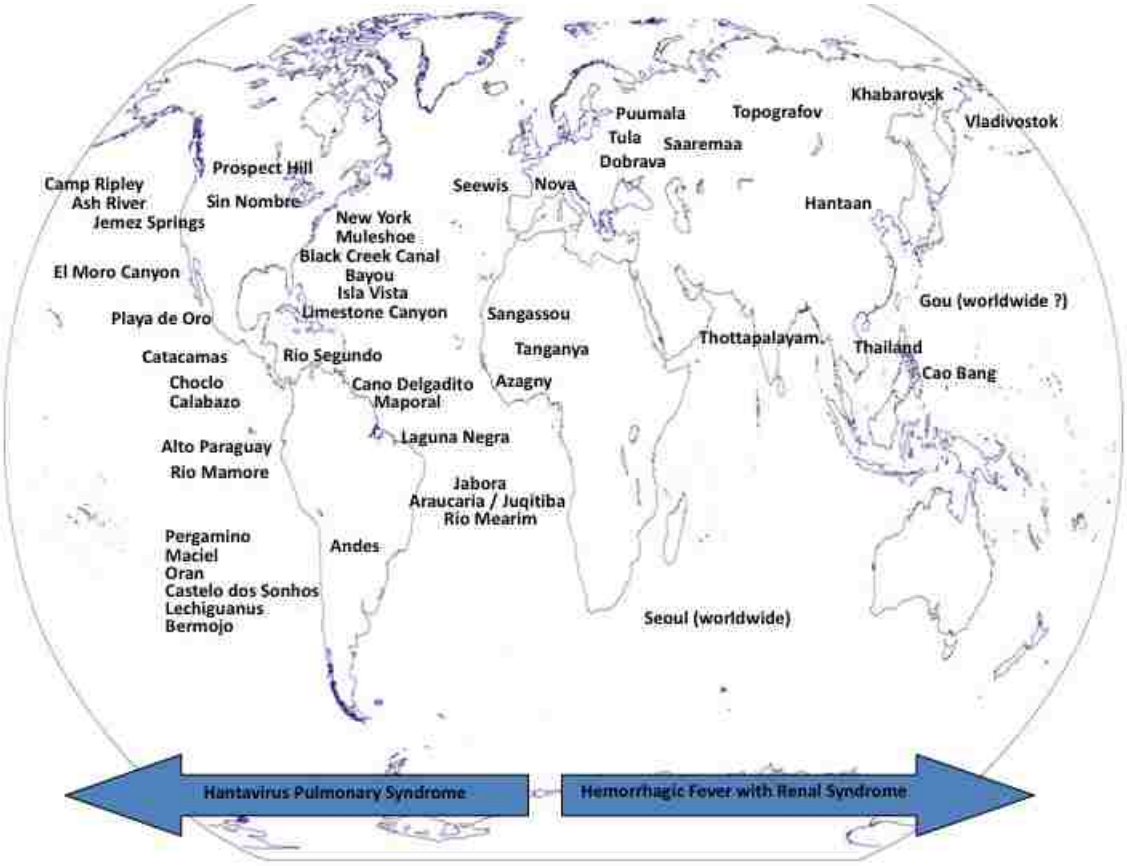

Figure 1-1: Hantavirus Global Distribution Map.

The map above depicts the global distribution of hantaviruses which is closely mirrored with the ranges of their natural hosts. Used with permission of Dr. David Safronetz 2016.

Hantaviruses: History

Based on historical descriptions of symptoms for a variety of human ailments, it has been suggested that hantaviruses have been responsible for human disease for at least a century (Khaiboullina et al. 2005). However, the first well-documented emergence of hantavirus-induced disease in humans occurred during the Korean War where several thousand troops from the United Nations contracted an acute febrile illness, which was initially designated “Korean hemorrhagic fever” (Jonsson et. al 2010). This disease, now referred to as hemorrhagic fever with renal syndrome (HFRS), is characterized by intense headaches, fever, chills, and abdominal pain followed by later symptoms which can include low blood pressure, kidney failure, vascular leakage and other hemorrhagic manifestations (Linderholm et al. 2001). The causative agent was later identified (ca. 1970s) as a virus from field mice (*Apodemus agrarius)* (Lee et al.1978). The virus was named Hantaan Virus from the region where the host rodent was captured - near the Hantaan River between North and South Korea, close to the 38th parallel (Lee et al. 1982; Lindkvist et al. 2008).

Today, hantaviruses continue to be among some of the most concerning etiological agents of emergent zoonotic infectious diseases and are considered a significant threat worldwide. The viruses are maintained in reservoirs of natural hosts. Geographic distribution of these natural hosts and epidemiology associated with these viruses are congruent.

In the Old World, the majority of hantavirus cases have been reported in Europe (e.g., Russia) and Eastern Asia. For example, hantavirus is widespread

in China. Some reports estimate that hantavirus-induced disease in China accounts for ~90% of the world's recorded cases per year (Liu et al. 2012).

The Hubei province of central China has often recorded the highest annual occurrence of HFRS in the world. Over the last 20 years, rapid urbanization in China has undoubtedly contributed to the increasing rate of HFRS. Rapid socioeconomic growth, modern transportation, and extensive migration from rural to urban areas has contributed to an expanded geographic range for natural hantavirus hosts. The concomitant increase in human population density in Chinese cities has resulted in an increase in the frequency of cases in Northern China (e.g., Beijing and Mongolia), a region where cases have historically been rare to nonexistent (Liu et al. 2012).

In the New World, at least two highly virulent strains of hantavirus have been well-characterized. Although hantavirus diseases occur less frequently in the Americas, the lethality rates can reach as high as 40% with some of the more pathogenic strains (Safronetz et al. 2009).

Since the isolation of the first New World hantavirus in 1993 (Nichol et al. 1993), cases of hantavirus-induced human disease have been reported in the western region of the United States and several countries in Central and South America. However, disease induced by New World hantaviruses differs significantly from that caused by hantaviruses in Europe and Asia. Specifically, New World hantavirus results in vascular leakage in the lungs, pulmonary edema, and cardiovascular collapse. The disease is called Hantavirus

Cardiopulmonary Syndrome (HCPS). Two species of the virus are responsible for HCPS: Sin Nombre Virus (SNV) and Andes Virus (ANDV).

SNV was isolated in 1993 in the southwestern United States (i.e., New Mexico). SNV is highly virulent, resulting in up to 35% lethality (Schonrich et al. 2008). ANDV was subsequently isolated in 1997 after an outbreak in Chile and was later responsible for additional HCPS outbreaks in both Chile and Argentina (Lopez et al. 1997). Of the 22 described hantaviruses, ANDV is considered one of the "most deadly" hantaviruses (Schonrich et al. 2015). Outbreaks of ANDV have been reported to cause case fatality rates exceeding 40%. It is also the only hantavirus capable of transmission directly from human–to–human (Martinez-Valdebenito et al. 2014).

Hantavirus-induced diseases including HFRS and HCPS continue to emerge (and re-emerge) worldwide with annual documented case totals in Europe and Asia reaching up to 200,000 and case fatality rates as high as 10% (Safronetz et al. 2011). Despite causing unique symptomology, both hantavirus-induced diseases cause vascular leakage and thrombocytopenia. North American and South American strains of SNV and ANDV continue to be the most devastating in terms of mortality (Clement et al 2014).

Response to Hantaviruses

The clinical manifestation of hantavirus infection in humans is acute affecting renal, cardiac, pulmonary, central nervous system, and endocrine function. Two separate hantavirus-induced diseases in humans have been

defined and studied – HFRS caused by Old World hantavirus and HCPS caused by New World hantavirus. HFRS is characterized by a high fever which can be accompanied by headache, abdominal pains, backache and kidney dysfunctions. HFRS disease is highly variable and is thought to depend heavily on the hantavirus genotype and host genetic factors (Vaheri et al. 2013). HCPS is also a severe disease which is often associated with respiratory failure from pulmonary edema (Duchin et al. 1994). Despite causing separate diseases, Old World and New World hantaviruses share common features at the cellular level leading to detrimental impact on the host. These effects include an acute phase characterized by the aforementioned symptoms but may also lead to long-term or "chronic" debilitation. Specifically, hantaviruses infect and replicate primarily in endothelial cells increasing capillary permeability. Altering capillary permeability results in extravasation of fluid into alveolar spaces causing hypotension, pulmonary edema, and thrombocytopenia (Vaheri et al. 2013). Interestingly, the viral infection does not appear to cause notable cytopathic effects or cellular necrosis. Instead, it is proposed that infection of endothelial layers modulates an immuno-pathological response that is activated by infected endothelial cells and migrating blood mononuclear cells (Khaiboullina et al. 2005). Frankly, the exact mechanism by which immunopathology ensues after hantavirus infection is poorly understood and continues to be a core focus of current research (Schonrich et al. 2008).

Natural Reservoirs of Hantaviruses

Hantaviruses have a unique coevolutionary history with their specific host rodent species. This relationship between the virus and the natural host species has resulted in persistence of the virus which causes no signs of disease in the rodent population. It appears that the virus/host systems are highly co-evolved and viral persistence, in the absence of disease, is mediated by both the virus and the host to ensure the survival by both. (Easterbrook et al. 2008). Laboratory-infected animals are not able to completely clear the virus, and it is speculated that low levels of viral replication persist over the lifetime of the animal accompanied with the persistent shedding of that virus in the host urine, feces and saliva.

It appears that the hantavirus infection is spread through the rodent population when exposed to contaminated dust from the excretia of other infected rodents, as well as biting and scratching behavior between individuals. The grooming habits of animals and their cagemates has also been observed and thought to contribute as well (Hw et al. 1981). Maternal transmission to offspring is not thought to be a major route of transmission due to passive transfer of protective maternal antibodies though milk (Botten et al. 2002; Dohmae et al. 1995).

Of the most common strains of hantavirus that occur in the old world the natural reservoirs tend to be associated with 2 families of rodents *Murinae* and *Arvicolinae.* The new world hantavirus typically has hosts in the rodent subfamily *Sigmodontinae* (Schonrich et al. 2008; Khaiboullina et al. 2005). In that only a

small percentage of rodents and insectovore species have been tested for hantavirus infection, it is expected that many more species of hantavirus will be discovered in the future (Plyusnin et al. 1996).

Biology and Life Cycle of Hantaviruses

The viral RNA segments are designated as the S, L, and M segments and encode the nucleocapsid protein (NP), the viral RNA polymerase (RdRp), and the glycoprotein precursor (GPC), respectively (Löber et al. 200).

Hantaviruses are thought to enter their host cells via a clathrin dependent endocytosis mechanism (Jin et al. 2002) through the use of integrin receptors (Wickham et al. 1994; Roivainen et al. 1994; Jackson et al. 1997; Guerrero et al. 2000). The human integrin alpha(v)beta(3) is one receptor that is expressed on human endothelial cells and allows entry of HFRS and HCPs causing hantavirus species (Gavrilovskaya et al. 2008). Interestingly, several species of non-pathogenic hantaviruses have been shown to use the alpha(v)beta(1) integrin (Gavrilovskaya et al. 2002).

After the virus particle is internalized the RNA segments are released into the cytoplasm accompanied by an RNA dependent RNA polymerase (RdRp) which initiates primary transcription of viral proteins (Jin et al. 2002). Once the production of viral components have accumulated to the proper levels, the remaining morphogenesis, which is not completely understood, is completed as the virus buds in the Golgi (Figure 1-2) (Plyusnin et al. 1996).

The majority of hantavirus species that cause HFRS and HPS do not cause cytopathic effects in most cell types. Therefore, virus-induced disease is likely caused by indirect mechanisms including the host immune response and disregulation of the chemokine and cytokine responses. Because the main target cells are endothelial cells, it is thought that hantavirus disease and pathogenesis is linked to the cellular response activated in endothelial cells.

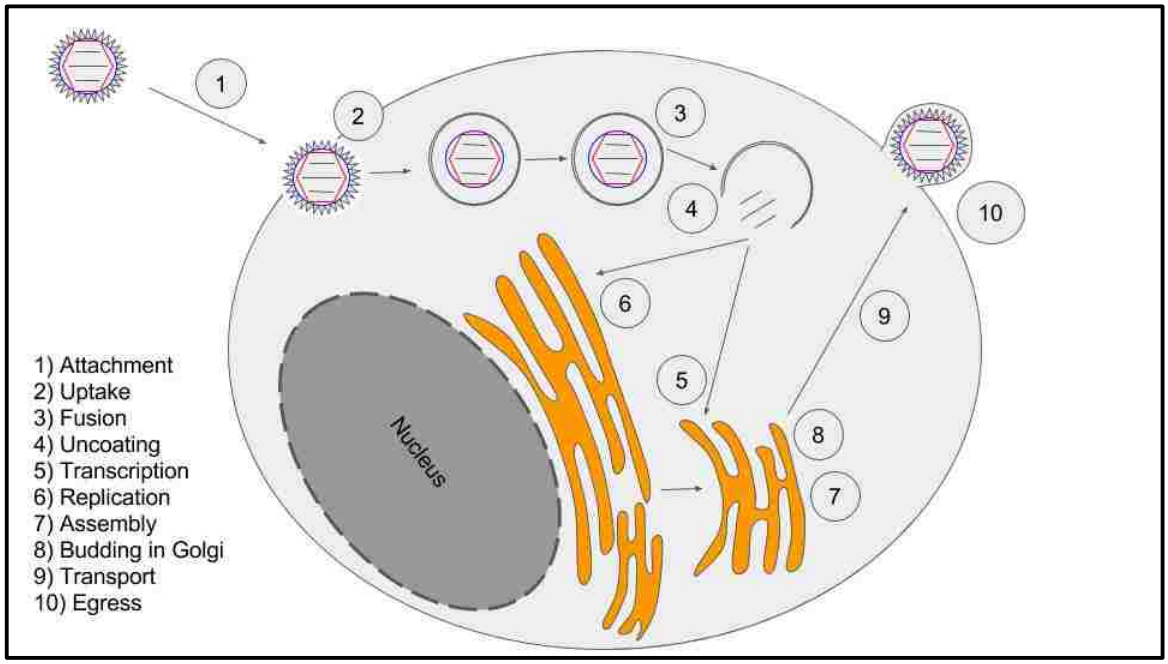

Figure 1-2: Hantavirus life cycle schematic.

Represents the major steps in the lifecycle of a hantavirus in the host cell.

The viral glycoprotein is synthesized as a glycoprotein precursor molecule by ribosomes bound to the endoplasmic reticulum (Pensiero et al. 1992; Schmaljohn et al. 1987; Khaiboullina et al. 2005). The precursor molecule is then translocated to the ER via a signal peptide where it is cleaved by the signal peptidase at a cut site with conserved amino acid sequence of "WAASA" into the two glycoprotein subunits G1 and G2 (Löber et al. 2001). Formation of the G1/G2 complex allows translocation to the Golgi where the final maturation steps take place and become ready for viral assembly. The cytoplasmic tails of the G1/G2 complex interact with the nuclear protein/viral RNA complex and initiate viral budding from the Golgi. The newly-formed viral particles are then transported to the plasma membrane where viral egress takes place (Vaheri et al. 2013).

Some of the major hurdles in studying hantavirus infection and its influence on the host immune response is the limited availability of animal models. The only adult small animal disease model currently available is the ANDV infection of the Syrian golden hamster (Safronetz et al. 2012). While the use of a STAT1 knock out mouse model was explored in chapter 2 it was not intended for use as a lethal ANDV challenge model. An immunocompromised mouse model would be valuable as a model for rVSV vector efficacy in stimulating the immune system when paired with commonly available reagents for exploring the in vivo immune response in murine models. Thus, chapter 2 explores the use of the STAT1 knock out mouse model and forms the basis for the decision to use the hamster model in the research presented in Chapter 3. In addition, two nonhuman primate disease models have

been described, one for Sin nombre virus (Safronetz et al. 2014) and the other one for Puumala virus (Groen et al. 1995), but neither are well established at this time. This leaves the ANDV hamster disease model as the currently best choice for hantavirus vaccine efficacy testing.

Vesicular Stomatitis Virus (VSV) Background

VSV as a Vaccine Platform

As our knowledge of the immune response and vaccines increase, the scientific and medical fields will have a greater ability to design tools against human disease. Vaccines that are specifically designed to mediate an effective immune response and elicit a custom tailored response to a specific disease can be more efficient and have reduced side effects. Viruses that are designed to stimulate specific immune responses are getting more attractive and rVSV has been proven to be a very promising platform to develop viral therapeutics.

Because VSV is an RNA virus it is thought that problems associated with DNA virus based platforms could be circumvented with the RNA based rVSV platform by avoiding viral replication in a cell's nucleus. DNA virus-based therapeutics have come under scrutiny because of the possibility of the virus to cause mutations to the host cell's DNA in addition to the disruption of host cell transcription processes.

Because of the wide distribution of phosphatidylserine (the host membrane component which VSV glycoprotein (G) binds to in order to facilitate entry) in host cells across the domains of life the VSV has an exceptionally broad tissue tropism. Replacement of the native VSV-G with other viral glycoproteins allows the engineering of recombinant vectors (rVSV) that have more specific host tissue/cellular targeting and thus likely less side effects.

Since the reverse genetics system allows precise tailoring of the virus, it can be attenuated by careful manipulation of the genome and the deletion of viral pathogenicity factors to reduce side effects while still being replication competent. The rVSV vaccine vectors have many other advantages, such as the ability to be administered as a single dose and given through other routes such as mucosal membranes that do not involve injection but are still able to elicit a robust protective immune response (Schlereth et al. 2000; Roberts et al. 1998).

The ability to manipulate and grow the virus in the lab is straightforward and the virus is easily grown rapidly and to high titers in tissue culture. It grows so well that it has been suggested in the rVSV-HIV literature that one liter culture of supernatant volume could generate enough vaccine material to vaccinate over 1 billion people (Rose et al. 2001). Because of these advantages, this study focuses on using a rVSV based vector as a vaccine against lethal Andes virus Infection.

VSV Introduction

VSV is a non-segmented negative stranded RNA virus in the family of *Rhabdoviridae*. Approximately 170 members of the *Rhabdoviridae* have been discovered and these members have an extremely wide host range which allow infection across two kingdoms of life (Ghanem et al. 2012).

VSV has a small and simple genome structure which is approximately 11 kb pairs and is composed of five genes. The genes are arranged in the 3' to 5' direction and are arranged in the conserved order N-P-M-G-L. The first gene (N)

encodes the nucleoprotein which is responsible for forming the nucleocapsid around the viral RNA. The P gene encodes the phosphoprotein, which, in combination with N, is used for RNA synthesis. The matrix protein (M gene) provides the structural system between the nucleocapsid and the viral envelope. The G gene encodes the glycoprotein which allows attachment to target cells and initiates fusion of the virus particle the host cell membrane. The L gene encodes for the viral RNA polymerase which acts as both a viral transcriptase to produce viral mRNAs and a replicase which is responsible for producing successive copies of the genome. The three prime end of the N gene contains a transcriptional start sequence which initiates transcription until the polymerase encounters a series of U residues at the end of the gene. These residues cause the polymerase to stutter back-and-forth producing a poly-A tail before the mRNA is discharged. The nearby downstream restart signal causes the polymerase to re-engage and continue transcription of the next downstream gene. Production of a decreasing gradient of mRNAs is produced from the viral genome, where those near the 3' end of the genome are produced in much higher quantities. Because the polymerase always starts at the 3’ end of the genome and has a chance to dissociate at the end of each gene after transcription, and genes that are closer to the 5' end of the genome are produced much less abundantly (Figure 1-3).

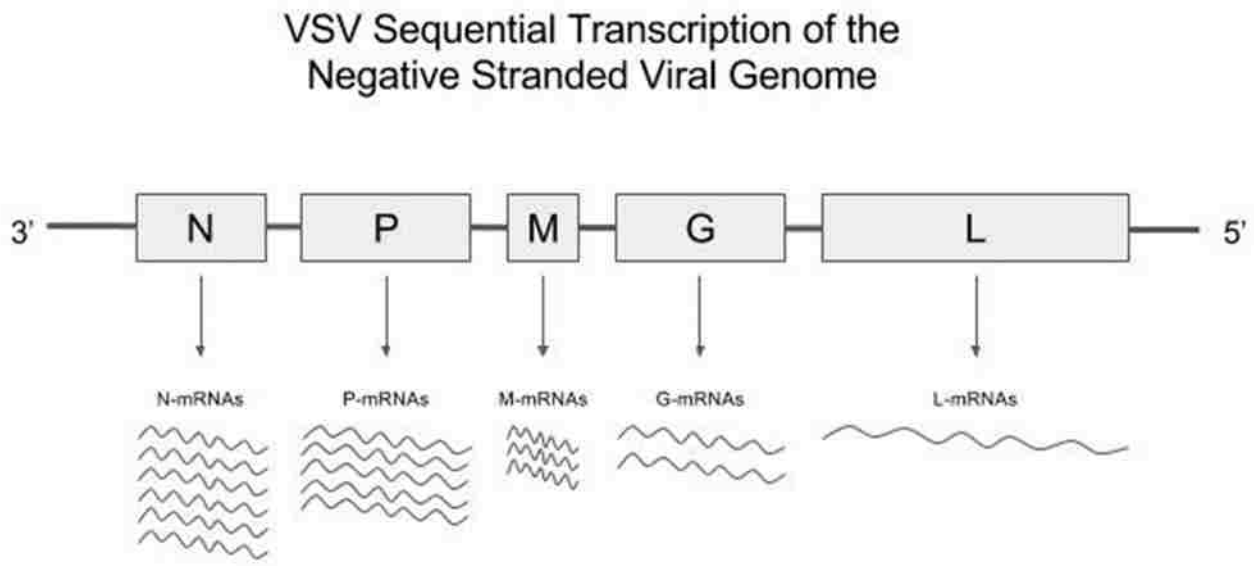

Figure 1-3: VSV Transcription.

Represents the sequential transcription of viral genes in the VSV genome.

The order of these genes is highly conserved in this group of viruses and is a simple and efficient way to regulate viral gene expression levels. After viral N and P proteins have accumulated to high enough levels in the host cell, the polymerase is able to synthesize full-length, positive strand, anti-genome RNAs. The anti-genome, in turn, serves as a template for synthesis of full-length genomes which are incorporated into new virus particles and then bud out of the host cell to complete the viral replication cycle.

VSV Infection & Disease in Animals and Humans

Rhabdoviridae hosts include insects, fish, plants, and vertebrates. VSV can cause vesicular lesions in domestic animals (cattle, sheep, horses, pigs) which self-resolve, and, after healing, leave the animals with no long term ill effects. The early clinical symptoms of VSV infection closely mimic the symptoms of hoof-and-mouth disease and the mis-diagnosis has the potential to cause economic loss.

Information supporting VSV's role in human disease is rare. Although VSV has been shown to be able to infect humans, it is generally believed to be largely asymptomatic with the viral infection self-resolving (Lichty et al. 2004). VSV is endemic in regions ranging from the southern portion of Mexico to the northern part of South America, and also occurs as a livestock animal pathogen in several states in the US (Flanagan et al. 2001). Although human seroprevalence against

VSV is reported in these endemic regions, the clinical disease (described as a flu-like illness) rates attributed to VSV are low (Roberts et al. 1999).

rVSV Reverse Genetics System

Members of the Rhabdoviridae family have proven to be important biomedical tools. Particularly, in the case of VSV, they can easily be genetically engineered to become useful research tools such as vectors, vaccines, viral tracers in neurotropism studies, and basic viral lifecycle studies. One of the primary features that makes VSV so useful is its ability to be manipulated using a reverse genetics system.

Although there are several systems established for rescuing members of the *Rhabdoviridae* family, I used a rescue plasmid encoded T7 RNA polymerase system which uses 6 “helper” plasmid containing the genes for N, P, M, G, L and the T7 polymerase.

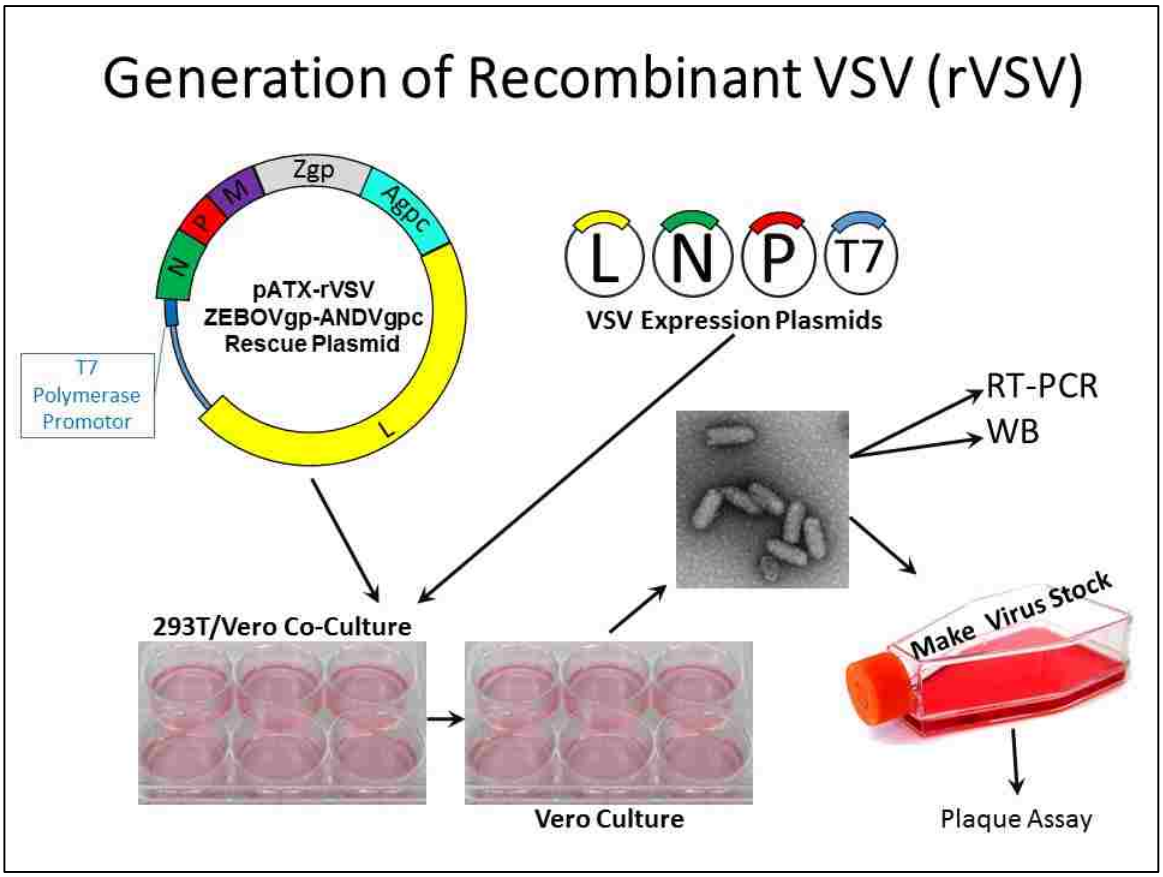

Figure 1-4: VSV Reverse Genetics System.

The bivalent vaccine vector rVSVΔG-ZEBOVgp-ANDVgpc was rescued using the VSV reverse genetics system by amplifying and cloning the ZEBOVgp and ANDVgpc open reading frames into the pATX plasmid downstream of the M gene.

Detailed description of the rVSV rescue system have been described at length in previous literature (Figure 1-4) (Garbutt et al. 2004; Tsuda et al. 2011; Brown et al. 2011). Briefly, a mixed culture of Vero cells and 293T cells were grown to a confluence of 90%. The cell co-culture was then transfected with the rVSV rescue plasmid in addition to the 6 helper plasmids listed above. The transfected co-cultures were then maintained for 12-14 days after transfection until cytopathic effects (CPE) were observed indicating successful viral rescue and replication.

In this study we explored the ability of using a combination of glycoproteins from the vastly different virus strains, Andes virus and Ebola virus, to design a more potent and effective vaccine platform that may have broad applications for future vaccine development.

Objectives and Hypothesis

A previous vaccine efficacy study (Brown et al. 2011) focusing on the protection of an rVSV vector expressing the ANDV glycoprotein precursor (rVSVΔG-ANDVgpc) against lethal ANDV infection used an unrelated rVSV vector expressing the ZEBOV glycoprotein (rVSVΔG-ZEBOVgp) as a negative control which was expected to not provide any protection in the lethal ANDV infection study. Interestingly, rVSVΔG-ZEBOVgp provided varying degrees of protection in hamsters against ANDV infection when administered between 7 days prior and 3 days after ANDV challenge. This data indicates that rVSV vector vaccination can result in unspecific protective efficacy against heterologous challenge.

We hypothesize that the ZEBOVgp will target important immune cells like monocytes, macrophages and dendritic cells, which can lead to a rapid and enhanced immune response that is broadly protective against heterogeneous viral challenge. This response will be a valuable trait for vectors used in peri-exposure prophylaxis where a rapid, but short lived protective response may suppress viral replication and give the adaptive immune response (if an appropriate immunogen is provided) a better chance to kick in and offer a longer term immunity.

In this study, bivalent vaccine vectors (rVSVΔG-ZEBOVgp-ANDVgpc; rVSVΔG-ANDVgpc-ZEBOVgp) including the ZEBOVgp will be utilized based on the idea that ZEBOVgp may target the vaccine vectors to important immune cells, such as monocytes, macrophages and dendritic cells, strongly enhancing

innate and adaptive immune responses and, thus improve protective efficacy against a lethal heterologous ANDV challenge. The study further looks at the effects of the insertion and position of the foreign viral glycoproteins (here ZEBOV and ANDV) into the genome of rVSV and how it influences the rVSV when used as a vaccine utilizing the ANDV hamster challenge model.

To generate the vaccine vector an Indiana strain of VSV is modified by removing the native glycoprotein and replacing it with two other viral proteins, ZEBOV gp and ANDV gpc. Two monovalent (rVSVΔG-ANDVgpc and rVSVΔG-ZEBOVgp) and two bivalent [rVSVΔG-ANDVgpc-ZEBOVgp (Tsuda et al. 2011) and rVSVΔG-ZEBOVgp-ANDVgpc] vectors are compared in this study and the results are described in chapter 3.

While the hamster model is sufficient for testing the protective effects of vaccines and survival, the shortcomings become very evident when wanting to explore the specific mechanisms involved in the resulting changes in survival. There is a lack of commercial reagents specific to the hamster which makes it very difficult to study the immune response mechanism involved in protection. It would therefore be desirable to have a mouse model available because of the available reagents to study response mechanisms in mice. Since it has been shown before that immunosuppression of hamsters can result in a lethal model for SNV (Brocato et al. 2014) it would be ideal to demonstrate a lethal model of ANDV in immunocompromised mice to facilitate the study of the mechanism of immune response in mice since there are many more commercial reagents available to study immune mechanisms in the murine models. Unfortunately,

such a murine model does not yet exist. However, as a first step towards developing such a model we investigated the possibility of testing selected rVSV-based vaccine candidates in such immunocompromised mouse models and the results of this work are discussed in chapter 2.

Because hantaviruses are continually emerging as zoonotic pathogens the threat of significant disease in humans have increased the interest in studying hantavirus not only to research viral pathogenesis but also to evaluate possible medical interventions such as vaccines or other therapeutics. In particular, a fast acting vaccine would be particularly important for the Andes virus as it has been shown to successfully be transmitted from human-to-human.

References

Botten, Jason, Katy Mirowsky, Chunyan Ye, Keith Gottlieb, Melissa Saavedra, Liana Ponce, and Brian Hjelle. 2002. 'Shedding and Intracage Transmission of Sin Nombre Hantavirus in the Deer Mouse (Peromyscus maniculatus) Model', *J Virol*, 76: 7587-94.

Brocato, Rebecca L., Christopher D. Hammerbeck, Todd M. Bell, Jay B. Wells, Laurie A. Queen, and Jay W. Hooper. 2014. 'A lethal disease model for hantavirus pulmonary syndrome in immunosuppressed Syrian hamsters infected with Sin Nombre virus', *J Virol*, 88: 811-19.

Brown, K. S., D. Safronetz, A. Marzi, H. Ebihara, and H. Feldmann. 2011. 'Vesicular stomatitis virus-based vaccine protects hamsters against lethal challenge with Andes virus', *J Virol*, 85: 12781-91.

Clement, Jan, Piet Maes, and Marc Van Ranst. 2014. 'Hemorrhagic Fever with Renal Syndrome in the New, and Hantavirus Pulmonary Syndrome in the old world: Paradi(se)gm lost or regained?', *Virus Research*, 187: 55-58.

Colleen, B. Jonsson, Figueiredo Luiz Tadeu Moraes, and Vapalahti Olli. 2010. 'A Global Perspective on Hantavirus Ecology, Epidemiology, and Disease', *Clinical Microbiology Reviews*, 23: 412-41.

Dohmae, K., and Y. Nishimune. 1995. 'Protection against hantavirus infection by dam's immunity transferred vertically to neonates', *Arch Virol*, 140: 165-72.

Duchin, J. S., F. T. Koster, C. J. Peters, G. L. Simpson, B. Tempest, S. R. Zaki, T. G. Ksiazek, P. E. Rollin, S. Nichol, and E. T. Umland. 1994. 'Hantavirus pulmonary syndrome: a clinical description of 17 patients with a newly recognized disease. The Hantavirus Study Group', *N Engl J Med*, 330: 949-55.

Easterbrook, Judith D., and Sabra L. Klein. 2008. 'Immunological Mechanisms Mediating Hantavirus Persistence in Rodent Reservoirs', *PLoS Pathog*, 4: e1000172.

Flanagan, E. Brian, Joann M. Zamparo, L. Andrew Ball, Luis L. Rodriguez, and Gail W. Wertz. 2001. 'Rearrangement of the Genes of Vesicular Stomatitis Virus Eliminates Clinical Disease in the Natural Host: New Strategy for Vaccine Development', *J Virol*, 75: 6107-14.

Gavrilovskaya, I. N., T. Peresleni, E. Geimonen, and E. R. Mackow. 2002. 'Pathogenic hantaviruses selectively inhibit beta3 integrin directed endothelial cell migration', *Arch Virol*, 147: 1913-31.

Gavrilovskaya, Irina N., Elena E. Gorbunova, Natalie A. Mackow, and Erich R. Mackow. 2008. 'Hantaviruses direct endothelial cell permeability by sensitizing cells to the vascular permeability factor VEGF, while angiopoietin 1 and sphingosine 1-phosphate inhibit hantavirus-directed permeability', *J Virol*, 82: 5797-806.

Ghanem, Alexander, and Karl-Klaus Conzelmann. 2012. 'Reverse Genetics of Rhabdoviruses.' in, *Reverse Genetics of RNA Viruses* (John Wiley & Sons, Ltd).

Guerrero, C. A., E. Méndez, S. Zárate, P. Isa, S. López, and C. F. Arias. 2000. 'Integrin alpha(v)beta(3) mediates rotavirus cell entry', *Proc Natl Acad Sci U S A*, 97: 14644-49.

Hooper, J. W., T. Larsen, D. M. Custer, and C. S. Schmaljohn. 2001. 'A lethal disease model for hantavirus pulmonary syndrome', *Virology*, 289: 6-14.

Hw, Lee, Lee Pw, Baek Lj, Song Ck, and Seong Iw. 1981. 'Intraspecific transmission of Hantaan virus, etiologic agent of Korean hemorrhagic fever, in the rodent Apodemus agrarius', *The American journal of tropical medicine and hygiene*, 30: 1106-12.

Jackson, T., A. Sharma, R. A. Ghazaleh, W. E. Blakemore, F. M. Ellard, D. L. Simmons, J. W. Newman, D. I. Stuart, and A. M. King. 1997. 'Arginine-glycine-aspartic acid-specific binding by foot-and-mouth disease viruses to the purified integrin alpha(v)beta3 in vitro', *J Virol*, 71: 8357-61.

Jin, Mirim, Junghyun Park, Sungwook Lee, Boyoun Park, Jinwook Shin, Ki-Joon Song, Tae-In Ahn, Sue-Yun Hwang, Byung-Yoon Ahn, and Kwangseog Ahn. 2002. 'Hantaan virus enters cells by clathrin-dependent receptor-mediated endocytosis', *Virology*, 294: 60-69.

Jonsson, Colleen B., Luiz Tadeu Moraes Figueiredo, and Olli Vapalahti. 2010. 'A Global Perspective on Hantavirus Ecology, Epidemiology, and Disease', *Clinical Microbiology Reviews*, 23: 412-41.

Khaiboullina, Svetlana F., S. P. Morzunov, and Stephen C. St. Jeor. 2005. 'Hantaviruses: Molecular Biology, Evolution and Pathogenesis', *Current Molecular Medicine*, 5: 773-90.

Lee, H. W. 1982. 'Hemorrhagic fever with renal syndrome (HFRS)', *Scand.J.Infect.Dis.Suppl*, 36: 82-85.

Lichty, Brian D., Anthony T. Power, David F. Stojdl, and John C. Bell. 2004. 'Vesicular stomatitis virus: re-inventing the bullet', *Trends in Molecular Medicine*, 10: 210-16.

Linderholm, M., and F. Elgh. 2001. 'Clinical characteristics of hantavirus infections on the Eurasian continent', *Curr Top Microbiol Immunol*, 256: 135-51.

Lindkvist, Marie, and Umeå Universitet. 2008. 'Genetic and serologic characterization of a Swedish human hantavirus isolate'.

Liu, Jing, Dong-Ying Liu, Wen Chen, Jin-Lin Li, Fan Luo, Qing Li, Jia-Xin Ling, Yuan-Yuan Liu, Hai-Rong Xiong, Xiao-Hua Ding, Wei Hou, Yun Zhang, Shi-Yue Li, Jie Wang, and Zhan-Qiu Yang. 2012. 'Genetic analysis of hantaviruses and their rodent hosts in central-south China', *Virus Research*, 163: 439-47.

Löber, Christian, Bärbel Anheier, Steffi Lindow, Hans-Dieter Klenk, and Heinz Feldmann. 2001. 'The Hantaan Virus Glycoprotein Precursor Is Cleaved at the Conserved Pentapeptide WAASA', *Virology*, 289: 224-29.

Martinez-Valdebenito, Constanza, Mario Calvo, Cecilia Vial, Rita Mansilla, Claudia Marco, R. Eduardo Palma, Pablo A. Vial, Francisca Valdivieso, Gregory Mertz, and Marcela Ferrés. 2014. 'Person-to-Person Household and Nosocomial Transmission of Andes Hantavirus, Southern Chile, 2011', *Emerging Infectious Diseases*, 20: 1629-36.

Michael, Garbutt, Liebscher Ryan, Wahl-Jensen Victoria, Jones Steven, Möller Peggy, Wagner Ralf, Volchkov Viktor, Klenk Hans-Dieter, Feldmann Heinz, and Ströher Ute. 2004. 'Properties of Replication-Competent Vesicular Stomatitis Virus Vectors Expressing Glycoproteins of Filoviruses and Arenaviruses', *J Virol*, 78: 5458-65.

Nichol, S. T., C. F. Spiropoulou, S. Morzunov, P. E. Rollin, T. G. Ksiazek, H. Feldmann, A. Sanchez, J. Childs, S. Zaki, and C. J. Peters. 1993. 'Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness', *Science*, 262: 914-7.

Pensiero, M. N., and J. Hay. 1992. 'The Hantaan virus M-segment glycoproteins G1 and G2 can be expressed independently', *J Virol*, 66: 1907-14.

Plyusnin, A., O. Vapalahti, and A. Vaheri. 1996. 'Hantaviruses: genome structure, expression and evolution', *The Journal of general virology*, 77 (Pt 11): 2677-87.

Roberts, A., L. Buonocore, R. Price, J. Forman, and J. K. Rose. 1999. 'Attenuated vesicular stomatitis viruses as vaccine vectors', *J Virol*, 73: 3723-32.

Roberts, A., E. Kretzschmar, A. S. Perkins, J. Forman, R. Price, L. Buonocore, Y. Kawaoka, and J. K. Rose. 1998. 'Vaccination with a recombinant vesicular stomatitis virus expressing an influenza virus hemagglutinin provides complete protection from influenza virus challenge', *J Virol*, 72: 4704-11.

Roivainen, M., L. Piirainen, T. Hovi, I. Virtanen, T. Riikonen, J. Heino, and T. Hyypiä. 1994. 'Entry of coxsackievirus A9 into host cells: specific interactions with alpha v beta 3 integrin, the vitronectin receptor', *Virology*, 203: 357-65.

Rose, N. F., P. A. Marx, A. Luckay, D. F. Nixon, W. J. Moretto, S. M. Donahoe, D. Montefiori, A. Roberts, L. Buonocore, and J. K. Rose. 2001. 'An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants', *Cell*, 106: 539-49.

Safronetz, D., N. R. Hegde, H. Ebihara, M. Denton, G. P. Kobinger, S. St Jeor, H. Feldmann, and D. C. Johnson. 2009. 'Adenovirus vectors expressing hantavirus proteins protect hamsters against lethal challenge with andes virus', *J Virol*, 83: 7285-95.

Schlereth, B., J. K. Rose, L. Buonocore, V. ter Meulen, and S. Niewiesk. 2000. 'Successful vaccine-induced seroconversion by single-dose immunization in the presence of measles virus-specific maternal antibodies', *J Virol*, 74: 4652-57.

Schmaljohn, C. S., A. L. Schmaljohn, and J. M. Dalrymple. 1987. 'Hantaan virus M RNA: coding strategy, nucleotide sequence, and gene order', *Virology*, 157: 31-9.

Schönrich, Günther, Detlev H. Krüger, and Martin J. Raftery. 2015. 'Hantavirus-induced disruption of the endothelial barrier: neutrophils are on the payroll', *Front Microbiol*, 6: 222.

Schönrich, Günther, Andreas Rang, Nina Lütteke, Martin J. Raftery, Nathalie Charbonnel, and Rainer G. Ulrich. 2008. 'Hantavirus-induced immunity in rodent reservoirs and humans', *Immunological Reviews*, 225: 163-89.

Svetlana, Khaiboullina, S. Morzunov, and Jeor Stephen St. 2005. 'Hantaviruses: Molecular Biology, Evolution and Pathogenesis', *Current Molecular Medicine*, 5: 773-90.

Tsuda, Y., D. Safronetz, K. Brown, R. LaCasse, A. Marzi, H. Ebihara, and H. Feldmann. 2011. 'Protective efficacy of a bivalent recombinant vesicular stomatitis virus vaccine in the Syrian hamster model of lethal Ebola virus infection', *J Infect Dis*, 204 Suppl 3: S1090-7.

Vaheri, Antti, Tomas Strandin, Jussi Hepojoki, Tarja Sironen, Heikki Henttonen, Satu Mäkelä, and Jukka Mustonen. 2013. 'Uncovering the mysteries of hantavirus infections', *Nature Reviews Microbiology*, 11: 539-50.

Wickham, T. J., E. J. Filardo, D. A. Cheresh, and G. R. Nemerow. 1994. 'Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization', *The Journal of Cell Biology*, 127: 257-64.

CHAPTER 2 STAT1-DEFICIENT MICE ARE NOT AN APPROPRIATE MODEL FOR EFFICACY TESTING OF RECOMBINANT VESICULAR STOMATITIS VIRUS-BASED FILOVIRUS VACCINES

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Abstract

STAT1-defiecient mice lack a response to interferon α, β and γ and therefore allowing for replication of non-adapted wild-type (wt) Ebola- and Marburgviruses. We sought to establish a mouse model for efficacy testing of live-attenuated recombinant vesicular stomatitis virus (rVSV)-based filovirus vaccine vectors using wt Ebola- and Marburgvirus challenge strains. While infection of immunocompetent mice with different rVSV-based filovirus vectors did not cause disease, infection of STAT1-deficient mice with the same vectors resulted in systemic infection and lethal outcome for the majority of tested rVSVs. Despite differences in viral loads, organ tropism was remarkably similar between rVSV filovirus vaccine vectors and VSVwt with the exception of the brain. In conclusion, STAT1-deficient mice are not an appropriate immunocompromised mouse model for efficacy testing of live-attenuated, replication-competent rVSV vaccine vectors.

Introduction

Members of the family Filoviridae, Ebola- and Marburgviruses, are pathogens causing viral hemorrhagic fever in nonhuman primates and humans (Feldmann et al. 2013). While outbreaks of filovirus hemorrhagic fever have occurred mainly in their endemic area in Central Africa, currently, the biggest documented Ebola virus (EBOV) outbreak is still ongoing in West African countries such as Guinea, Liberia and Sierra Leone. Not only has the virus spread from there into neighboring Mali, it was also introduced to Senegal, Nigeria, Spain, the United Kingdom and the United States where further spread of the virus was successfully stopped (WHO 2015). Despite tremendous effort being made to accelerate Phase 1 Clinical trials for experimental vaccines and therapeutics (www.ClinicalTrials.gov), there is still no approved countermeasure available. One of the experimental vaccine platforms currently in Phase 1 Clinical trials is based on recombinant vesicular stomatitis virus (rVSV). The rVSV vaccines for EBOV, strain Mayinga (EBOV-May) and Marburg virus (MARV), strain Musoke (MARV-Mus) are attenuated and have been shown to protect rodents and nonhuman primates from lethal disease (Jones et al. 2005) and subsequently vaccine vectors for other MARV isolates and ebolavirus species have been developed (Marzi et al. 2011).

Protective efficacy testing of vaccine candidates against challenge with filoviruses isolated directly from human or wildlife (wild-type (wt) isolates) can only be performed in macaques or immunocompromised rodents (i.e. STAT1-/- or interferon α/β receptor (IFNAR)-/- mice), where infection causes disease

without previous adaptation (Bray et al. 2001; Raymond et al. 2011). While STAT1-/- mice have been shown to develop lethal disease after infection with the majority of wt filovirus isolates (Raymond et al. 2011), IFNAR-/- mice seem more resistant to infection with wt filoviruses i.e. Reston virus (RESTV) (Bray 2001). As the rVSV vector for EBOV-May did not cause disease in immunocompromised NOD-SCID mice (Jones et al. 2007), we sought to evaluate the usefulness of STAT1-/- mice as a model for the initial evaluation of the protective efficacy of new or improved rVSV filovirus vaccine vectors.

We hypothesized that the attenuated rVSV vectors would also be attenuated in this immunocompromised mouse model, in which rVSVwt causes lethal disease (Katzenell et al. 2014). We found, however, that vaccination of STAT1-/- mice with 4 different monovalent rVSV vectors individually expressing different filovirus glycoproteins (GP) resulted in systemic infection with lethal outcome for most of the rVSV vectors. In conclusion, while STAT1-/- mice are an animal model for wt Ebola- and Marburgvirus infections, this animal model can likely not be utilized for efficacy testing of live-attenuated vaccines such as the rVSV-based vectors.

Methods

Animal ethics and safety statements.

Research was approved and conducted in compliance with the guidelines of the NIAID/RML Institutional Animal Care and Use Committee (IACUC). The facility where this research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and has an approved Office of Laboratory Animal Welfare (OLAW) Assurance (#A4149-01). All procedures were conducted by trained personnel under the supervision of veterinarians, and all invasive clinical procedures were performed while animals were anesthetized. Early endpoint criteria, as specified by the IACUC approved scoring parameters, were used to determine when animals should be humanely euthanized. rVSV vectors are classified as biosafety level 2 (BSL2) pathogens and all work with the live vectors was approved by the Institutional Biosafety Committee (IBC) under BSL2 conditions.

Virus recovery, propagation and purification:

The following rVSVs were recovered from full-length plasmids and propagated as described previously (Lawson et al. 1995; Garbutt et al. 2004; Marzi et al. 2011): rVSVwt, rVSV/EBOV-May-GP, rVSV/RESTV-Pen-GP (RESTV, strain Pennsylvania), rVSV/MARV-Mus-GP, and rVSV/MARV-Ang-GP (MARV, strain Angola). Viruses were subsequently purified through a 20% sucrose cushion, resuspended in DMEM (Sigma, St. Louis, MO) containing 10% FBS (Life

Technologies, Carlsbad, CA), aliquotted and stored at -80ºC. Viral titers were determined performing classical plaque assay on Vero cells (Marzi et al. 2011) and the median tissue culture infectious dose (TCID50) using the Reed-Muench method (Reed et al. 1938).

Growth kinetics.

Vero E6 cells were seeded in a 24-well plate the day before the experiment. Infection was performed with rVSVwt, rVSV/EBOV-May-GP, rVSV/RESTV-Pen-GP, rVSV/MARV-Mus-GP and rVSV/MARV-Ang-GP at a multiplicity of infection of 0.001 for one hour. Following washes with plain DMEM, cells in each well were covered with 1 ml DMEM/2% FBS. At time points 0, 6, 12, 24, 36, 48, 72, and 96 hours post infection 30 µl supernatant were collected. Samples were stored at -80ºC before titration on Vero E6 cells.

Mouse experiments.

Four to six week old 129S6/SvEv control and 129S6/SvEv-STAT1-/- mice (Harlan Laboratories) were inoculated with the indicated dose of rVSV (diluted in sterile DMEM) by intraperitoneal (i.p.) injection in a total volume of 200 µl. The animals were monitored for signs of illness and weighed daily. On necropsy days, mice were anesthetized, bled, euthanized and tissue samples were taken and stored at -80ºC. Surviving mice were euthanized 21 days after infection, and serum was collected for antibody titrations.

Titrations.

Vero E6 cells were seeded the day before titration in 96-well plates. Tissue samples were homogenized in DMEM, cleared from debris by centrifugation and serial dilutions were prepared. Blood and cell culture supernatants were thawed and serial dilutions were prepared. A confluent layer of Vero E6 cells was infected in triplicates per dilution for one hour at 37ºC, then 100 µl DMEM/2% FBS were added. After 2-6 days the plates were analyzed for VSV specific cytopathic effect (CPE) and the median tissue culture infectious doses (TCID50) were calculated using the Reed-Muench method (Reed et al. 1938).

Results & Discussion

For the development of filovirus vaccines, promising candidates will require efficacy testing, which involves challenging immunized animals with infectious wt Ebola- and Marburgviruses. In the case of small rodent models, only the use of immunocompromised mouse strains or rodent-adapted viruses that cause severe disease are suitable for adequately evaluating vaccine efficacy. We sought to establish a mouse screening model for efficacy testing of live, attenuated vaccines, in which wt stocks of Ebola or Marburg virions could be used to challenge immunized animals. We chose STAT1-/- mice, which lack a response to interferon α, β and γ, and have previously been shown to be largely susceptible to wt filovirus infections (Raymond et al. 2011). The potential feasibility of this approach was supported by the self-limiting replication of the first rVSV filovirus vector, rVSV/EBOV-May-GP, in immunocompromised NOD-

SCID mice resulting in an asymptomatic phenotype (Jones et al. 2007). In contrast, VSVwt infection is known to cause lethal disease in STAT1-/- mice (Katzenell et al. 2014), but replication of rVSV vectors expressing filovirus GPs instead of the VSV glycoprotein is known to be attenuated in vitro and in vivo (Garbutt et al. 2004; Jones et al. 2005).

In a first experiment we compared the growth kinetics of rVSVwt, rVSV/EBOV-May-GP, rVSV/RESTV-Pen-GP, rVSV/MARV-Ang-GP and rVSV/MARV-Mus-GP in Vero E6 cells over 72 hours. As expected, 3 of the 4 different rVSV vectors replicated similarly in cell culture and were strongly attenuated (several log10) compared to rVSVwt, but reached the same end titer at 72 hours. The exception was rVSV/MARV-Ang-GP, which was less attenuated over the same time period (Fig. 2-1A). This finding is surprising and further investigations need to address potential unique features of the MARV-Ang-GP that may influence infectivity, as the glycoprotein mediates virus entry and is the only difference among all vectors.

We next moved on to perform in vivo characterization of the rVSV vectors. First, groups of 3 129S6/SvEv control mice (immunocompetent) were i.p. infected (usual route of rVSV vaccination in mice) with 10^7 pfu of the 4 rVSV filovirus vectors and rVSVwt; none of the animals developed weight loss or other signs of disease (data not shown). Second, groups of 3 STAT1-/- mice were i.p. infected with different doses (ranging from 10^4-10^7 pfu/animal) of the rVSV vectors and monitored daily for weight change and disease progression. As expected, rVSVwt caused lethal outcome in STAT1-/- mice at all doses tested (Fig. 2-1B).

Surprisingly, the only clearly attenuated vector was rVSV/MARV-Mus-GP, for which infection resulted in 67-100% survival (Fig. 2-1C right panel). While most of the mice survived, all infected animals displayed signs of disease including weight loss (data not shown). Interestingly, rVSV/EBOV-May-GP was only slightly attenuated at the two lower doses with all animals succumbing to infection in a dose-dependent manner (Fig. 2-1D left panel). Similarly, rVSV/RESTV-Pen-GP was only minimally attenuated in the groups receiving the two lower doses with increased time to euthanasia (Fig. 2-1D right panel). Interestingly, rVSV/MARV-Ang-GP was advanced in disease progression with all animals succumbing to infection slightly faster than rVSVwt infected mice independent of the dose (Fig. 2-1C left panel).

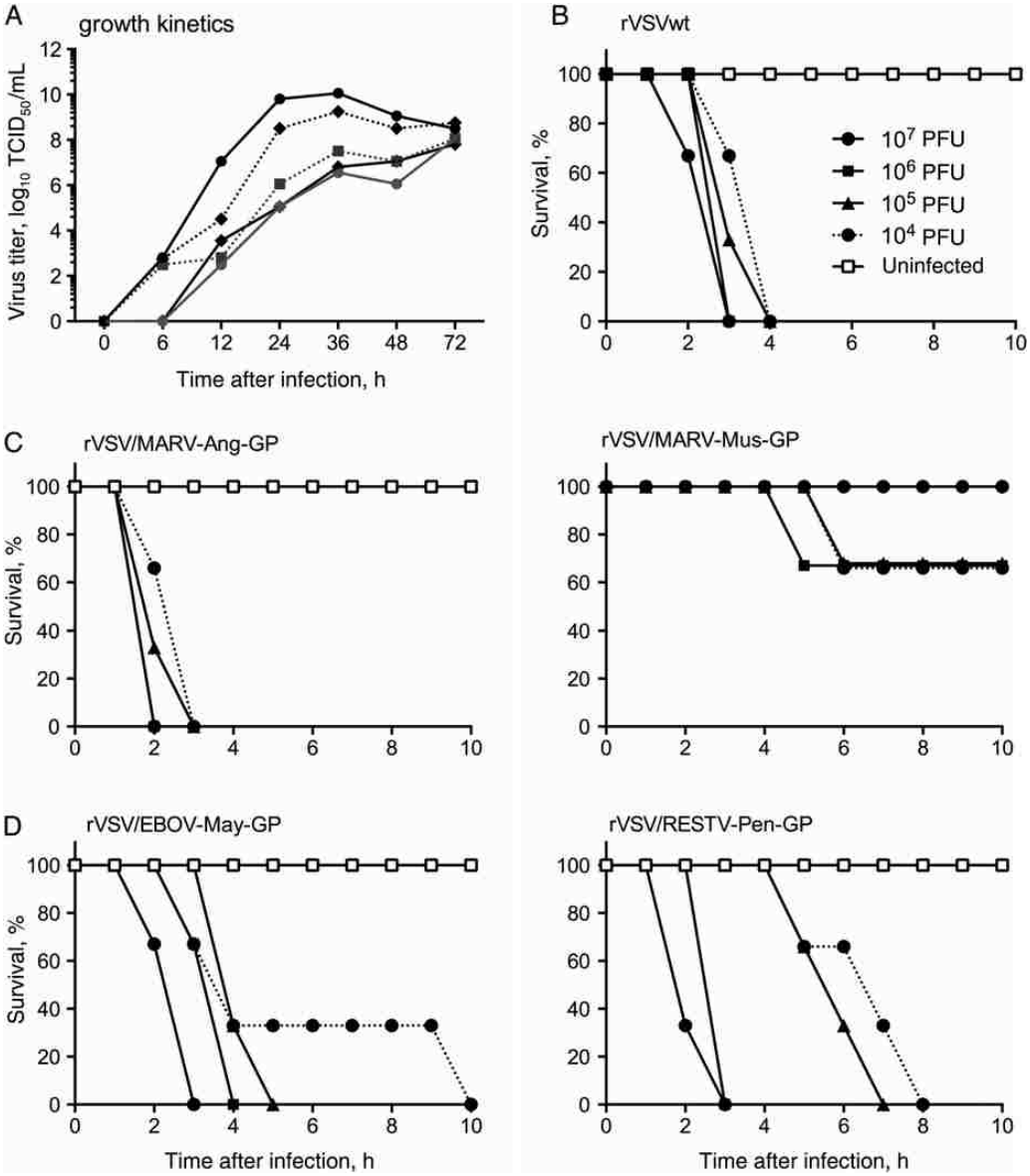

Figure 2-1: Wild-type recombinant vesicular stomatitis virus (rVSVwt) and rVSV filovirus vaccines replicate comparably in vitro and can cause lethal infection in Stat1−/− mice.

A, In vitro growth kinetics on Vero E6 cells. Infection with a multiplicity of infection of 0.001 resulted in the depicted growth curves for rVSVwt (black circle on a black solid line), rVSV/EBOV-May-GP (Ebola virus strain Mayinga; black square on a black dotted line), rVSV/RESTV-Pen-GP (Reston virus strain Pennsylvania; grey circle on a grey solid line), rVSV/MARV-Ang-GP (Marburg virus strain Angola; black solid diamond on a black dotted line), and rVSV/MARV-Mus-GP (Marburg virus strain Musoke; black diamond on a black solid line). B–D, Groups

of 3 mice were infected with the indicated vector and monitored daily for weight loss and signs of illness. Survival curves are shown. Abbreviations: PFU, plaque-forming units; TCID50, 50% tissue culture infectious dose.

All surviving immunocompetent control and STAT-1 knockout mice were euthanized on day 21 after infection and serum was collected for the analysis of humoral immune responses. Every animal seroconverted to VSV with titers ≥102,400 confirming that all mice were indeed infected with rVSV (data not shown). In addition, we compared GP-specific IgG responses in rVSV/MARV-Mus-GP infections of immunocompetent control and STAT1-/- mice, which developed IgG titers up to 25,600 and 6,400, respectively (data not shown). The lower titers detected in STAT1-/- mice are not unexpected, as these animals are defective in cell signaling pathways that contribute to T- and B-cell responses. In macaques, however, a MARV-Mus-GP-specific IgG titer of 640 has been sufficient for protection against lethal MARV-Mus challenge (Jones et al. 2005; Mire et al. 2014), suggesting that the rVSV-immunized STAT1-/- mice might be protected from lethal filovirus challenge.

While it became obvious that STAT1-/- mice are not an appropriate model for testing protective efficacy of rVSV filovirus vectors, we sought to characterize the disease caused by all the rVSV vectors in these mice. In order to determine the tissue tropism and organ virus load, we infected groups of 10 STAT1-/- mice with 10^7 pfu of the different rVSV vectors and euthanized 5 animals each at 12 and 24 hours post inoculation. At the time of euthanasia, blood, lymph node, lung, heart, liver, spleen, kidney, and brain samples were collected and frozen for virus titration. As expected, the highest virus loads in all tissues were obtained for

rVSVwt infected animals with titers comparable to previously published data from VSV infected STAT1-/- or IFNAR-/- mice (Katzenell et al. 2014; Honke et al. 2012). Notably, the lowest amounts of rVSVwt were consistently found in the brain of all mice (Fig. 2-2A). Viremia and tissue titers were comparable for rVSVwt and rVSV/MARV-Ang-GP infected mice at both time points (Fig. 2-2A, B left panel), confirming systemic infection and similar disease progression (Fig. 2-1B, C right panel). The only notable difference was found for brain tissue, in which rVSV/MARV-Ang-GP could only be detected in 1/5 mice at each time point (Fig. 2-2B left panel).

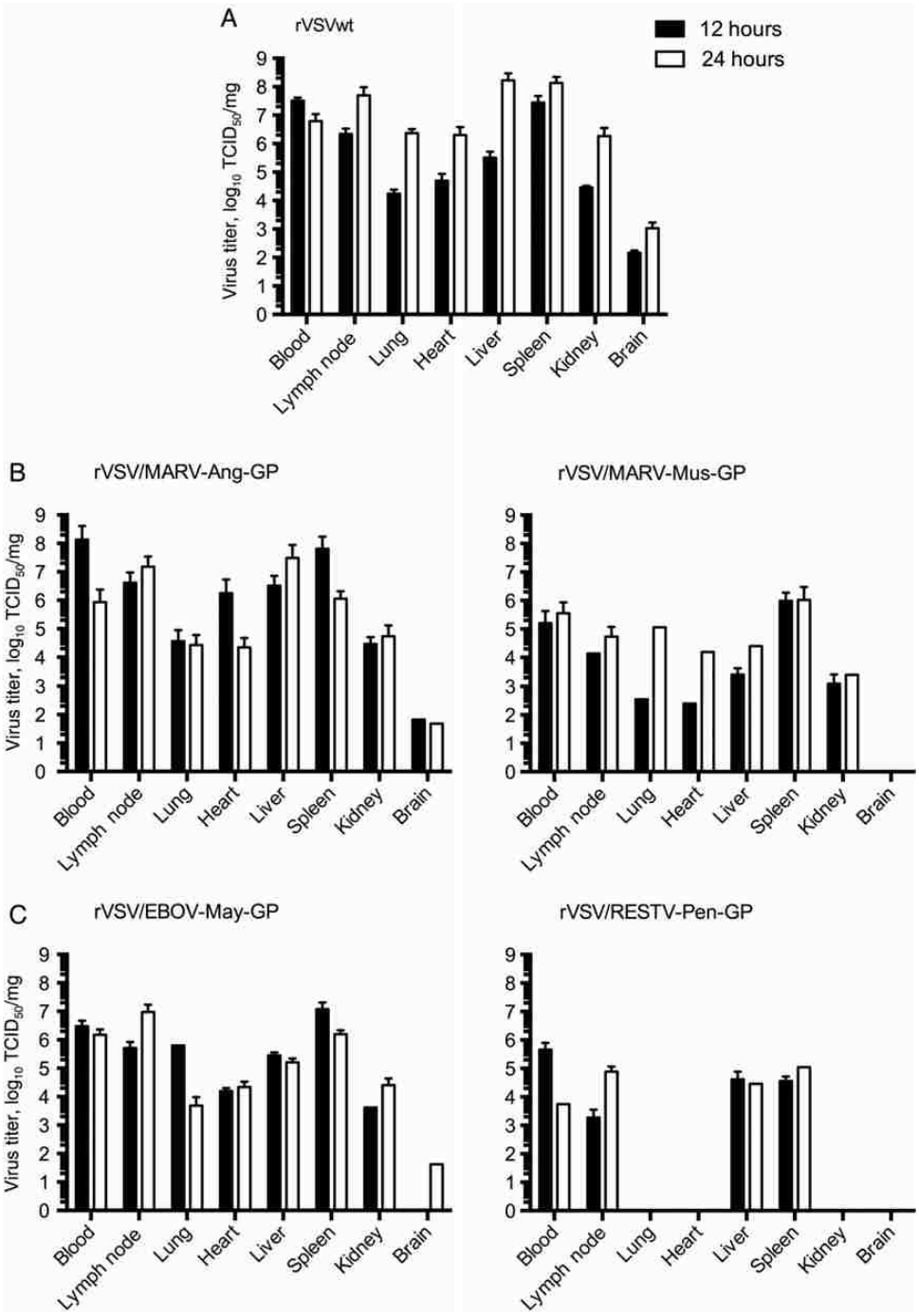

Figure 2-2: Recombinant vesicular stomatitis virus (rVSV) vectors replicate systemically in Stat1−/− mice.

Groups of 5 mice were infected with 10^7 plaque- forming units of the respective rVSV vector and euthanized at the indicated time points. Tissue samples were collected, and virus titers were determined. The organ loads of rVSVwt (wild-type

rVSV; A), rVSV/MARV-Ang-GP (Marburg virus strain Angola; B, left panel), rVSV/MARV-Mus-GP (Marburg virus strain Musoke; B, right panel), rVSV/EBOV-May-GP (Ebola virus strain Mayinga; C, left panel), and rVSV/RESTV-Pen-GP (Reston virus strain Pennsylvania; C, right panel) per group are shown. Error bars represent standard errors of the mean. Abbreviation: TCID50, 50% tissue culture infectious dose.

Slightly lower virus titers were detected in blood and tissues of rVSV/EBOV-May-GP infected mice (Fig. 2-2C left panel), mirroring the slightly slower disease progression compared to rVSVwt and rVSV/MARV-Ang-GP infected animals (Fig. 2-1B, C left panel, D left panel). As noted with rVSV/MARV-Ang-GP infected animals, rVSV/EBOV-May-GP was only found to replicate in the brain of 1 mouse (Fig. 2C left panel). Infection of $STAT1^{-/-}$ mice with rVSV/RESTV-Pen-GP resulted in viremia similar to animals infected with rVSV/EBOV-May-GP, but virus tissue loads were lower and only detectable in liver, spleen and lymph node (Fig. 2-2C right panel). This finding is in contrast to the lethal phenotype in $STAT1^{-/-}$ mice (Fig. 2-1C right panel) and warrants further investigation. rVSV/MARV-Mus-GP infection resulted in the lowest viremia and in reduced viral tissue loads of all rVSV vectors (Fig. 2-2B right panel), which is in line with the attenuation of this vector (Fig. 1D right panel), but it still caused systemic infection without reaching the brain.

Notably, rVSV infected immunocompetent control mice did not show any signs of disease and virus was only occasionally isolated from lymph nodes, liver or spleen samples of mice infected with any of the rVSV vectors including rVSVwt. Overall, infection of immunocompetent control mice did not result in productive rVSV replication, but was sufficient to generate humoral immune

responses (discussed above). For rVSV/EBOV-May-GP these antibody responses have previously been shown to protect mice from lethal mouse-adapted EBOV challenge (Jones et al. 2007).

While immunocompetent mice do not develop disease after infection with wt *Ebola*- and *Marburgviruses*, it has been shown that STAT1$^{-/-}$ mice are susceptible to infection with these pathogens leading to uniform lethality (Bray 2001; Raymond et al. 2011). Although kinetics of disease progression differed among wt filoviruses, replication was similar for all with key target organs being lymph nodes, liver and spleen (Raymond et al. 2011). Overall, organ tropism of the rVSV filovirus vaccine vectors studied here in STAT1$^{-/-}$ mice was similar and confirms a key role of GP in determining cell and tissue tropism (Feldmann et al. 2013). Interestingly, also the organ tropism of rVSVwt was similar (Katzenell et al. 2014; Honke et al. 2012), which may be explained by the in general systemic nature of infections in STAT1$^{-/-}$ mice caused by wt filoviruses, rVSV filovirus vaccine vectors and VSVwt. The only exception was the brain, which seems to be only a major target organ of VSVwt infection.

While STAT1$^{-/-}$ mice are not an appropriate animal model for evaluating the protective efficacy of live-attenuated vectors such as the rVSV filovirus vaccines, this animal model might be useful for further studies characterizing the function of filovirus GPs. The remarkably different phenotypes displayed by the two rVSV/marburgvirus-GP and the two rVSV/ebolavirus-GP vectors in STAT1$^{-/-}$ mice are likely caused by different properties of their GPs, warranting further investigations.

Funding

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References

1. Feldmann H, Sanchez A, Geisbert TW. Filoviridae: Marburg and Ebola viruses In: Knipe DM, Howley, P.M., ed. Fields Virology. Philadelphia: Lippincott Williams & Wilkins, **2013**:923-56.
2. WHO. Ebola situation report, 11 March 2015. Geneva, Switzerland: World Health Organization, **2015**.
3. Jones SM, Feldmann H, Stroher U, et al. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. Nature medicine **2005**; 11:786-90.
4. Marzi A, Feldmann H, Geisbert TW, Falzarano D. Vesicular Stomatitis Virus-Based Vaccines for Prophylaxis and Treatment of Filovirus Infections. Journal of bioterrorism & biodefense **2011**; S1.
5. Bray M. The role of the Type I interferon response in the resistance of mice to filovirus infection. The Journal of general virology **2001**; 82:1365-73.
6. Raymond J, Bradfute S, Bray M. Filovirus infection of STAT-1 knockout mice. The Journal of infectious diseases **2011**; 204 Suppl 3:S986-90.
7. Jones SM, Stroher U, Fernando L, et al. Assessment of a vesicular stomatitis virus-based vaccine by use of the mouse model of Ebola virus hemorrhagic fever. The Journal of infectious diseases **2007**; 196 Suppl 2:S404-12.
8. Katzenell S, Chen Y, Parker ZM, Leib DA. The differential interferon responses of two strains of Stat1-deficient mice do not alter susceptibility to HSV-1 and VSV in vivo. Virology **2014**; 450-451:350-4.
9. Lawson ND, Stillman EA, Whitt MA, Rose JK. Recombinant vesicular stomatitis viruses from DNA. Proceedings of the National Academy of Sciences of the United States of America **1995**; 92:4477-81.
10. Garbutt M, Liebscher R, Wahl-Jensen V, et al. Properties of replication-competent vesicular stomatitis virus vectors expressing glycoproteins of filoviruses and arenaviruses. Journal of virology **2004**; 78:5458-65.
11. Marzi A, Ebihara H, Callison J, et al. Vesicular stomatitis virus-based Ebola vaccines with improved cross-protective efficacy. The Journal of infectious diseases **2011**; 204 Suppl 3:S1066-74.
12. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. American Journal of Hygiene **1938**; 27:493-7.
13. Mire CE, Geisbert JB, Agans KN, et al. Durability of a vesicular stomatitis virus-based marburg virus vaccine in nonhuman primates. PloS one **2014**; 9:e94355.
14. Honke N, Shaabani N, Cadeddu G, et al. Enforced viral replication activates adaptive immunity and is essential for the control of a cytopathic virus. Nature immunology **2012**; 13:51-7.

CHAPTER 3 EVALUATION OF POST-EXPOSURE PROTECTION CONFERRED BY VESICULAR STOMATITIS VIRUS-BASED VACCINES.

Introduction

Hantaviruses are a group of zoonotic pathogens comprising the genus *Hantavirus* within the *Bunyaviridae* family. They are enveloped particles containing a tri-segmented, single stranded, negative sense RNA genome. The three viral RNA segments are designated small (S), medium (M) and large (L) and encode the nucleocapsid protein (NP), the glycoprotein precursor (GPC) and the viral RNA polymerase (RdRp), respectively (Löber et al. 2001). Hantavirus are rodent or insectivore-borne pathogens and primary transmission to humans is thought to largely occur by inhalation of dried excreta (Jonsson et al. 2010). Hantavirus infections in humans are associated with two clinical syndromes; hemorrhagic fever with renal syndrome (HFRS) and hantavirus (cardio)pulmonary syndrome (HCPS or here HPS), which are caused by Old World and New World hantaviruses, respectively (Clement et al. 2014). Of the 22 described human-pathogenic hantaviruses, Andes virus (ANDV) is one of the most virulent New World hantaviruses (Schonrich et al. 2015). ANDV has a HPS case fatality rate of >40% and is the only reported hantavirus that is known to transmit directly from human–to–human (Martinez-Valdebenito et al. 2014). Therefore, among all New World hantaviruses a vaccine would be of particular interest for ANDV, for which a hamster disease model has been established (Hooper et al. 2001).

In a previous study we have demonstrated the potency of a live-attenuated, recombinant vesicular stomatitis virus (rVSV)-based vaccine vector expressing the ANDV glycoprotein precursor in place of the VSV glycoprotein (rVSVΔG-

ANDVgpc) with complete and partial (90%) protection in the Syrian Golden hamsters against lethal ANDV challenge when administered as close as 3 days prior to and 24 hours post infection, respectively (Brown et al. 2011). Surprisingly, a similar rVSV vaccine vector expressing the Ebola virus glycoprotein (rVSVΔG-ZEBOVgp), used as a negative control, also mediated partial protection against lethal ANDV challenge when administered between 7 days prior to 3 days post infection (Brown et al. 2011). Detection of strong induction of innate immune responses, particularly through rVSVΔG-ZEBOVgp (Brown et al. 2011), lead to the hypothesis that a bivalent vector expressing ANDV GPC on the backbone of rVSVΔG-ZEBOVgp) would be a more potent ANDV vaccine.

In this study we investigated the protective efficacy of two monovalent (rVSVΔG-ANDVgpc and rVSVΔG-ZEBOVgp) and two bivalent rVSV vaccine vectors (rVSVΔG-ANDVgpc-ZEBOVgp (Tsuda et al. 2011) and rVSVΔG-ZEBOVgp-ANDVgpc (this study)) in hamsters against lethal ANDV challenge. In addition, two routes of immunization, intraperitoneal and intranasal, were compared. The two bivalent vectors both mediated full protection when administered even 3 days prior to ANDV challenge independent of the route of immunization. The monovalent rVSVΔG-ANDVgpc was similarly protective but intranasal immunization showed reduced efficacy when administered close to challenge. The bivalent rVSVΔG-ZEBOVgp-ANDVgpc was most potent in post-exposure treatment, followed by the monovalent rVSVΔG-ZEBOVgp. The monovalent rVSVΔG-ANDVgpc was least potent in post-exposure treatment.

Intraperitoneal immunization was superior over the intranasal route in post-exposure treatment. Our data indicates the benefit of bivalent rVSV vaccine vectors based on the rVSVΔG-ZEBOVgp backbone with the second immunogen expressed in the downstream position in peri-exposure application.

Materials and Methods

Cells and Viruses:

Vero E6 (African green monkey kidney) and HEK 293T (human embryonic kidney) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% L-Glutamine, penicillin/streptomycin. BHK-21 cells were maintained in Gibco Minimum Essential Media (MEM) supplemented with 10% FBS, 1% L-Glutamine, penicillin/streptomycin . ANDV, strain Chile 9717869 (Meissner et al. 2001) (kindly provided by C. Schmaljohn, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD), was grown and titrated in Vero E6 cells as previously described (Safronetz et al. 2009).

Generation of rVSV Vectors:

The generation, growth and titration of rVSVs expressing the Ebola virus glycoprotein (rVSVΔG-ZEBOVgp), the ANDV glycoprotein precursor (rVSVΔG-ANDVgpc) or both (rVSVΔG-ANDVgpc-ZEBOVgp) has been described elsewhere (Garbutt et al. 2004, Brown et al. 2011; Tsuda et al. 2011). For the

generation of rVSVΔG-ZEBOVgp-ANDVgpc), the open reading frame of the ANDV glycoprotein gene was amplified by iProof High-Fidelity DNA Polymerase (Bio-Rad) from plasmid DNA using the primers 5'-GTA*GTCGAC*CACCATGGAAGGGTGGTATCTGGTTGCTCTTGG-3' (Sal1restriction site underlined) and 5'-TAC*GCTAGC*TACCTATTAGACAGTTTTCTTGTGTCCTCTCCTGGG-3' (Nhe1 restriction site underlined). The PCR product was digested with XhoI & NheI, purified using a PCR purification kit (Qiagen) and cloned into the pATX-VSVΔG-ZEBOVgp plasmid downstream of the ZEBOVgp ORF (Garbutt et al. 2004). The resulting plasmid, pATX-VSVΔG-ZEBOVgp-ANDVgpc, was sequence-confirmed prior to use. rVSVΔG-ZEBOVgp-ANDVgpc was rescued as described previously (Garbutt et al. 2004). Briefly, Vero E6 and 293T cells were seeded together into six-well plates at 1:6 and 1:4 dilutions, respectively. When the co-cultures reached 90% confluence, cells were transfected with pATX-VSVΔG-ZEBOVgp-ANDVgpc (2μg) along with the VSV helper plasmids [SV-T7 (2.5μg), VSV-N (0.5μg), VSV-L (0.25μg plasmid DNA), VSV-P (1.25μg), and pCAGGS-VSVG (2μg)] using Lipofectamine 2000 transfection reagent (Invitrogen) and incubated for 24 hours at 37^0C (5% CO_2). DMEM supplemented with 3% FBS, L-Glutamine, penicillin/streptomycin (1ml/well) was added 24 hours after transfection. Every 12 hours, 20% of the medium was replaced to maintain the co-culture until cytopathic effects (CPE) was observed 12-14 days post-transfection. Supernatant (500μl) of CPE-positive cells was removed and placed onto fresh Vero cells for an additional passage of the rescued rVSV. Supernatant was

collected from both, the transfected co-culture and blind passage on fresh Vero cells, to verify virus rescue by immunoblot (rVSV proteins) and RT-PCR (rVSV genome) as described previously (Brown et al. 2011; Tsuda et al. 2011).

In vitro growth studies (Tissue Culture Infectious Dose 50 (TCID50) assay):

Vero cells were grown to 90% confluence and triplicates were inoculated with rVSVs at an MOI of 0.1 and 0.001 for 1 hour at 37°C. Subsequently, the inoculum was replaced with fresh DMEM supplemented with 3% fetal bovine serum. At predetermined time points 250µl supernatant was collected from each of the triplicate wells and frozen at -80°C. For titration, samples were serially diluted ten-fold and the dilutions were used to inoculate 90% confluent Vero cells (triplicates) for 1 hour at 37°C. Subsequently, the inoculum was removed and replaced with fresh DMEM supplemented with 3% fetal bovine serum. After 4 days if incubation at 37°C wells were analyzed by light microscopy for CPE. The resulting $TCID_{50}$ values were calculated using the Reed-Muench method.

rVSV pathogenesis studies:

Syrian Golden hamsters (female, 4-6 weeks of age; Charles River) were anesthetized and inoculated intraperitoneally (i.p.) with either culture medium (DMEM) (Mock control) or 1x10^5 plaque forming units (pfu) of the different rVSV vectors [rVSVΔG-ANDVgpc, rVSVΔG-ZEBOVgp, rVSVΔG-ANDVgpc-ZEBOVgp, rVSVΔG-ZEBOVgp-ANDVgpc, and VSV-wt] (8 animals per group). Four animals per group were euthanized on day 2 post infection for collection of

blood and tissues (e.g., spleen, liver, lung, kidney, brain) to determine viral growth. The remaining animals (4 hamsters per group) were monitored for disease progression until 14 days post-infection.

Vaccine efficacy studies:

Syrian Golden hamsters (female, 5-6 weeks of age, Charles River) were challenged i.p. with a lethal dose of ANDV (200 focus forming units (ffu)) following vaccination or prior to treatment with rVSV vectors (1x10^5 pfu) by the i.p. or intranasal (i.n.) route. Three animals per group (9 animals in total) were euthanized on day 8 post-infection and blood and organ samples were collected for virology. The remaining six animals per group were monitored for survival for up to 40 days post challenge. For the post-exposure experiment a group of DMEM alone treated hamsters was included as a negative control group.

Ethics and safety:

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Rocky Mountain Laboratories (RML). RML is an American Association for Accreditation of Laboratory Animal Care (AAALAC) approved facility. This study was performed in accordance with the recommendations described in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, the Office of Animal Welfare, and the United States Department of Agriculture. Trained personnel carried out all procedures under isoflurane anesthesia. The Institutional Biosafety Committee (IBC) at RML

approved all standard operating protocols (SOPs) for work with infectious rVSV and ANDV.

Statistical analysis:

Group numbers were selected to achieve a statistical significance with a minimum statistical power of 80%. All tests were one-tail tests at 95% confidence intervals and took into account the possibility of having unexpected survivors or mortality in the treated or control groups (www.dssresearch.com).

Results

rVSV rescue:

The bivalent vaccine vector rVSVΔG-ZEBOVgp-ANDVgpc was rescued in this study using the rVSV reverse genetics system as previously established (Lawson et al. 1995; Garbutt et al. 2004). For this, the ZEBOVgp and ANDVgpc open reading frames were amplified and cloned into the pATX plasmid downstream of the M gene in the first (bp 3093-31625'; MluI and 3' AvrII restriction sites) and second cloning positions (bp 3217-3231; 5' SalI and a 3' NheI restriction sites) (Figure 3-1A). The resulting genome plasmid pATX-rVSVΔG-ZEBOVgp-ANDVgpc was sequence-confirmed and used to rescue the corresponding rVSV on a mixture of Vero and 293 cells. Cell cultures exhibited CPE after 12 to 14 days post-transfection indicating successful rescue of rVSVΔG-ZEBOVgp-ANDVgpc. Further confirmation was obtained by RT-PCR-amplification of the ZEBOVgp and ANDVgpc open reading frames (Figure 1B)

and by immunoblot using ZEBOVgp and ANDVgpc-specific antibodies on material derived from cell culture supernatants (Figure 3-1C). A virus seed stock was prepared by infecting Vero cells yielding a titer of 4x10^6 plaque forming units (pfu) as determined by standard plaque assay. The seed stock was sequenced for final confirmation to exclude the appearance of mutations during rescue and cell culture passage.

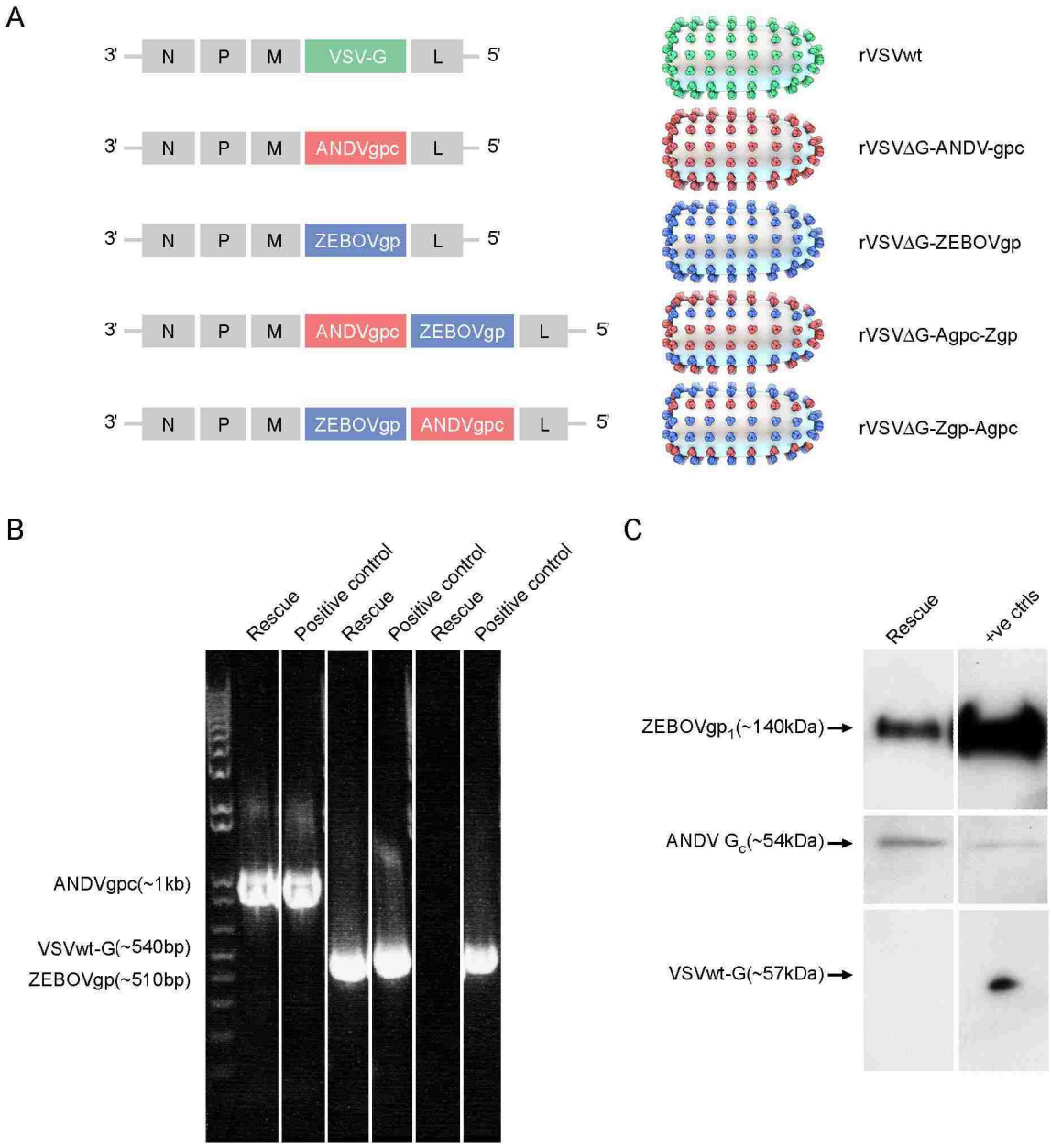


Figure 3-1: Rescue and confirmation of gene expression of rVSVΔG-ZEBOVgp-ANDVgpc.

(A) rVSV vectors that express either monovalent [ANDV glycoprotein precursor (rVSVΔG-ANDVgpc) or Zaire ebolavirus glycoprotein (rVSVΔG-ZEBOVgp)] or

bivalent (ANDV and ZEBOV glycoproteins; rVSVΔG-ZEBOVgp-ANDVgpc; rVSVΔG-ANDVgpc-ZEBOVgp) immunogens in place of the native VSV glycoprotein (VSVwt). (B) RT-PCR amplification of a part of the ZEBOVgp and ANDVgpc open reading frames from rescued rVSVΔG-ZEBOVgp-ANDVgpc. (C) Immunoblot using ZEBOVgp and ANDVgpc-specific antibodies on material derived from cell culture supernatants infected with rVSVΔG-ZEBOVgp-ANDVgpc.

rVSVs are attenuated in vitro:

As our in vivo vaccine efficacy model is the Syrian Golden hamster, in vitro testing was started in BHK cells. For this, BHK-21 cells were infected with rVSVΔG-ZEBOVgp-ANDVgpc, rVSVΔG-ANDVgpc-ZEBOVgp, rVSVΔG-ZEBOVgp, rVSVΔG-ANDVgpc, or VSVwt at an MOI of 0.001 and supernatants were collected over the course of 65 hours. VSVwt replicated to the highest titers at all time points of the experiments with >4 log differences to any rVSV within the first 24 hours (Figure 3-2A). Up to 24 hours post infection the rVSVs showed minor differences in replication kinetics with the bivalent vector rVSVΔG-ANDVgpc-ZEBOVgp displaying the lowest replication rate. After 24 hours, all of the rVSVs showed comparable virus growth kinetics reaching similar endpoint titers at 65 hours post infection, about 1 log lower compared to VSVwt (Figure 3-2A). Virus growth kinetics for all rVSVs were also performed in Vero cells in comparison to VSVwt resulting in similar replication kinetics (data not shown). Altogether, the introduction of the foreign glycoprotein genes into the rVSVΔG genome resulted in in vitro attenuation of the resulting vectors without major differences between monovalent and bivalent vectors.

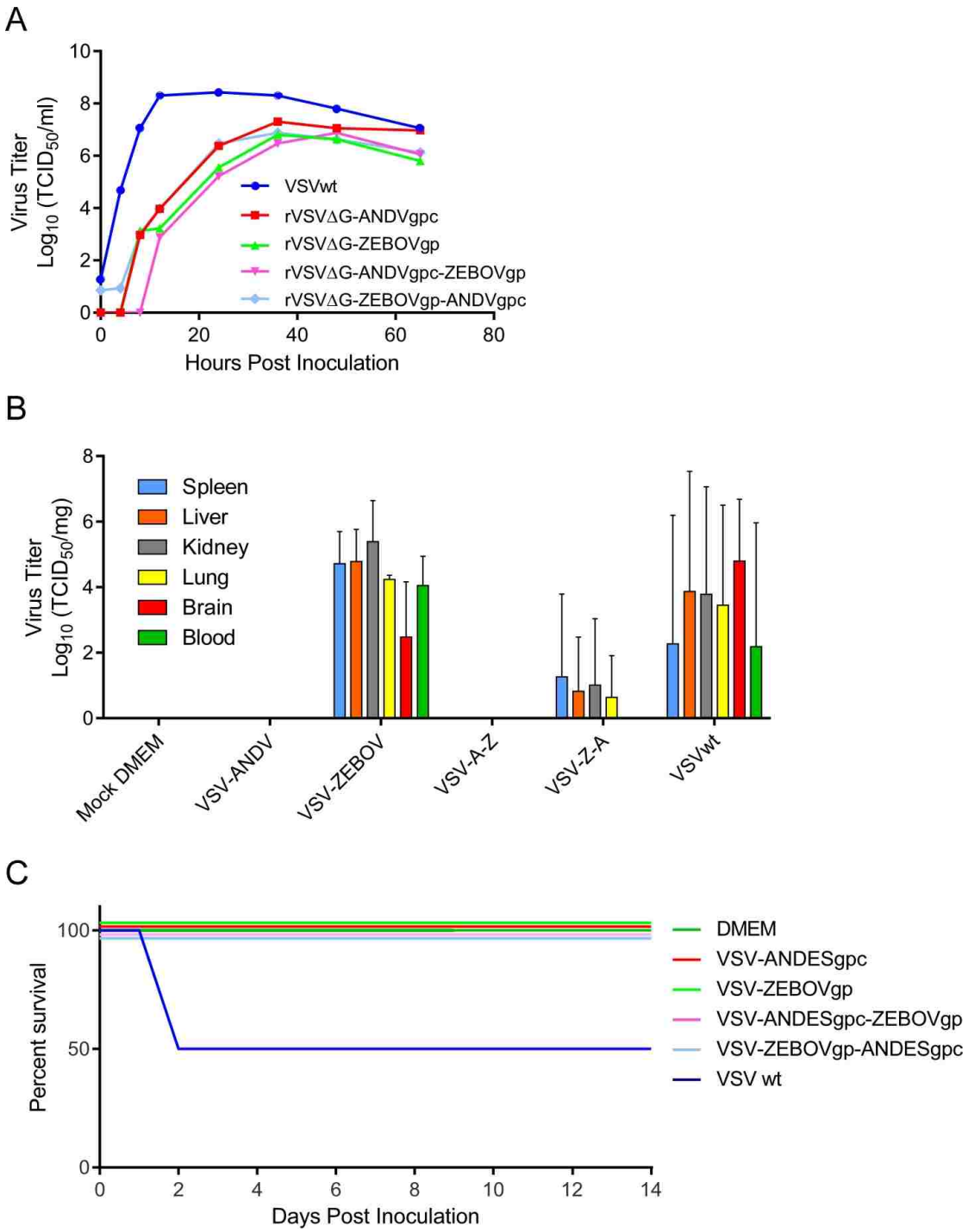


Figure 3-2: rVSVs are attenuated in cell culture and in the hamster disease model compared to VSVwt.

(A) BHK-21 cells were infected with rVSVΔG-ZEBOVgp-ANDVgpc, rVSVΔG-ANDVgpc-ZEBOVgp, rVSVΔG-ZEBOVgp, rVSVΔG-ANDVgpc, or VSVwt at an MOI of 0.001 and supernatants were collected over the course of 65 hours. Supernatants were analyzed using a TCID50 assay. (B) Hamsters (groups of 8 animals) were inoculated i.p. with a single dose of rVSV∆G-ANDVgpc, rVSV∆G-ZEBOVgp, rVSV∆G-ZEBOVgp-ANDVgpc, rVSV∆G-ANDVgpc-ZEBOVgp or VSVwt (10^5 PFU/animal). Blood and tissue samples (brain, liver, lung, kidney, and spleen) were taken from 4 hamsters in each group 2 days after inoculation and infectious titers were analyzed using a TCID50 assay. DMEM-inoculated animals served as non-inoculated controls. (C) The remaining 4 animals of each group were monitored for survival over the course of 14 days.

rVSV vectors are in vivo attenuated:

Syrian Golden hamsters (groups of 8 animals) were inoculated i.p. with a single dose of rVSVΔG-ANDVgpc, rVSVΔG-ZEBOVgp, rVSVΔG-ZEBOVgp-ANDVgpc, rVSVΔG-ANDVgpc-ZEBOVgp and VSVwt (10^5 PFU/animal). A DMEM-inoculated animals served as non-inoculated controls. The inoculation with VSVwt at this dose resulted in lethality of 50%. In contrast, animals in all other rVSV inoculated groups showed no or very limited disease signs (temporary minor weight loss of up to 3% over the first three days) and survived the inoculation (Figure 3-2C). To assess rVSV replication, blood and tissue samples (brain, liver, lung, kidney, and spleen) were taken from 4 hamsters in each group 2 days after inoculation and infectious titers were analyzed using a TCID50 assay (Figure 3-2B). VSVwt inoculated animals were positive in all tissues tested. To assess survival after rVSV inoculation the remaining 4 hamsters in each group were monitored for symptoms of disease such as weight loss, ruffled fur, reduced movement, and others for 14 consecutive days or until clinical signs indicated a humane endpoint (Figure 3-2C). Of all rVSV groups only animals inoculated with rVSVΔG-ZEBOVgp were clearly positive for viral infectivity with organ titers similar to VSVwt inoculated animals. Organ titers around the detection limit were found in hamsters inoculated with rVSVΔG-ZEBOVgp-ANDVgpc, whereas no viral infectivity was found in organs or blood of animals inoculated with rVSVΔG-ANDVgpc or rVSVΔG-ANDVgpc-ZEBOVgp (Figure 3-2B). Overall, all rVSV vectors are attenuated in the hamster disease model compared to VSVwt warranting vaccine efficacy studies in this model.

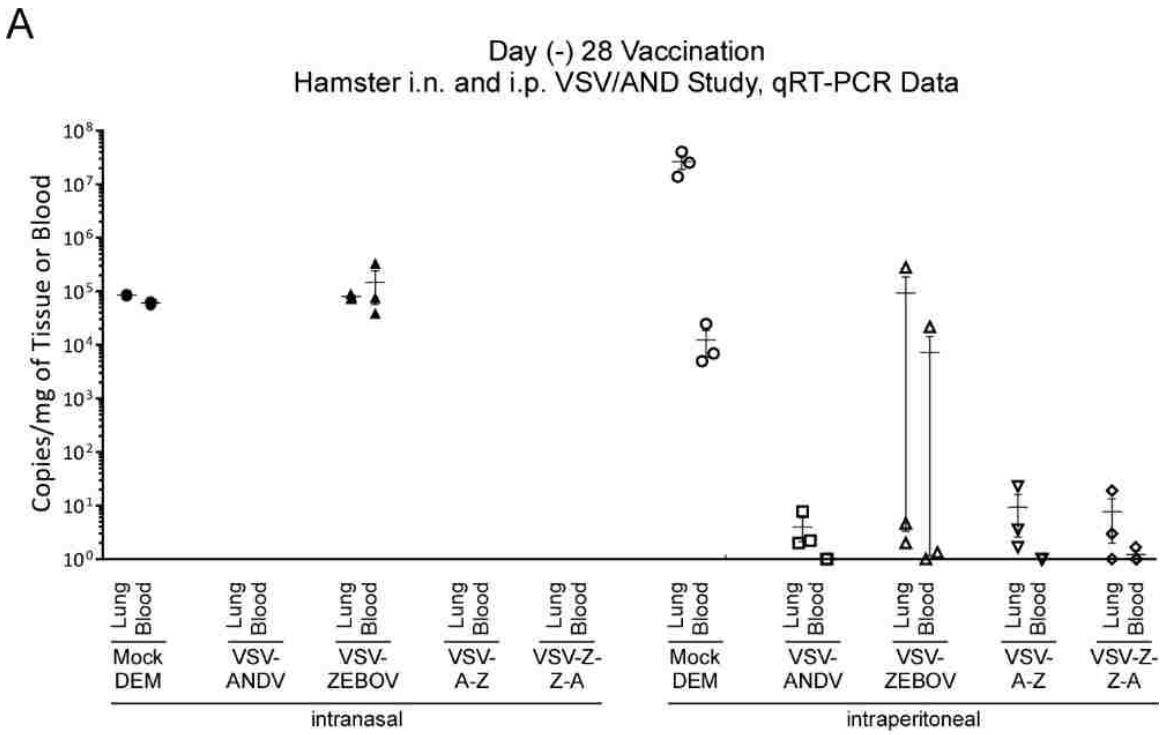
rVSV vectors protect against ANDV challenge when administered pre-exposure.

For pre-exposure efficacy testing of the rVSV vectors, hamsters (group of 9 animals) were immunized i.p. or i.n. with a single dose of 10^5 PFU 28 days before lethal ANDV challenge (200 FFU/animal). Three animals of each group were euthanized on day 8 post challenge for lung (main target organ for ANDV infection) and blood titration. All animals remained clinically asymptomatic during the vaccination period indicating a lack of adverse effects to immunization with the rVSV vectors in this animal species. The animals immunized with the monovalent non-specific rVSVΔG-ZEBOVgp vector, expressing the ZEBOV glycoprotein were, as expected, unprotected and succumbed to infection between days 7 to 11 post ANDV challenge (Table 3-1). Animals immunized with the monovalent specific rVSVΔG-ANDVgpc vector were expected to be protected and showed 100% (i.n. vaccination) and 83% (i.p. vaccination) survival. Animals immunized with the two bivalent vectors, rVSVΔG-ZEBOVgp-ANDVgpc and rVSVΔG-ANDVgpc-ZEBOVgp were completely protected independent of route of vaccination. The survival data is supported by organ and blood titers as shown in Figure 3-3A.

Vaccine Vector	Pre-exposure Immunization				Post-exposure Immunization					
	28 days		3 days		1 day		3 days		5 days	
	i.p.	i.n.	i.p.	i.n.	i.p.	i.n.	i.p.	i.n.	i.p.	i.n.
rVSVΔG-ZEBOVgp-ANDVgpc	100% (6/6)	100%	100%	100%	100%	100%	83% (5/6)	0%	0%	0%
rVSVΔG-ANDVgpc-ZEBOVgp	100%	100%	100%	100%	100%	50% (3/6)	17% (1/6)	0%	0%	0%
rVSVΔG-ANDVgpc	83% (5/6)	100%	100%	33% (2/6)	33% (2/6)	0%	0%	0%	17%	0%
rVSVΔG-ZEBOVgp	0% (0/6)	0%	33% (2/6)	0%	100%	83% (5/6)	67% (4/6)	0%	0%	0%
DMEM	ND	ND	ND	ND	0%	0%	ND	ND	ND	ND

Table 3-1: Bivalent rVSVΔG-ZEBOVgp-ANDVgpc offers better post-exposure protection than previous bivalent and monovalent constructs.
Hamsters (group of 6 animals) were immunized i.p. or i.n. with a single dose of 10^5 PFU before (-28 days and -3 days pre-exposure) or after (days +1, +3 and +5 post-exposure) lethal ANDV challenge (200 FFU/animal). Animals were monitored for survival up to 40 days post challenge.

In order to address the potential for rapid immunization we next immunized animals with a single dose of 10^5 PFU 3 days prior to lethal ANDV challenge (200 FFU/animal). Animals immunized by the i.n. route with the monovalent specific rVSVΔG-ANDVgpc vector were only partially protected (33%), whereas animals immunized i.p. showed full protection (100%) (Table 1). Interestingly, survival increased from none to 33% in the group that was immunized i.p. with the non-specific monovalent rVSVΔG-ZEBOVgp vector (Table 3-1). All animals immunized by either route with the two bivalent vectors were fully protected against lethal ANDV challenge. As with the day 28 immunization, the survival data is supported by organ and blood titers shown in Figure 3-3B.


A
Day (-) 28 Vaccination
Hamster i.n. and i.p. VSV/AND Study, qRT-PCR Data
Copies/mg of Tissue or Blood
Lung
Blood
Mock DEM
VSV-ANDV
VSV-ZEBOV
VSV-A-Z
VSV-Z-Z-A
intranasal
intraperitoneal

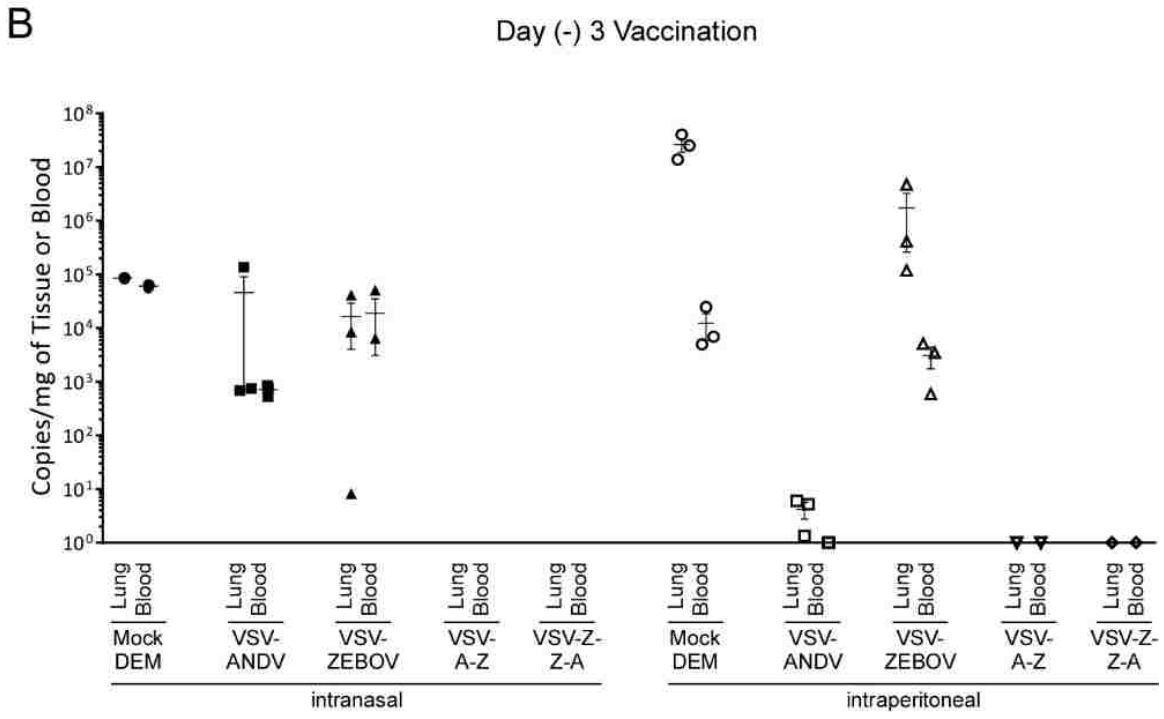

B
Day (-) 3 Vaccination
Copies/mg of Tissue or Blood
Lung
Blood
Mock DEM
VSV-ANDV
VSV-ZEBOV
VSV-A-Z
VSV-Z-Z-A
intranasal
intraperitoneal

Figure 3-3: Blood and lung tissue data support survival data in pre-exposure treatment with rVSVs.

Hamsters (group of 9 animals) were immunized i.p. or i.n. with a single dose of 10^5 PFU before (-28 days and -3 days) lethal ANDV challenge (200 FFU/animal). (A) lung tissue and blood was taken from 3 animals of each group vaccinated 28 days prior to challenge and analyzed for viral replication using RT-PCR. (B) Lung tissue and blood were taken from 3 animals of each group vaccinated 3 days prior to challenge and analyzed for viral replication using RT-PCR.

rVSV vectors protect against ANDV challenge when administered shortly after exposure:

For post-exposure efficacy testing of the rVSV vectors, hamsters (group of 9 animals) were immunized i.p. or i.n. with a single dose of 10^5 PFU on days +1, +3 and +5 after lethal ANDV challenge (200 FFU/animal). As with the pre-exposure study, three animals of each group were euthanized on day 8 post challenge for organ (lung) and blood titration. None of the animals immunized i.p. or i.n. on day 5 or immunized i.n. on day 3 post ANDV challenge were protected. Disease progression was almost identical to DMEM-treated controls resulting in death (Table 3-1). In contrast, all hamsters in the groups immunized i.p. 1 day post ANDV challenge with the two bivalent vectors as well as the non-specific monovalent rVSVΔG-ZEBOVgp vector survived ANDV challenge, whereas surprisingly survival was only 33% in the group immunized i.p. with the monovalent rVSVΔG-ANDVgpc vector (Table 3-1). The i.n. route of immunization was less efficient but showed a similar trend with only the hamsters in the rVSVΔG-ZEBOVgp-ANDVgpc group showing 100% survival, followed by 83% and 50% survival when immunized i.n. with rVSVΔG-ZEBOVgp and rVSVΔG-

ANDVgpc-ZEBOVgp, respectively. Again, the monovalent specific rVSVΔG-ANDVgpc vector did not perform well with no survival after single-shot i.n. immunization. A similar result was observed with i.p. immunization on day 3 post ANDV challenge. The bivalent rVSVΔG-ZEBOVgp-ANDVgpc vector performed best with 83% survival followed by the rVSVΔG-ZEBOVgp vector with 67% survival. The other two vectors did not provide any protection from disease and death. Infectivity data on lung tissue and blood confirmed ANDV infection of the animals, but did not show significant distinction among the vaccine groups (Figure 3-4).

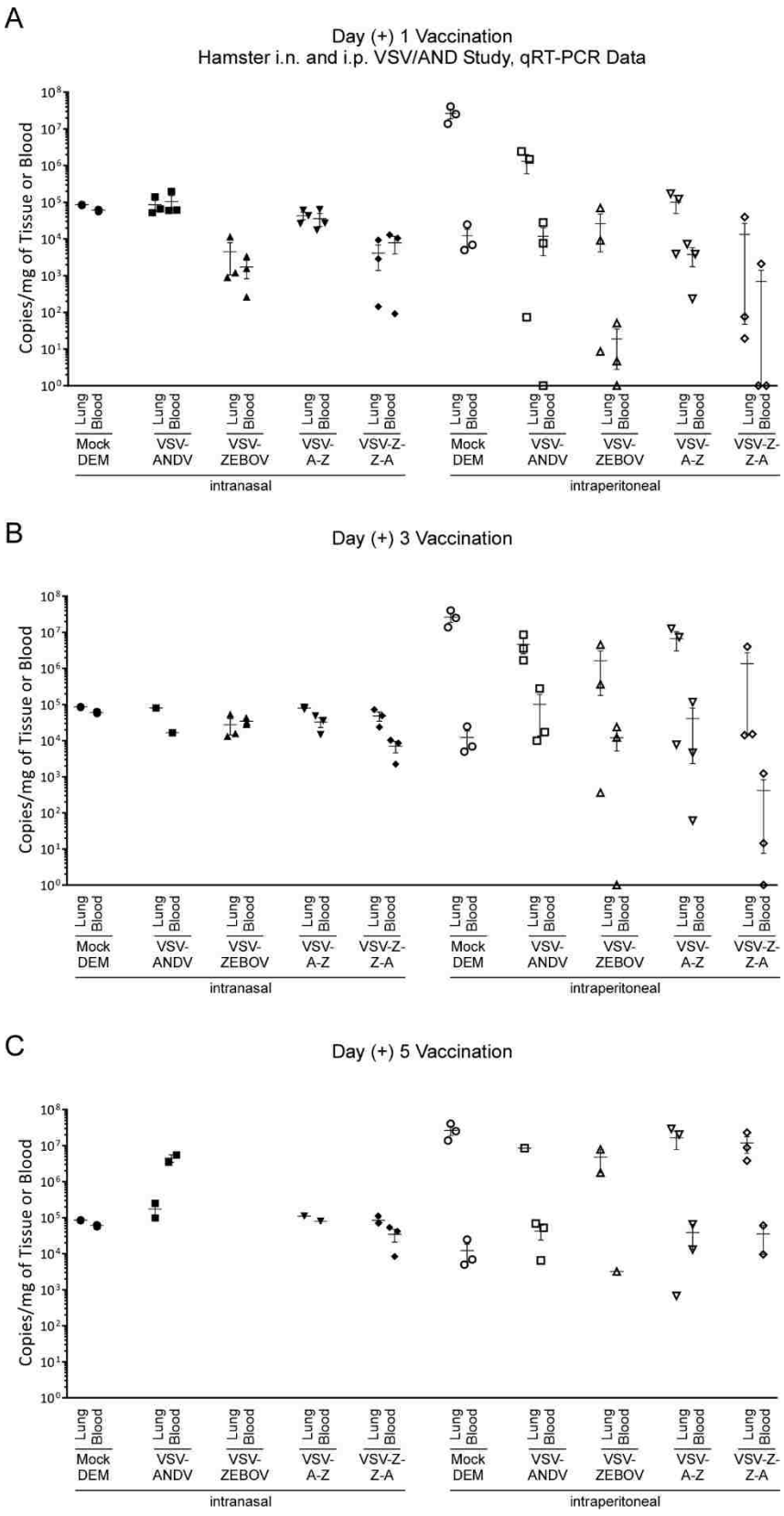

A
Day (+) 1 Vaccination
Hamster i.n. and i.p. VSV/AND Study, qRT-PCR Data
B
Day (+) 3 Vaccination
C
Day (+) 5 Vaccination
Copies/mg of Tissue or Blood
Lung
Blood
Mock DEM
VSV-ANDV
VSV-ZEBOV
VSV-A-Z
VSV-Z-Z-A
intranasal
intraperitoneal

Figure 3-4: Post-exposure treatment with rVSVs does not prevent ANDV Infection.

Hamsters (group of 9 animals) were immunized i.p. or i.n. with a single dose of 10^5 PFU after lethal ANDV challenge (200 FFU/animal). (A) Three animals of each group inoculated 1 day after challenge were euthanized on day 8 post challenge for organ (lung) and blood titration using RT-PCR. This was repeated for groups inoculated 3 days (B) and 5 days (C) after challenge.

Overall, best performance in post-exposure vaccination was achieved with the two bivalent vectors with rVSVΔG-ZEBOVgp-ANDVgpc being superior in efficacy. Interestingly, the monovalent non-specific rVSVΔG-ZEBOVgp vector was almost as efficacious against ANDV challenge if administered shortly after post-exposure. Surprisingly, the monovalent specific rVSVΔG-ANDVgpc vector performed extremely poorly in post-exposure vaccination. The i.p. immunization route was superior over the i.n. route for post-exposure vaccination.

Discussion

Although the annual cases of ANDV remain low in South America when compared to incidences of Old World strains in Europe and Asia, the high lethality rate and the potential for human-to-human transmission illustrates that a vaccine and other treatments for the disease need to be developed.

Historically, rVSV has been used extensively as a laboratory tool for studying virus and cell biology, but more recently its potential as a therapeutic and vaccine tool has been realized. Here we have constructed and rescued a bivalent rVSV vaccine vector that expresses the glycoproteins from ZEBOV and ANDV in place of the native VSV glycoprotein. The rationale for the bivalent

vaccine vector was based on evidence that the ZEBOVgp targets the vaccine vectors to important immune cells strongly enhancing innate immune responses (Prescott et al. 2015). For this study we used the Syrian hamster model to test vaccine safety and efficacy. While rVSVwt caused systemic infection in Syrian hamsters resulting in severe or lethal outcome, the rVSV-based vaccine candidates temporarily replicated in Syrian hamsters without causing signs of clinical disease. We showed that the rVSV-based vaccine candidates are attenuated when compared to the VSVwt in hamsters and tissue culture cells.

The protective efficacy of the rVSV vectors was also tested following administration prior to or after ANDV challenge in the HPS hamster model in order to define the most potent candidate vaccine for prophylactic vaccination and post-exposure treatment. The results indicated that the organization of the ZEBOV and ANDV genes can alter the protection in the hamster. Importantly, the efficacy of post-exposure protection was increased by including the ZEBOVgp upstream of the ANDVgpc immunogen in the bivalent rVSVΔG-ZEBOVgp-ANDVgpc. This may be due to increased ZEBOVgp production, expression or presentation to the innate immune system.

Although ANDV results in systemic infection in hamsters, the lungs are the primary site of viral replication and the only organ which develops pathological abnormalities during the course of disease (Safronetz et al. 2011). We correctly expected that, similar to i.p. immunization, administration of the ANDVgpc

containing rVSV vaccines via the i.n. route would provide 100% protection when given 28 days pre-exposure. While we hypothesized that i.n. immunization would provide increase post-exposure protection by eliciting a strong innate immune response directly in the lungs of hamsters, the results showed a trend that indicated that the i.p. route was more effective and that the efficacy of mucosal immunization post-challenge may not offer the same protection as a systemic i.p. inoculation.

The results of these experiments further defined the protective efficacy of the recombinant rVSV vaccine candidates in the Syrian hamster model when challenged with a lethal dose of ANDV. Importantly, using the novel rVSV vectors we were able to demonstrate an increase in efficacy of post-exposure protection in the lethal hamsters model of HPS (Brown et al. 2011; Tsuda et al. 2011).

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References

Bray, M. 2001. 'The role of the Type I interferon response in the resistance of mice to filovirus infection', *J Gen Virol*, 82: 1365-73.

Brown, K. S., D. Safronetz, A. Marzi, H. Ebihara, and H. Feldmann. 2011. 'Vesicular stomatitis virus-based vaccine protects hamsters against lethal challenge with Andes virus', *J Virol*, 85: 12781-91.

Clement, Jan, Piet Maes, and Marc Van Ranst. 2014. 'Hemorrhagic Fever with Renal Syndrome in the New, and Hantavirus Pulmonary Syndrome in the old world: Paradi(se)gm lost or regained?', *Virus Research*, 187: 55-58.

Colleen, B. Jonsson, Figueiredo Luiz Tadeu Moraes, and Vapalahti Olli. 2010. 'A Global Perspective on Hantavirus Ecology, Epidemiology, and Disease', *Clinical Microbiology Reviews*, 23: 412-41.

Feldmann, H., A. Sanchez, and T.W. Geisbert 2013. 'Filoviridae: Marburg and Ebola viruses ' in D.M. Knipe, Howley, P.M. (ed.), *Fields Virology* (Lippincott Williams & Wilkins: Philadelphia).

Garbutt, M., R. Liebscher, V. Wahl-Jensen, S. Jones, P. Moller, R. Wagner, V. Volchkov, H. D. Klenk, H. Feldmann, and U. Stroher. 2004. 'Properties of replication-competent vesicular stomatitis virus vectors expressing glycoproteins of filoviruses and arenaviruses', *J Virol*, 78: 5458-65.

Honke, N., N. Shaabani, G. Cadeddu, U. R. Sorg, D. E. Zhang, M. Trilling, K. Klingel, M. Sauter, R. Kandolf, N. Gailus, N. van Rooijen, C. Burkart, S. E. Baldus, M. Grusdat, M. Lohning, H. Hengel, K. Pfeffer, M. Tanaka, D. Haussinger, M. Recher, P. A. Lang, and K. S. Lang. 2012. 'Enforced viral replication activates adaptive immunity and is essential for the control of a cytopathic virus', *Nat Immunol*, 13: 51-7.

Jones, S. M., H. Feldmann, U. Stroher, J. B. Geisbert, L. Fernando, A. Grolla, H. D. Klenk, N. J. Sullivan, V. E. Volchkov, E. A. Fritz, K. M. Daddario, L. E. Hensley, P. B. Jahrling, and T. W. Geisbert. 2005. 'Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses', *Nat Med*, 11: 786-90.

Jones, S. M., U. Stroher, L. Fernando, X. Qiu, J. Alimonti, P. Melito, M. Bray, H. D. Klenk, and H. Feldmann. 2007. 'Assessment of a vesicular stomatitis virus-based vaccine by use of the mouse model of Ebola virus hemorrhagic fever', *J Infect Dis*, 196 Suppl 2: S404-12.

Jonsson, Colleen B., Luiz Tadeu Moraes Figueiredo, and Olli Vapalahti. 2010. 'A Global Perspective on Hantavirus Ecology, Epidemiology, and Disease', *Clinical Microbiology Reviews*, 23: 412-41.

Katzenell, S., Y. Chen, Z. M. Parker, and D. A. Leib. 2014. 'The differential interferon responses of two strains of Stat1-deficient mice do not alter susceptibility to HSV-1 and VSV in vivo', *Virology*, 450-451: 350-4.

Lawson, N. D., E. A. Stillman, M. A. Whitt, and J. K. Rose. 1995. 'Recombinant vesicular stomatitis viruses from DNA', *Proc Natl Acad Sci U S A*, 92: 4477-81.

Lee, H. W. 1982. 'Hemorrhagic fever with renal syndrome (HFRS)', *Scand.J.Infect.Dis.Suppl*, 36: 82-85.

Linderholm, M., and F. Elgh. 2001. 'Clinical characteristics of hantavirus infections on the Eurasian continent', *Curr Top Microbiol Immunol*, 256: 135-51.

Lindkvist, Marie, and Umeå Universitet. 2008. 'Genetic and serologic characterization of a Swedish human hantavirus isolate'.

Liu, Jing, Dong-Ying Liu, Wen Chen, Jin-Lin Li, Fan Luo, Qing Li, Jia-Xin Ling, Yuan-Yuan Liu, Hai-Rong Xiong, Xiao-Hua Ding, Wei Hou, Yun Zhang, Shi-Yue Li, Jie Wang, and Zhan-Qiu Yang. 2012. 'Genetic analysis of hantaviruses and their rodent hosts in central-south China', *Virus Research*, 163: 439-47.

Löber, Christian, Bärbel Anheier, Steffi Lindow, Hans-Dieter Klenk, and Heinz Feldmann. 2001. 'The Hantaan Virus Glycoprotein Precursor Is Cleaved at the Conserved Pentapeptide WAASA', *Virology*, 289: 224-29.

Martinez-Valdebenito, Constanza, Mario Calvo, Cecilia Vial, Rita Mansilla, Claudia Marco, R. Eduardo Palma, Pablo A. Vial, Francisca Valdivieso, Gregory Mertz, and Marcela Ferrés. 2014. 'Person-to-Person Household and Nosocomial Transmission of Andes Hantavirus, Southern Chile, 2011', *Emerging Infectious Diseases*, 20: 1629-36.

Marzi, A., H. Ebihara, J. Callison, A. Groseth, K. J. Williams, T. W. Geisbert, and H. Feldmann. 2011. 'Vesicular stomatitis virus-based Ebola vaccines with improved cross-protective efficacy', *J Infect Dis*, 204 Suppl 3: S1066-74.

Marzi, A., H. Feldmann, T. W. Geisbert, and D. Falzarano. 2011. 'Vesicular Stomatitis Virus-Based Vaccines for Prophylaxis and Treatment of Filovirus Infections', *J Bioterror Biodef*, S1.

Mire, C. E., J. B. Geisbert, K. N. Agans, B. A. Satterfield, K. M. Versteeg, E. A. Fritz, H. Feldmann, L. E. Hensley, and T. W. Geisbert. 2014. 'Durability of a vesicular stomatitis virus-based marburg virus vaccine in nonhuman primates', *PLoS One*, 9: e94355.

Nichol, S. T., C. F. Spiropoulou, S. Morzunov, P. E. Rollin, T. G. Ksiazek, H. Feldmann, A. Sanchez, J. Childs, S. Zaki, and C. J. Peters. 1993. 'Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness', *Science*, 262: 914-7.

Plyusnin, A., O. Vapalahti, and A. Vaheri. 1996. 'Hantaviruses: genome structure, expression and evolution', *The Journal of general virology*, 77 (Pt 11): 2677-87.

Prescott, Joseph, Blair L. Debuysscher, Friederike Feldmann, Donald J. Gardner, Elaine Haddock, Cynthia Martellaro, Dana Scott, and Heinz Feldmann. "Single-dose Live-attenuated Vesicular Stomatitis Virus-based Vaccine Protects African Green Monkeys from Nipah Virus Disease." Vaccine 33.24 (2015): 2823-829.

Raymond, J., S. Bradfute, and M. Bray. 2011. 'Filovirus infection of STAT-1 knockout mice', *J Infect Dis*, 204 Suppl 3: S986-90.

Reed, L.J., and H. Muench. 1938. 'A simple method of estimating fifty percent endpoints', *American Journal of Hygiene*, 27: 493-97.

Safronetz, D., M. Zivcec, R. Lacasse, F. Feldmann, R. Rosenke, D. Long, E. Haddock, D. Brining, D. Gardner, H. Feldmann, and H. Ebihara. 2011. 'Pathogenesis and host response in Syrian hamsters following intranasal infection with Andes virus', *PLoS Pathog*, 7: e1002426.

Schönrich, Günther, Detlev H. Krüger, and Martin J. Raftery. 2015. 'Hantavirus-induced disruption of the endothelial barrier: neutrophils are on the payroll', *Front Microbiol*, 6: 222.

Vaheri, Antti, Tomas Strandin, Jussi Hepojoki, Tarja Sironen, Heikki Henttonen, Satu Mäkelä, and Jukka Mustonen. 2013. 'Uncovering the mysteries of hantavirus infections', *Nature Reviews Microbiology*, 11: 539-50.

WHO. 2015. "Ebola situation report, 4 March 2015." In. Geneva, Switzerland: World Health Organization.

www.dssresearch.com. 'Statistical Power Calculators | Health Care Research Calculators'.

CHAPTER 4 CONCLUSIONS

Summary

Although the annual cases of ANDV remains low in South America, when compared to incidences of Old World strains of Europe and Asia, the high lethality rate and the potential for human-to-human transmission illustrates that a vaccine and other treatments for the disease need to be developed. This dissertation describes the development of a rVSV based vaccine platform that is effective in protecting Syrian hamsters in peri-exposure challenge with a lethal dose of ANDV. In addition, constructs that contain the ANDVgpc are also useful when used as a conventional vaccine 28 days before challenge.

In summary, these data have shown that rVSV presents an efficient system for tailoring specific vaccines for ANDV. First, chapter 2 investigates the use of STAT1 KO mice as an animal model to test the tolerance of rVSV constructs as vaccine vectors. The results of chapter 2 have shown that mice lacking an innate immune response involving interferon alpha, beta, and gamma do not comprise an animal model which is suited for testing rVSV vaccine vectors. Specifically, the rVSVs cause severe disease in the immunocompromised mouse model therefore disqualifying it as a useful model in my studies. Second, chapter 3 describes the successful design and rescue of a bivalent rVSV that expresses both ANDV and ZEBOV glycoproteins in place of

the deleted VSV glycoprotein. The results in chapter 3 describe how the insertion of glycoproteins from ZEBOV and ANDV effectively attenuates the pathogenicity of the rVSV vector thereby reducing negative symptoms in the hamster model while still allowing enough replication for the rVSVs to induce a robust and protective immune response after only one inoculating dose. The data support that the rVSV∆G-ZEBOVgp-ANDVgpc vaccine vector was more protective than both monovalent vectors and the bivalent vector rVSV∆G-ANDVgpc-ZEBOVgp. The new bivalent vector rVSV∆G-ZEBOVgp-ANDVgpc was able to protect 83% of animals with a single i.p. inoculation 3 days after a lethal ANDV challenge. This surpasses all other previously developed rVSV vaccines designed for hantavirus vaccination in the Syrian hamster model. The protections studies show that, for overall survival, the bivalent vectors with both the ZEBOV and ANDV glycoprotein inserts are very effective. It may be shown in future studies that look more closely at the specific immune response that the ZEBOVgp is effective at initiating a broadly protective short term innate response while the presence of the ANDVgpc allows the formation/establishment of a long lived and specific adaptive response in a single dose immunization. The survival results of these protection studies also suggest that the placement of the glycoprotein gene in the rVSV genome can play a significant role in the survival outcome of lethally inoculated animals. In general, having the ZEBOVgp gene in the most upstream cloning position was more protective in the post-exposure vaccination when animals were challenged with a lethal dose of ANDV in both the monovalent and bivalent genome configurations. The data showing the viral

load on blood and lung from lethally inoculated animals is interesting but difficult to make any conclusions about without more in-depth experiments that examine the mechanism of the immune response. It may indicate that survival may not be as dependent on the ability of the rVSV vector to induce an immune state that reduces the overall viral load (in post exposure studies) but may be another mechanism that is more influential in survival. This may be due to the vector's ability to modulate the immune response to reduce pathological and destructive immune responses. One of the limitations of these studies is the lack of established and commercially available reagents to examine the mechanism of the hamster immune response but, as the hamster model sees increased use as a disease model these materials will become more available.

In light that rVSV constructs, which contain an unrelated antigen to the hantavirus family, can provide protection against a heterologous virus challenge is striking. It has been hypothesized that rVSV is able to produce a very robust, non-specific, innate response which serves to suppress the levels of the challenge virus until the adaptive immune response, which would otherwise be too slow, establishes itself enough to clear the virus.

Significance

These studies have the potential to further the understanding of vaccine action and may provide insights that lead to the development of better vaccines that are fast acting, are effective without multiple boosts and can offer protection in extremely short durations before exposure. Such scenarios could be critical in

an ANDV outbreak where human-to-human transmission has been documented, as well as, post exposure treatments that could arise in the field or in laboratory conditions. The ability to administer a vaccination that can protect in the extreme short term and after exposure have proved to be extremely useful. Examples of this can be found in the current Rabies vaccine and the use of the experimental rVSV-ZEBOV vaccine in the recent West African Ebola outbreak where a VSV-based vaccine was successfully used in emergency ring vaccination. (Henao-Restrepo et al. 2016) (Dean et al. 2015). I anticipate that this research will provide useful insight that allows the future development of these types of vaccines and reduce the number of deaths that result from these ever increasing and dangerous zoonotic pathogens as our world becomes less constrained by borders and more mobile.

Future Directions

In conclusion of my research project, I have gathered data that will generate renewed and lasting interest in the phenomenon that allows the ZEBOVgp to protect against unrelated viral diseases when used as a peri-exposure vaccination. In particular it will be interesting what insights will be discovered regarding the mechanism of the immune response as newer more powerful tools become available. For future directions the mechanism of the immune responses responsible for the peri-exposure protection should be defined and evaluated. Unfortunately, many of the advanced tools and reagents are still underdeveloped or unavailable to study this in the hamster model.

Hopefully my study will further the use of the Syrian hamster model in the Hantavirus field and initiate more commercial reagent development. It is thought that the inclusion of the ZEBOVgp in the first cloning position of the rVSV genome is able to induce a rapid innate immune response that enhances the effect of the vaccine. Unfortunately, I was not able to explore the immune response to determine the mechanism of this short-term peri-exposure protection. However, future work towards this goal should be pursued. Defining the immunological mechanism would be an important milestone and would be valuable as it could be applied broadly to direct future vaccine development.

With the recent publication of the hamster genome/transcriptome it could be valuable to explore methods that use deep sequencing to look at changes in transcription profiles in hamsters and used to look at differences in the transcriptomes / transcriptional changes of animals that get different vaccines to identify differential gene expression that could influence the immune responses in the hamster model. This could provide key insights to investigate the idea that ZEBOVgp targets the vaccine vectors to important immune cells strongly enhancing innate and adaptive immune responses and, thus improve protective efficacy which could, in turn, allow for further optimization of the vectors (and maybe unrelated vaccine platforms) to increase the protective efficacy and/or extend the window of time in which the vaccine could be effectively administered.

Second, since the protection studies were performed with only 6 animals per group these experiments should be reproduced using larger group sizes to enhance the confidence in the improvement of the rVSVΔG-ZEBOVgp-ANDVgpc

over the other bivalent and monovalent rVSVs. In addition, the results should be confirmed in a second animal model if one is developed. Preferentially, the second animal model would be a nonhuman primate model to evaluate safety and immunology before advancing to human trials

Overall this study has greatly advanced the understanding that the inclusion of a second glycoprotein from a completely unrelated virus (ZEBOVgp) can stimulate the immune system in such a way that there is an enhancement of the post-exposure protection of hamsters from a ANDV challenge. Finally, it may also be important in answering questions about the overall use of vaccines and other therapeutics that are used in post-exposure protection from other viral illnesses.

References

Dean, Natalie E., Norheim, Gunnstein, Carroll, Miles W., Watson, Conall H., Draguez, Bertrand, Edmunds, W. J., Røttingen, John-Arne, et al. "Efficacy and Effectiveness of an rVSV-Vectored Vaccine Expressing Ebola Surface Glycoprotein: Interim Results from the Guinea Ring Vaccination Cluster-Randomised Trial." Elsevier, http://dx.doi.org/10.7892/boris.71001.

Henao-Restrepo, Ana Maria, Anton Camacho, Ira M. Longini, Conall H. Watson, W. John Edmunds, Matthias Egger, Miles W. Carroll, et al. 2016. "Efficacy and Effectiveness of an rVSV-Vectored Vaccine in Preventing Ebola Virus Disease: Final Results from the Guinea Ring Vaccination, Open-Label, Cluster-Randomised Trial (Ebola Ça Suffit!)." The Lancet. doi:10.1016/S0140-6736(16)32621-6.