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Title of Dissertation: "Evaluation of the Protective Efficacy of Recombinant Vesicular Stomatitis Virus Vectors Against Marburg Hemorrhagic fever in Nonhuman Primate Models"

Name of Candidate: Kathleen Daddario-Dicaprio
Doctor of Philosophy Degree
19 January 2007

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FINAL EXAMINATION FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

Name of Student: Kathleen Daddario-Dicaprio

Date of Examination: 19 January 2007

Time: 12:15

Place: Lecture Room A

DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
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Kathleen Daddario Dicaprio

Kathleen Daddario-Dicaprio
Department of Pathology
Uniformed Services University

Evaluation of the protective efficacy of recombinant vesicular stomatitis virus vectors against Marburg hemorrhagic fever in nonhuman primate models

By

Kathleen Daddario-DiCaprio

Dissertation submitted to the Faculty of the Program in Molecular Pathobiology of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2006.

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Kathleen Daddario-DiCaprio

Department of Pathology

Uniformed Services University of the Health Sciencecs

Abstract

Title of Dissertation: Evaluation of the protective efficacy of recombinant vesicular stomatitis virus vectors against Marburg hemorrhagic fever in nonhuman primate models

Kathleen Daddario-DiCaprio

Doctor of Philosophy, 2006

Dissertation directed by:

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Marburg virus (MARV) causes a severe and often fatal hemorrhagic disease in both humans and nonhuman primates (NHPs) for which there are no proven therapies or vaccines. The need for efficacious interventions is underscored both by the recent MARV outbreak in Angola and by the risks of potential MARV laboratory exposures. Unfortunately, the majority of attempts at developing effective vaccines and therapeutics against MARV HF have been unsuccessful. While several vaccine platforms have demonstrated utility in rodent models, these platforms have been inconsistent in transitioning protective responses into NHP models. In this study, we investigated whether live, attenuated, replication-competent recombinant vesicular stomatitis virus vectors (rVSVs), which express the transmembrane glycoprotein of MARV strain Musoke (rVSV-MARV), could protect NHPs against MARV challenge. This study evaluated the utility of rVSV-MARV as both preventative and postexposure treatment strategies against homologous and heterolgous MARV challenge in NHPs. Results showed that rVSV-MARV provided complete protective responses when used as a preventative vaccine as well as a postexposure treatment and that protection associated with both strategies were composed primarily of MARV-specific antibody responses that constituted only low to moderate induction of neutralizing antibodies. Additional evaluations illustrated the absence of detectable T-lymphocyte responses, thus highlighting the protective role of humoral immunity. Although the protective mechanism of the rVSV-MARV vaccine in NHPs remains to be determined, the study design for evaluating postexposure protection against MARV in NHPs provides a paradigm to outline correlates of immunity for which other interventions could apply. These studies suggest that survival is weighted heavily on antibody responses; however,

the possibility that other mechanisms of immunity are contributing to viral clearance cannot be excluded. Possible mechanisms include direct viral interference, induction of a robust innate response, or a "jump-start" of the adaptive immune response. Accordingly, these results provide critical insight into the nature of effective anti-MARV immunity.

“ Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses”

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I would first off like to thank the Department of Pathology for providing me with the means to get to this point in my scientific career. More specifically I'd like to express my appreciation to my thesis committee. Dr. Kagan: I thank you for my USUHS lab experience and your continued guidance. Dr. Dveksler, Dr. Ottolini, and Dr. Friedman: thank you for your continued support and for always challenging and helping me develop into a better scientist.

I believe that the events that unfold throughout our lives are preplanned and we are left to experience them and make the most of them. Living by this belief, I look back to a day that has changed my life both professionally and personally. On the day that Tom Geisbert spoke to the Department of Pathology in fall of 2003. I knew right then and there that this was something that I wanted to be part of. Tom: I can't tell you how happy I am that I decided to make the most of that experience and gain enough courage to talk to you about joining your lab. I have developed not only as a scientist but as a person, under your watch. Thank you for being not only the essence of mentorship to me, but a father figure during times.

For all of the gratitude and appreciation that I express to Tom, I hold the same for Dr. Lisa Hensley. Lisa: from day one I knew I was joining a team of great minds. At first meeting, I had limited experiences in a laboratory setting and had worked with few scientists. I was amazed at how successfully you could manage several projects. I eventually got used to your fast pace instruction and very quickly realized that inside that head of yours was a brilliant mind. Thank you for your continued guidance, support, and friendship.

They say that behind every great man stands an even greater woman. This may be an old adage, but I strongly believe that this fits here. Joan Geisbert: I can't express to you how grateful I am for all that you have done in making me the scientist that I am today. Simply put, you make work fun. Through months of shadowing you in the BSL-4 lab during necropsies and monkey bleeds, I watched your every move and was amazed at the care and precision with which you execute your everyday tasks. I knew from the first moment that I watched you in action that this was something that I could develop a passion for and what better mentor to have in the lab than you. Thank you for all that you do in this field.

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To my Dad, Mom, and Matt: words can not express how grateful I am for your continued love and support in EVERYTHING I do, whether you agree or disagree. I am who I am today because of you, thank you. To Mike: you are my love and my balance in life. KYEOTP.

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LIST OF ABBREVIATIONS

MARV	Marburg virus
EBOV	Ebola virus
HF	Hemorrhagic fever
VSV	Vesicular stomatitis virus
NHP	Nonhuman primate
rVSV	Recombinant vesicular stomatitis virus
rVSV-MARV	Recombinant vesicular stomatitis virus expressing MARV strain Musoke glycoprotein
GP	Glycoprotein
rVSV-EBOV	Recombinant vesicular stomatitis virus expressing EBOV species Zaire glycoprotein
BSL-4	Biosafety level 4 containment
ICEBOV	Ivory Coast species of Ebola virus
REBOV	Reston species of Ebola virus
SEBOV	Sudan species of Ebola virus
ZEBOV	Zaire species of Ebola virus
NP	Nucleoprotein
VP30	Viral protein 30
VP35	Viral protein 35
L	L protein, RNA-dependent RNA polymerase
VP24	Viral protein 24
VP40	Viral matrix protein 40

PT	Prothrombin time
APTT	Activated partial thromboplastin time
RT-PCR	Reverse transcriptase-polymerase chain reaction
ELISA	Enzyme-linked immunosorbant assay
DIC	Disseminated intravascular coagulation
VLP	Virus-like particles
VEEV	Venezuelan equine encephalitis virus
Ad5	Adenovirus serotype 5
Ig	Immunoglobulin
MARV-Musoke	MARV strain Musoke
MARV-Angola	MARV strain Angola
MARV-Ravn	MARV strain Ravn
MARV-Ci67	MARV strain Ci67
SIV	Simian immunodeficiency virus
ADCC	Antibody-dependent cell-mediated cytotoxicity
CDC	Complement-dependent cytotoxicity
VSV G	Vesicular stomatitis virus glycoprotein
RSV	Respiratory syncytial virus
PBMC	Peripheral blood mononuclear cells
APC	Antigen presenting cell
cADVax	Complex adenovirus vector
TI	T-lymphocyte independent
IFN	Interferon

IL	Interleukin
MCP	Monocyte chemotactic protein
PFU	Plaque forming unit
TNF	Tumor necrosis factor

I. Background and Significance

Significance

Marburg (MARV) and Ebola (EBOV) viruses cause severe and often fatal hemorrhagic diseases for which there are currently no vaccines or therapies approved for human use. The reported potential of filoviruses as biological weapons (Alibek, Handelmann 1999; Miller J 2001; Borio, Inglesby et al. 2002) and the recent attention drawn to outbreaks of emerging and re-emerging viruses, such as the 2004-2005 epidemic of MARV hemorrhagic fever (HF) in Angola (WHO 2005; Towner, Khristova et al. 2006; CDC 2005), has significantly increased public recognition of these deadly pathogens.

The development of effective treatments and therapies has been an ongoing challenge since these viruses were first discovered. The requirement for biosafety level (BSL)-4 containment has served as a major impediment towards the development of therapeutics. To date, the only available form of treatment for filoviral HF is intensive supportive care. With continual developments of biocontainment laboratories nationwide, additional and more promising means of treatment and prevention are needed for laboratory workers. Furthermore, the recent MARV outbreak in Angola, with case fatality rates approaching 90 percent, desperately calls attention to the fact that there is a critical and pressing need for effective countermeasures against the filoviruses.

This thesis entails evaluating in nonhuman (NHP) models a live, attenuated, recombinant vesicular stomatitis virus (rVSV) vector platform expressing the transmembrane glycoprotein (GP) gene of MARV as both a preventative vaccine and

postexposure treatment strategy against MARV HF. The thesis research demonstrated is significant because countermeasures against MARV HF are desperately needed both for aid in outbreaks and for accidental laboratory exposures. Furthermore, elucidation of the immunological mechanisms associated with rVSV therapeutic intervention may define potential correlates of immunity and identify critical pathogenic processes for the rational design and development of additional countermeasures.

Background

History

Filoviral disease introduced itself in early August 1967 when simultaneous outbreaks of hemorrhagic fever (HF) occurred in both Marburg and Frankfurt, Germany and later in Belgrade, Yugoslavia. The causative agent was named Marburg virus after the town where illness was initially observed. This outbreak was linked to exposure of laboratory workers and scientists to infected African green monkeys (*Cercopithecus aethiops*) imported from the Lake Kyoga region of Uganda (WHO 1967). In total there were 32 patients affected and a case fatality rate of 22 percent (Martini, Knauff et al. 1968). This newly emergent virus would later be classified as the first recognized member of the virus family *Filoviridae* (Kiley, Eddy et al. 1982). The second and only other member of the *Filoviridae* family was discovered in the Democratic Republic of the Congo (formally known as Zaire) (WHO 1978a) and Sudan in 1976 (WHO 1978b; Bowen, Platt et al. 1980). This virus was named Ebola virus (EBOV) after the river near the place of its first outbreak in the former Zaire. EBOV is perhaps the more notorious

member of the *Filoviridae* family as it has been given considerable attention by the popular press.

Since the initial outbreaks in 1967, MARV has only surfaced in sporadic episodes in South Africa (Gear, Cassel et al. 1975), Kenya (Smith, Johnson et al. 1982; Johnson, Johnson et al. 1996), Democratic Republic of the Congo (WHO 1999), and Angola (WHO 2005). Although it was confirmed that MARV caused each of these outbreaks, several different strains of MARV have been identified. These occurrences between 1967 and 1987 suggested that MARV may be less pathogenic than EBOV. However, a large outbreak of MARV HF in northeastern Democratic Republic of the Congo between October of 1998 and September of 2000 offered the first hint that MARV may be just as lethal in primates as its more famous cousin, EBOV. In this outbreak there were 154 cases of which 48 were laboratory confirmed and 106 were suspected. Among the 48 laboratory confirmed cases, the case fatality rate was 56 percent (Borchert, Boelaert et al. 2000; Borchert, Mulangu et al. 2005) while the mortality rate was about 83 percent when all cases, laboratory confirmed and suspected cases, were considered (Bausch, Nichol et al. 2006). Whereas the previous episodes of MARV HF each involved a single distinct strain of MARV, this outbreak involved at least nine genetically distinct strains of MARV. The second, and most recent outbreak, occurred in Angola during 2004-2005. This Angolan MARV outbreak is not only the largest outbreak to date, but is also associated with the highest case fatality rate of around 90 percent (WHO 2005; Towner, Khristova et al. 2006).

Up until the 1998 MARV outbreak in the Democratic Republic of the Congo, case fatality rates caused by the several strains of MARV identified led to confirmed case

fatality rates ranging from about 22 percent to slightly greater than 50 percent (Martini G 1971; Gear, Cassel et al. 1975; Colebunders, Sleurs et al. 2004). However, the recent Angolan outbreak was caused by a new strain of MARV responsible for an even more significant mortality. An initial report by the World Health Organization noted that the epidemic killed 329 people of the 374 that were infected. The outbreak was recently declared over, and these numbers were revised, with the updated report noting 227 deaths among the 252 reported cases (ISID, 2005). The reasons for the increased lethality of this new strain of MARV are presently unknown, but are of significant concern.

Nomenclature, taxonomy, and structure

Marburgvirus (MARV) and *Ebolavirus* (EBOV) comprise the two genera that make up the virus family *Filoviridae* in the phylogenetic order *Mononegavirales* (Feldmann, Geisbert 2004). Within the MARV genus there is a single species, *Lake Victoria marburgvirus* that contains at least nine strains. The genus *Ebolavirus* is slightly more complex with four recognized species: *Ivory Coast ebolavirus* (ICEBOV), Reston *ebolavirus* (REBOV), Sudan *ebolavirus* (SEBOV), and the Zaire *ebolavirus* (ZEBOV). A detailed outline of the current taxonomy is listed in Table 1.

The genome of all filoviruses consists of a nonsegmented, single negative stranded linear RNA molecule, which is organized as shown in Figure 1. The average size of a filoviral genome is 19 kb with MARV being slightly larger than that of EBOV (MARV 19.1 kb, EBOV 18.9 kb). Briefly, the MARV genome is transcribed into seven individual mRNAs that encode seven structural proteins. Four proteins, nucleoprotein (NP), viral protein 30 (VP30), viral protein 35 (VP35), and the catalytic subunit L of the

RNA-dependent RNA polymerase (L), are associated with the viral genomic RNA in the ribonucleoprotein complex. The three remaining structural proteins are membrane associated; viral protein 24 (VP24) and viral protein 40 (VP40) are matrix proteins, while the glycoprotein (GP) is a membrane glycoprotein that is located at the surface of MARV-infected cells and forms the virion spikes. EBOV also produces GP in a secreted form during viral infection as a result of transcriptional editing .

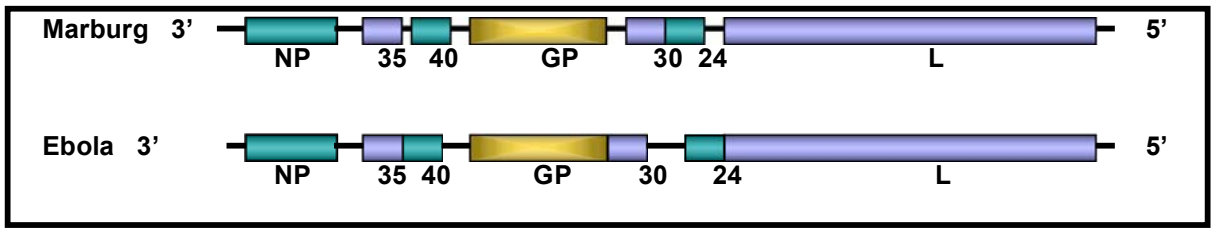

Figure 1. Organization of filoviral genomes in the genera *marburgvirus* (MARV) and *ebolavirus* (EBOV). The linear gene order is conserved and shown here in antigenomic orientation as nucleoprotein (NP), viral protein 35(35), viral protein 40 (40), glycoprotein (GP), viral protein 30 (30), viral protein 24 (24), and the RNA-dependent-RNA polymerase (L).

Table 1. Taxonomy for the virus family *Filoviridae.*

Genus	Species	Strain	Location, Year Isolated
Marburgvirus	*Lake Victoria marburgvirus*		
		Ratayczak	West Germany, 1967
		Ci67	West Germany, 1967
		Popp	West Germany, 1967
		Voege	Yugoslavia, 1967
		Ozolin	Zimbabwe, 1975
		Musoke	Kenya, 1980
		Ravn	Kenya, 1987
		Angola	Angola, 2005
Ebolavirus	*Ivory Coast ebolavirus*	Ivory Coast	Tai Forest, 1994
	Reston ebolavirus	Reston	USA, 1989
		Phillipines	Phillipines, 1989
	Sudan ebolavirus	Boniface	Sudan, 1976
		Gulu	Uganda, 2000
	Zaire ebolavirus	Mayinga	Zaire, 1976
		Zaire	Zaire, 1976
		Kikwit	DRC, 1995

There are a number of different strains of MARV that make up the *Lake Victoria marburgvirus* species. These MARV strains include the Ci67 (Siegert, Shu et al. 1968) and the Popp strain (Bukreyev, Volchkov et al. 1995), two different strains isolated from the first MARV outbreak in the 1967 episodes in Marburg, Germany; the Ozolin strain from a case in 1975 in South Africa (Gear, Cassel et al. 1975); the Musoke strain from a 1980 case in Kenya (Smith, Johnson et al. 1982); the Ravn strain from the 1987 case in Kenya (Johnson, Johnson et al. 1996); and the recently isolated Angola strain from the latest outbreak in 2004-2005 (CDC 2005). Comparative analyses of the GP and VP35 genes of these MARV strains showed that there are two distinct lineages within the single species. The original MARV isolates, Popp and Ci67, the Ozolin strain, the Musoke strain, and the Angola strain comprise one lineage (Towner, Khristova et al. 2006). The Ravn strain represents a second genetic lineage within the species (21-23% amino acid difference) (Sanchez, Trappier et al. 1998). However, the two lineages are not separate species to the same extent as the difference that separates the EBOV species, where there is 37-41% difference in amino acid sequences among the four species.

Biohazard classification

All members of the family *Filoviradae* are classified as "Risk Group 4" agents by the American Biological Safety Association (WHO, www.absa.org) based on their high mortality rates, person-to-person transmission, potential aerosol infectivity, and absence of proven vaccines and chemotherapy (Sanchez A 2001). Due to their high risk, work with infectious filoviruses must be performed in a certified BSL-4 laboratory. A detailed description of biosafety aspects and work in a BSL-4 laboratory can be found in

"Biosafety in Microbiological and Biomedical Laboratories" available through the Centers for Disease Control and Prevention (http://www.cdc.gov/OD/OHS/biosfty/bmbl4/bmbl4toc.htm).

Transmission and natural reservoir

Since the initial outbreak in 1967, filoviruses have been primarily limited to regions of Central Africa having only surfaced sporadically and unpredictably (Gear, Cassel et al. 1975; WHO 1978a; WHO 1978b; Smith, Johnson et al. 1982; Johnson, Johnson et al. 1996; Georges, Renaut et al. 1999; Feldmann H 2006). Very little is known about the natural history of filoviruses. Despite attempts to identify an animal reservoir and/or anthropod vectors, the natural reservoir of these presumed zoonotic pathogens remains unknown (Leirs, Kreb et al. 1999). Bats have been speculated to play a role in the maintenance of the virus in nature (Leroy, Kumulungui et al. 2005); however, at present no filovirus has been isolated from a bat.

Transmission of filoviruses between humans is poorly understood. Person to person transmission has been documented among those caring for infected individuals or among those preparing the dead for burial (Smith, Johnson et al. 1982). It is likely that these transmissions occur as a result of close contact to infected bodily fluids. In addition to nosocomial transmission, the reuse of unsterilized needles and syringes has been a significant contributor to secondary transmission (WHO 1978a; WHO 1978b) and highlights how medical settings where hygiene practices are poorly understood or ignored can provide an environment for devastating and rapid spread of filoviral disease. Also, other routes of transmission, such as aerosol, oral, and conjunctival, have been

documented in primates under experimental conditions (Jaax, Davis et al. 1996; Sergeev, Lub et al. 1995). In the field, establishment of strict quarantine measures preventing further virus transmission are still the only way to fight the infections.

Human clinical presentation and diagnosis

The incubation period for filoviral HF is thought to range from four to ten days and the collection of clinical manifestations varies in severity depending on viral virulence, routes of exposure, dose of infection, and host factors (Geisbert, Jahrling 2004). Patients with filoviral HF usually encounter an abrupt onset of nonspecific symptoms such as fever, myalgia (muscle pain), and chills (Martini G 1971; Sanchez A 2001). As disease progresses, multisystemic involvement has been noted and includes systemic, gastrointestinal (anorexia, nausea, vomiting, abdominal pain, diarrhea), respiratory (chest pain, shortness of breath, cough), vascular (conjunctival, hypotension, edema), and neurologic (headache, confusion, coma) manifestations (Sanchez A 2001). Commonly severe hemorrhagic manifestations develop and are manifested as petechiae, ecchymoses, uncontrolled bleeding from venipunture sites, and mucosal hemorrhages (Sanchez A 2001). Note however, that the loss of blood is not sufficient enough to account for death. A macropapular rash often develops around day five that is often classified as a filoviral manifestation; often used as a valuable differential diagnostic feature (Sanchez A 2001). Terminal stages of disease are associated with convulsions, shock, and diffuse coagulopathy (often manifested as disseminated intravascular coagulation, DIC) (Martini G 1971; Geisbert, Jahrling 2004). Clinical laboratory findings include thrombocytopenia, leukopenia, neutrophilia, and lymphopenia. Liver

enzymes such as aspartate transaminase are elevated and coagulation assays such as prothrombin time (PT) and activated partial thromboplastin time (APTT) are often prolonged. In addition, patients demonstrating DIC show elevated fibrin degradation products and decreased fibrinogen.

A differential diagnosis usually begins with that of acute febrile illness and can become more definitive with a patient's history of travel to an endemic region. More definitive diagnoses of filoviral HF entails a combination of manifestations to include an acute illness for up to three weeks in the absence of either associated trauma, another focal disease process, or a response to antibiotics or antimalarial agents (Bausch, Nichol et al. 2006). A positive diagnosis requires presentation of at least three of the aforementioned multisystemic signs and symptoms in addition to a positive laboratory test for filovirus infection. Laboratory tests used to detect filovirus infection include RT-PCR, virus isolation, ELISA antigen detection, or immunohistochemical analysis (Bausch, Nichol et al. 2006; CDC 1998).

Animal models of Marburg hemorrhagic fever

The use of animal models has been invaluable for studying the pathogenesis of numerous infectious diseases as well as for testing the efficacy of experimental prophylactic and therapeutic vaccine and/or drug regimens. Animal models that adequately reproduce human MARV HF are needed to gain further insight into the pathogenesis of these diseases and to test the efficacy of promising interventions. Guinea pigs, mice, hamsters, rabbits, sheep, and NHPs have been employed to study MARV HF and each has demonstrated susceptibility to virus infection (Pokhodiaev,

Gonchar et al. 1991; Ryabchikova, Kolesnikova et al. 1996; Lee, Groebner et al. 2006; Jones, Feldmann et al. 2005). However, NHPs display disease characteristics such as clinical disease, and related pathology that are most reminiscent of those described in human MARV HF. Although rodents are useful as a first screen for evaluating antiviral drugs and vaccine strategies, and genetically engineered mice can help in understanding specific host-pathogen interactions; efficacy of vaccine and/or therapeutic interventions do not always transition similarly into the primate models. Use of filoviruses in rodent models requires adaptation of virus by serial passage. Furthermore, neither mice nor guinea pigs exhibit the hemorrhagic manifestations that characterize NHP and human MARV infections. Also associated with human and NHP MARV infections includes bystander lymphocyte apoptosis (discussed below), which is was neither a prominent feature of MARV infections in mice nor guinea pigs (Geisbert, Hensley et al. 2000).

Nonhuman model of Marburg hemorrhagic fever

In evaluating MARV HF, the NHP model demonstrates the most resemblance to that of human MARV disease. However, despite this resemblance, careful consideration must be given to the choice of species, sex, and age of the NHP model. Among the several NHP species that have been employed to model MARV HF, cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) are predominately used. Notably, MARV disease demonstrates very similar courses and characteristics between the two models. The only observable difference to date is that disease course in rhesus macaques is one to two days longer than that in cynomolgus macaques . The current standard for choosing a rhesus or cynomolgus macaque model

relies heavily on the study objectives and historical data. The majority of studies employing a NHP model to evaluate vaccine platforms have relied heavy on the cynomolgus macaque model. This is mostly because historical data involving classical vaccine approaches used this model and the consistency of using the same model allows for more insight and integration of data for studying vaccine platforms. In contrast, studies evaluating therapeutics predominately use the rhesus macaque model. Uniformly, it has been accepted that studies evaluating therapeutics employ a rhesus macaque model because many pharmaceutical companies or corporations use rhesus macaques for clinical drug toxicity studies. It is therefore, beneficial to carry data over using the same animal model.

Aside from genetic variation, the nature of the route of infection, challenge dose, and the nature of the challenge dose itself is also carefully considered. The design of our NHP models comprises MARV isolates that are of low passage and that have been shown to be free of endotoxin. The MARV challenge dose selected is 1000 plaque forming units (pfu) and given by intramuscular injection, that reflects a likely laboratory exposure and which is uniformly lethal.

Marburg pathogenesis and cellular tropism

Filovirus infection often results in fulminant hemorrhagic disease in humans and NHPs (Schnittler, Feldmann 1999; Geisbert, Jahrling 2004). Indeed, among all viral HFs those caused by filoviruses are regarded as the most severe, typically associated with coagulation disorders and multisystemic disease. The factors contributing to the development of lesions in EBOV and MARV HF are unknown and, at present, there is no

evidence that the infections differ in their underlying mechanisms. Futhermore, the severity of these lesions are commonly not enough to account for the shock-like death of the host and, therefore, suggests that immunomodulators and inflammatory mediators may have an important role in disease. Filoviral infections of human and NHPs are associated with a lack of inflammation in infected tissues, marked lymphopenia, and severe lymphoid degeneration (Sanchez A 2001; Geisbert, Jahrling 2004). In addition, a deregulated and overwhelming inflammatory cytokine and chemokine response is observed (Geisbert, Hensley et al. 2003; Hensley, Young et al. 2002; Villinger, Rollin et al. 1999; Baize, Leroy et al. 2002).

Once the host is infected, filoviral replication occurs in the cytoplasm of target cells. Morphological studies of EBOV- and MARV-infected primate tissues show that cells of the mononuclear phagocyte system are primary sites of replication (Jaax, Davis et al. 1996; Zaki and Goldsmith 1999; Geisbert, Pushko et al. 2002; Davis, Anderson et al. 1997; Geisbert, Jahrling et al. 1992; Geisbert, Hensley et al. 2003). In addition, cultured human monocytes/ macrophages are highly susceptible to filoviral infections resulting in cell death and massive production of infectious particles (Hensley, Young et al. 2002; Stroher, West et al. 2001). The primary cellular tropism of filoviruses appears to be monocytes/ macrophages as well as dendritic cells (Geisbert, Hensley et al. 2003). The primary organ tropism (lymphoid tissues, liver) may be explained by the direct access of virions to sessile macrophages without penetration of the cell or the tissue barrier (Schnittler, Feldmann 1999). It is likely that extravasation of infected circulating monocytes/ macrophage is the mechanism for spread of the virus. Endothelial cells, various epithelial cells (including hepatocxytes and adrenal cortical cells), fibroblasts,

and fibroblastic reticular cells are also targeted by filoviruses (Jaax, Davis et al. 1996; Davis, Anderson et al. 1997; Zaki, Goldsmith 1999; Geisbert, Hensley et al. 2003; Geisbert, Jahrling et al. 1992; Geisbert, Jaax 1998; Geisbert, Young et al. 2003); this pantropism of filoviral infections is typically associated with the late stages of disease and remains unexplained mechanistically.

During MARV HF the development of immunosuppression is evidenced by the characteristic lymphoid degeneration and lymphopenia. Lymphopenia is a consistent finding during MARV disease despite the non-permissiveness of lymphocytes to support MARV replication both *in vitro* and *in vivo*. The progressive lymphopenia and lymphoid degeneration may, in part, be explained by evidence of large scale lymphocyte apoptosis in humans and NHPS (Geisbert, Jahrling 2004). To date, the exact mechanism for the loss of bystander lymphocytes through apoptosis is unknown. Moreover, the ability of MARV to infect mononuclear phagocytic cells and antigen presenting cells suggests that the virus may disrupt the host's ability to mount an effective adaptive response (Bosio, Aman et al. 2003). Indeed, a number of viruses are thought to induce immunosuppression by infecting and impairing the function of dendritic cells, thereby enhancing their chance to escape immune surveillance (Kruse, Rosorius et al. 2000; Raftery, Schwab et al. 2001).

The extensive loss of lymphocytes may impair the host's ability to mount an appropriate adaptive immune response. The role of cell-mediated responses via T-lymphocytes and humoral (antibody) responses via B-lymphocytes in protection against MARV infection is not well understood. Several studies in mouse, guinea pig, and NHP models have evaluated the protective role of both T-lymphocyte and antibody responses

induced by vaccination and postexposure treatment platforms (Warfield, Swenson et al. 2004; Riemenschneider, Garrison et al. 2003; Jones, Feldmann et al. 2005; Wang, Schmaljohn et al. 2006; Lee, Groebner et al. 2006). Among the successful vaccine platforms evaluated in rodent models, induction of both T-lymphocyte and antibody responses were evident and suggest an important role for adaptive responses in protection against MARV HF. However, in successful platforms evaluated in the NHPs models, T-lymphocyte responses were absent and only antibody responses were induced (Hevey, Negley et al. 1998; Jones, Feldmann et al. 2005). Based on these studies the exact components of the adaptive immune responses that are necessary for protection against MARV HF is unclear and may vary among animal models.

MARV HF is also associated with a deregulated and overwhelming cytokine response that, in part, could influence disease pathogenesis. Indeed, the ability of viruses to influence such deregulation by increasing levels of interferon (IFN)-α and the overproduction of reactive oxygen species has been demonstrated and has been associated with bystander lymphocyte apoptosis (Kayagaki, Yamaguchi et al. 1999; Takabayashi, Kawai et al. 2000). The symptoms of MARV HF are comparable to those of cytokine-induced systemic inflammatory response syndrome that is a surplus reaction of the host triggered by pathogens or their products, and also include development of coagulation abnormalities (Levi, de Jonge et al. 2001). The inflammatory and procoagulant host responses to infection are inextricably intertwined. Inflammatory cytokines (e.g., tumor necrosis factor (TNF) -α, interleukin (IL) -1β, and IL-6) and cytokine receptors are capable of activating coagulation and inhibiting fibrinolysis, whereas the procoagulant thrombin is capable of stimulating multiple inflammatory

pathways (Hirasawa, Oda et al. 2004; Shinozawa, Xie et al. 2004; Grignani and Maiolo 2000; Levi, de Jonge et al. 2001; Boontham, Chandran et al. 2003).

Therapy

Currently the only form of treatment for filoviral HF is intensive supportive care. In a developed hospital setting, supportive management of infected patients involves particular attention to maintenance of hydration, circulatory volume, blood pressure, and the provision of supplemental oxygen (Casillas, Nyamathi et al. 2003; Stille, Bohle et al. 1968). During various outbreaks a number of treatments were administered in an attempt to reduce severity of the disease. For example, the beneficial use of convalescence sera was reported during a MARV outbreak in Frankfurt, Germany (Slenczka W 1999). Since the coagulation cascade is deregulated during MARV HF (leading to DIC), treatments to alleviate microthrombi formation have been attempted. The use of heparin to counterbalance coagulopathies was associated with successful treatment of two MARV-infected patients (Peters, Khan 1999). Guinea pig and NHP models have been developed for MARV HF (Jones, Feldmann et al. 2005; Ignat'ev, Strel'tsova et al. 1994; Sergeev, Lub et al. 1995; Ignat'ev, Strel'tsova et al. 1996; Agafonova, Viazunov et al. 1997; Hevey, Negley et al. 1998; Ignatyev, Agafonov et al. 1996; Kolokol'tsov, Davidovich et al. 2001) and several immunomodulatory drugs including desferal, ridostin, and polyribonate have been evaluated in these models demonstrating partial protection and slight increases in mean time to death (Ignat'ev, Strel'tsova et al. 1996; Ignat'ev; Sergeev, Lub et al. 1995). Despite the ability of several of these drugs to induce protective responses in guinea pigs, the efficacy and action of these immunomodulators in NHPs

has yet to be determined. In addition, evaluation of drugs such as IFN and ribavirin showed that neither approach had therapeutic potential against MARV infection in NHP and guinea pig models, respectively (Ignat'ev, Strel'tsova et al. 1996; Sergeev, Lub et al. 1995; Kolokol'tsov, Davidovich et al. 2001).

Vaccines

Vaccine developments against MARV HF have encompassed a variety of approaches in both rodent and NHP models. Classical approaches have exploited either inactivated whole virus or attenuated virus strains. Also, a variety of approaches including DNA-based vaccines (Riemenschneider, Garrison et al. 2003), baculovirus recombinants (Hevey, Negley et al. 1998; Hevey, Negley et al. 1997), virus-like particles (VLPs) (Warfield, Swenson et al. 2004), inactivated whole virions (Hevey, Negley et al. 1997), replication-defective adenovirus-based (Wang, Schmaljohn et al. 2006), and recombinant vesicular stomatitis virus (rVSV) vectors (Garbutt, Liebscher et al. 2004) have been used as mechanisms to deliver the GP, VP40, and NP. The efficacy and specific details of these intervention strategies have been recently reviewed in detail (Feldmann, Jones et al. 2003; Hart M 2003; Geisbert, Jahrling 2003). GP, VP40, and NP proteins have been predominantly utilized as antigen targets for vaccine development as VP40 and NP are abundantly expressed inside the viral particle and GP is the only known viral protein exposed on the surface of the viral particle. One important point to note is that results using small animal models are not always predictive of outcome in NHPs (Geisbert, Pushko et al. 2002).

In developing vaccines against any virus, there are always concerns about broad protection and the ability to protect against different strains or isolates of the same virus. Of particular concern regarding cross-protection among the MARV strains are results reported using a platform based on Venezuelan equine encephalitis virus (VEEV) replicons. This study by Hevey *et al.* showed that cynomolgus monkeys vaccinated with VEEV expressing MARV GP or VEEV expressing MARV NP replicons based on strain Musoke (MARV-Musoke) were protected against lethal homologous challenge (Hevey, Negley et al. 1998), but not against a challenge with the Ravn strain of MARV (MARV-Ravn (Hevey, Pushko et al. 2001). This result raises the concern that the 21-23% difference in amino acids between the two MARV lineages may be significant enough to affect the ability of a candidate vaccine to confer cross-protection against the different MARV strains. Indeed, in a recent study by Wang *et al.* a multivalent vaccine based on a replication-defective adenovirus backbone expressing antigenic proteins from the different strains of MARV induced complete protective responses in guinea pigs against the Musoke, Ravn, and Ci67 strains of MARV (Wang, Schmaljohn et al. 2006). Use of adenoviral vector platforms (Ad5) has been, however, controversial due to the significant percentage (approximately 40-60 percent) of U.S. population that demonstrates preexisiting immunity to Ad5 and therefore raises questions regarding its vaccine efficiency (Brandt, Kim et al. 1969; Schulick, Vassalli et al. 1997; Piedra, Poveda et al. 1998). It has therefore been suggested that a more efficient vaccine should utilize a viral backbone that is less likely to have problems with preexisting immunity.

Vaccines as postexposure treatments

The capacity of vaccines as postexposure treatments is demonstrated by the management of a number of viral infections including rabies (Rupprecht, Hanlon et al. 2004), hepatitis B (Yu, Cheung et al. 2004), smallpox (Massoudi, Barker et al. 2003; Mortimer P 2003), and herpes simplex virus 2 (Stanberry L 2004). For example, rabies is responsible for a large extent of morbidity and mortality in humans worldwide. The only means to escape rabies death is to receive the postexposure prophylaxis with the rabies vaccine as soon after infection as possible. The only form of protection against rabies infection is through preventative measures; it is preventable but incurable. Although definitive correlates of protection associated with both preventative and postexposure vaccination is unclear, it has become widely accepted that development of antibody and neutralizing antibody early enough in the disease course is favorable (Rupprecht, Gibbons 2004). The importance of humoral responses in protection against rabies is highlighted by current postexposure treatment standards, which outline the use of either two intradermal regimens with the rabies vaccine in concert with immunoglobulin (Ig) injection at potential inoculation sites (Wilde, Khawplod et al. 2005). Comparatively, it is suggested that postexposure prophylaxis for hepatitis B infection entails passive immunization with hepatitis B Ig (HBIG) in concert with the hepatitis B vaccine (Yu, Cheung et al. 2004; Harris, Daly-Gawenda et al. 1991). The management of smallpox infection is, however, less defined as a consequence of its global eradication in the late 1980's. The increased concern about a potential bioterrorism attack has initiated studies to evaluate the efficacy of smallpox vaccination as a managerial strategy for postexposure prophylaxis. Similar to that outlined for rabies and hepatitis, the mechanisms associated with protective immune response against smallpox is unclear. Indeed, data available

regarding the efficacy of the smallpox vaccine as a postexposure therapy illustrates its partial protective effects up to four days following exposure (Massoudi, Barker et al. 2003; Mortimer P 2003; Kennedy, Frey et al. 2004).

Recombinant vesicular stomatitis virus system

Vesicular stomatitis (VS) is a disease that predominately affects livestock and is caused by the vesicular stomatitis virus (VSV). There are several serotypes of VSV, two of which (Indiana and New Jersey) infect domestic animals and man. Clinical signs associated with VSV in livestock strongly resemble that of foot-and–mouth disease and are characterized by extensive vesicular and erosive lesions on the dorsal surface of the tongue, frequently accompanied by vesicles in the gums, lips, coronary band, and teats (Letchworth, Rodriguez et al. 1999). In humans, infection with VSV produces uniformly non-fatal influenza-like illness. Human infection with VSV appears to be relatively common in endemic areas of Central America and is usually related to direct contact with affected animals (Johnson, Tesh et al. 1969; Reif, Webb et al. 1987; Tesh, Peralta et al. 1969).

VSV is a nonsegmented, negative-stranded RNA virus that belongs to the family *Rhabdoviridae*, genus *Vesiculovirus (*Letchworth, Rodriguez et al. 1999; Wagner R 2001). Morphologically, VSV has a bullet-shaped appearance. Infection with VSV results in a strong IFN response and the production of nitric oxide, both of which have been demonstrated to play major protective roles in defending against infection (Bi, Reiss 1995; Komatsu, Bi et al. 1996; Gresser, Tovey et al. 1975; Gresser, Tovey et al. 1976; Steinhoff, Muller et al. 1995).

As the prototype virus of the *Rhabdoviridae* family, VSV has been extensively studied in the laboratory serving as an effective tool in both cellular and molecular biology due to its simple structure and rapid-high titer growth in mammalian and many other cell types. After the establishment of the VSV reverse genetics system, the utility of VSV reached new levels, as this system provided a way to genetically manipulate VSV to express a variety of foreign genes (Lawson, Stillman et al. 1995; Schnell, Buonocore et al. 1996). The VSV genome is highly flexible and can tolerate insertion of foreign transcription units and genes. Indeed, it has been shown that the VSV genome can functionally replace its single surface glycoprotein gene, which is responsible for attachment and fusion to the host cell, with a foreign transmembrane glycoprotein (Kretzschmar, Buonocore, et al. 1997). These characteristics make this system suitable for studying the role of foreign soluble and transmembrane GP in the context of infectious viral particles.

The infectious clone system for VSV provides the capacity to construct infectious VSV viral particles that express foreign GP in place of VSV glycoprotein (G). Garbutt, *et al.* , utilized this system to construct replication-competent rVSV vectors that express either the GP of MARV (rVSV-MARV) or the GP of EBOV (rVSV-EBOV) (Garbutt, Liebscher et al. 2004). The GPs of MARV and EBOV were cloned into the plasmid, VSVXN2ΔG, which contains the VSV genome lacking the VSV G gene. The four VSV genes and either MARV or EBOV GP genes are flanked by the bacteriophage T7 promoter, the VSV leader, the hepatitis delta virus ribozyme, and the T7 terminator sequence. Infectious virus was generated by co-transfection of BHK-T7 cells with VSVXNΔG expressing either EBOV or MARV GP and plasmids expressing the VSV

nucleoprotein, phosphoprotein, and polymerase. Transcription of all plasmids is under the control of the bacteriophage T7 RNA promoter. Supernatants from transfected cells were blind passaged onto Vero E6 cells and rescued virus was passaged on Vero E6 cells to obtain a viral stock. A schematic drawing of the infectious clone system utilized to construct rVSV viral particles expressing the MARV and EBOV GP is represented in Figure 2.

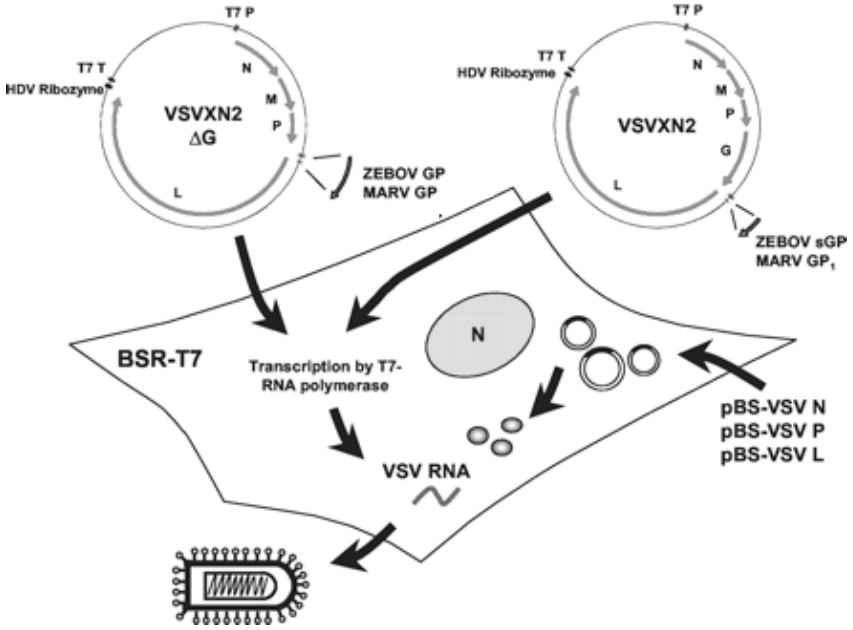

Figure 2. Schematic drawing of the infectious clone system for vesticular stomatitis vius (VSV), Indiana subtype (Garbutt, Liebscher et al. 2004).

II. Objectives

Marburg virus (MARV) causes a severe and often fatal hemorrhagic disease in both humans and NHPs for which there are no proven therapies or vaccines. The need for efficacious interventions is underscored both by the recent MARV outbreak in Angola and by the risks of potential MARV laboratory exposures. Unfortunately, the majority of attempts at developing effective vaccines and therapeutics against MARV HF have been unsuccessful. While several vaccine platforms have demonstrated utility in rodent models, these platforms have been inconsistent in transitioning protective responses into the more robust NHP models. The utility of a replication deficient adenovirus vector has been controversial as a large percentage of the population demonstrates immunity against adenovirus strains and suggests that such a preexisting immunity may interfere with filovirus-targeted protective immune responses. It has been implied, therefore, that a more useful vector platform should entail a viral backbone for which there is less likelihood of preexisting immune responses in populations. Another considered virus vector system, VSV, illustrates such characteristics and presents as a candidate vaccine platform. As a pathogen commonly associated with disease in livestock and very infrequently causing disease in humans outside of endemic regions, VSV demonstrates candidacy as a virus for which preexisting immunity is unlikely in the U.S. population. In addition, a reverse genetics systems for VSV (Indiana strain) has been developed that allows insertion of foreign genes into its genome. Indeed, rVSV platforms that are live, attenuated, replication competent and express the GP genes of either the Zaire species of EBOV or the Musoke-MARV have been established. Therefore, the first project outlined in this thesis was to determine whether the live-attenuated replication competent rVSV

vector expressing the transmembrane GP of MARV-Musoke could protect against a high dose lethal MARV-Musoke challenge in a cynomolgus macaque model. Also, an important goal of this study, was to determine what correlates of immunity are associated with the rVSV platform *in vivo*.

The capacity of vaccines to offer broad cross-reactive immunity and protection are desirable. The identification of several MARV strains that demonstrate differences in disease course and lethality highlights the need for a single antigenically matched vaccine platform that can offer cross-protective responses against these different strains. The second project outlined in this thesis was to determine in a cynomolgus macaque model whether rVSV-MARV, which expresses the GP of the Musoke strain, could induce protective response against heterologous MARV strains.

The use of vaccines as postexposure treatment strategies is used in the management of several viral diseases. Therefore, the third project outlined in this thesis was to determine whether rVSV-MARV could induce protective responses as a postexposure treatment in a rhesus macaque model against MARV HF. The importance of this project is twofold; 1) a postexposure treatment strategy is desperately needed not only to aid to outbreaks but also to manage laboratory-acquired exposures and 2) development of a postexposure treatment strategy will provide a model for elucidating protective correlates of immunity and provide a novel platform for identifying mechanisms of MARV pathogenesis in a NHP model.

The overall hypothesis of this thesis is that rVSV-MARV holds utility as both a preventative and postexposure treatment strategy and that use of rVSV-MARV will provide a potential model for not only studying correlates of immunity associated with

protection against MARV HF but also facilitating the elucidation of the mechanisms of MARV pathogenesis.

III. Live attenuated recombinant vaccine protects non-human primates against either Ebola virus or Marburg virus (*Nature Medicine* July 2005 11(7):786-790)

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Abstract

Vaccines and therapies are urgently needed to address public health needs on emerging pathogens and biological threat agents such as the filoviruses, Ebola virus (EBOV) and Marburg virus (MARV). Here, we developed replication-competent vaccines against EBOV and MARV based on attenuated recombinant vesicular stomatitis virus vectors expressing either the EBOV glycoprotein or MARV glycoprotein. A single intramuscular injection of the EBOV or MARV vaccine elicited completely protective immune responses in non-human primates against lethal EBOV or MARV challenges (n = 4 for each). Importantly, vaccine vector shedding was not detectable in the monkeys and none of the animals developed fever or other symptoms of illness associated with vaccination. The EBOV vaccine induced humoral and apparent cellular immune responses in all vaccinated monkeys, while the MARV vaccine induced a stronger humoral than cellular immune response. No evidence of EBOV or MARV replication was detected in any of the protected animals after challenge. Our data suggests that these vaccine candidates are safe and highly efficacious in a relevant animal model.

Results and Discussion

Ebola virus (EBOV) and Marburg virus (MARV), family *Filoviridae*, are emerging/re-emerging pathogens causing hemorrhagic fever with high mortality rates in humans and nonhuman primates[1-3]. The public health concern about filoviruses has increased in recent years as a result of increased awareness and frequency of occurrences in Central Africa as evidenced by the current outbreak of MARV in Angola[4] and also because filoviruses are considered to be potential agents of bioterrorism[5]. Currently, there are no EBOV or MARV vaccines or therapies approved for human use. Recently, we generated live attenuated recombinant vesicular stomatitis viruses (rVSV) expressing the transmembrane glycoprotein (GP) of *Zaire ebolavirus* (ZEBOV) (VSVΔG/ZEBOVGP) and MARV (VSVΔG/MARVGP)[6]. Here, we evaluated the utility of these rVSV vectors as candidate vaccines for EBOV and MARV using the cynomolgus macaque model.

Filovirus vaccine research has been extensively reviewed in the past and has primarily focused on EBOV[7,8]. The first EBOV vaccine to protect non-human primates was a DNA prime-adenovirus boost approach using both the GP and nucleoprotein (NP) as target antigens[9]. However, this approach required several months for immunity to develop which limits the utility of this strategy. More recently an accelerated vaccine was described. A single immunization of non-human primates with $2x10^{12}$ particles of an equal mixture of human adenovirus 5 vectors carrying either the ZEBOV GP or NP genes resulted in complete protection against ZEBOV[10]. Despite the intriguing success of the adenovirus vaccine, pre-existing immunity rates of between 40-60% have been reported

to adenovirus in the human population and this may eventually limit the utility of this approach[11-13].

A smaller number of efforts have focused on developing vaccines against MARV. Alphavirus replicons expressing MARV proteins protected cynomolgus monkeys from homologous MARV challenge[14]. However, subsequent studies evaluating this platform as a vaccine for EBOV were less encouraging as the EBOV counterpart of this alphavirus replicon platform was unable to protect any animal against lethal EBOV challenge, under similar test conditions[7]. The ideal vaccine would protect humans from infection from all four EBOV species (ZEBOV, *Sudan ebolavirus* [SEBOV], *Reston ebolavirus*, *Ivory Coast ebolavirus*) and MARV. While the adenovirus-based vaccine platform has completely protected non-human primates against ZEBOV[9,10], and the platform based on alphavirus replicons protected monkeys against MARV[14], no platform has demonstrably protected non-human primates against both of these viruses.

Vaccines based on live attenuated rVSV have been highly effective in animal models and are particularly attractive because they can be mucosally administered[15-18]. Furthermore, VSV infections in humans occur fairly rarely worldwide, mainly in the enzootic regions of the Americas and consequently global pre-existing immunity is negligible[19]. Preliminary immunization studies in mice[6] and guinea pigs (S. Jones, unpublished data) indicated the usefulness of VSVΔG/ZEBOVGP as a vaccine delivery system against ZEBOV. However, rodent models are not generally predictive for efficacy of vaccines and antiviral drugs against filoviral infections in non-human primates[7]. Thus, in this

study, we tested the protective efficacy of the replicating rVSV vector in non-human primates.

We employed twelve cynomolgus macaques, of which six animals were immunized by intramuscular (i.m.) injection with a single dose of VSVΔG/ZEBOVGP (animal #105, #332, #480, #508, #725, #790) and the remaining six with a single dose of VSVΔG/MARVGP (#190, #338, #462, #652, #770, #831). The animals were monitored closely for clinical symptoms and shedding of rVSVs (**Fig. 1**). Following vaccination none of the non-human primates displayed any signs of clinical symptoms indicating that the rVSVs are non-pathogenic for these animals. All twelve animals were subsequently challenged on d 28 post-immunization by i.m. injection with a high dose ($1x10^3$ pfu) of either ZEBOV (#105, #332, #462, #508, #652, #725) or MARV, strain Musoke (#190, #338, #480, #770, #790, #831). The two VSVΔG/MARVGP-immunized animals (#462, #652), which served as controls in the ZEBOV challenge, started to show clinical signs of disease on d 3 post-challenge and succumbed to the infection on d 6. In contrast, none of the VSVΔG/ZEBOVGP-immunized macaques became sick and all four animals were fully protected against the ZEBOV challenge. The two VSVΔG/ZEBOVGP-immunized animals (#480, #790), which served as controls for the MARV challenge, showed first signs of disease on d 4 post-challenge and both succumbed to the MARV infection on d 9. In contrast, none of the VSVΔG/MARVGP-immunized macaques became sick and all four animals were fully protected against the MARV challenge. None of the protected animals in either challenge experiment displayed any clinical signs or visual symptoms of

EBOV or MARV disease. Results of blood chemistry and hematology did not differ substantially from pre-challenge values and historical controls (data not shown).

To determine if viremia or shedding of the rVSV's occurs following immunization, whole blood and swab samples were analyzed. A mild viremia was detected on d 2 post-immunization by virus isolation (**Fig. 2a, c**) and RT-PCR (data not shown) in all six VSVΔG/ZEBOVGP-immunized animals and four of the six VSVΔG/MARVGP-immunized monkeys. Virus was undetectable in all remaining blood and swab samples with the exception of RT-PCR positives in a single blood and single nasal swab sample taken on d 6 from animal #190 vaccinated with VSVΔG/MARVGP; however, the same specimens were negative by virus isolation. Thus, inoculation led to transient viremia in most of the animals and probably resulted from localized virus replication at as yet undetermined sites. However, there is no compelling evidence to suggest that occasional virus shedding would lead to transmission. The inoculation dose was high (10^7 pfu) and three logs greater than the doses successfully used to immunize mice[6] and guinea pigs (S. Jones, unpublished data) against ZEBOV. Thus, it seems feasible to reduce or even avoid transient viremia by using a lower immunization dose.

ZEBOV and MARV replication and shedding was analyzed from the blood and swab samples taken after the challenges (**Fig. 2b, d**). The two control animals of the ZEBOV challenge study developed high EBOV titers in blood (up to ~10^4 pfu/ml) by d 3 (**Fig. 2b**) and organs (10^4-10^8 pfu/g) after death (data not shown). Similarly, the controls of the MARV challenge experiment showed high viremia levels (10^6-10^8 pfu/ml by d 6 and 9)

(**Fig. 2d**) and organ titers (10^3-10^9 pfu/g) (data not shown). In contrast, neither ZEBOV or MARV viremia (blood) (**Fig. 2b and 2d**) nor ZEBOV or MARV shedding (data not shown) was detectable in the protected animals, which were immunized with VSVΔG/ZEBOVGP and VSVΔG/MARVGP, respectively.

By the day of ZEBOV challenge (d 0) all VSVΔG/ZEBOVGP-immunized animals had developed low to moderate level IgG antibody titers against ZEBOV GP (**Fig. 3a**). Interestingly, neutralizing antibody titers to ZEBOV were not detectable prior to challenge but became positive (1:80 to 1:320) 14 and 28 d after challenge (**Fig. 3b**). It remains unclear why the neutralizing antibody titers in two animals fell off after an initial rise. However, this has been seen in previous studies in non-human primates infected with ZEBOV (T. Geisbert, unpublished observation). Interestingly, the cellular responses in the VSVΔG/ZEBOVGP-immunized animals of this study mirrored the neutralizing antibody responses, as the specific production of IFN-γ and TNF-α was not detectable before ZEBOV challenge (**Fig. 4**). After challenge, all VSVΔG/ZEBOVGP-immunized animals responded positively ranging between 0.05 to 6% positive IFN-γ- or TNF-α-positive CD8 cells and 0.02 and 0.4% positive CD4 cells. It should be noted that some subjects (e.g., #508; **Fig. 4**) displayed a strong cellular response. In animals immunized with the GP/NP-expressing adenovirus vaccine the highest cellular response detected was approximately 1.5% of CD8 cells producing IFN-γ using an identical assay[10]. This indicates that the VSVΔG/ZEBOVGP appears to be a potent stimulator of cellular immunity.

By the day of MARV challenge (d 0) all animals vaccinated with VSVΔG/MARVGP had developed moderate IgG antibody titers against the MARV GP (**Fig. 3c**). Neutralizing antibody titers to MARV (1:80) were only detected in two animals (**Fig. 3d**). Consistent with results in the ZEBOV portion of this study, the cellular responses in the MARV-immunized animals initially mirrored their neutralizing antibody responses, as the specific production of IFN-γ and TNF-α were not detectable before MARV challenge. However, in contrast to the ZEBOV results (**Fig. 4**), no evidence of a cellular immune response was detected after MARV challenge (data not shown). This indicates that protection of these animals against MARV might be due to indices other than cellular immunity or neutralizing antibodies and may be partly associated with non-neutralizing antibodies.

Finally, we tested the protective efficacy against a re-challenge with a heterologous virus strain. All animals which were protected from the lethal ZEBOV (strain Kikwit) challenge, were re-challenged with $1x10^3$ pfu of SEBOV (strain Gulu) 234 d after initial challenge (**Figs. 1a, b**). Three of the four animals succumbed to the SEBOV infection on d 6 and 7 post-re-challenge with viremias ranging from 10^7-10^8 pfu/ml. Only one animal survived the re-challenge showing transient viremia of ~10^3 pfu/ml on d 6 (data not shown). This single survivor cannot necessarily be attributed to vaccine protection since the SEBOV macaque model is not uniformly lethal (T. Geisbert, unpublished data). The lack of cross protection was not unexpected as the EBOV species differ from one another by 37-41% at the nucleotide and amino acid levels[20]. All VSVΔG/MARVGP-immunized macaques, which were protected against the lethal MARV, strain Musoke challenge,

were re-challenged with a 1 x 10^3 pfu of MARV, strain Popp, 113 d after initial challenge (**Figs. 1a, c**). In contrast to the SEBOV re-challenge, all four animals remained healthy and survived the re-challenge without showing clinical symptoms. The re-challenge results indicated that cross-protection can only be achieved against heterologous strains from the same virus species. Indeed, the MARV strains used in this study are genetically similar. Homology between nucleotide sequences of these two strains is 93.9%[21].

Although protection of monkeys by the rVSV EBOV vaccine appeared to be associated with humoral and cellular immune responses (**Figs. 3a, b and 4**), protection of monkeys by the rVSV MARV vaccine appeared to be primarily associated with the humoral immune response (**Figs. 3c, d**). Notably, neutralizing antibodies were poorly induced, suggesting that protection may be due to rather higher levels of non-neutralizing antibodies in these animals. It is possible that the *in vitro* neutralization assay is not detecting the same neutralizing antibodies required to neutralize the ability of MARV or ZEBOV to infect their primary *in vivo* targets. However, the MARV results in the current study are not without precedent. In similar studies to evaluate alphavirus replicons expressing MARV genes including GP, cynomolgus monkeys were protected from homologous MARV challenge despite the absence of neutralizing antibody titers in pre-challenge sera[14]. The potential importance of non-neutralizing antibodies to protection against MARV has also been noted in another study. Specifically, neutralizing antibodies were not detected in rhesus monkeys immunized with an inactivated whole virion preparation. Although cellular responses were not detected in these animals, three of these six monkeys survived a lethal MARV challenge[22]. Importantly, the investigators

were unable to associate protection with the humoral or cellular immune response and concluded that protective immunity is determined by the indices of nonspecific immunity[22].

This current study is the first to show that non-human primates can be protected with a single-dose immunization using a vector expressing solely the ZEBOV GP. In addition, this is the first vaccine platform to demonstrate the ability to protect non-human primates against EBOV and MARV, which is an important first step toward developing a pan-filovirus vaccine. Protection is dependent on immunization with an attenuated, replication competent virus, which may raise questions regarding the safety of live attenuated vectors. However, there has been no evidence of pathogenicity in four species of animals (mouse, guinea pig, goat, non-human primate) tested so far (S. Jones/H. Feldmann, unpublished data). Most importantly, we demonstrated here that despite a short-term viremia rVSV replication and shedding were not detectable in non-human primates and that the animals did not develop fever or other symptoms, nor were there changes in blood chemistry or hematology.

The use of replicating rVSV-based vectors, shown here for EBOV and MARV, has proven to be a potent and promising concept for future vaccine development against these aggressive pathogens, and may be equally applicable to other lethal emerging/re-emerging viruses.

Methods

Vaccine vectors and viruses. The recombinant VSV expressing the GPs of ZEBOV (strain Mayinga) and MARV (strain Musoke) were generated as described recently using the infectious clone for the VSV, Indiana serotype (kindly provided by J. Rose)[6]. Briefly, the appropriate open reading frames for the GPs were generated by PCR, cloned into the VSV genomic vectors lacking the VSV GP gene, sequenced confirmed, and originally rescued using the method described earlier[6,23]. ZEBOV (strain Kikwit) was isolated from a patient of the EBOV outbreak in Kikwit 1995[24] while SEBOV (strain Gulu) was isolated from a patient of the EBOV outbreak in Gulu 2000[25]. MARV strain Musoke was isolated from a human case in 1980 in Kenya[26] and strain Popp was isolated from a patient of the first MARV outbreak in 1967[21].

Animal studies. Twelve healthy adult cynomolgus macaques (*Macaca fascicularis*) (4-6 kg) were used for these studies. For the EBOV portion of this study, four animals were immunized i.m. with 10^7 pfu of VSVΔG/ZEBOVGP (#105, #332, #508, #725) and two animals with ~ $5x10^7$ pfu of VSVΔG/MARVGP (#462, #652; controls). The six cynomolgus macaques were challenged i.m. 28 d after the single dose immunization with $1x10^3$ pfu of ZEBOV. For the MARV portion of this study, four animals were immunized i.m. with ~ $5x10^7$ pfu (#190, #338, #770, #831) and two animals with 10^7 pfu of VSVΔG/ZEBOVGP (#480, #790; controls). These six cynomolgus macaques were challenged i.m. 28 d after the single dose immunization with $1x10^3$ pfu of MARV, strain Musoke. The re-challenge of the VSVΔG/ZEBOVGP-immunized animals, which were protected against the challenge with ZEBOV (#105, #332, #508, #725), was performed i.m. 234 d after initial challenge with $1x10^3$ pfu of SEBOV. The re-challenge of the

VSVΔG/MARVGP-immunized animals, which were protected against the challenge with MARV, strain Musoke (#190, #338, #770, #831), was done i.m. 113 d after initial challenge with $1x10^3$ pfu of MARV, strain Popp. Swab samples (oral, nasal, rectal, vaginal) and blood were taken as indicated (**Fig. 1a**). Animal studies were performed in BSL-4 biocontainment at USAMRIID and approved by the USAMRIID Laboratory Animal Care and Use Committee. Animal research was conducted in compliance with the Animal Welfare Act and other Federal statues and regulations relating to animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility used is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Virus detection. RNA was isolated from blood and swabs using appropriate RNA isolation kits (QIAGEN). For the detection of VSV we used a RT-PCR assay targeting the matrix gene (nt position 2355 – 2661, NC_001560). ZEBOV and MARV RNA were detected using primer pairs targeting the L genes [ZEBOV (AF 272001): RT-PCR - nt position 13344 – 13622; nested PCR – nt position 13397 - 13590; MARV (X 68494): RT-PCR – nt position 1966 - 2243; nested PCR – nt position 2017 – 2213]. Virus titration was performed by plaque assay on Vero E6 cells from all blood and selected organ (adrenal, ovary, lymph nodes, liver, spleen, pancreas, lung, heart, brain) and swab samples[24]. Briefly, increasing 10-fold dilutions of the samples were adsorbed to Vero E6 monolayers in duplicate wells (0.2 ml per well); thus, the limit for detection was 25 pfu/ml.

Immune responses. IgG antibodies against ZEBOV and MARV were detected with an Enzyme-Linked Immunosorbent Assay (ELISA) using purified virus particles as an antigen source[10]. Neutralization assays were performed by measuring plaque reduction in a constant virus:serum dilution format as previously described[27]. Briefly, a standard amount of ZEBOV or MARV (~ 100 pfu) was incubated with serial two-fold dilutions of the serum sample for 60 min. The mixture was used to inoculate Vero E6 cells for 60 min. Cells were overlayed with an agar medium, incubated for 8 d, and plaques were counted 48 h after neutral red staining. Endpoint titers were determined by the dilution of serum, which neutralized 80% of the plaques ($PRNT_{80}$).

Cellular immune responses. The method for assessment of T-cell responses to EBOV was previously published[10]. Briefly, peripheral blood mononuclear cells were isolated from cynomolgus macaque whole-blood samples by separation over Ficoll. Approximately 1x 10^6 cells were stimulated in 200 µl RPMI medium (GIBCO) for 6 h at 37 °C with anti-CD28 and anti-CD49d antibodies and either DMSO or a pool of 15-nucleotide peptides spanning the ZEBOV GP (Mayinga strain) or the MARV GP (Musoke strain) open reading frames in the presence of brefeldin A. The peptides were 15 amino acids in length, overlapping by 11 and spanning the entire ZEBOV GP or MARV GP at a final concentration of 2 µg/ml. Cells were fixed and permeabilized with FACS lyse (Becton Dickinson) supplemented with Tween-20, and stained with a mixture of antibodies against lineage markers (CD3-PE, CD4-PerCP, CD8-FITC) and either TNF-α-APC or IFN-γ-APC. Samples were run on a FACSCalibur and analyzed using the

software FlowJo. Positive gating for lymphocytes using forward versus side scatter was followed by CD3+/CD8- and CD3+/CD4- gating, and specific populations were further defined by anti-CD4 and anti-CD8 positivity, respectively. Cytokine-positive cells were defined as a percentage within these individual lymphocyte subsets, and at least 200,000 events were analyzed for each sample.

Acknowledgments

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References

1. Sanchez, A. *et al*. Filoviridae: Marburg and Ebola Viruses. in *Fields Virology* (eds. Knipe, D.M. & Howley, P.M.) 1279-1304 (Lippincott Williams & Wilkins, Philadelphia, 2001).
2. Feldmann, H., Jones, S., Klenk, H.D. & Schnittler, H.J. Ebola virus: from discovery to vaccine. *Nat Rev Immunol* **3**, 677-85 (2003).
3. Geisbert, T.W. & Jahrling, P.B. Exotic emerging viral diseases: progress and challenges. *Nat Med* **10(12 Suppl)**, S110-21 (2004).
4. Centers for Disease Control and Prevention (CDC). Outbreak of Marburg virus hemorrhagic fever--Angola, October 1, 2004-March 29, 2005. *MMWR Morb Mortal Wkly Rep* **54**, 308-9 (2005).
5. Borio, L. *et al*. Hemorrhagic fever viruses as biological weapons: medical and public health management. *Jama* **287**, 2391-405 (2002).
6. Garbutt, M. *et al*. Properties of replication-competent vesicular stomatitis virus vectors expressing glycoproteins of filoviruses and arenaviruses. *J Virol* **78**, 5458-65 (2004).
7. Geisbert, T.W. *et al*. Evaluation in nonhuman primates of vaccines against Ebola virus. *Emerg Infect Dis* **8**, 503-7 (2002).
8. Geisbert, T.W. & Jahrling, P.B. Towards a vaccine against Ebola virus. *Expert Review of Vaccines* **2**, 777-789 (2003).
9. Sullivan, N.J., Sanchez, A., Rollin, P.E., Yang, Z.Y. & Nabel, G.J. Development of a preventive vaccine for Ebola virus infection in primates. *Nature* **408**, 605-9 (2000).

10. Sullivan, N.J. *et al*. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature* **424**, 681-4 (2003).

11. Brandt, C.D. *et al*. Infections in 18,000 infants and children in a controlled study of respiratory tract disease. I. Adenovirus pathogenicity in relation to serologic type and illness syndrome. *Am J Epidemiol* **90**, 484-500 (1969).

12. Piedra, P.A., Poveda, G.A., Ramsey, B., McCoy, K. & Hiatt, P.W. Incidence and prevalence of neutralizing antibodies to the common adenoviruses in children with cystic fibrosis: implication for gene therapy with adenovirus vectors. *Pediatrics* **101**, 1013-9 (1998).

13. Schulick, A.H. *et al*. Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries. Potential for immunosuppression and vector engineering to overcome barriers of immunity. *J Clin Invest* **99**, 209-19 (1997).

14. Hevey, M., Negley, D., Pushko, P., Smith, J. & Schmaljohn, A. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. *Virology* **251**, 28-37 (1998).

15. Roberts, A., Buonocore, L., Price, R., Forman, J. & Rose, J.K. Attenuated vesicular stomatitis viruses as vaccine vectors. *J Virol* **73**, 3723-32 (1999).

16. Roberts, A. *et al*. Vaccination with a recombinant vesicular stomatitis virus expressing an influenza virus hemagglutinin provides complete protection from influenza virus challenge. *J Virol* **72**, 4704-11 (1998).

17. Schlereth, B., Rose, J.K., Buonocore, L., ter Meulen, V. & Niewiesk, S. Successful vaccine-induced seroconversion by single-dose immunization in the presence of measles virus-specific maternal antibodies. *J Virol* **74**, 4652-7 (2000).

18. Rose, N.F. *et al.* An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell* **106**, 539-49 (2001).

19. Wagner, R.R. & Rose, J.K. Rhabdoviridae: The Viruses And Their Replication. in *Fields Virology*, Vol. 1 (eds. Knipe, D.M. & Howley, P.M.) (Lippincott Williams & Wilkins, Philadelphia, 1996).

20. Sanchez, A., Trappier, S.G., Mahy, B.W., Peters, C.J. & Nichol, S.T. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. *Proc Natl Acad Sci USA* **93**, 3602-7 (1996).

21. Bukreyev, A.A., Volchkov, V.E., Blinov, V.M., Dryga, S.A. & Netesov, S.V. The complete nucleotide sequence of the Popp (1967) strain of Marburg virus: a comparison with the Musoke (1980) strain. *Arch Virol* **140**, 1589-600 (1995).

22. Ignatyev, G.M., Agafonov, A.P., Streltsova, M.A. & Kashentseva, E.A. Inactivated Marburg virus elicits a nonprotective immune response in Rhesus monkeys. *J Biotechnol* **44**, 111-8 (1996).

23. Schnell, M.J., Buonocore, L., Kretzschmar, E., Johnson, E. & Rose, J.K. Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses are incorporated efficiently into virus particles. *Proc Natl Acad Sci U S A* **93**, 11359-65 (1996).

24. Jahrling, P.B. *et al*. Evaluation of immune globulin and recombinant interferon-alpha2b for treatment of experimental Ebola virus infections. *J Infect Dis* **179 Suppl 1**, S224-34 (1999).

25. Sanchez, A. *et al*. Analysis of human peripheral blood samples from fatal and nonfatal cases of Ebola (Sudan) hemorrhagic fever: cellular responses, virus load, and nitric oxide levels. *J Virol* **78**, 10370-7 (2004).

26. Smith, D.H. *et al*. Marburg-virus disease in Kenya. *Lancet* **1**, 816-20 (1982).

27. Jahrling, P.B. Filoviruses and Arenaviruses. in *Manual of Clinical Microbiology* (ed. Murray, P.R.) 1125-1136 (ASM Press, Washington, DC, 1999).

Figures

Figure 1

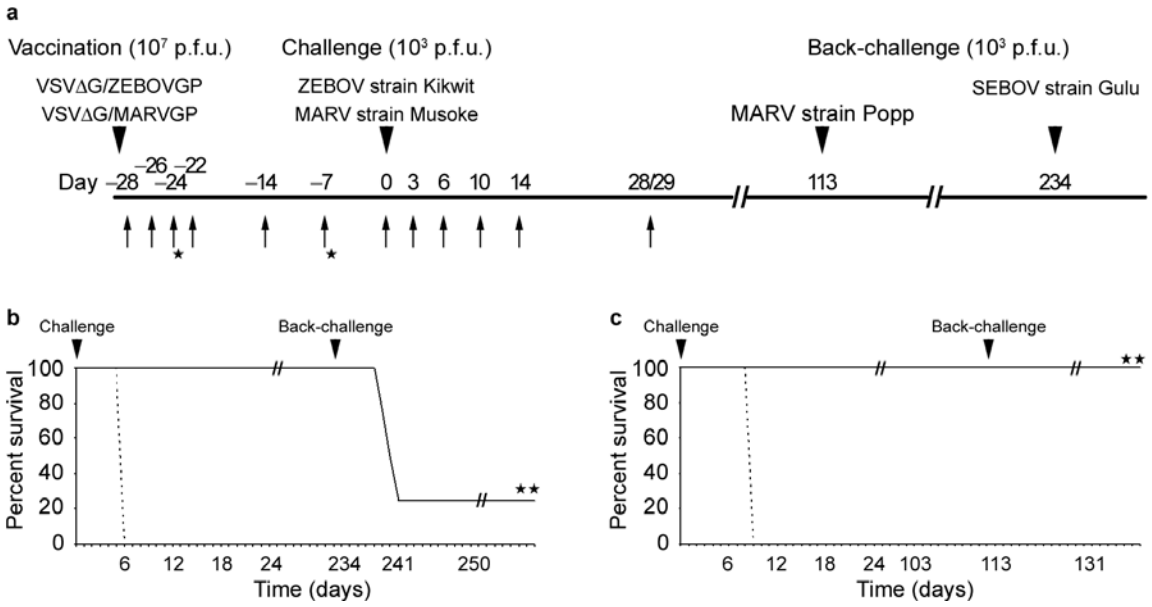

Figure 1. Immunization, challenge and re-challenge of non-human primates. (**a**) Flow chart of experimental design. Arrows indicate days of sampling (blood and swabs), * indicates additional sampling days for the ZEBOV study. (**b**) Kaplan-Meier mortality chart of the EBOV vaccine study. Dotted line, animals immunized with VSVΔG/MARVGP and challenged with ZEBOV; solid line, animals immunized with VSVΔG/ZEBOVGP, challenged with ZEBOV, and re-challenged with SEBOV. (**c**) Kaplan-Meier mortality chart of the MARV vaccine study. Dotted line, animals immunized with VSVΔG/ZEBOVGP and challenged with MARV, strain Musoke; solid line, animals immunized with VSVΔG/MARVGP, challenged with MARV, strain Musoke, and re-challenged with MARV, strain Popp.

Figure 2

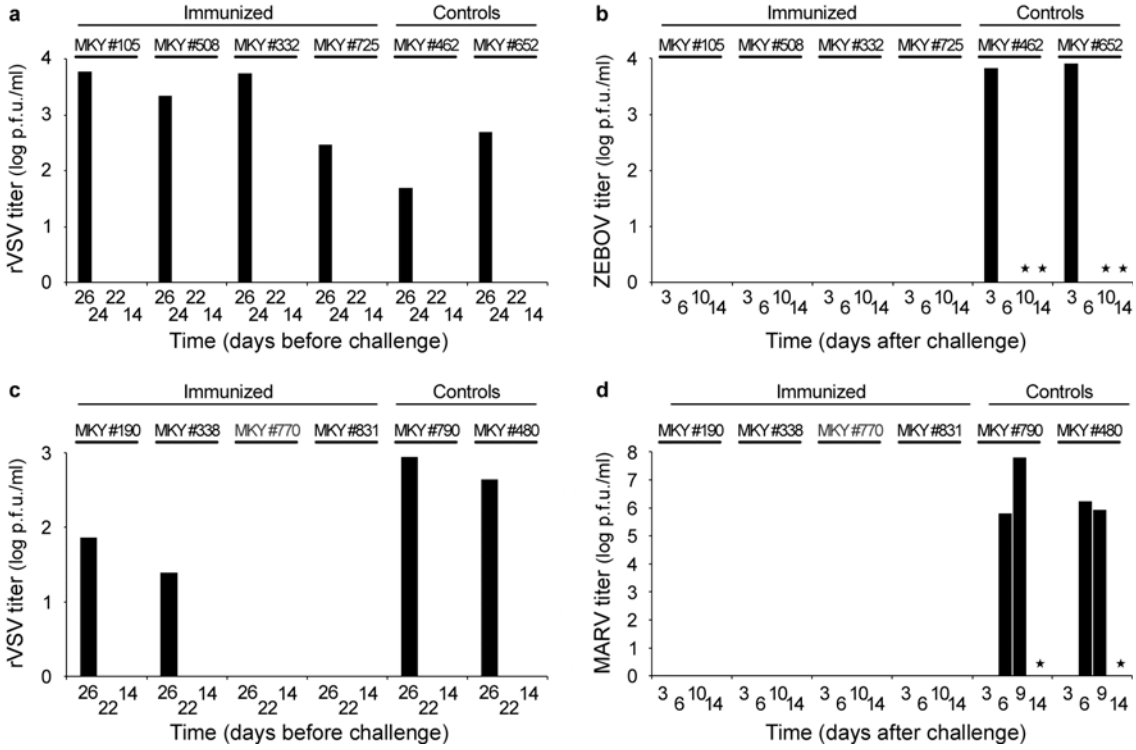

Figure 2. Viremia levels in non-human primates after immunization and challenge. VSV viremia levels were determined after immunization with VSVΔG/ZEBOVGP (**a)** or VSVΔG/MARVGP **(c)** and ZEBOV and MARV viremia levels after challenge with ZEBOV(**b**), or MARV, strain Musoke **(d)**, from plasma taken at the indicated time points (**Fig. 1a**). Note: animals MKY #105, MKY #508, MKY #332, and MKY #725 were vaccinated with VSVΔG/ZEBOVGP while animals MKY #462 and MKY #652 served as the controls in this study and were vaccinated with VSVΔG/MARVGP; all were challenged with ZEBOV. Animals MKY #190, MKY #338, MKY #770, and MKY #831 were vaccinated with VSVΔG/MARVGP while animals MKY #790 and MKY #480 served as the controls in this study and were vaccinated with VSVΔG/ZEBOVGP; all were challenged with MARV, strain Musoke. *sample was not available (animal had died).

Figure 3

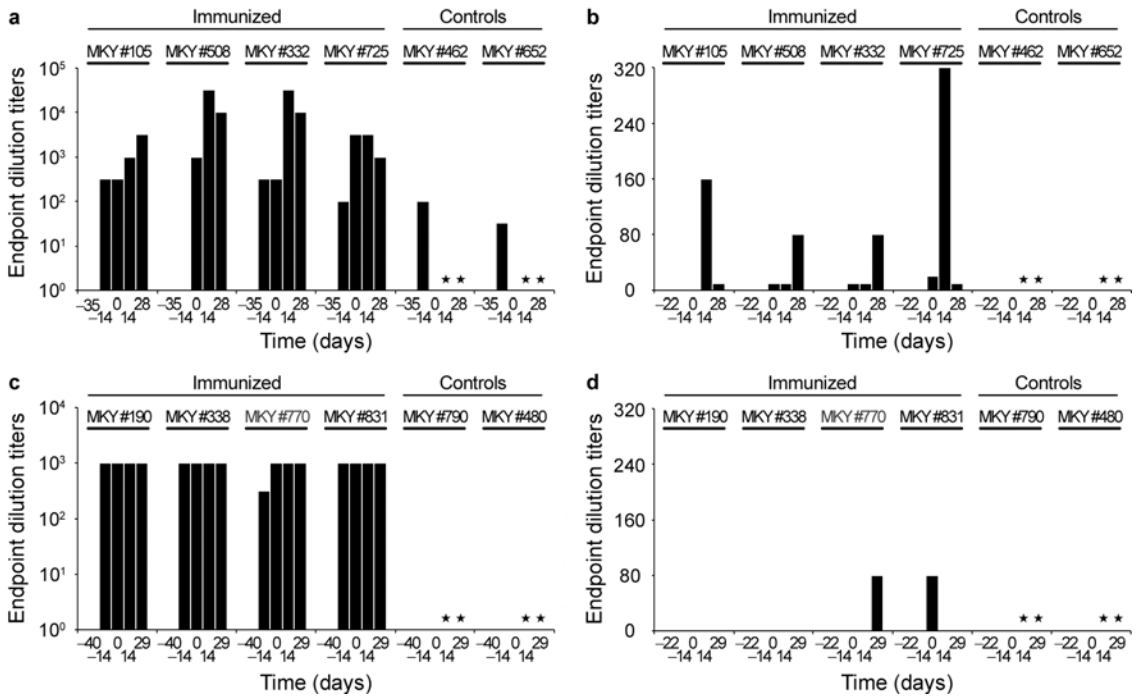

Figure 3. Humoral immune response in non-human primates to ZEBOV and MARV before and after challenge. (**a**) IgG response to ZEBOV. (**b**) ZEBOV neutralizing antibodies. (**c**) IgG response to MARV. (**d**) MARV neutralizing antibodies. IgG responses were measured using an established ELISA (see under 'Methods'). Titers are presented as endpoint dilutions. Neutralizing antibodies were detected using a plaque reduction neutralization assay ($PRNT_{80}$) as described under 'Methods'. Titers are presented as endpoint dilutions. –, days prior to challenge; 0, day of challenge; +, days after challenge; *sample was not available (animal had died).

Figure 4

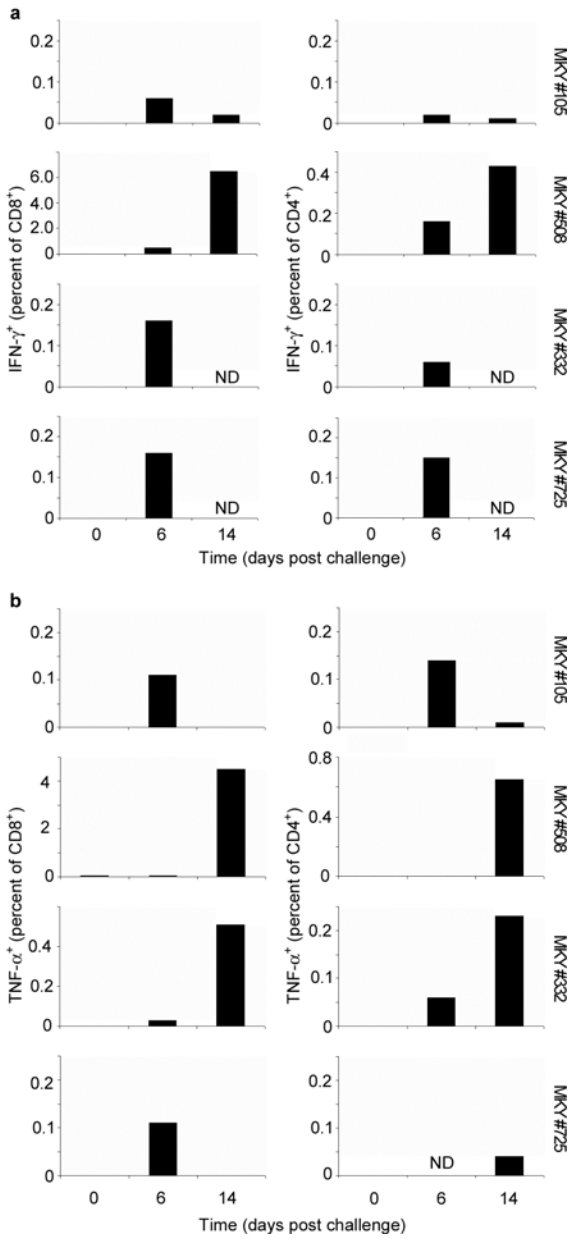

Figure 4. Cellular immune response in non-human primates prior to and after challenge with ZEBOV. (**a**) interferon (IFN)-γ. (**b**) tumor necrosis factor (TNF)-α. Intracellular levels of IFN-γ and TNF-α were determined in CD4- and CD8-positive T-cell populations as described under 'Methods'. Cellular responses following re-stimulation with a GP peptide library were seen in all animals after challenge. One animal (MKY #508) had relatively high levels of circulating CD8-positive T-cells producing IFN-γ and TNF-α at day 14 post-challenge.

IV. Cross Protection against Marburg Virus Strains using a Live Attenuated Recombinant Vaccine

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Abstract

Marburg virus (MARV) has been associated with sporadic episodes of hemorrhagic fever including a recent highly publicized outbreak in Angola that produced severe disease and significant mortality in infected patients. MARV is also considered to have potential as a biological weapon. Recently, we reported the development of a promising attenuated, replication competent vaccine against MARV based on recombinant vesicular stomatitis virus (VSV) expressing the glycoprotein of the Musoke strain of MARV (VSVΔG/MARVGP-Musoke). We used this vaccine to demonstrate complete protection of cynomolgus monkeys against a homologous MARV challenge. While these results are highly encouraging, an effective vaccine would need to confer protection against all relevant strains of MARV. Here, we evaluated the protective efficacy of the VSVΔG/MARVGP-Musoke vaccine against two heterologous MARV strains, the seemingly more pathogenic Angola strain, and the more distantly related Ravn strain. In this study, seven cynomolgus monkeys were vaccinated with the VSVΔG/MARVGP-Musoke vector. Three of these animals were challenged with the Angola strain, three with the Ravn strain, and a single animal with the Musoke strain of MARV. Two animals served as controls and were each injected with a nonspecific VSV vector; these controls were challenged with the Angola and Ravn strains, respectively. Both controls succumbed to challenge by day 8. However, none of the specifically vaccinated animals showed any evidence of illness either from the vaccination or from the MARV challenges and all of these animals survived. These data suggest that the VSVΔG/MARVGP-Musoke vaccine should be sufficient to protect against all known MARV strains.

Introduction

Marburg virus (MARV) causes severe and often fatal infections in humans and nonhuman primates. Historically, several strains of MARV have produced confirmed case fatality rates ranging from 23 percent to slightly greater than 50 percent (4, 16). However, in 2004-2005 a new strain of MARV caused even more significant mortality during a large outbreak in Angola. Case fatality rates during this episode fluctuated around 90 percent. An initial report by the World Health Organization noted that the epidemic killed 329 people of the 374 that were infected; the outbreak was recently declared over and these numbers were revised with the updated report noting that there were 227 deaths among the 252 reported cases (12). The reasons for the increased lethality of this new strain of MARV are presently unknown, but are of significant concern.

While there are currently no licensed vaccines or antivirals to prevent or treat MARV infections, we recently described the development of a promising new replication competent vaccine against MARV based on recombinant vesicular stomatitis virus (VSV) (7,14). In one study, we demonstrated complete protection of cynomolgus macaques against a high dose (1000 plaque forming units [pfu]) lethal MARV challenge using a single injection of recombinant VSV vectors expressing the glycoprotein (GP) of the homologous Musoke strain of MARV (MARV-Musoke) (14). More recently, we demonstrated that the same vaccine vector used as postexposure treatment for rhesus monkeys (*Macaca mulatta*) infected with 1000 pfu of strain Musoke was also able to protect all animals from clinical disease and death (5).

MARV along with Ebola virus (EBOV) comprise the two genera that make up the family *Filoviridae* (6). There is a single species, *Lake Victoria marburgvirus*, within the MARV genus. There are a number of different strains of MARV. Comparative analyses of the GP and viral protein (VP) 35 genes of MARV strains showed that there are two distinct lineages within the *Lake Victoria marburgvirus* species of MARV. The original MARV isolates from the 1967 episodes in Marburg, Germany (Popp and Ratayczak strains), from a case in 1975 in South Africa (Ozolin strain), and from 1980 in Kenya (Musoke strain) comprise one lineage. An isolate from Kenya in 1987 (Ravn strain) represents a second genetic lineage within the *Lake Victoria marburgvirus* species (21-23% amino acid difference) (20).

MARV is composed of seven structural proteins and the nonsegmented negative-sense viral RNA genome. Four proteins (NP, VP35, VP30, and L) make up the helical nucleocapsid, which is surrounded by a matrix that is composed of the viral proteins VP40 and VP24. The surface of MARV virions is coated with spikes that consist of the structural GP. The GP plays a role in virus entry and pathogenesis and serves as a major and logical target for vaccine strategies including our recombinant VSV-based system (14).

While most strains of MARV produce lethal infections in nonhuman primates, the disease course varies among the strains and is in general more protracted than what is seen with EBOV. Recent studies have suggested that the new Angola isolate of MARV, appears to produce a disease in nonhuman primates that is more rapid and severe than other MARV strains, and in fact, appears to be as virulent as *Zaire ebolavirus* (ZEBOV) in rhesus macaques (T.W. Geisbert, unpublished observation).

In developing vaccines against any virus, there are always concerns about broad protection and the ability to protect against different strains or isolates of the same virus. Of particular concern regarding cross-protection among the MARV strains are results reported using a different vaccine vector system. Notably, a study using a platform based on Venezuelan equine encephalitis virus (VEEV) replicons, showed that cynomolgus monkeys vaccinated with VEEV (MARV GP) or VEEV (MARV NP) replicons based on strain Musoke were protected against lethal homologous challenge (9), but not against a challenge with the Ravn strain of MARV (MARV-Ravn) (10). This result raises the concern that the 21-23% difference in amino acids between the two MARV lineages may be significant enough to affect the ability of a candidate vaccine to confer cross-protection against the different MARV strains. Here, we tested the ability of our recombinant VSV vaccine expressing the MARV-Musoke strain GP to protect nonhuman primates against a lethal challenge with either the more genetically diverse Ravn strain or the seemingly more pathogenic Angola strain (MARV-Angola).

Materials and Methods

Vaccine vectors and viruses. The recombinant VSVs expressing the GPs of ZEBOV (VSVΔG/ZEBOVGP) and MARV-Musoke (VSVΔG/MARVGP) were generated as described recently using the infectious clone for the VSV, Indiana serotype (7,14). Briefly, the appropriate open reading frames for the GPs were generated by PCR, cloned into the VSV genomic vectors lacking the VSV GP gene, sequenced confirmed, and originally rescued using the method described earlier. MARV-Musoke was isolated from a human case in 1980 in Kenya (21), MARV-Ravn was isolated from a human case

in 1987 in Kenya (13), while MARV-Angola was isolated from a patient of the recent outbreak in Angola in 2005 (25).

Sequencing and phylogenetic analysis. MARV-Angola was isolated from clinical specimens (whole blood) from several patients. The sequence of the GP gene was determined using primers based on the GP sequences of the Musoke strain. The deduced amino acid sequence of the Angola GP open reading frame (ORF) was compared with the amino acid sequences of different filovirus GPs which were retrieved from GenBank. Phylogenetic analysis was performed with MEGA version 3.1 (www.megasoftware.net) using a neighbor-joining tree and 1000 bootstrap replicates. The protein sequences included in the analysis were: NP_042028 (MARV Popp strain) ABA87127 (MARV Musoke strain), ABE27085 (MARV Durba strain), AAQ55258 (MARV Ozolin strain), ABE27071 (MARV Ravn strain), ABE27092 (MARV Durba strain), AAB37093 (*Ivory Coast ebolavirus* [CIEBOV] Ivory Coast strain), BAB69006 (*Reston ebolavirus* [REBOV], Reston strain), AAU43887 (*Sudan ebolavirus* [SEBOV], Gulu strain), AAC54889 (REBOV, Philippine strain), AAB37096 (SEBOV, Boniface strain), AAC54882 (SEBOV, Maleo strain), AAC57992 (*Zaire ebolavirus* [ZEBOV], Eckron strain), AAQ55048 (ZEBOV, Zaire strain), AAN37507 (ZEBOV, Mayinga strain), and AAL25818 (ZEBOV, Gabon strain).

Animal studies. Nine healthy adult cynomolgus macaques (*Macaca fascicularis*) (5-9 kg) were used for these studies. Seven of these animals were vaccinated intramuscularly (i.m.) with ~ $2x10^7$ pfu of VSVΔG/MARVGP while two animals served as experimental controls and received ~ $2x10^7$ pfu of VSVΔG/ZEBOVGP. All animals were challenged 28 days after the single dose immunization with 1000 pfu of MARV as

follows: one group of three macaques that were vaccinated with the VSVΔG/MARVGP was challenged with MARV-Ravn. A second group of three animals vaccinated with the VSVΔG/MARVGP was challenged with MARV-Angola, while the remaining macaque that was vaccinated with the VSVΔG/MARVGP was challenged with MARV-Musoke. One of the control animals receiving the VSVΔG/ZEBOVGP was challenged with MARV-Ravn while the second control animal was challenged with MARV-Angola.

Swab samples (oral, nasal, rectal) and/or blood were taken before vaccination (day -28) and at days 3 (day -25), 6 (day -22), 14 (day -14), and 28 (day 0) after vaccination and at days 3, 6, 10, 14, and 28 after the MARV challenges. Animal studies were performed in BSL-4 biocontainment at USAMRIID and were approved by the USAMRIID Laboratory Animal Care and Use Committee. Animal research was conducted in compliance with the Animal Welfare Act and other Federal statues and regulations relating to animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility used is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Hematology and serum biochemistry. Total white blood cell counts, white blood cell differentials, red blood cell counts, platelet counts, hematocrit values, total hemoglobin, mean cell volume, mean corpuscular volume, and mean corpuscular hemoglobin concentration were determined from blood samples collected in tubes containing EDTA, by using a laser-based hematologic Analyzer (Coulter Electronics, Hialeah, FL, USA). The white blood cell differentials were performed manually on Wright-stained blood smears. Serum samples were tested for concentrations of albumin

(ALB), amylase (AMY), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), glucose (GLU), cholesterol (CHOL), total protein (TP), total bilirubin (TBIL), urea nitrogen (BUN), and creatinine (CRE) by using a Piccolo Point-Of-Care Blood Analyzer (Abaxis, Sunnyvale, CA, USA).

Virus detection. RNA was isolated from blood and swabs using appropriate RNA isolation kits (QIAGEN, Mississauga, ON, Canada). For the detection of VSV we used a RT-PCR assay targeting the matrix gene (nt position 2355 – 2661, NC_001560). MARV RNA was detected using primer pairs targeting the L gene (X 68494): RT-PCR – nt position 1966 - 2243; nested PCR – nt position 2017 – 2213]. The low detection limit for this MARV assay is 0.1 pfu/ml of plasma. Virus titration was performed by plaque assay on Vero E6 cells from all blood and selected organ (adrenal, ovary, lymph nodes, liver, spleen, pancreas, lung, heart, brain) and swab samples. Briefly, increasing 10-fold dilutions of the samples were adsorbed to Vero E6 monolayers in duplicate wells (0.2 ml per well); thus, the limit for detection was 25 pfu/ml.

Humoral immune responses. IgG antibodies against MARV were detected with an Enzyme-Linked Immunosorbent Assay (ELISA) using purified virus particles as an antigen source (14). Neutralization assays were performed by measuring plaque reduction in a constant virus:serum dilution format as previously described (Sullivan, Geisbert et al. 2003). Briefly, a standard amount of MARV-Musoke (~ 100 pfu) was incubated with serial two-fold dilutions of the serum sample for 60 minutes. The mixture was used to inoculate Vero E6 cells for 60 minutes. Cells were overlayed with an agar medium, incubated for 8 days, and plaques were counted 48 hours after neutral red

staining. Endpoint titers were determined by the dilution of serum, which neutralized 50% of the plaques ($PRNT_{50}$).

Cellular immune responses. The method for assessment of T-cell responses to MARV was previously published (14). Briefly, peripheral blood mononuclear cells were isolated from cynomolgus macaque whole-blood samples by histopaque gradient (Sigma, St Louis, MO, USA). Approximately $1x10^6$ cells were stimulated in 200 µl RPMI medium (Gibco, Invitrogen, Carlsbad, CA, USA) for 6 hours at 37 °C with anti-CD28 and anti-CD49d antibodies and either DMSO or a pool of MARV-Musoke GP-specific peptides in the presence of brefeldin A. The peptides were 15 amino acids in length, overlapping by 11 and spanning the entire MARV GP at a final concentration of 2 µg/ml. Cells were fixed and permeabilized with FACSlyse™ (Becton Dickinson, San Jose, CA, USA) supplemented with Tween-20, and stained with a mixture of antibodies against lineage markers (CD3-PE, CD4-PerCP, CD8-FITC) and either TNF-α-APC or IFN-γ-APC. Samples were run on a FACSCalibur and analyzed using the software FlowJo. Positive gating for lymphocytes using forward versus side scatter was followed by $CD3^+$/CD8- and $CD3^+$/CD4- gating, and specific populations were further defined by anti-CD4 and anti-CD8 positivity, respectively. Cytokine-positive cells were defined as a percentage within these individual lymphocyte subsets, and at least 200,000 events were analyzed for each sample.

Results

Sequence Analysis

At the beginning of this study the GP sequence of MARV-Angola was not determined. Therefore, we sequenced the GP ORF of several isolates which were obtained in Winnipeg by inoculation of Vero E6 cells with whole blood collected from patients during the MARV-Angola outbreak. Comparative analysis showed that all GP gene sequences were identical. Thus, the deduced amino acid sequence of the GP ORF of one of the isolates was used for phylogenetic analysis with the deduced amino acid sequences of several EBOV and MARV GPs including two recently deposited sequences from the MARV outbreak in Durba/Watsa (Fig. 1). The phylogenetic analysis demonstrated that MARV strains separated into two major branches, one of which included strains from 1987 (Ravn) and 1998-2000 (Durba/Watsa). The second major branch again separated into two further branches with strains from 1975 (Ozolin) and 1998-2000 (Durba/Watsa) separated from strains originating in 1967 (Popp, Ratayczak), 1980 (Musoke), and 2004-2005 (Angola). The close relationship between the Angola and Musoke strains indicated a likelihood of cross protection by the VSVΔG/MARVGP-Musoke vaccine against a heterologous challenge with the Angola strain. However, cross protection against a challenge with the more distantly related Ravn strain seemed less likely. We recently demonstrated that the VSVΔG/MARVGP-Musoke vaccine does not provide any cross protection against ZEBOV strains (14).

Clinical Observations

A total of nine cynomolgus monkeys were used to evaluate whether a preventative vaccination strategy employing a single injection of the recombinant VSV expressing the GP of MARV-Musoke (VSVΔG/MARVGP) could cross-protect against

MARV hemorrhagic fever (HF) caused by the Ravn and Angola strains (Fig. 2A). We vaccinated seven animals by i.m. injection of VSVΔG/MARVGP vectors (Subjects #1-7) and two control animals with nonspecific recombinant VSV expressing the GP of ZEBOV (VSVΔG/ZEBOVGP) (Control #1 and Control #2). Animals were challenged 28 days after the single dose vaccine with either heterologous MARV-Ravn (Subjects #1-3 and Control # 1) or heterologous MARV-Angola (Subjects #4-6 and Control #2). One animal (Subject #7) was challenged with homologous MARV-Musoke and served as an internal vaccine control as we have previously shown that the VSVΔG/MARVGP-Musoke vaccine can provide complete protection against a homologous MARV challenge (14).

Animals were monitored closely after both vaccination and MARV challenge for clinical symptoms of illness, viremia from either the vaccine or MARV, and shedding of the recombinant VSVs. None of the animals vaccinated with VSVΔG/MARVGP-Musoke showed any evidence of clinical illness either after vaccination or after the MARV challenge. Importantly, all of the animals vaccinated with VSVΔG/MARVGP-Musoke survived against either a heterologous challenge with MARV-Ravn or MARV-Angola with no observable clinical changes. In contrast, both the MARV-Ravn (Control #1) and the MARV-Angola (Control #2) control animals followed a typical disease course and developed fevers, macular rashes and signs of depression by day 6 postinfection, and succumbed to MARV HF on day 8 postinfection (Fig. 2B).

Viremia and blood chemistry

To determine whether viremia or shedding of the recombinant VSVs occurred after immunization whole blood and swab samples from all nine of the vaccinated animals were analyzed by RT-PCR and virus isolation. A transient and low level (≤ 1.7 log10 pfu/ml) recombinant VSV viremia was detected by virus isolation at day 3 after vaccination in plasma from four of the VSVΔG/MARVGP-vaccinated animals (Subject #1, 4, 5, and 7) (Fig. 3A). Also, a low level of VSVΔG/ZEBOVGP was detected by virus isolation from a nasal swab of one of the control animals at day 3. However, this animal had evidence of self-inflicted bleeding around the nares so we are uncertain as to the exact significance of this particular finding. In addition, we did not detect VSVΔG/ZEBOVGP in plasma of this control animal by virus isolation further questioning the importance of the low level of recombinant virus detected from the nasal swab.

Blood samples were also analyzed after MARV challenge for evidence of MARV replication and shedding by plaque assay (Fig. 3B) and by RT-PCR (data not shown). By day 6, both the MARV-Ravn control animal (Control # 1) and the MARV-Angola control animal (Control #2) developed high MARV titers in the blood as detected by plaque assay (> 10^7 log pfu/ml). RT-PCR was more sensitive and showed evidence of MARV in plasma of these two control animals by day 3 postinfection. In contrast, no MARV was detected in the plasma by virus isolation or RT-PCR in any of the animals vaccinated with VSVΔG/MARVGP.

Analysis of blood chemistry and hematology was performed before and after the MARV challenges. Again, in accordance with no clinical symptoms none of the animals vaccinated with VSVΔG/MARVGP showed any evidence of changes in blood chemistry

and hematology either after vaccination or after the MARV challenges. In contrast, while neither of the control animals showed any of evidence of changes in blood chemistry or hematology after vaccination with VSVΔG/ZEBOVGP, both controls developed lymphopenia and thrombocytopenia after the MARV challenges. In addition, both of these controls showed significant elevations in circulating levels of enzymes (ALP, AST, and ALT) associated with impairment of the liver by day 6 postinfection.

Evaluation of antibody and cellular immune response

The antibody responses of the cynomolgus macaques immunized with VSVΔG/MARVGP were evaluated after vaccination (day -14, day 0) and after MARV challenge (day 14, day 28) by IgG ELISA and by plaque neutralization tests ($PRNT_{50}$). All of the animals developed high anti-MARV IgG antibody levels (≥ 1:1000) by the day of the MARV challenges (day 0) (Fig. 4A). Low levels of anti-MARV neutralizing antibodies (1:10 to 1:20) were observed in four of the seven animals vaccinated with VSVΔG/MARVGP at the day of MARV challenge (Fig. 4B). All seven of these animals developed low levels of neutralizing antibodies (1:10 to 1:80) by day 28 after the MARV challenges (Fig. 4B).

To better understand the cellular responses of T-cell populations found in peripheral blood mononuclear cell fractions of specifically and nonspecifically vaccinated animals in mediating protection against MARV challenge, flow cytometry was employed during the course of study. There was no evidence of either IFN-γ or TNF-α production in CD4 or CD8 T-cell populations either before or after the MARV challenges in any of the animals employed in this study (data not shown).

Discussion

The recent outbreak of MARV in Angola generated significant interest from the popular press and reinforces the danger of emerging viruses such as MARV as significant public health threats. However, MARV also poses a threat as a potential biological weapon (1). It is primarily for this reason that there has been an increased investment in developing countermeasure against this highly lethal pathogen.

Significant advances have been made over the last decade in developing countermeasures against the filoviruses particularly regarding the creation of promising vaccines (14, 22, 23). Recently, we showed that a replication competent vaccine based on recombinant VSV expressing either the GP of EBOV or the GP of MARV could completely protect nonhuman primates against a homologous filovirus challenge (14). While the EBOV vaccine based on the Zaire species GP completely protected macaques against a lethal ZEBOV challenge, it was unable to protect macaques against challenge with another EBOV species, *Sudan ebolavirus* (SEBOV). This was not an unexpected finding since macaques that survive experimental challenge with wild type SEBOV are not protected against a subsequent back-challenge with ZEBOV (2). Indeed, there is a 37-44% difference between SEBOV and ZEBOV at the nucleotide and amino acid levels (19) further supporting the view that there would be little if any cross-protection among these species of EBOV.

From the perspective of vaccine development it is apparent that a vaccine that would protect against both SEBOV and ZEBOV would likely need to include SEBOV-specific antigens as well as ZEBOV-specific antigens. While there is only a single species of MARV, as noted previously there are two genetically disparate lineages of

MARV with 21-23% difference in amino acids (20). This variation raised concern that the recombinant VSV vaccine based on MARV-Musoke may not protect against more divergent strains of MARV. Importantly, we show in the current study that the VSVΔG/MARVGP vector does in fact protect nonhuman primates against a lethal challenge with one of the most divergent MARV strain, Ravn, and also against challenge with the more closely related but ostensibly most pathogenic strain of MARV, Angola. Thus, the VSV-based approach seems to be superior over the only other successful approach using VEEV MARV GP and/or VEEV MARV NP which protected nonhuman primates against a lethal homomogous challenge (9), but failed to protect against a lethal heterologous challenge using the Ravn strain (10).

Regarding the mechanism by which the VSVΔG/MARVGP vaccine protects, results of the current effort are consistent with our original vaccination study (14) as well as a recent postexposure treatment study (5) as protection of monkeys by the VSVΔG/MARVGP vaccine in both cases appeared to be associated primarily with the humoral but not the cellular immune response. Notably, neutralizing antibodies were poorly induced in the animals in this study as well as in both previous studies (5, 14), suggesting that protection may in part be due to non-neutralizing antibodies which were present at high levels in these animals.

The replication competent VSV-based vaccine platform has a number of advantages over successful but replication-defective systems such as the adenovirus system using human adenovirus 5 vectors (22, 23). First, a significant concern regarding many vector-based vaccine systems, in particular adenovirus 5, is anti-vector immunity (3). Importantly, there is a very low percentage of VSV seropositivity in the general

population (24). In addition, any anti-vector immunity that may be present in a very small number of individuals may not be important at all because in VSV infections neutralizing antibodies are directed against the VSV glycoprotein, which is not expressed using the recombinant vector employed here (7). Second, durability is a major concern of any vaccine platform, in particular, for vaccines designed to be used against exotic pathogens such as viral hemorrhagic fevers, which are primarily found in remote geographic locations where boosting is often difficult and not practicable. In general, live attenuated vaccines give long-lasting immunity after a single administration. For example, the highly attenuated yellow fever vaccine confers near complete protection that persists for 30 or more years (15). While replication-defective viruses have a number of advantages over classical inactivated virion vaccine approaches, the durability of such replication-defective vaccines compared to live attenuated vaccines is largely unknown. Safety is of course a serious concern that is associated with the use of any live vaccine. Currently, replication-defective VSV vectors capable of only a single cycle of replication are being developed as an alternative to replication-competent VSV vectors (17). Whether these vectors can confer protective immunity against highly lethal pathogens such as the hemorrhagic fever viruses, and whether there will be any trade off regarding durability for potential safety remains to be determined.

Not only have live attenuated VSV-based vaccines shown promise in nonhuman primate models of EBOV and MARV infections, but we also recently demonstrated the success of this platform in protecting monkeys against a lethal Lassa virus challenge (8). Others are using replication competent VSV-based systems as candidate vaccines for a variety of viruses including HIV. In fact, a candidate recombinant VSV-based HIV

vaccine was shown to prevent AIDS-like disease in monkeys (18). Moreover, in an effort to address safety concerns with live vaccines, Wyeth is currently engineering VSV vectors that are highly attenuated in animals. The company plans to begin human trials with these vectors expressing HIV antigens within a year (11).

The use of attenuated recombinant VSV-based vectors has proven to be an effective and promising platform for the development of preventive vaccines against a number of pathogenic viruses. Findings from the current study taken together with observations from our previous work (5, 14) suggest that a vaccine that would confer protection against all relevant species or strains of filoviruses would likely require three antigens including those specific for the ZEBOV GP, the SEBOV GP, and the MARV-Musoke GP.

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recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

References

1. **Borio, L., T. Inglesby, C. J. Peters, A. L. Schmaljohn, J. M. Hughes, P. B. Jahrling, T. Ksiazek, K. M. Johnson, A. Meyerhoff, T. O'Toole, M. S. Ascher, J. Bartlett, J. G. Breman, E. M. Eitzen, Jr., M. Hamburg, J. Hauer, D. A. Henderson, R. T. Johnson, G. Kwik, M. Layton, S. Lillibridge, G. J. Nabel, M. T. Osterholm, T. M. Perl, P. Russell, and K. Tonat.** 2002. Hemorrhagic fever viruses as biological weapons: medical and public health management. Jama **287:**2391-405.
2. **Bowen, E. T., G. S. Platt, G. Lloyd, R. T. Raymond, and D. I. Simpson.** 1980. A comparative study of strains of Ebola virus isolated from southern Sudan and northern Zaire in 1976. J Med Virol **6:**129-38.
3. **Chirmule, N., K. Propert, S. Magosin, Y. Qian, R. Qian, and J. Wilson.** 1999. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther **6:**1574-83.
4. **Colebunders, R., H. Sleurs, P. Pirard, M. Borchert, M. Libande, J. P. Mustin, A. Tshomba, L. Kinuani, L. A. Olinda, F. Tshioko, and J. J. Muyembe-Tamfum.** 2004. Organisation of health care during an outbreak of Marburg haemorrhagic fever in the Democratic Republic of Congo, 1999. J Infect **48:**347-53.

5. **Daddario-Dicaprio, K. M., T. W. Geisbert, U. Stroher, J. B. Geisbert, A. Grolla, E. A. Fritz, L. Fernando, E. Kagan, P. B. Jahrling, L. E. Hensley, S. M. Jones, and H. Feldmann.** 2006. Postexposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment. Lancet **367:**1399-1404.

6. **Feldmann, H., T. W. Geisbert, P. B. Jahrling, H. D. Klenk, S. V. Netesov, C. J. Peters, A. Sanchez, R. Swanepoel, and V. E. Volchkov.** 2004. Filoviridae, p. 645-653. *In* C. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (ed.), Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London.

7. **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.

8. **Geisbert, T. W., S. Jones, E. A. Fritz, A. C. Shurtleff, J. B. Geisbert, R. Liebscher, A. Grolla, U. Stroher, L. Fernando, K. M. Daddario, M. C. Guttieri, B. R. Mothe, T. Larsen, L. E. Hensley, P. B. Jahrling, and H. Feldmann.** 2005. Development of a new vaccine for the prevention of Lassa fever. PLoS Med **2:**e183.

9. **Hevey, M., D. Negley, P. Pushko, J. Smith, and A. Schmaljohn.** 1998. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. Virology **251:**28-37.

10. **Hevey, M., D. Negley, A. Staley, and A. Schmaljohn.** 2001. Determination of vaccine components required for protecting cynomolgus macaques against genotypically divergent isolates of Marburg virus, Abstract No. W36-4, 20th Annual Meeting of the American Society for Virology, Madison, Wisconsin, USA.

11. **International AIDS Vaccine Initiative.** 2005. Annual AIDS vaccine meeting highlights recent data from clinical trials and lessons on recruitment and retention of volunteers. International AIDS Vaccine Initiative, http://www.iavireport.org/Issues/Issue9-4/promise.asp.

12. **International Society for Infectious Diseases.** 2005. Marburg Hemorrhagic Fever-Angola. http://www.promedmail.org Archive No. 20051108.3269).

13. **Johnson, E. D., B. K. Johnson, D. Silverstein, P. Tukei, T. W. Geisbert, A. N. Sanchez, and P. B. Jahrling.** 1996. Characterization of a new Marburg virus isolated from a 1987 fatal case in Kenya. Arch Virol Suppl **11:**101-14.

14. **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.

15. **Lefeuvre, A., P. Marianneau, and V. Deubel.** 2004. Current Assessment of Yellow Fever and Yellow Fever Vaccine. Curr Infect Dis Rep **6:**96-104.

16. **Martini, G.** 1971. Marburg Virus Disease. Clinical Syndrome, p. 1-9. *In* M. GA and R. Siegert (ed.), Marburg Virus Disease. Springer-Verlag, New York.

17. **Publicover, J., E. Ramsburg, and J. K. Rose.** 2005. A single-cycle vaccine vector based on vesicular stomatitis virus can induce immune responses comparable to those generated by a replication-competent vector. J Virol **79:**13231-8.

18. **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.

19. **Sanchez, A., S. G. Trappier, B. W. Mahy, C. J. Peters, and S. T. Nichol.** 1996. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. Proc Natl Acad Sci U S A **93:**3602-7.

20. **Sanchez, A., S. G. Trappier, U. Stroher, S. T. Nichol, M. D. Bowen, and H. Feldmann.** 1998. Variation in the glycoprotein and VP35 genes of Marburg virus strains. Virology **240:**138-46.

21. **Smith, D. H., B. K. Johnson, M. Isaacson, R. Swanapoel, K. M. Johnson, M. Killey, A. Bagshawe, T. Siongok, and W. K. Keruga.** 1982. Marburg-virus disease in Kenya. Lancet **1:**816-20.

22. **Sullivan, N. J., T. W. Geisbert, J. B. Geisbert, L. Xu, Z. Y. Yang, M. Roederer, R. A. Koup, P. B. Jahrling, and G. J. Nabel.** 2003. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. Nature **424:**681-4.

23. **Sullivan, N. J., A. Sanchez, P. E. Rollin, Z. Y. Yang, and G. J. Nabel.** 2000. Development of a preventive vaccine for Ebola virus infection in primates. Nature **408:**605-9.

24. **Wagner, R. R., and J. K. Rose.** 1996. Rhabdoviridae: The Viruses and their Replication, p. 1121-1135. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology. Lippincott Williams & Wilkins, Philadelphia.

25. **World Health Organization.** 2005. Marburg haemorrhagic fever, Angola. Wkly Epidemiol Rec **80:**158-9.

Figures

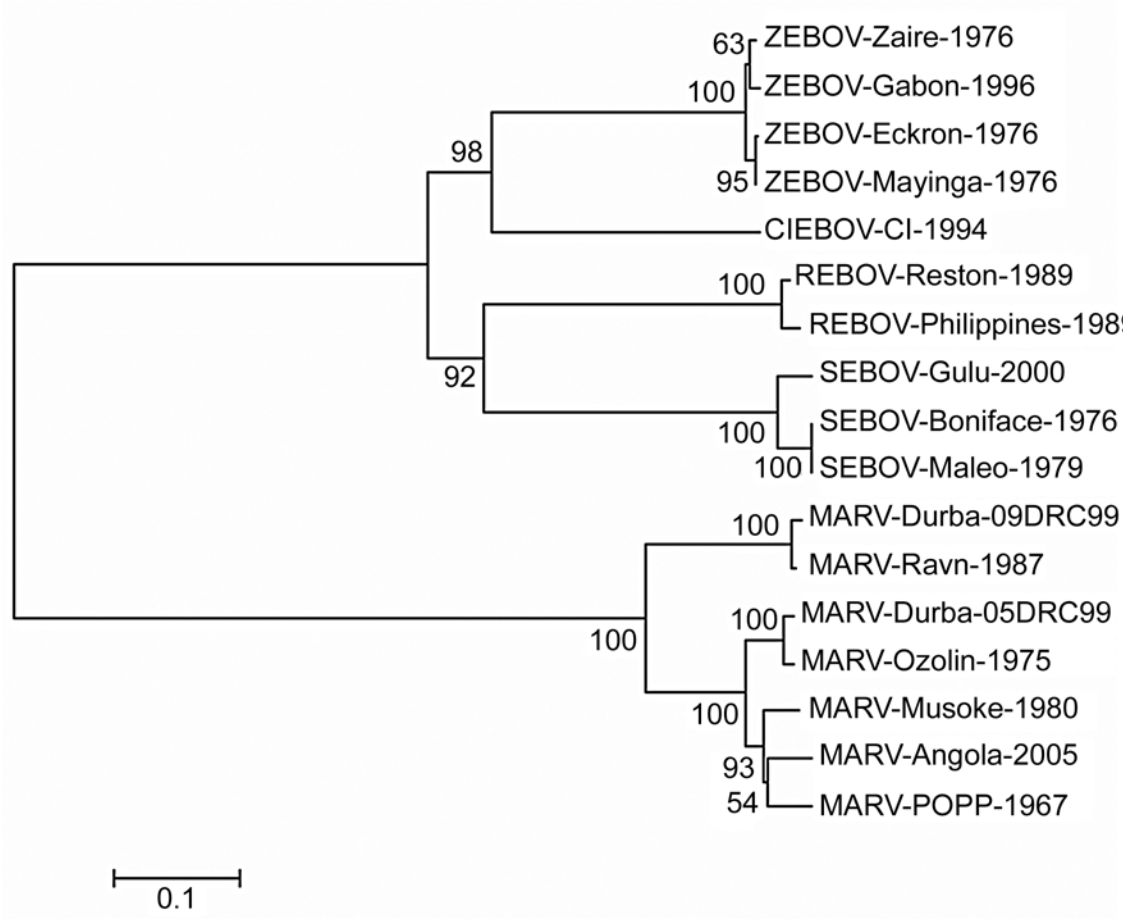

Figure 1. Phylogenetic tree analysis for the GPs of filoviruses. The amino acid sequences of filovirus GPs present in the protein database of GenBank were analyzed using MEGA version 3.1 (www.megasoftware.net). A neighbor-joining tree and 1000 bootstrap replicates for branch points was prepared. The analysis shows that the GP of MARV-Angola is more closely related to MARV-Musoke than to MARV-Ravn and has substantial differences with ZEBOV species. With the exception of the two Durba strains, the assigned abbreviations are from Virus Taxonomy, The Eighth Report of the International committee on Taxonomy of Viruses (6).

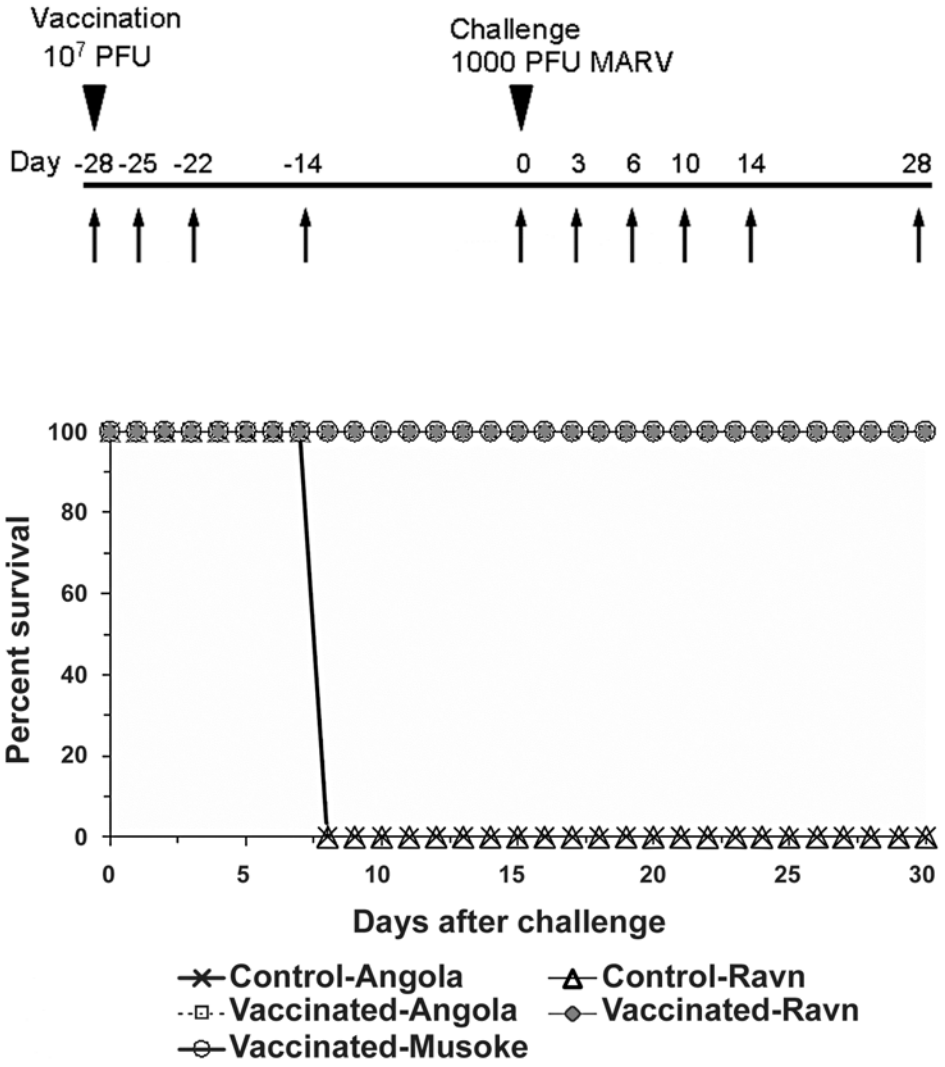

Figure 2. Vaccination and challenge of nonhuman primates. (A) Flow chart of the experimental design. Arrows indicate the days of sampling (blood and swabs). (B) Kaplan-Meier mortality chart of the MARV vaccine study. Open triangle, animal vaccinated with VSVΔG/EBOVGP and challenged with MARV-Ravn (Control #1); x, animal vaccinated with VSVΔG/EBOVGP and challenged with MARV-Angola (Control # 2); solid diamond, animals vaccinated with VSVΔG/MARVGP and challenged with MARV-Ravn (Subjects #1-3); open square, animals vaccinated with VSVΔG/MARVGP and challenged with MARV-Angola (Subjects #4-6); and open circle, animal vaccinated with VSVΔG/MARVGP and challenged with MARV-Musoke.

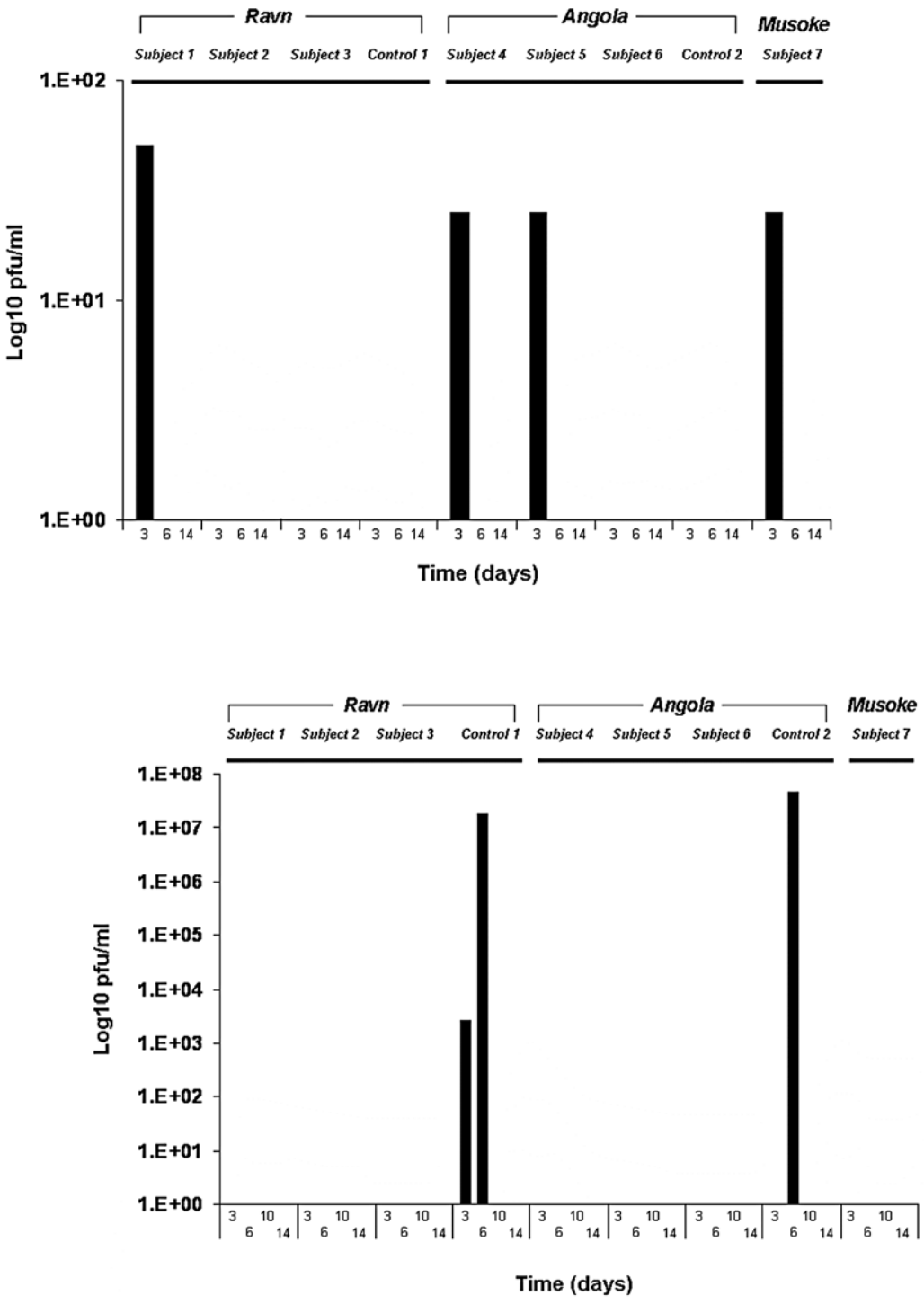

Figure 3. Viremia levels in nonhuman primates after vaccination and MARV challenge. (A) VSV viremia levels were determined after vaccination with VSVΔG/MARVGP (Subjects #1-7) or VSVΔG/ZEBOVGP (Controls # 1 and 2). (B) MARV viremia levels after challenge with MARV-Ravn, MARV-Angola, or MARV-Musoke from plasma taken at the indicated time points. Viremias were determined by plaque assay.

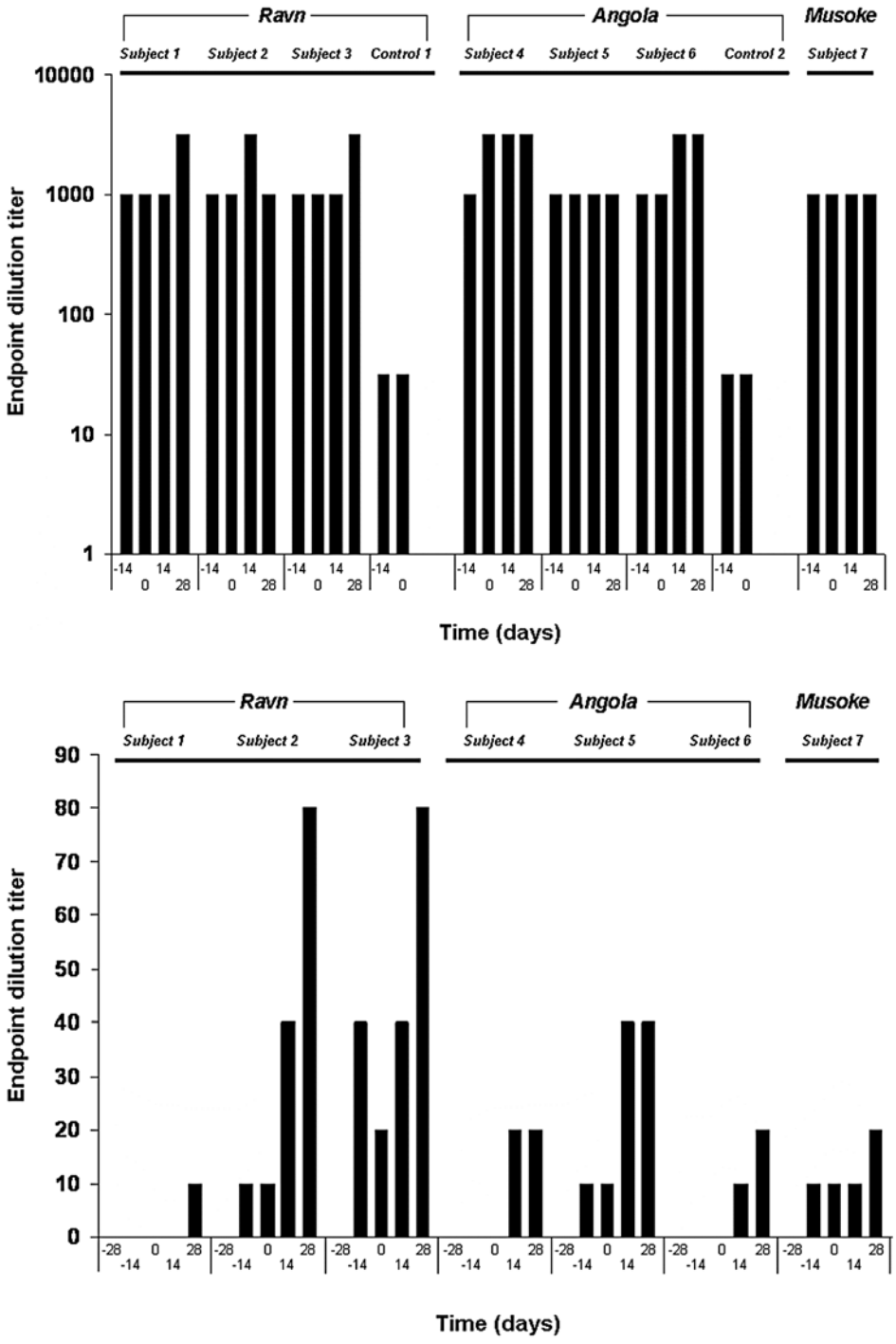

Figure 4. Humoral immune response in nonhuman primates to MARV before and after challenge. (A) IgG responses to MARV measured using an established ELISA (see under 'Methods'). Titers are presented as endpoint dilutions. (B) MARV neutralizing antibodies detected using a plaque reduction neutralization assay ($PRNT_{50}$) as described under 'Methods'. Titers are presented as endpoint dilutions. –, days prior to challenge; 0, day of challenge.

V. **Postexpsoure protection against Marburg hemorrhagic fever with recombinant vesicular stomatitis virus vectors in nonhuman primates: an efficacy assessment.** ***Lancet.*** **Apr 29;367 (9520): 1399-404**.

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Abstract

Background Effective countermeasures are urgently needed to prevent and treat infections caused by biological threat agents such as Marburg virus (MARV). Recently, we showed that a single injection of a replication-competent vaccine based on attenuated recombinant vesicular stomatitis virus (rVSV), elicited completely protective immune responses in nonhuman primates against a lethal MARV challenge. Here, we tested the concept that this rVSV-based MARV vaccine may also have utility as a postexposure treatment for MARV hemorrhagic fever (HF).

Methods We used a rhesus macaque model of MARV HF that produced 100% mortality. We administered rVSV vectors expressing the MARV Musoke strain glycoprotein to five macaques approximately 20 minutes after a high-dose lethal injection of homologous MARV. Three animals served as MARV-positive controls and received nonspecific rVSV vectors.

Findings All five rhesus monkeys that were treated with the rVSV MARV vectors as a postexposure treatment survived a high dose lethal challenge of MARV. None of these five animals developed clinical symptoms consistent with MARV HF. Importantly, all three of the control animals developed fulminant MARV HF and succumbed to the MARV challenge.

Interpretation This first demonstration of postexposure protection against MARV in nonhuman primates provides a new paradigm for the treatment of MARV HF. Indeed,

these data suggest that rVSV-based filoviral vaccines may not only have potential as preventive vaccines but may be equally useful for postexposure treatment of filoviral infections.

Introduction

Marburg virus (MARV) causes a severe and often fatal hemorrhagic disease for which there is currently no vaccine or therapy approved for human use. The reported potential of MARV as a biological weapon[1] and the recent attention drawn to outbreaks of emerging and re-emerging viruses, such as the 2005 epidemic of MARV hemorrhagic fever (HF) in Angola[2], has significantly increased public recognition of this deadly pathogen.

The recent MARV outbreak in Angola, with case fatality rates approaching 90 percent, calls attention to the fact that there is a critical and pressing need for effective countermeasures against the filoviruses. To date, the only available form of treatment for MARV HF is intensive supportive care. The development of effective treatments and therapies for MARV HF has been an ongoing challenge since the disease was first discovered. The requirement for biosafety level (BSL)-4 containment has served as a major impediment towards the development of MARV therapeutics.

Guinea pig and nonhuman primate (NHP) models have been developed for MARV HF [3-10]. While several studies have employed these models to evaluate candidate vaccines, relatively few studies have examined postexposure interventions. Several immunomodulatory drugs including desferal, ridostin, and polyribonate were evaluated in guinea pig models of experimental MARV infection; partial protection and slight increases in mean time to death were observed in these studies[4,10]. Despite the ability of several of these drugs to induce protective responses in guinea pigs, the efficacy and action of these immunomodulators in NHPs has yet to be determined. In addition, evaluation of drugs such as interferon and ribavirin showed that neither approach had

therapeutic potential against MARV infection in NHP and guinea pig models, respectively [4, 8,10].

Notwithstanding the slow progress in developing therapies against MARV HF, important advances in developing preventive vaccines have been made. Recently, we described the generation and evaluation of a live, attenuated, recombinant vesicular stomatitis virus (rVSV) expressing the transmembrane glycoprotein (GP) of MARV (VSVΔG/MARVGP) [9,11] and demonstrated that vaccination with this vector completely protected NHPs against a lethal MARV challenge[9]. The rVSV vaccine platform demonstrates great potential as a preventive strategy against MARV infection; however, despite the potential of rVSV as a preexposure prophylaxis, the efficacy of this system as a postexposure prophylaxis has yet to be determined.

The capacity of vaccines as postexposure treatments is demonstrated by the management of a number of viral infections including rabies[12,13], hepatitis B [14], and smallpox [15,16]. The utility of postexposure vaccination in managing these viral diseases and the success of our VSVΔG/MARVGP vector as a preventative one-shot vaccine has lead us to evaluate the therapeutic efficacy of a postexposure vaccination strategy employing VSVΔG/MARVGP vectors in a NHP model of lethal MARV HF.

Materials and Methods

Recombinant vectors and virus

The rVSV expressing the GPs of MARV strain Musoke (MARV-Musoke) and Zaire EBOV (ZEBOV) (strain Mayinga) were generated as described recently using the

infectious clone for the VSV Indiana serotype (kindly provided by John Rose, Yale University, New Haven, Connecticut, United States)[11]. Briefly, the appropriate open reading frames for the GPs were generated by PCR, cloned into the VSV genomic vectors lacking the VSV surface glycoprotein (G) gene, sequenced-confirmed, and rescued using the method described earlier[11,17]. The recombinant viruses expressing MARV-Musoke GP and ZEBOV GP were designated VSVΔG/MARVGP and VSVΔG/ZEBOVGP, respectively. MARV-Musoke was isolated from a human case in 1980 in Kenya[18].

Animal studies

Eight healthy rhesus macaques (*Macaca mulatta*), 4-6 yrs old and weighing between 3 kg and 6 kg were employed in this study. Animals were inoculated intramuscularly (i.m.) with 1000 plaque forming units (pfu) of MARV-Musoke. Approximately 20 min after MARV challenge, five of the eight animals were treated i.m. with a total of 1×10^7 pfu of VSVΔG/MARVGP vectors divided among four different anatomical locations (right and left tricep, and right and left caudal thigh). The three remaining animals served as experimental controls and were treated with an equivalent dose of VSVΔG/ZEBOVGP vectors. Animals were closely monitored for evidence of clinical symptoms. Blood was collected before MARV challenge and on d 3, 6, 10, 14, 22, and 37 after MARV challenge. Animal studies were performed in a BSL-4 containment laboratory at USAMRIID and approved by the USAMRIID Laboratory Animal Care and Use Committee. Animal research was conducted in compliance with the Animal Welfare Act and other Federal statues and regulations relating to animals and experiments involving

animals, and adheres to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility used is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Virus detection

RNA was isolated from blood using the appropriate RNA isolation kits (QIAGEN, Ontario, Canada). For the detection of VSV we used a RT-PCR assay targeting the matrix gene (nt position 2355-2661, NC_001560)[9]. The low detection limit for this assay is 100 pfu per ml of plasma. MARV viral RNA was detected using primer pairs targeting the L gene[9]. The low detection limit for this MARV assay is 0.1 pfu per ml of plasma. Levels of infectious MARV were determined by plaque assay on Vero E6 cells from all blood samples[19]. In brief, we adsorbed increasing 10-fold dilutions of plasma to Vero E6 monolayers in duplicate wells of a standard 6-well plate (0.2 ml per well); thus, the limit for detection of this plaque assay was 25 pfu/ml.

Hematology and serum biochemistry

Total white blood cell counts, red blood cell counts, platelet counts, hematocrit values, total hemoglobin, mean cell volume, mean corpuscular volume, and mean corpuscular hemoglobin concentration were determined from blood samples collected in tubes containing EDTA, by using a laser-based hematologic analyzer (Coulter Electronics, Hialeah, Florida, United States). The white blood cell differentials were performed manually on Wright-stained blood smears. Serum samples were tested for concentrations

of albumin (ALB), amylase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), glucose, cholesterol, total protein, total bilirubin (TBIL), urea nitrogen (BUN), and creatinine (CRE) by using a Piccolo Point-Of-Care Blood Analyzer (Abaxis, Sunnyvale, California, United States).

Humoral immune response

IgG and IgM antibodies against MARV were detected by an ELISA using purified viral particles as an antigen source[9]. Neutralization assays were performed by measuring plaque reduction in a constant virus-serum dilution format as previously described[9]. Briefly, we incubated a standard amount of MARV (~ 100 pfu) with serial dilutions (two-fold) of serum samples for 60 min. The mixture was then used to inoculate Vero E6 cells for 60 min. Cells were overlaid with an agar medium, incubated for 8 d, and plaques were counted 48 h after neutral red staining. Endpoint titers were determined by the dilution of serum which neutralized 50% of the plaques ($PRNT_{50}$).

Cellular immune responses

The method for assessment of T-cell responses to MARV was previously shown[9]. Briefly, peripheral blood mononuclear cells (PBMC) from rhesus macaques were isolated by histopaque gradient (Sigma, St. Louis Missouri). Approximately 1 $x10^6$ cells were stimulated in 200 µl of RPMI (Gibco) for 6 h at 37ºC with antibodies specific for CD28 and CD49d and either dimethylsulfoxide or a pool of 15-nucleotide coding sequences for peptides spanning the open reading frames for the gene encoding the MARV-Musoke GP

in the presence of brefeldin A. The peptides were 15 amino acids in length, overlapping by 11, and were used at a final concentration of 2 μg/ml. Cells were fixed and permeabilized with FACS lyse (Becton Dickinson) supplemented with Tween-20, and then stained with a mixture of antibodies against lineage markers (CD3, CD4, CD8) and either TNF-α or IFN-γ. Samples were run on a FACSCalibur and analyzed using the software FlowJo. Cytokine-positive cells were defined as a percentage within individual lymphocyte subsets, and at least 200,000 events were analyzed for each sample.

Results

Clinical Illness

A total of eight rhesus monkeys were used to evaluate the therapeutic efficacy of a postexposure treatment strategy employing the VSVΔG/MARVGP vectors against MARV HF. We treated five rhesus monkeys with i.m. injections of VSVΔG/MARVGP vectors (Subjects #1 through # 5) and four rhesus monkeys with nonspecific VSVΔG/ZEBOV vectors (controls) (Subjects #6 through # 8). Treatments were initiated approximately 20 min after challenge with MARV.

Three of the five animals challenged with MARV and subsequently treated with the VSVΔG/MARVGP vectors became febrile by d 6; however, body temperatures returned to prechallenge values by d 10. Importantly, all five of these animals survived the MARV challenge. In contrast, one of the three control animals (treated with nonspecific VSVΔG/ZEBOVGP vectors) developed a fever at d 6 and the remaining two control animals became febrile by d 10. All three control animals developed macular

rashes by d 10 and succumbed to the MARV challenge with one animal expiring on d 11 and two animals expiring on d 12 (figure 1).

To determine whether viremia of the rVSV vectors occurred after treatment, whole blood samples from all eight treated animals were analyzed by RT-PCR (data not shown). A transient rVSV viremia was detected in four of the five VSVΔG/MARVGP-treated animals and two of the three control animals on d 3. MARV replication was also analyzed from blood samples taken after MARV challenge and rVSV vector treatment (figure 2). All three of the control animals developed high MARV titers by d 6 (~ 10^3 – 10^5 pfu/ml). In contrast, no MARV was detected in plasma by plaque assay at any time point from any of the five animals treated with the VSVΔG/MARVGP vectors after the MARV challenge. However, RT-PCR showed a transient MARV viremia at d 3 in four of these five specifically treated animals.

Analysis of blood chemistry and hematology was performed before and on d 3, 6, 10, and 14 after the MARV challenge. No substantial changes (greater than threefold change over prechallenge values) were detected in any of five animals treated with the VSVΔG/MARVGP vectors during the course of this study. In contrast, all three control animals developed leukocytosis with concurrent neutrophilia at end-stage disease. In addition, the three control animals showed considerable increases in circulating levels of ALP, ALT, AST, GGT, and TBIL at d 10 suggesting severe damage to the liver. Two of the three control animals also showed substantial increases in levels of BUN at d 10 and decreases in levels of AMY at d 10, indicating possible injury of the kidneys and pancreas, respectively.

Humoral and Cellular Immune Response

The serological response profile of MARV infection after treatment was evaluated by plaque reduction neutralization test ($PRNT_{50}$) and by IgM and IgG ELISAs. All five of the animals challenged with MARV and treated with VSVΔG/MARVGP vectors demonstrated low to moderate levels of IgM by d 6 (≥ 1:32) (figure 3A); moderate levels of IgG were seen in four of the five treated animals (≥ 1:100) at d 10 (figure 3B). Plaque reduction neutralization tests demonstrated neutralizing antibody titers (≥ 1:10) at d 6 through d 37 in all five animals treated with the VSVΔG/MARVGP vectors (figure 4).

To better understand the function of T lymphocytes in mediating protection against MARV challenge, flow cytometry was employed during the course of the study. Intracellular staining of PBMC fractions showed an absence of IFN-γ and TNF-α induction in all animals, suggesting an absence of T lymphocyte activation (data not shown).

Discussion

Use of the rVSV-based vector system as a countermeasure against MARV HF demonstrates dual utility both as a potential preventive vaccine[9] and now as a possible postexposure treatment. Here, we demonstrated that the rVSV-based vectors expressing the GP of MARV were capable of mediating postexposure protection against a homologous MARV challenge in NHPs. The mechanism and correlates of protection against MARV remain to be determined. There are a number of possibilities including interference or competition for target cells caused by the rVSV vectors. Noble and colleagues described a noninfectious, defective interfering influenza A virus that

interfered with the replication of a virulent influenza A virus when the viruses were administered simultaneously to mice[20]. In addition, this interference prevented clinical disease in the mice. Our rVSV vectors, which exploit the MARV GP for binding and entry, could in fact interfere with MARV replication as they target the same host cells as wild type MARV. Even an alteration or delay in the disease course may be enough to tip the balance in favor of the host. In addition to interference, other possible protective mechanisms may include activation of the innate immune system or specific activation of cellular or humoral immune responses.

Our results suggest that the VSVΔG/MARVGP vectors induced protection at least partially through responses to the surface GP, presumably by stimulation of GP-specific neutralizing antibodies. Specifically, low to moderate levels of neutralizing antibodies were detected in sera by d 6 after challenge. Although these data suggest that neutralizing antibodies may play a role in postexposure protection, the contribution of non-neutralizing antibodies and the therapeutic activity of antibody-mediated effector mechanisms also merit consideration. Indeed, recent studies evaluating VSVΔG/MARVGP vectors as a preventative one-shot vaccine against MARV suggested that protection was associated with humoral immune responses, notably by non-neutralizing antibodies as neutralizing antibodies were poorly induced[9]. There are many examples of preventative vaccine approaches linking protective immune responses with non-neutralizing antibodies. For example, monoclonal antibodies specific for the VSV G protein that had no neutralizing activity against VSV *in vitro* were shown to completely protect mice against a lethal VSV challenge[21]. Also, relevant to our current results is a study employing mice vaccinated with a similar rVSV vector lacking the VSV G gene[22] .

In this study the VSV G gene was replaced with the fusion G gene of respiratory syncytial virus (RSV); mice were then vaccinated with this rVSV vaccine. Importantly, the vaccine induced detectable serum antibodies against RSV by ELISA, but no detectable neutralizing antibodies, yet still protected the mice from RSV challenge.

The importance of the antibody response in the success of the VSVΔG/MARVGP vectors is further supported by the lack of detectable cellular immune responses observed in the VSVΔG/MARVGP-treated animals. The lack of T lymphocyte activation observed in this study suggests that antibody production may in part occur through a T lymphocyte-independent (TI) mechanism. Protective levels of antibodies have been observed for a number of viral agents including VSV[23], influenza[24-26], and polyomavirus[27] in T lymphocyte-deficient mice. TI production of IgM may occur via strong cross-linking of the B cell receptor. Antigens that are capable of induction of antibody in a TI fashion are often composed of rigidly arranged repetitive antigenic determinants spaced 5-10 nm apart. Previous work with VSV suggested that particle-associated G protein, rather than soluble portions of the G protein or the G protein alone, is necessary for TI antibody induction[28,29]. Because our rVSV vectors are relatively uncomplicated GP exchange vectors, it would not be surprising if the MARV GP were incorporated into the VSV virion structure in a manner similar to authentic VSV G; therefore, MARV GP in the background of the rVSV vector may also be capable of inducing antibody production by a TI mechanism.

Clearly, more studies are needed to unravel the mechanisms by which VSVΔG/MARVGP vectors mediate protective immune responses as a postexposure treatment. However, it also clear that these results have important clinical implications

and offer a new approach for treating MARV HF and perhaps in tackling other viral HFs. In addition, these results suggest that single-shot vaccination regimens using these rVSV vectors as preventive vaccines (currently 28 d between vaccination and filovirus challenge) can be significantly reduced.

Acknowledgments

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References

1. Borio L, Inglesby T, Peters CJ, et al. Hemorrhagic fever viruses as biological weapons: medical and public health management. *Jama* 2002;**287**(18)**:**2391-405.
2. World Health Organization. Marburg haemorrhagic fever, Angola. *Wkly Epidemiol Rec* 2005;**80**(18)**:**158-9.
3. Ignat'ev GM, Strel'tsova MA, Agafonov AP, et al. [The immunological indices of guinea pigs modelling Marburg hemorrhagic fever]. *Vopr Virusol* 1994;**39**(4)**:**169-71.

4. Sergeev AN, Lub M, P'Iankova O G, Kotliarov LA. [The efficacy of the emergency prophylactic and therapeutic actions of immunomodulators in experimental filovirus infections]. *Antibiot Khimioter* 1995;**40**(5)**:**24-7.

5. Ignatyev GM, Agafonov AP, Streltsova MA, Kashentseva EA. Inactivated Marburg virus elicits a nonprotective immune response in Rhesus monkeys. *J Biotechnol* 1996;**44**(1-3)**:**111-8.

6. Agafonova OA, Viazunov SA, Zhukov VA, et al. [Relationship between the level of specific antibodies with disease outcome in Cercopithecus aethiops monkeys in experimental Marburg disease]. *Vopr Virusol* 1997;**42**(3)**:**109-11.

7. Hevey M, Negley D, Pushko P, Smith J, Schmaljohn A. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. *Virology* 1998;**251**(1)**:**28-37.

8. Kolokol'tsov AA, Davidovich IA, Strel'tsova MA, Nesterov AE, Agafonova OA, Agafonov AP. The use of interferon for emergency prophylaxis of marburg hemorrhagic fever in monkeys. *Bull Exp Biol Med* 2001;**132**(1)**:**686-8.

9. Jones SM, Feldmann H, Stroher U, et al. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. *Nat Med* 2005;**11**(7)**:**786-90.

10. Ignat'ev GM, Strel'tsova MA, Agafonov AP, Kashentseva EA, Prozorovskii NS. [Experimental study of possible treatment of Marburg hemorrhagic fever with desferal, ribavirin, and homologous interferon]. *Vopr Virusol* 1996;**41**(5)**:**206-9.

11. Garbutt M, Liebscher R, Wahl-Jensen V, et al. Properties of replication-competent vesicular stomatitis virus vectors expressing glycoproteins of filoviruses and arenaviruses. *J Virol* 2004;**78**(10)**:**5458-65.

12. Rupprecht CE, Hanlon CA, Hemachudha T. Rabies re-examined. *Lancet Infect Dis* 2002;**2**(6)**:**327-43.

13. Rupprecht CE, Gibbons RV. Clinical practice. Prophylaxis against rabies. *N Engl J Med* 2004;**351**(25)**:**2626-35.

14. Yu AS, Cheung RC, Keeffe EB. Hepatitis B vaccines. *Clin Liver Dis* 2004;**8**(2)**:**283-300.

15. Massoudi MS, Barker L, Schwartz B. Effectiveness of postexposure vaccination for the prevention of smallpox: results of a delphi analysis. *J Infect Dis* 2003;**188**(7)**:**973-6.

16. Mortimer PP. Can postexposure vaccination against smallpox succeed? *Clin Infect Dis* 2003;**36**(5)**:**622-9.

17. Schnell MJ, Buonocore L, Kretzschmar E, Johnson E, Rose JK. Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses are incorporated efficiently into virus particles. *Proc Natl Acad Sci U S A* 1996;**93**(21)**:**11359-65.

18. Smith DH, Johnson BK, Isaacson M, et al. Marburg-virus disease in Kenya. *Lancet* 1982;**1**(8276)**:**816-20.

19. Jahrling PB, Geisbert TW, Geisbert JB, et al. Evaluation of immune globulin and recombinant interferon-alpha2b for treatment of experimental Ebola virus infections. *J Infect Dis* 1999;**179 Suppl 1:**S224-34.

20. Noble S, McLain L, Dimmock NJ. Interfering vaccine: a novel antiviral that converts a potentially virulent infection into one that is subclinical and immunizing. *Vaccine* 2004;**22**(23-24)**:**3018-25.

21. Lefrancois L. Protection against lethal viral infection by neutralizing and nonneutralizing monoclonal antibodies: distinct mechanisms of action in vivo. *J Virol* 1984;**51**(1)**:**208-14.

22. Kahn JS, Roberts A, Weibel C, Buonocore L, Rose JK. Replication-competent or attenuated, nonpropagating vesicular stomatitis viruses expressing respiratory syncytial virus (RSV) antigens protect mice against RSV challenge. *J Virol* 2001;**75**(22)**:**11079-87.

23. Fehr T, Bachmann MF, Bluethmann H, Kikutani H, Hengartner H, Zinkernagel RM. T-independent activation of B cells by vesicular stomatitis virus: no evidence for the need of a second signal. *Cell Immunol* 1996;**168**(2)**:**184-92.

24. Sha Z, Compans RW. Induction of CD4(+) T-cell-independent immunoglobulin responses by inactivated influenza virus. *J Virol* 2000;**74**(11)**:**4999-5005.

25. Lee BO, Rangel-Moreno J, Moyron-Quiroz JE, et al. CD4 T cell-independent antibody response promotes resolution of primary influenza infection and helps to prevent reinfection. *J Immunol* 2005;**175**(9)**:**5827-38.

26. Graham MB, Braciale TJ. Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J Exp Med* 1997;**186**(12)**:**2063-8.

27. Szomolanyi-Tsuda E, Welsh RM. T cell-independent antibody-mediated clearance of polyoma virus in T cell-deficient mice. *J Exp Med* 1996;**183**(2)**:**403-11.

28. Bachmann MF, Zinkernagel RM. The influence of virus structure on antibody responses and virus serotype formation. *Immunol Today* 1996;**17**(12)**:**553-8.

29. Bachmann MF, Hengartner H, Zinkernagel RM. T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: role of antigen patterns in B cell induction? *Eur J Immunol* 1995;**25**(12)**:**3445-51.

Figures

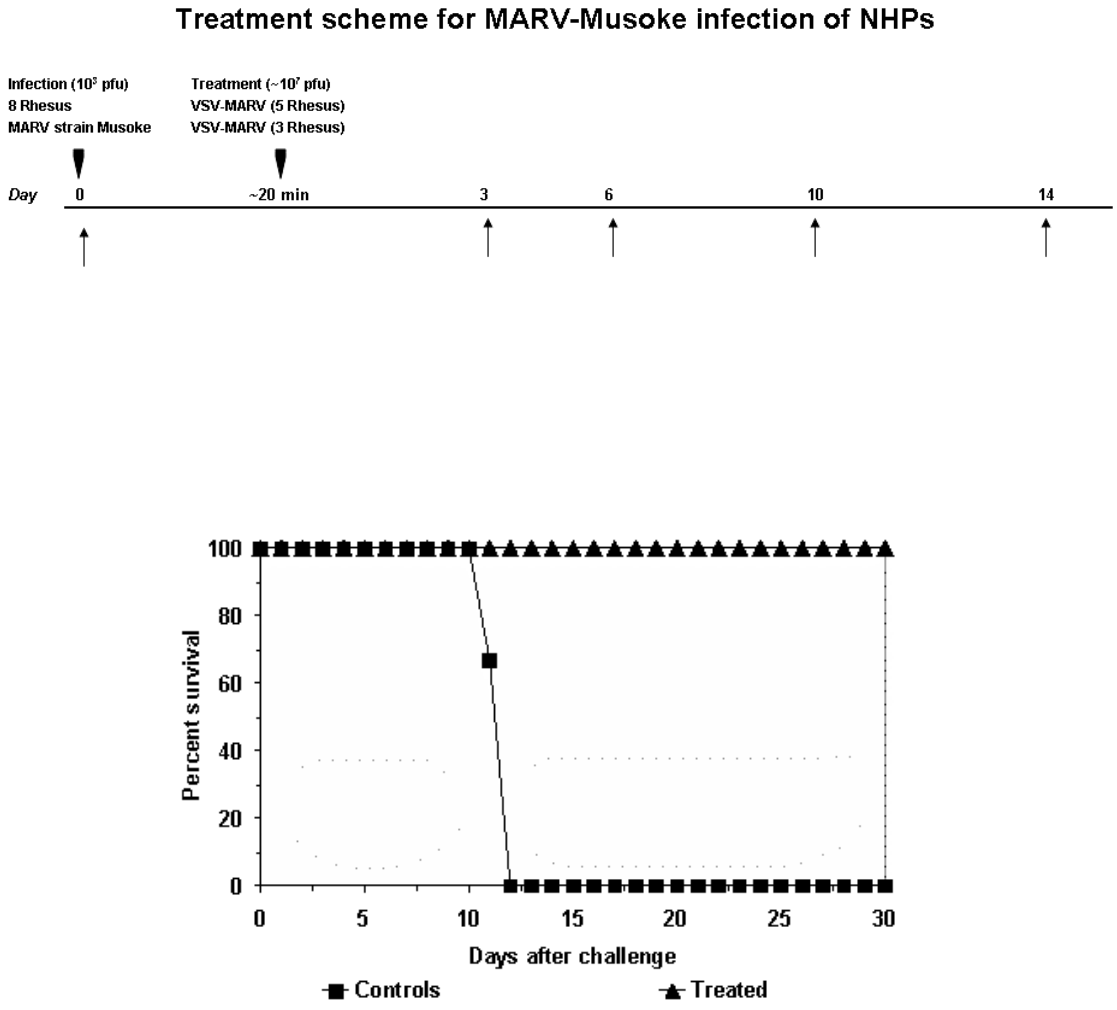

Figure 1. (A) Flow chart of challenge and treatment experiment design. Arrows indicate days of sampling (blood and swabs). Kaplan Meier mortality charts representing (B) VSV-MARV therapy against MARV-Musoke challenge Solid squares, animals treated with irrelevant VSV-EBOV (controls) following challenge; solid triangles, animals treated with VSV-MARV following challenge.

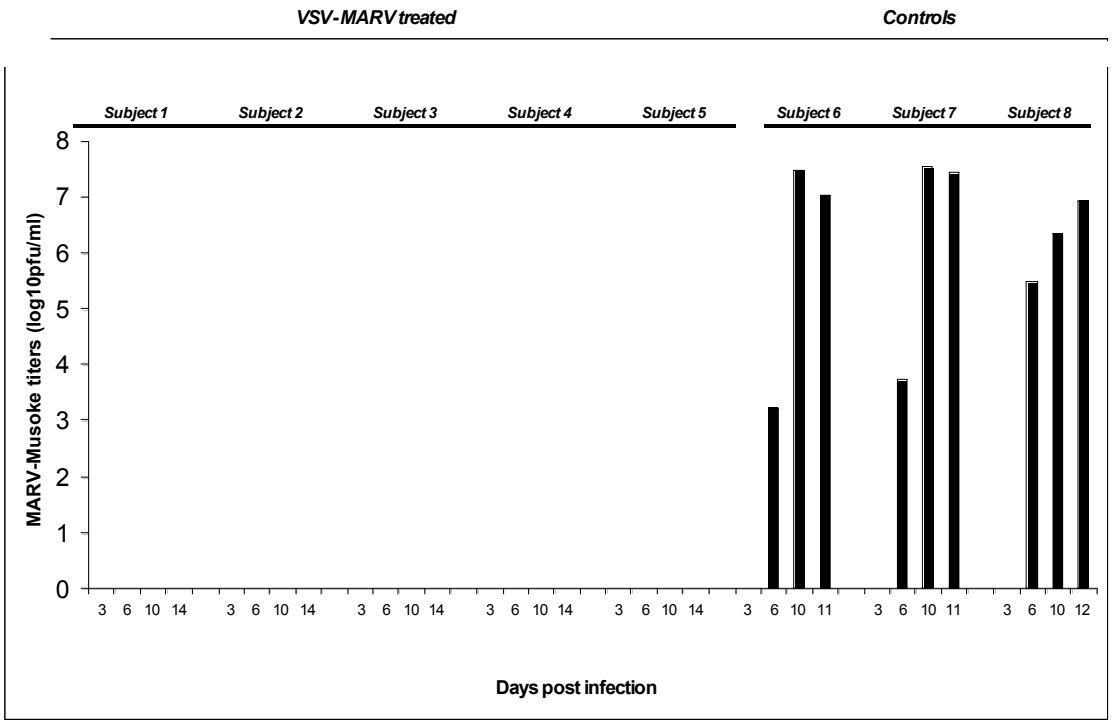

Figure 2. Plasma MARV viremia of NHPs after challenge with MARV and treatment with rVSV vectors. Five animals were treated with VSV-MARV vectors approximately 20 min after challenge with a lethal dose of MARV. Three controls animals were treated with nonspecific VSV-EBOV vectors after MARV challenge. Viremia was determined by plaque assay at the indicated time points.

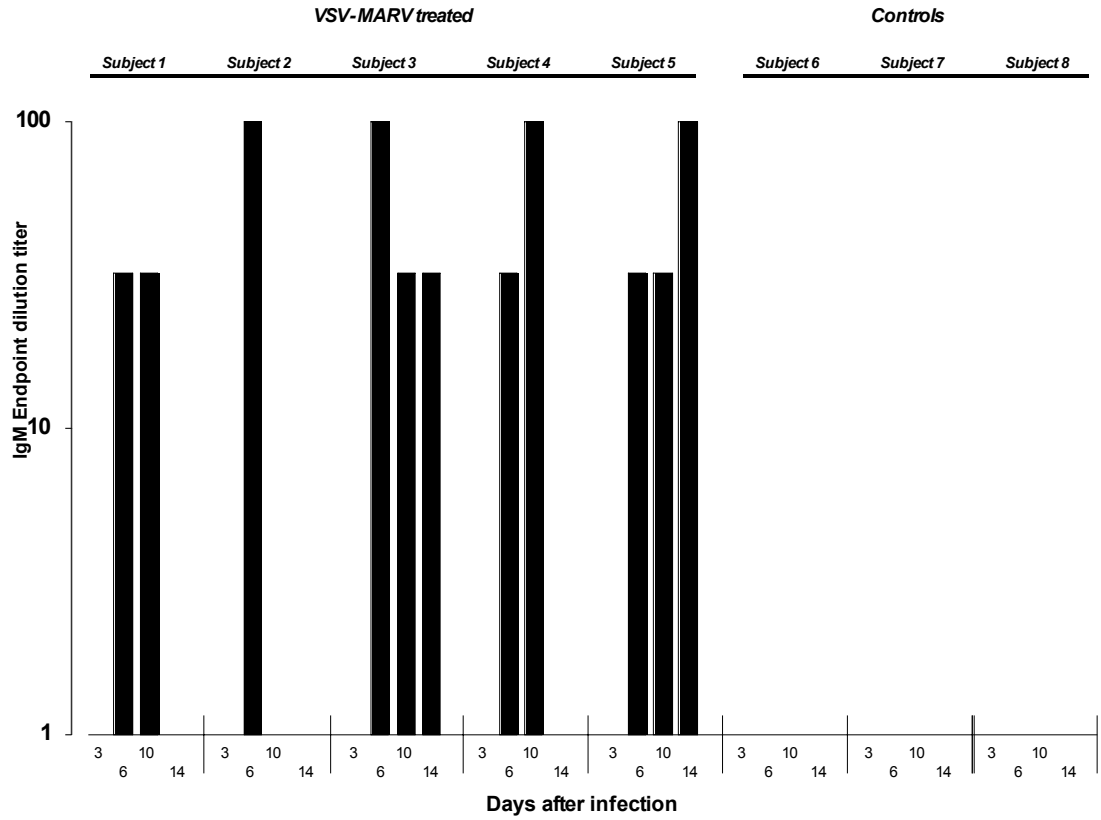

Figure 3A. A) The serological response profile of MARV infection after treatment with rVSV vectors. IgM response profiles of animals treated with VSV-MARV vectors after challenge with MARV. Subjects #1 to Subject #5 represent animals treated with VSV-MARV following challenge with MARV. Subjects #6 to Subject #8 represent control animals that were treated with irrelevant VSV-EBOV following challenge with MARV.

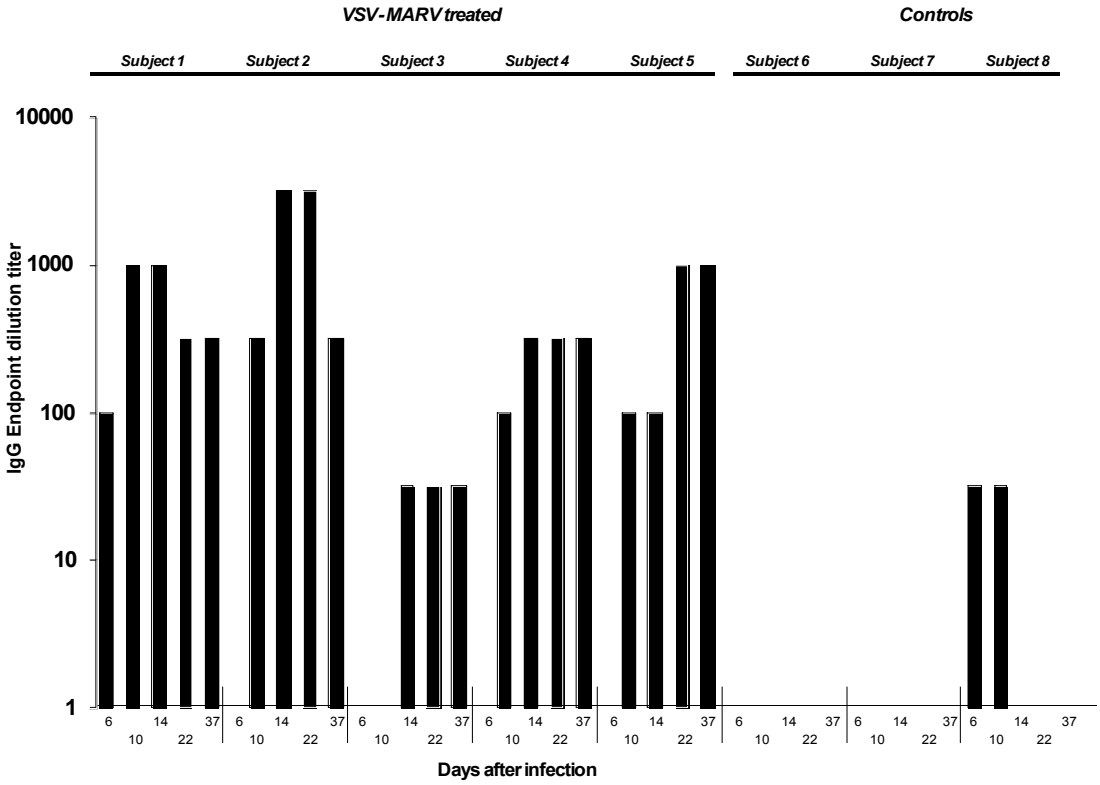

Figure 3B. B) The serological response profile of MARV infection after treatment with rVSV vectors. IgG response profiles of animals treated with VSVΔG/MARVGP vectors after challenge with MARV. Subjects #1 to Subject #5 represent animals treated with VSV-MARV following challenge with MARV. Subjects #6 to Subject #8 represent control animals that were treated with irrelevant VSV-EBOV following challenge with MARV

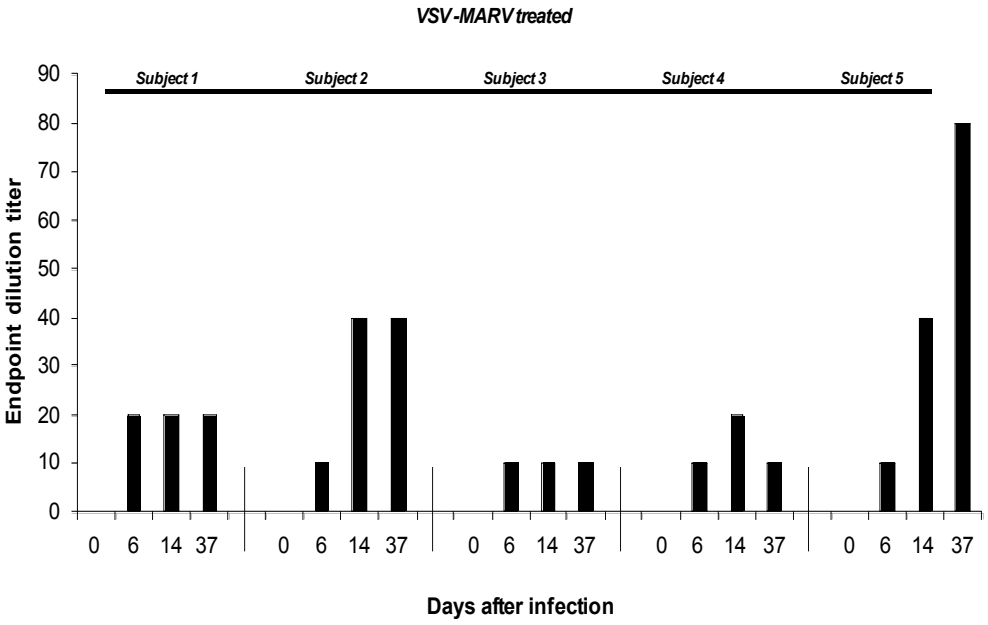

Figure 4. MARV neutralizing antibodies. Development of neutralizing antibodies in animals treated with VSV-MARV vectors after challenge with MARV. Neutralizing antibodies were detected using a plaque reduction neutralizing assay ($PRNT_{50}$). Titers are presented as endpoint dilutions.

VI. Discussion

MARV causes a severe and often fatal hemorrhagic disease in both humans and NHPs for which there are currently no licensed therapies or vaccines. The need for efficacious interventions is underscored both by the recent MARV outbreak in Angola with a fatality rate of 90 percent and by the risks of potential MARV laboratory exposures. Several MARV vaccine and therapeutic platforms have demonstrated significant to moderate efficacy in rodent models, however, their utility in NHP models has either proved unsuccessful or has yet to be determined. This thesis presents the results of three specific aims: 1) to determine whether the live-attenuated replication competent rVSV vector expressing the transmembrane GP of MARV strain Musoke (rVSV-MARV) could protect NHPs against a homologous MARV challenge and elucidate protective correlates of immunity *in vivo*; 2) to determine in NHPs whether immunity induced by rVSV-MARV could provide protection against heterologous MARV strains; and 3) to evaluate the efficacy of rVSV-MARV as a postexposure treatment strategy against MARV. This discussion attempts to integrate these results, in conjunction with supplemental data, with findings from other MARV vaccine and treatment platforms.

The animal models outlined in this thesis entail both cynomolgus and rhesus macaques because these animals appear to most accurately reproduce human MARV disease. NHPs are phylogenetically closely related to humans and most genes, serum proteins, and other factors that require analysis, are biochemically and antigenically similar to those in humans. Rodent models have been developed, and clearly have utility,

but require serial adaptation of the virus to produce lethal disease (Hevey, Negley et al. 2003). Furthermore, because of differences in disease pathology (Geisbert, Jahrling 2004), such as the absence of several characteristic features of MARV HF including lymphocyte apoptosis and hemorrhagic manifestations (Geisbert, Hensley et al. 2000), there are numerous scientific questions that cannot be satisfactorily addressed by an evaluation only in rodents. Several species of NHPs have been used to model MARV HF, and which species most accurately reproduces human disease is debatable. Nonetheless, the cynomologus and rhesus macaques appear to be excellent animal models for human MARV HF and are therefore deemed the most relevant models to evaluate efficacy and correlates of immunity associated with vaccines and therapies.

The goal of a NHP study is to model the human infection and disease course as accurately as possible; however, little is known regarding what comprises the average dose and route of exposure in human MARV infections. Although filovirus transmission has been documented to occur through several routes of exposure (Jaax, Davis et al. 1996; Sergeev, Lub et al. 1995), our NHP studies employ a MARV challenge via the intramuscular route as representation through needlestick. The reuse of syringes and needles have been associated with the propagation and worsening of filovirus outbreaks (WHO 1978a; WHO 1978b). Also, we employ a challenge dose of approximately 1000 plaque forming units (pfu) because it is likely that this dose would be associated with a needlestick , which exposes a person to high titer bodily fluids such as blood or blood products. Indeed, viremias associated with terminal stages of human filovirus disease have been recorded to reach levels as high as 10^7 pfu/ml (Jahrling, Geisbert et al. 1999; WHO 1978b).

Several characteristics support the potential for VSV as a highly attenuated vaccine vector for the delivery of heterologous viral proteins. rVSVs are able to accommodate large inserts and multiple genes into their genomes (Roberts, Buonocore et al. 1999), which offers advantages over other RNA virus vectors such as poliovirus (Morrow, Ansardi et al. 1994) and alphavirus (Xiong, Levis et al. 1989; Hevey, Negley et al. 1998). The utility of rVSV vectors as vaccine candidates is strengthened by their ability to elicit strong humoral and cellular immune responses *in vivo* and to induce both mucosal and systemic immunity through mucosal immunization (Kuno-Sakai, Kimura et al. 1994; Moldoveanu, Clements et al. 1995; Rose, Marx et al. 2001). Furthermore, naturally occurring infections with VSV are rare and there is a low seroprevalence of VSV antibodies in the general population (Wagner, Rose 1996). It is thus suggested that interference with preexisting immunity is unlikely.

In the studies outlined in this thesis, two different species of primates were used; cynomolgus macaques and rhesus macaques, and animals were exposed to high doses (approximately 10^7 pfu) of live attenuated rVSV-MARV vectors. Notably, we did not detect any signs of clinical illness associated with rVSV-MARV infection, indicating that rVSVs are non pathogenic for these animals. Although, very low levels of rVSV-MARV were detected in plasma and on nasal swabs by RT-PCR in only a few animals, we were unable to detect infectious rVSV-MARV by virus isolation.

Infection of NHPs with rVSVs did not show any signs of clinical illness or discomfort, strongly suggesting that these vaccines are safe for immunocompetent mammals including humans. However, replication competent rVSVs may cause undesirable side effects in immunocompromised individuals. Therefore, studies will

need to address whether rVSV may cause clinical illness and/or ineffectively provide protection against MARV HF in immunosuppressed NHP models. For example, human immunodeficiency virus (HIV) infection is notably recognized as causing immunosuppression in infected individuals due to its cellular tropism for lymphocytes. Therefore, a justifiable model for immunsupression could be one associated with HIV infection. To model human HIV infection, simian immunodeficiency virus (SIV)-infected NHPS could be used as an immunocompromised NHP model on which the rVSV platform could be evaluated as both preventative and postexposure measures against MARV HF. Other alternatives that could address the potential, although unlikely, pathogenesis of the replication-competent rVSV vectors could be to evaluate vaccine strategies with either replication defective VSV vehicles (Majid, Ezelle et al. 2006) or single-cycle rVSV vectors (Publicover, Ramsburg et al. 2005).

Results from Chapter 2 demonstrate, for the first time, that the rVSV-MARV vaccine induces 100 percent protection in a NHP model against MARV HF; that correlates of protection comprise strong humoral immunity in association with an undetectable cell-mediated response; and that cross-protection could be achieved against a heterolgous strain of MARV. The study outlined was performed using a cynomolgus macaque model, which is the NHP species most commonly used in vaccine studies, and entailed a 28 day period after vaccination (before challenge) during which parameters of both cellular and humoral immune responses were evaluated. An immunization schedule consisting of 28 days prior to challenge was chosen because this time frame was the shortest successful timeframe used by a different vaccine platform against filoviruses in NHPs (Sullivan, Geisbert et al. 2003). These same parameters were additionally

estimated following challenge with a high lethal dose of MARV strain Musoke. By day 14 following vaccination (14 days before MARV challenge) animals immunized with rVSV-MARV developed moderate IgG antibody titers against the MARV GP and these levels were sustained through MARV challenge on day 0 and up to day 29 following challenge. This suggests that rVSV-MARV was able to induce a complete antibody response before challenge. Detection of neutralizing antibodies was, however, absent in all but two animals both of which developed neutralizing antibody levels that were low and transient; on day 0 and day 29, respectively. Furthermore, there was no evidence of a cellular immune response before or after MARV challenge as demonstrated by absence of specific IFN-γ or TNF-α production from T-lymphocyte populations *in vivo*. These findings suggest that preventative protection against MARV strain Musoke in a NHP model may be dependant on humoral immune responses. Also, it suggests that antibody responses may, in part, act through a non-neutralizing mechanism or through mechanisms other than classical neutralization.

Antibody Responses: Quantitative and Qualitative

The definitive role of antibody responses in providing protection against MARV HF is unclear. The beneficial use of convalescence sera was reported during a MARV outbreak in Frankfurt, Germany (Slenczka W 1999; Stille, Bohle et al. 1968) suggesting that MARV-specific antibodies are involved in protection. Experimentally, passive immunotherapy studies have been performed in guinea pig models to evaluate whether antibodies alone could protect against a lethal MARV challenge. Notably, passive immunotherapy with serum from either convalescent guinea pigs immunized with an

avirulent plaque-purified derivative of MARV or from MARV virion-immunized guinea pigs provided complete protection when administered two hours before lethal MARV challenge (Hevey, Negley et al. 1997). These studies also showed that protection was dependent on the dose of immune serum given, as lower doses resulted in incomplete protection with delays in death (Hevey, Negley et al. 1997). Additionally, passive transfer experiments have been conducted to evaluate the protective efficacy of several MARV-specific monoclonal antibodies. In contrast, these studies showed incomplete protective responses with delays in death (Hevey, Negley et al. 2003).

Evaluations of MARV-specific antibody responses are commonly used to determine the effectiveness of MARV vaccines; however, these responses are not always indicative of a protective vaccine against MARV challenge. For example, in their evaluations of inactivated MARV as a vaccine in rhesus macaques, Ignatyev *et al.* show that although levels of MARV-specific antibodies were essentially the same in all animals vaccinated, only three out of the six animals survived (Ignatyev, Agafonov et al. 1996). In the three surviving animals, there was a significant rise in MARV-specific antibody titers compared to pre-challenge levels; therefore, suggesting that vaccination induced incomplete immunity prior to challenge and that protection required an additive antibody response (Ignatyev, Agafonov et al. 1996). Evaluations using a VEE alphavirus replicon vaccine platform demonstrated complete protective responses in a cynomolgous macaque model; however, protection was associated with slight to moderate boosts in MARV-specific antibody responses (Hevey, Negley et al. 1998). Although is can be suggested from results of these studies that there may be a specific quantitative correlate of protection (i.e., titer of MARV-specific IgG antibodies), consideration about the

qualitative nature of the antibody responses should also be included. For example, in addition to their neutralizing properties, antibodies can mediate host effector functions and facilitate virus clearance from the host. It has been illustrated in mice that the Fc portion of immunoglobulin G2a (IgG2a) interacts with complement components (Neuberger, Rajewsky 1981) and Fc receptors (Gessner, Heiken et al. 1998) with stronger affinity than that of IgG1 (Nimmerjahn, Ravetch 2005). The qualitative difference between these two IgG isotypes in providing antibody-mediated protection has been demonstrated for several viral infections. More efficient clearing of viruses such as influenza (Huber, McKeon et al. 2006), Ebola (Wilson, Hevey et al. 2000) and yellow fever (Schlesinger, Chapman 1995; Lefeuvre, Marianneau et al. 2004) has been associated with monoclonal antibodies of the IgG2a than to monoclonal antibodies of the IgG1, despite both isotypes displaying similar antigenic specificities. Resolving rVSV-MARV vaccine-induced IgG antibody subclasses are ongoing studies in our laboratory.

Antibody response data from our rVSV-MARV preventative vaccine strategy combined with that from other MARV vaccine studies suggest that there may be a threshold of humoral responses that must be met in order to induce protective responses. However, the quantitative (i.e., the titers of MARV-specific antibody) and qualitative (i.e., the effector functions of the MARV-specific antibodies) are unclear. Interestingly, all MARV vaccine platforms to date, which have been evaluated in a NHP model, have demonstrated the absence of neutralizing antibodies (Ignatyev, Agafonov, et al. 1996; Hevey, Negley et al. 2001; Jones, Feldmann et al. 2005). These data suggest that perhaps antibody actions are through mechanisms other than classical neutralization.

Neutralizing antibody plays a crucial role in antiviral protection. Neutralization of a virus by antibody binding is often represented by loss of viral infectivity when bound by these antibodies. Indeed, *in vitro* assays that detect neutralizing antibodies to evaluate the ability of antibodies present in serum samples to inhibit infectivity of a known concentration of virus. Although mechanisms of neutralization have been debated over the years, the binding of antibody free virus particles, as well as to infected cells, can mediate several antiviral activities (Burton D 2002). Antibody Fc-mediated effector systems can lead to cell lysis or clearance by antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Activation of complement by antibodies can lead to the deposition of complement components on the virion surface, which in collaboration with bound antibodies, can enhance neutralization. Complement activation can also lead directly to virus lyses. Additionally, complement and Fc receptors can bind antibody-complement-coated viruses which lead to phagocytosis followed by inactivation of the virion in an intracellular compartment of the phagocyte. Additional antibody antiviral activities including inhibition of viral replication inside cells (Fujinami, Oldstone 1979; Levine, Hardwich et al. 1991), inhibiting the release of viral progeny (Gerhard W 2001), and cell-cell transmission of viruses (Pantaleo, Demarest et al. 1995; Burioni, Williamson et al. 1994) have been documented. There are many examples of preventative vaccine approaches linking protective immune responses with preseumable non-neutralizing antibodies. For example, monoclonal antibodies specific for the VSV G protein that had no neutralizing activity against VSV *in vitro* were shown to completely protect mice against a lethal VSV challenge (Lefrancois L 1984). Also, relevant to our current results is a study employing mice vaccinated with a similar

rVSV vector lacking the VSV G gene (Kahn, Roberts et al. 2001). In this study the VSV G gene was replaced with the fusion G gene of respiratory syncytial virus (RSV); mice were then vaccinated with this rVSV vaccine. Importantly, the vaccine induced detectable serum antibodies against RSV by ELISA, but no detectable neutralizing antibodies, yet still protected the mice from RSV challenge.

T-lymphocyte responses

The primary markers of MARV vaccine effectiveness have been either the antibody titer to MARV antigens or the measurement of antibody function such as antiviral neutralizing activity. Relatively few vaccine platforms have incorporated the measurements of T-lymphocyte function in conjunction with or independent of antibody measurements to assess MARV vaccine efficacy. However, these studies were performed in different animal models and provide inconsistent results, therefore making it difficult to correlate data. For example, NHPs vaccinated with inactivated MARV that survived showed no significant changes in their lymphocyte proliferation responses following live MARV challenge (Ignatyev, Agafonov et al. 1996). Also, guinea pigs vaccinated with adenovirus vectors expressing MARV GP demonstrated IFN-γ production from T-lymphocyte populations following immunization, thus supporting vaccine-induced T-lymphocyte responses (Wang, Schmaljohn et al. 2006). Although this study illustrates in guinea pigs the ability of MARV GP-expressing adenovirus vaccines to induced T-lymphocyte responses, they did not evaluate such responses following MARV challenge. In our studies, we utilized a flow cytometric method to measure *in vitro* IFN-γ production of both CD4+ and CD8+ T-lymphocyte populations in peripheral

blood mononuclear cells (PBMCs) exposed to a pool of 15 amino acid peptides that span the entire GP gene of MARV strain Musoke. IFN-γ is produced by activated T-lymphocytes as part of the adaptive cell-mediated immune response and functions mainly to promote the activity of other cell-mediated effector cells such as cytotoxic T-lymphocytes. Using this assay to measure T-lymphocyte activation, we were unable to detect any IFNγ production and thus suggest that in response to GP peptides, T-lymphocyte activation was absent.

Despite the inability to detect a T-lymphocyte response in our preventative vaccine model, we cannot definitively conclude the absence of a cell-mediated response. The use of peptide pools to elucidate lymphocyte responses raises questions about whether native protein processing and presentation on MHC molecules is required. To address these concerns we also evaluated IFN-γ production of T-lymphocytes in response to *in vitro* presentation of VSV-MARV from autologous transformed antigen presenting cells (APCs) (unpublished data) For these studies APCs were isolated from NHPs prior to vaccination and transformed with *Herpesvirus papio*. Each day, on which assays were performed, transformed cells from each animal were infected with VSV-MARV and at 12 hours after infection. Transformed cells from each animal were individually mixed with PBMC fractions from the respective animals. Notably, measurements of IFN-γ were the same regardless of T-lymphocyte stimulation with either VSV-MARV-infected transformed APCs or MARV GP peptide pools.

Role of adaptive immunity

Cell-mediated and antibody responses are central to protection, but there are many uncertainties and controversies. Results outlined in this thesis suggest that rVSV-MARV vectors induce antibody responses that play a major protective role against MARV HF. Furthermore, results demonstrate the absence of detectable T-lymphocyte responses in the presence of strong humoral immunity, thus suggesting a possible T-lymphocyte independent (TI) response. Protective levels of antibodies have been observed for a number of viral agents including VSV (Fehr, Bachmann et al. 1996), influenza (Sha, Compans 2000; Lee, Rangel-Moreno et al. 2005; Szomolanyi-Tsuda, Welsh 1996) and polyomavirus (Graham, Braciale 1997) in T lymphocyte-deficient mice. TI production of IgM may occur via strong cross-linking of the B cell receptor. Antigens that are capable of inducing antibody responses in a TI fashion are often composed of rigidly arranged repetitive antigenic determinants spaced 5-10 nm apart. Previous work with VSV suggested that particle-associated G protein, rather than soluble portions of the G protein or the G protein alone, is necessary for TI antibody induction (Bachmann, Hengartner et al. 1995; Bachmann, Zinkernagel 1996). Because our rVSV vectors are relatively uncomplicated GP exchange vectors, it would not be surprising if the MARV GP were incorporated into the VSV virion structure in a manner similar to authentic VSV G; therefore, MARV GP in the background of the rVSV vector may also be capable of inducing antibody production by a TI mechanism.

While there are several approaches to evaluate antibody responses in rodent models (e.g., through the use of genetically engineered mouse models), approaches in NHPs are primarily limited to either passive transfer experiments or B-lymphocyte depletion studies. To examine the postexposure protective role of humoral immunity

induced by rVSV-MARV, recent pilot studies were aimed to directly examine the role of B-lymphocytes by depleting four rhesus macaques of B-lymphocytes prior to MARV strain Musoke challenge. To accomplish this depletion of B lymphocytes, Rituxan, an anti-CD20 monoclonal antibody that effectively depletes CD20-expressing B lymphocytes in monkeys and humans was used. The use of Rituxan is well established in NHPs (Schroder, Azimzadeh et al. 2003). Rituxan was administered intravenously to three of the four animals prior to MARV challenge and the animals received a total of two treatments of Rituxan prior to MARV challenge (pre-challenge treatments) and two treatments after challenge (post-challenge treatments) as outlined in Appendix figure 3. The control animal served as a virus control. Throughout the study, B-lymphocyte assessment in peripheral blood was carried out using flow cytometry. For these assays anti-CD19 antibody was used for staining to account for possible interference between Rituxan and CD20 antibodies. CD19 is expressed less optimally than CD20, therefore, on day –1 both CD20 and CD19 antibodies were used for comparison and confirmation of B-lymphocyte depletion (Appendix figure 4). One week after the final pre-challenge Rituxan treatment, at which time flow cytometric evaluation showed an approximate 99% reduction in B-lymphocytes, animals were challenge intramuscularly with 1000 pfu of MARV-Musoke. Approximately 20-30 minutes after MARV challenge, three animals received an intramuscular injection of rVSV-MARV vectors ($\sim 10^7$). The control animal was febrile on day 3 and by day 9 had developed macular rashes, dehydration, and anorexia. The control animal succumbed to MARV HF on day 11. At necropsy, the control animal showed lesions and pathological changes consistent with MARV HF (Rippey, Schepers et al. 1984; Geisbert, Jaax et al. 1998). In contrast, all three animals

treated with Rituxan survived MARV challenge. Aside from becoming febrile on days 4 and days 14 postinfection (Appendix figure 5), surviving animals did not demonstrate any significant changes in any other clinical parameters. Although flow cytometric data illustrates an approximate 99 percent B-lymphocyte depletion in peripheral blood (Appendix figure 6), additional confirmatory evaluation was needed. Therefore, ELISA was performed on surviving animals and demonstrated the presence of MARV-specific IgG antibodies. The development of MARV-specific antibodies strongly suggests that B-lymphocyte depletion was unsuccessful. Interestingly, MARV-specific antibody levels in animals treated with Rituxan were not significantly different from those observed in the animals from the original postexposure vaccination experiments outlines in chapter 5 (Appendix figure 7). Consequently, B-lymphocyte depletion studies need to be repeated using regimens that ensure depletion of B-lymphocytes in tissues as well as in circulation. Additional studies involving depletions of CD4 and CD8 T-lymphocytes may also provide insight into the nature of protective adaptive immune responses against MARV HF associated with postexposure treatment with rVSV-MARV.

Cross-protective responses

Vaccines that offer broad cross-reactive immunity are desirable as much time is required to identify and produce an antigenically well-matched vaccine. To evaluate whether protective immune responses induced in surviving cynomologus macaques vaccinated with rVSV-MARV and challenged with MARV strain Musoke could cross-protect against other MARV strains, we back-challenged with 1000 pfu of the Ci67 strain of MARV (MARV-Ci67). As outlined in Chapter 2, all animals back-challenged

survived without showing clinical symptoms. Notably, the nucleotide homology between these two strains of MARV is 93.9 percent (Bukreyev, Volchkov et al. 1995).

Marburgvirus contains a single species, *Lake Victoria marburgvirus*, consisting of viruses differing from one another by up to 21 percent at the nucleotide level. During the recent MARV outbreak in Angola, a new strain of MARV was identified which demonstrated less than a 7 percent difference in nucleotide homology between the other MARV strains (Towner, Khristova et al. 2006).

Comparative analyses of the GP and VP35 genes of the different MARV strains showed that there are two distinct lineages within the *Lake Victoria marburgvirus* species. The original MARV isolates, Popp and Ci67, the Ozolin strain, the Musoke strain, and the Angola strain comprise one lineage. The Ravn strain represents a second genetic lineage within the *Lake Victoria marburgvirus* species (21-23% amino acid difference) (Sanchez, Trappier et al. 1996). However, the two lineages are not separate species to the same extent as the difference that separates the EBOV species.

There is not enough heterology among the several strains of MARV to generate a new species. Although all animals back-challenged with the MARV-Ci67 survived, it was unclear as to whether cross protective responses were induced by rVSV-MARV, MARV challenge, or a combination of both. Results from Chapter 3 demonstrate that the rVSV-MARV vaccine which expressed the GP of MARV strain Musoke is an effective cross-protective vaccine against two different strains of MARV including, Ravn (MARV-Ravn) and the newly identified Angola strain (MARV-Angola).

Despite the optimistic cross protective results of our rVSV-MARV vaccine platform, there is concern that the 21-23 percent difference in amino acids between the

two lineages may be significant enough to affect the efficacy of candidate vaccines to confer cross protection against the different MARV strains. Using a platform based on Venezuelan equine encephalitis virus (VEEV) replicons, Hevey *et al.* showed that cynomolgus monkeys vaccinated with VEEV expressing MARV GP or VEEV expressing MARV NP replicons based on strain Musoke were protected against lethal homologous challenge (Hevey, Negley et al. 1998) but not against a challenge with MARV-Ravn (Hevey, Pushko et al. 2001). Such results suggest that separate vaccines expressing the individual MARV GP genes would be needed to address absence of cross protection in this alphavirus replicon vaccine system. In a recent study by Wang *et al.*, complex adenovirus (Ad)-based vaccine candidates (cADVax) were used to express individual MARV GPs from strains Ci67, Musoke, and Ravn and cross-reactive immune responses were evaluated in a mouse model (Wang, Schmaljohn et al. 2006). Interestingly, vaccination with each of the different MARV strain vaccines led to a cross reactive humoral immune response demonstrating that antibodies induced by each of the individual vaccines cross reacted against each other. These results corroborate with our results outlined in Chapter 3 that illustrate cross-protective immune responses associated with the rVSV-MARV vaccine that include induction of strong MARV-specific IgG antibody responses in conjunction with low levels of anti-MARV neutralizing antibodies (1:10 to 1:20). Furthermore, these findings show that is possible to elicit effective immunity against heterologous MARV strains potentially by stimulating the humoral arm of the immune system.

Postexposure protection

The immediate identification of postexposure treatment strategies against MARV HF is equally, if not more important, to the identification of preventative measures. The current utility of postexposure therapeutics to constitute immediate effective treatments during outbreaks as well as in the case of accidental laboratory exposures is noteworthy. Several experimental postexposure treatment strategies have been studied in both rodent and NHP models. Immunomodulatory drugs including desferal, ridostin, and polyribonate were evaluated in guinea pig models of experimental MARV infection; partial protection and slight increases in mean time to death were observed in these studies (Ignatyev, Agafonov et al. 1996; Sergeev, Lub et al. 1995). Despite the ability of several of these drugs to induce protective responses in guinea pigs, the efficacy and action of these immunomodulators in NHPs has yet to be determined. In addition, evaluation of drugs such as interferon and ribavirin showed that neither approach had therapeutic potential against MARV infection in NHP and guinea pig models, respectively (Sergeev, Lub et al. 1995; Kolokol'tsov, Davidovich et al. 2001; Ignat'ev, Strel'tsova et al. 1996).

Results from Chapter 5 demonstrate, for the first time, that rVSV-MARV can be used as an effective postexposure treatment to protect NHPs against MARV HF. Notably, rVSV-MARV also serves as a potential preventative measure, thus highlighting its dual utility against MARV HF. The effectiveness of postexposure vaccination is outlined in the management of several viral diseases including rabies, smallpox, and hepatitis B. We therefore wanted to assess whether our rVSV-based vectors expressing the GP of MARV-Musoke could mediate postexposure protection against a homologous MARV challenge in NHPs. The interval between MARV challenge and treatment was

20-30 minutes, which was chosen to represent a realistic amount of time consistent with the treatment of an accidental needlestick exposure involving a laboratory or health-care worker. With two recent laboratory accidents with the Zaire species of EBOV recorded (ISID 2004; ISID 2004) and the increased construction of new BSL-4 laboratories worldwide, the probability for such occurrences will increase greatly in the next decade. Following a 20-30 minute interval after MARV-Musoke, NHPs were treated with a single dose of rVSV-MARV and controls were treated with that of rVSV-EBOV. All rVSV-MARV-treated animals survived without showing any clinical illness, whereas control animals succumbed to MARV HF within the expected timeframe of 10 to 12 days after challenge. As seen with the preventative vaccine platform, the protective immune responses associated with the postexposure platform also consisted of strong antibody responses and absence of detectable T-lymphocyte mediated responses. In contrast to the preventative strategy, however, low to moderate levels of MARV neutralizing antibodies were seen in all animals treated with rVSV-MARV following MARV challenge.

Our results suggest that the rVSV-MARV vectors induced protection at least partially through responses to the surface GP, presumably by stimulation of GP-specific neutralizing antibodies. Specifically, low to moderate levels of neutralizing antibodies were detected in sera by day six after challenge. Although these data suggest that neutralizing antibodies may play a role in postexposure protection, the contribution of non-neutralizing antibodies and the therapeutic activity of antibody-mediated effector mechanisms also merit consideration.

Cross protective postexposure efficacy

The need for postexposure treatments against MARV HF is underscored by the recent MARV outbreak in Angola. In March of 2005, investigations began to identify the causative agent of viral HF in the Uige Province of northern Angola. The newly emergent MARV, designated strain Angola, was responsible for case fatality rates of 90% and deaths of approximately 227 people. While several strains of MARV have produced confirmed case fatality rates ranging from about 22 percent to slightly greater than 50 percent (Colebunders, Sleurs et al. 2004; Martini G 1971; Gear, Cassel et al. 2004), it is unknown as to why this newly emergent Angola strain was associated with increased lethality. Therefore, we hypothesized that by testing the limits of rVSV-MARV as a postexposure treatment, we could gain additional insight into mechanisms of protection associated with rVSV-MARV vectors. For these pilot studies, a total of four rhesus macaques were used, three experimental and one control, and were challenged intramuscularly with 1000 pfu of MARV-Angola. Following a 20-30 minute period, the three experimental animals were treated with rVSV-MARV and the control with rVSV-EBO. All four animals challenged with MARV-Angola succumbed to MARV HF; the control on day 7 and the rVSV-MARV-treated animals on days 8, 9, 10, respectively (Appendix figure 8). MARV HF in these animals displayed a much shorter disease course than that observed for MARV-Musoke and with more rapid and distinct demonstrations of MARV-associated pathologies (Appendix Table 2) Kinetics associated with MARV-Angola plasma viremia is also more rapid and distinct in NHPs than that demonstrated with MARV-Musoke (Appendix figure 9). Notably, by day 6 postinfection, viral titers were comparable between control and treated animals. Clinical findings such as dehydration, depression and anorexia developed at day 4 postinfection

for all animals. During the terminal stage of disease, significant changes in both hematological and biochemical parameters were observed as well as the development of macular rashes (Appendix figure 10). Pathological findings that differed to that found in MARV HF caused by strain Musoke included significantly more damage to the liver (Appendix figure 11) as well as more evident lymphadenopathy of inguinal and axillary lymph nodes (Appendix figure 12) . Although two of the three animals treated with rVSV-MARV demonstrated delays in death, clinical and pathological evidence suggests that the new Angola isolate of MARV, appears to produce a disease in NHPs that is more rapid and severe than other MARV strains, and in fact, appears to be as virulent as the Zaire species of EBOV.

We were surprised that rVSV-MARV did not protect because of its success as a preventative vaccine against MARV-Angola as well as its success as postexposure treatment against MARV-Musoke. Although, the differences between the GP genes of the two strains may account for differences in immune response specificity, this is unlikely, but not impossible. A recent study by Towner *et al*., demonstrated that nucleotide and protein identity of the entire genome between the two strains to be approximately 93 percent (Towner, Khristova et al. 2006). Notably, the gene with the greatest differences between strains was GP, demonstrating a 0.7-22.5% nucleotide difference. Therefore, subsequent studies were aimed to address whether the differences in disease courses associated with the different strains could explain the ineffectiveness of the rVSV-MARV vaccine to provide postexposure protection against MARV HF caused by the seemingly more lethal Angola strain. MARV HF caused by the Musoke strain appears to produce a disease that is more protracted than that caused by the Angola

strain when challenge doses are equal. Therefore, we hypothesized that by significantly lowering the challenge dose of MARV-Angola, we may be able to lengthen the associated disease course to more closely resemble that caused by MARV-Musoke. Employing the same postexposure pilot study design as mentioned earlier we challenged four rhesus macaques with 50 pfu MARV-Angola followed 20-30 minutes later with rVSV-MARV. In this study the single control animal developed viremia on day six (Appendix figure 13) and succumbed to MARV HF on day 10 postinfection (Appendix figure 14). This result suggested that protraction of MARV HF caused by MARV-Angola could be achieved by lowering the challenge dose. Importantly, all three animals challenged with the lower dose of MARV-Angola and treated with rVSV-MARV were protected against MARV HF without displaying any signs of clinical illness. Notably, studies to evaluate postexposure protection using a rVSV vector expressing the GP gene of strain Angola against 1000 pfu MARV-Angola challenge need to be performed.

Mechanisms of postexposure protection

Combined data regarding the utility of rVSV-MARV as preventative and postexposure countermeasures, in conjunction with their associated protective immune responses, provide critical insight into the nature of effective anti-MARV immunity. The study design for evaluating postexposure protection against MARV in NHPs provides a paradigm to outline correlates of immunity for which other interventions could apply. Furthermore, deciphering what antiviral responses MARV is sensitive to, will facilitate the elucidation of MARV mechanisms of pathogenesis. For example, it appears that humoral immunity plays a particularly important protective role which, in part, could be

through either neutralizing and/or non-neutralizing mechanisms. It is therefore likely that survival is dependent on the induction of a strong antibody response. Pathogenically, MARV HF is associated with lymphopenia and lymphoid degeneration (Geisbert, Hensley et al. 2000; Geisbert, Jaax 1998), which may account for the inability to produce an effective antibody response. Indeed, MARV may act in a manner similar to other viruses that are known to disable the adaptive immune response through bystander lymphocyte apoptosis. In HIV-1 infection, apoptosis of CD4 positive T-lymphocytes and CD19 positive B-lymphocytes was proposed to correlate with the lack of neutralizing activity found in the IgG fraction of infected patient's plasma and thus contributing to the loss of immune function (Samuelsson, Brostrom et al. 1997). Also, a strong correlation between extensive intravascular lymphocyte apoptosis and impaired humoral responses has been associated with fatal outcomes in EBOV-infected patients (Baize, Leroy et al. 1999). In contrast, EBOV survivors demonstrated early and continual development of EBOV-specific IgG that was followed by clearance of circulating viral antigen and activation of T cytotoxic lymphocytes (Baize, Leroy et al. 1999).

These results suggest that the rVSV-MARV vectors induce protection, at least partly, through responses to the surface GP, presumably by stimulation of GP-specific antibodies, however, the protective mechanism of the rVSV-MARV vaccine in NHPs remains to be determined. In order to dissect out which components of the immune response are necessary and required for protection from those that are merely beneficial but dispensable, additional studies are needed. Defining mechanisms by which the rVSV-MARV vectors provide protection is a major goal of ongoing studies. Although it is likely that survival is weighted heavily on antibody responses, it is possibility that

other immunological mechanisms contribute to viral clearance and, therefore, cannot be excluded. Possible mechanisms include direct viral interference, induction of a robust and effective innate response, or a "jump-start" of the adaptive immune response.

Viral interference

It is possible that rVSV-MARV vectors may be acting through a virus interference phenomenon. Superinfection exclusion, or homologous interference, is defined as the ability of an established virus infection to interfere with a homologous superinfecting virus. Superinfection exclusion has been described for a number of viruses, including retroviruses (Nethe, Berkhout et al. 2005) , VSV (Simon, Cardomone et al. 1990), borna disease virus (Formella, Jehle et al. 2000), and Sinbis virus (Karpf, Lenches et al. 1997). The mechanisms of interference have been identified at various stages of the viral life cycle, including receptor-mediated attachment, internalization and penetration of virus into infected cells (Van Hoeven, Miller 2005), and subsequent replication steps. The function of the MARV GP to mediate virus infection suggests that expression of MARV GP by rVSV would influence rVSV-MARV cellular tropism to act similarly to that of MARV. Therefore, it is likely that because both rVSV-MARV and MARV-Musoke express the same GP, they are targeting the same cell types for infection. If the superinfection exclusion model applies for this system, it can be proposed that infection with rVSV-MARV will interfere with subsequent "superinfection" with MARV, and possibly vice versa. In order to address potential interference between rVSV-MARV and MARV, recent studies based on dual infections were carried out in principal cell targets, primary human monocyte/macrophages and the ability of rVSV-

MARV to suppress MARV replication in these cells were evaluated by quantitative RT-PCR (unpublished data). At 24 and 48 hours postinfection, levels of rVSV-MARV were only slightly higher than that of MARV; however, both MARV and rVSV-MARV RNA transcript levels increased thus demonstrating active replication under these conditions. Although these data show the absence of viral interference between rVSV-MARV and MARV, additional studies are needed.

Innate responses

Recent studies indicate that cytokines produced by cells of the innate defense system play an essential role in directing the immune response towards a protective antiviral immunity (Steinman, Hemmi 2006). These cytokines might act to initiate the development of antiviral adaptive responses by acting as "danger signals" that alert the immune system. By promoting the differentiation and activation of immune effector cells (e.g., T-lymphocytes and B-lymphocytes), these cytokines serve as powerful natural adjuvants for the development of antiviral responses. Evaluating early host-virus interaction in the form of cytokine innate responses associated with rVSV-MARV protection against MARV HF may provide insight into underlying correlates of immunity. The fact that rVSV-EBOV-treated control animals succumbed to MARV HF within normal range strongly suggests that innate responses, by themselves, are not enough to account for protection. However, the role of innate responses to influence subsequent protective adaptive responses cannot be excluded. For example, type I IFNs have been demonstrated to link innate antiviral responses to subsequent effective adaptive responses and the clearance of virus (Cerutti, Qiao et al. 2005; Tough D 2004).

Several viruses such as EBOV (Basler, Mikulasova et al. 2003), influenza (Garcia-Sastre A 2001), and vaccinia (Symons, Alcami et al. 1995) have developed mechanisms to counteract the type I IFN responses thus highlighting their importance towards initiating protective immunity.

Recent studies were performed to evaluate innate responses associated with rVSV-MARV postexposure treatment. In these studies, cytokine responses were studied from animals challenged with either rVSV-MARV alone, MARV-Musoke alone, or MARV-Musoke followed by rVSV-MARV treatment after 20-30 minutes (Appendix figure 2). Cytokine comparisons between the three groups allowed us to decipher innate responses associated with each virus as part of the postexposure treatment strategy. For example, cytokine evaluations from animals infected with rVSV-MARV did not show any significant changes, aside from a moderate elevation of MCP-1 on day 1 post-infection.

The trends of cytokine changes in each group may reflect the development of protective responses that aid in the clearance of virus. A viremia developed as early as day 1 after challenge in animals infected with rVSV-MARV only, and was cleared by day 4. In comparison, viremia in animals infected with MARV alone did not develop until day 4. These data show that as rVSV-MARV is cleared from NHPs, MARV viremia is just beginning to develop (Appendix figure 1). These data suggest that in animals infected with MARV followed by treatment with rVSV-MARV, the early cytokine responses (i.e., day 1 to day 4 after challenge) may be attributed to responses against rVSV-MARV and later cytokine responses (i.e., day 4 to day 6 postinfection) may be attributed to responses against MARV. For example, in animals infected with

MARV alone and in animals infected with MARV followed by rVSV-MARV infection, expression of IFNα levels showed moderate increases at day 1 after challenge and suggest that a type I IFN responses is induced very early in these animals. By day 2 after challenge, both groups of animals show decreases in IFNα levels; however, only in animals infected with MARV alone did this decrease continue and return to baseline at day 4 after challenge. In contrast, on day 4 after challenge, animals infected with MARV followed by rVSV-MARV infection demonstrate a moderate increase in IFNα expression that remained elevated throughout study. These data suggest the possible role of IFNα to link innate responses to the development of protective adaptive responses. Although, the IFNα cytokine responses associated rVSV-MARV infection alone do not show any changes, such an absence could be due to the type I IFN sensitivity of rVSV-MARV (unpublished data). Indeed, the highly sensitive nature of wild-type VSV to type I IFNS is well accepted (Tough D 2004). Interestingly, cytokine responses associated rVSV-MARV infection alone do no show as significant changes; however, the highly attenuated nature of these recombinants may account for this finding (Roberts, Buonocore et al. 1999). These data suggest that the innate response induced by rVSV-MARV may influence adaptive responses that lead to virus clearance

Role of Marburg glycoprotein

In the studies outlined, it is suggested that the MARV-specific immune responses generated are, in large part, influenced by the MARV-GP expressed from rVSV vectors. Several studies have demonstrated the highly glycosylated nature of MARV GP accompanied by complex N- and O-linked carbohydrates (Feldmann, Will et al. 1991;

Geyer, Will et al.1992; Becker and Muhlberger 1996). To better understand the mechanisms of immunity associated with the rVSV-MARV platform, it may be noteworthy to evaluate cellular tropism and immunogenicity due to glycosylation of the GP structure in context of the rVSV-MARV as well as that of MARV strain Musoke and MARV strain Angola. Filovirus glycoproteins are likely responsible for the initial interaction of the filovirus particle with the immunological defense of the host. Differences in the glycosylation pattern of MARV GP in the context of rVSV-MARV, MARV strain Musoke, and MARV strain Angola may account for differences in protection elicited by rVSV-MARV. Also, inappropriate and/or altered glycosylation patterns within the GP of MARV strain Angola may influence selection of MARV strain Angola virulence.

Glycoprotein glycosylation has been showed to influence the structure, antigenicity, and immunogenicity of various viral glycoproteins. For example, the HIV envelope protein gp120 has been shown to consist of multiple carbohydrate chains that could contribute to HIV masking against neutralizing antibody *in vivo* (Quinones-Kochs, Buonocore et al. 2002). The glycosylation of EBOV GP has also been found to play critical immunogenic roles. Dowling et al., showed that mutation of one of the two N-linked EBOV GP2 glycosylation sites was detrimental to the antigenicity and immunigenicity of EBOV GP. They attributed such findings to the inability of such GP2 mutants to achieve conformational integrity of GP2 and GP1. These studies also demonstrated an enhancement of immunogenicity with mutation of two N-linked glycosylation sites of GP1, which is possibly a result of epitope unmasking (Dowling, Thompson et al. 2006).

The complexity of glycosylation patterns of a protein is dependent on several parameters including the polypeptide chain and the host cells and environment. Indeed, genomic studies performed on several different strains of MARV demonstrated that the gene with the greatest nucleotide and amino acid differences is the GP (Towner, Khristova et al. 2006). Such findings raise questions as to whether those genomic differences affect the downstream post-translational modifications of GP protein product and causing antigenic differences among the different strains. Furthermore, GP immunogenicity may be influenced by host-specific glycosylation. In the laboratory, rVSV vectors are produced in 293 cells (Garbutt, Liebscher et al. 2004) and propagated in Vero cells before use *in vivo* (Daddario-DiCaprio, Geisbert et al. 2006). MARV viruses are also propagated in Vero cells (Daddario-DiCaprio, Geisbert et al. 2006). The MARV GP glycosylation patterns are potentially influenced by the host cell system, which consists of a complex cascade of sequential enzyme-catalyzed reactions and such reactions may vary among the cell types used *in vitro* as well as the infected cells that propagate virus *in vivo (*Feldmann, Nichol et al. 1994; Geyer, Will et al.1992; Feldmann and Kiley 1999*)*. Notably, evaluation of MARV GP glycosylation patterns of viruses propagated in known primary cell types of MARV (e.g., human monocytes/ macrophages) may provide additional insight into the role of MARV GP glycosylation on protection.

Glycosylation patterns of MARV GP may not only influence the immunogenicity of GP, but also influence virus cellular tropism as GP plays a pivotal role in receptor binding and fusion of the virus with host cell membranes (Kuhn, Radoshitzky et al. 2006; Takada, Robison et al 1997). Although the exact receptors required for infection are not

known, several different receptors are probably able to mediate infection, which is most likely facilitated through endocytosis (Geisbert and Jahrling 1995). Indeed, the ability of MARV GP to bind lectin receptors on permissive cell types such as DC-SIGN (Bosio, Aman et al. 2003) and human macrophage C-type lectins specific for galactose/*N*-acetylgalactosamine (hMGL) (Takada, Fujioka et al. 2004) have been demonstrated. Engagement of MARV GP to these receptors may mediate infection either through the receptor itself or possible by preferentially targeting the virus to certain cell types early in the infection. For example, it has been speculated that binding of MARV GP to DC-SIGN may augment MARV infection of DC, which may possibly contribute to the immunosuppression and viral dissemination of the host (Marzi, Gramberg et al. 2004). The ability of highly glycosylated MARV GP to bind lectin receptors supports the importance of the GP glycosylation pattern. As stated earlier, several parameters such as genomic differences and host-specific glycosylation may influence and even alter the glycosylation patterns of MARV GP. It can therefore be speculated, that glycosylation differences between rVSV-MARV and MARV strain Angola may also influence the ability of these viruses to target similar cell types through lectin receptor binding.

Summary and final organizing hypothesis

The highly pathogenic potential of the *marburgviruses*, possible use as a bio-terrorist threat weapon, and more frequent outbreaks has hastened the need for vaccine and therapeutic interventions. A great deal of research has contributed to the knowledge base; however, the need for high level biocontainment facilities to conduct research with live virus has limited work to only those individuals with access to such laboratories.

This study provides contributory information regarding the protective role of rVSV-MARV-induced immune responses against MARV HF in NHPs. Replication-competent, attenuated, recombinant VSV vectors expressing the GP of MARV-Musoke demonstrated complete preventative and postexposure responses against homologous MARV-Musoke challenge in NHPs. As a preventative vaccine these rVSV-MARV vectors are also capable of protecting against heteologous MARV strains; however, postexposure protection against a more lethal strain of MARV required challenge with a lower virus dose. In each of these rVSV-MARV strategies against MARV HF in NHPs, the protective responses strongly suggest that humoral immunity plays a major role and that associated antibody responses may, in part, be mediated through either neutralizing or non-neutralizing mechanisms. Clearly additional studies are needed to decipher the different aspects of the rVSV-MARV-associated immune response. rVSV-MARV administered as a postexposure treatment may jump start the immune system to develop a specific response necessary to clear the virus. The expression of MARV GP by rVSV-MARV suggests that there is a similar cell tropism. Early targeting and infection of pivotal effector cells such as dendritic cells by rVSV-MARV will allow the host to begin to develop an immune response prior to MARV-Musoke-infecting dendritic cells and potentially preventing the host from mounting an effective response. At the same time rVSV may stimulate an effective innate response which helps hold the virus in check until an adaptive response can be developed.

The experimental NHP preventative and postexposure strategies using rVSV-MARV provide effective models for studying protective immune responses against MARV strains. The elucidation of these responses should provide insight in mechanisms

of MARV pathogenesis by which these viruses counteract, interfere, or avoid host immunity and should provide new targets for additional prophylactic and chemotherapeutic interventions. It is possible that multiple factors contribute to the success of rVSV vectors as both preventative and postexposure strategies. Dissecting out the importance of individual factors involved in mediating protection may be difficult as many of these factors may be necessary but not sufficient or neither necessary nor sufficient.

VII. Appendix

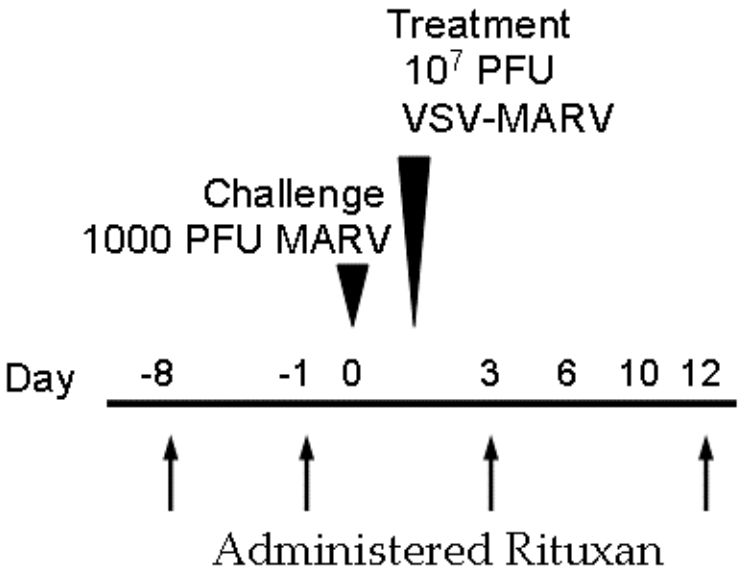

Figure 1. Flow chart of experimental design. Peripheral blood was taken on the days outlined for analysis. Arrows indicate days of Rituxan treatments of rhesus animals and arrowheads indicate challenge virus and dose.

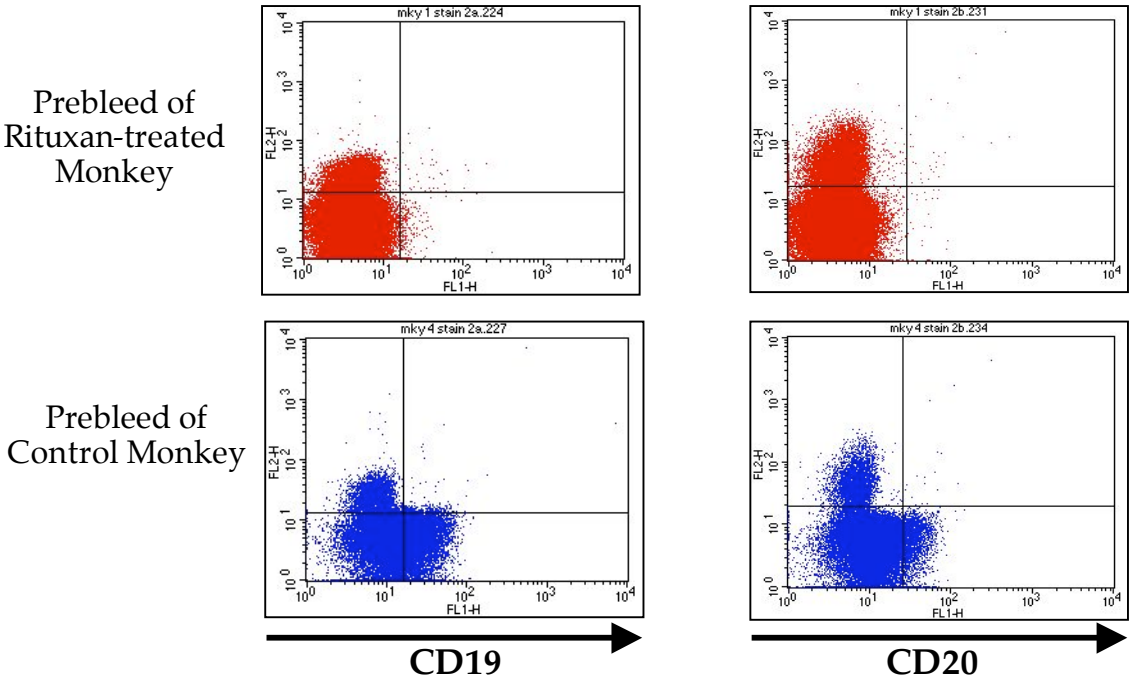

Figure 2. Flow cytometric analysis of PBMCs from Rituxan-treated NHP and control NHP before MARV-Musoke challenge. PBMCs were isolated from animals on day -1, stained for either CD19 or CD20, and evaluated by flow cytometry.

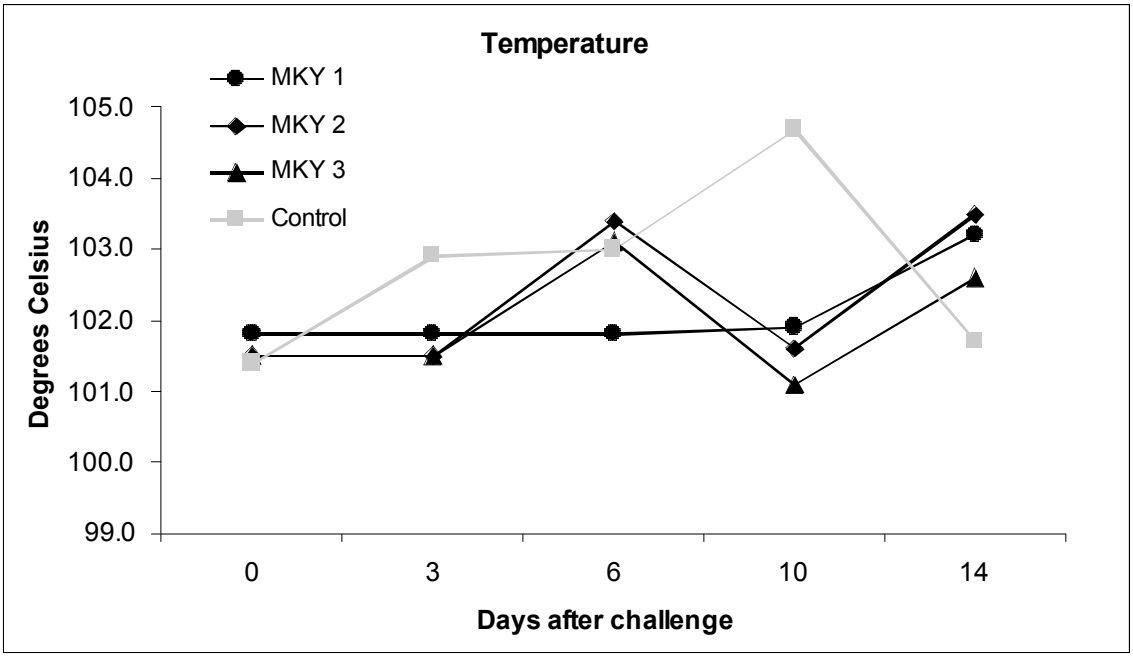

Figure 3. Temporal rectal temperatures following MARV-Musoke challenge in control NHP and NHPs treated with Rituxan.

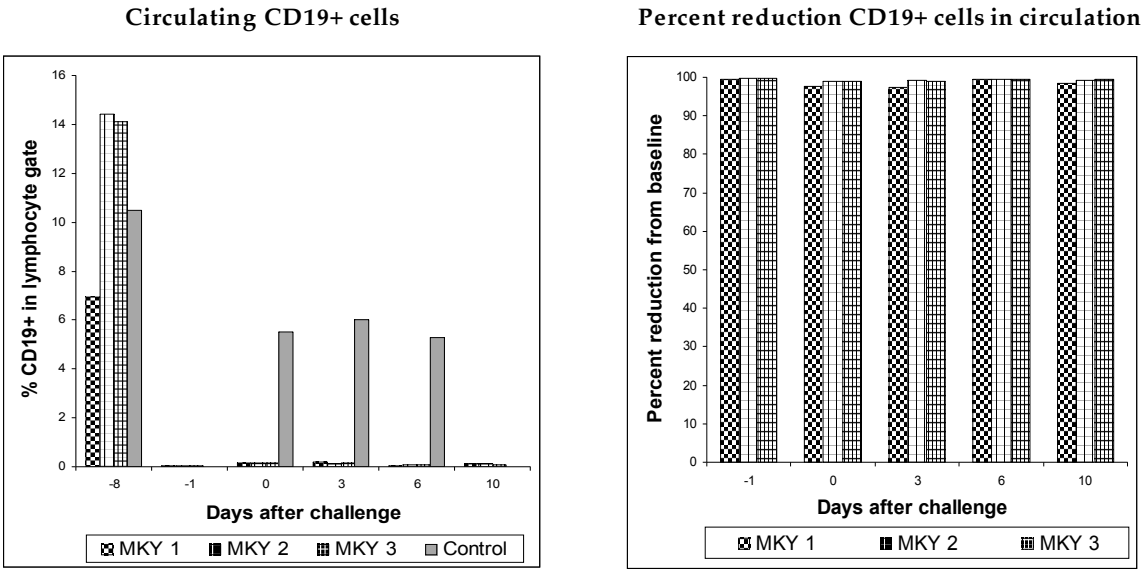

Figure 4. Flow cytometric values following Rituxan treatment and MARV challenge of circulating CD19+ cells circulating in peripheral blood (LEFT) and percent reduction of CD19+ cells in circulation (RIGHT). PBMCs were isolated from control and Rituxan treated animals on the days -8, -1, 0, 3, 6, 10 and stained with CD19 followed by flow cytometric analysis. Percent reduction was calculated from individual animal baseline values at day -8.

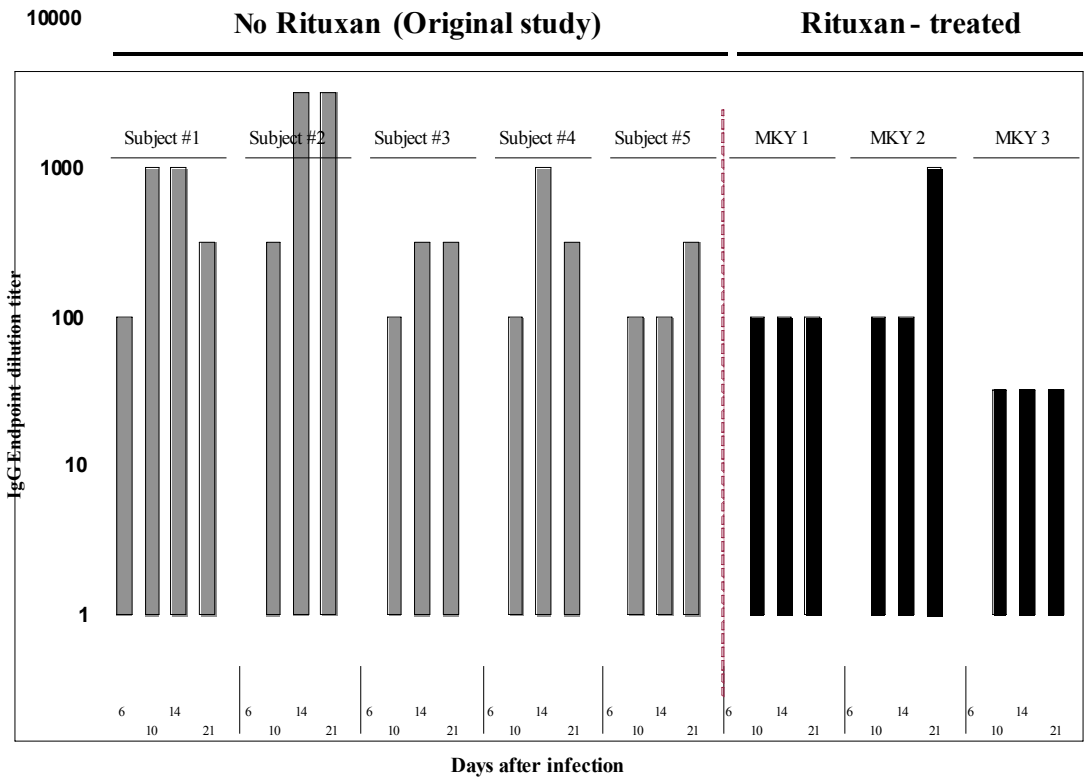

Figure 5. Serological IgG response profile of NHPs treated with Rituxan (black bars) in comparision to IgG titers of NHPs from the original study (Daddario-DiCaprio, Geisbert et al. 2005) (gray bars) outlined in chapter 5. On outlined days post-challenge, serum was isolated from each animal for frozen at -70ºC until completion of study. Serum samples were analyzed using a NHP IgG ELISA

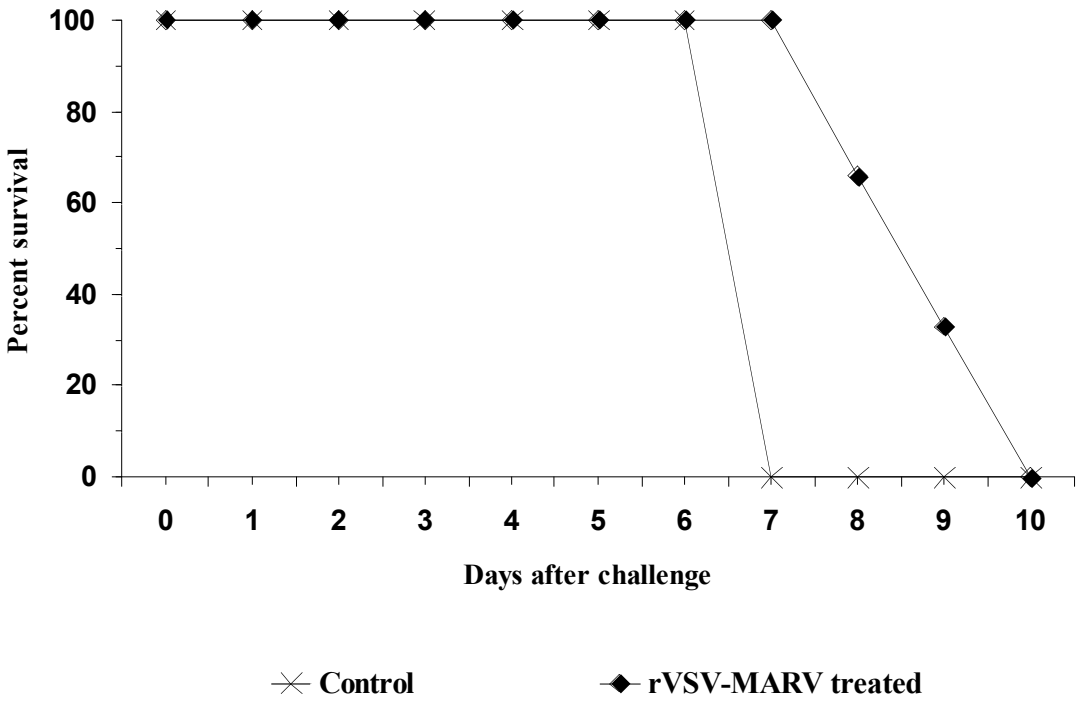

Figure 6. Kaplan-Meier mortality chart of rVSV-MARV treatment following MARV-Angola study design. ♦, animals challenged with MARV-Angola at 1000 pfu and treated with rVSV-MARV; X, animals challenged with MARV-Angola 1000 pfu and treated with irrelevant rVSV-EBOV.

Table 2. Scheme of clinical findings during MARV-Musoke and MARV-Angola HF in NHP models. Challenge dose of 1000 pfu produces a 100 percent lethal model.

Day of clinical findings in rhesus macaques		
	Musoke	Angola
Days after challenge		
Terminal stage	10-12	6-8
Asymptomatic	0-4	0-3
Viremia	4–12	3- 8
Fever	6–12	4-8
Rash	9–12	8
Dehydration	6–12	4-8
Depression	6-12	4-8
Anorexia	6–12	4-8

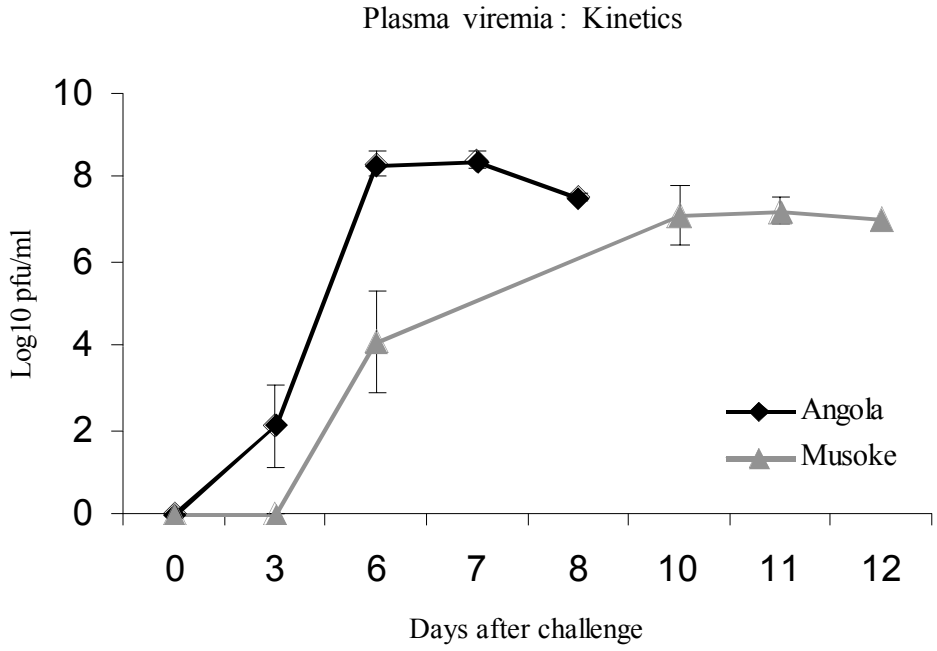

Figure 7. Temporal development of MARV-Angola and MARV-Musoke virus titers in NHP plasma. Plasma samples were collected on the days outlined and virus titers were evaluated by plaque assay. Note the protracted disease course associated with MARV-Musoke infection in comparison to that of MARV-Angola.

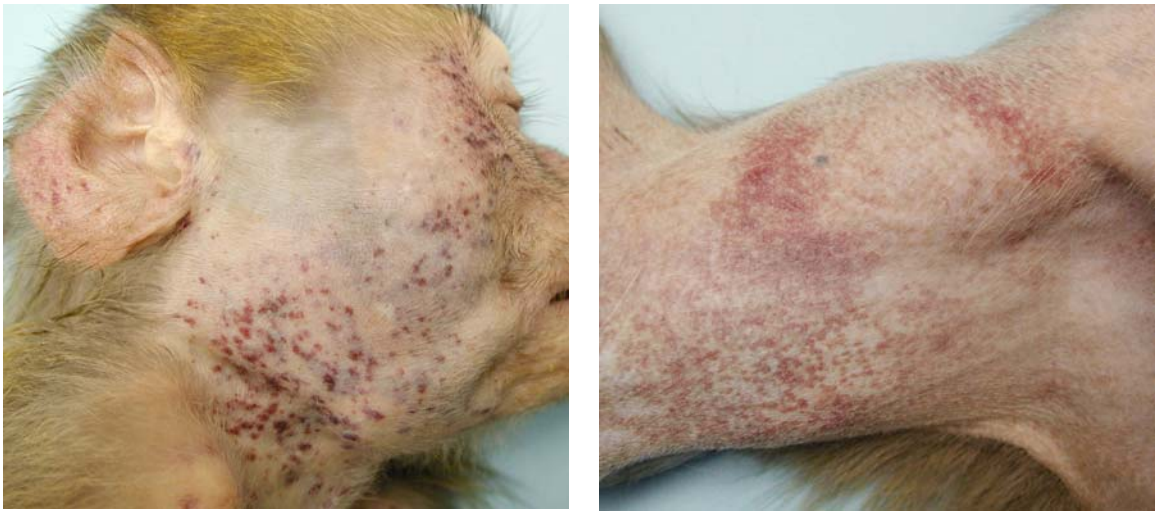

Figure 8. Representative cutaneous rashes from rhesus macaques experimentally infected with MARV-Angola. Characteristic maculopapular petechial rash of the face (LEFT) and right arm (RIGHT) at day 6 after challenge.

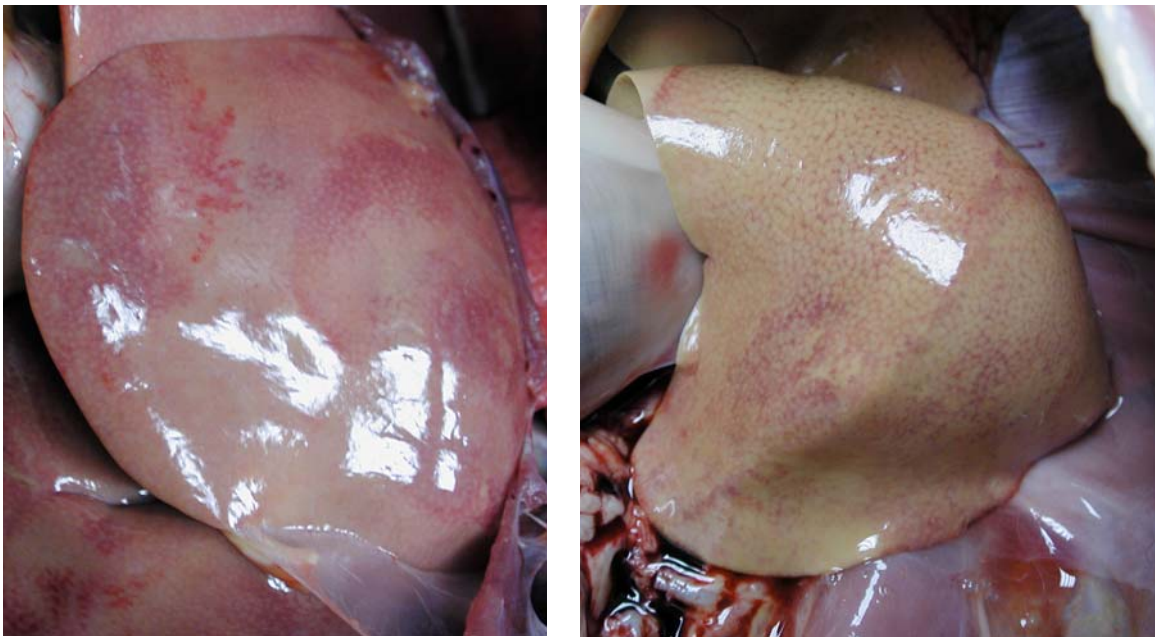

Figure 9. Representative gross necropsy lesions from rhesus macaques experimentally infected with either MARV-Musoke (LEFT) or MARV-Angola (RIGHT).

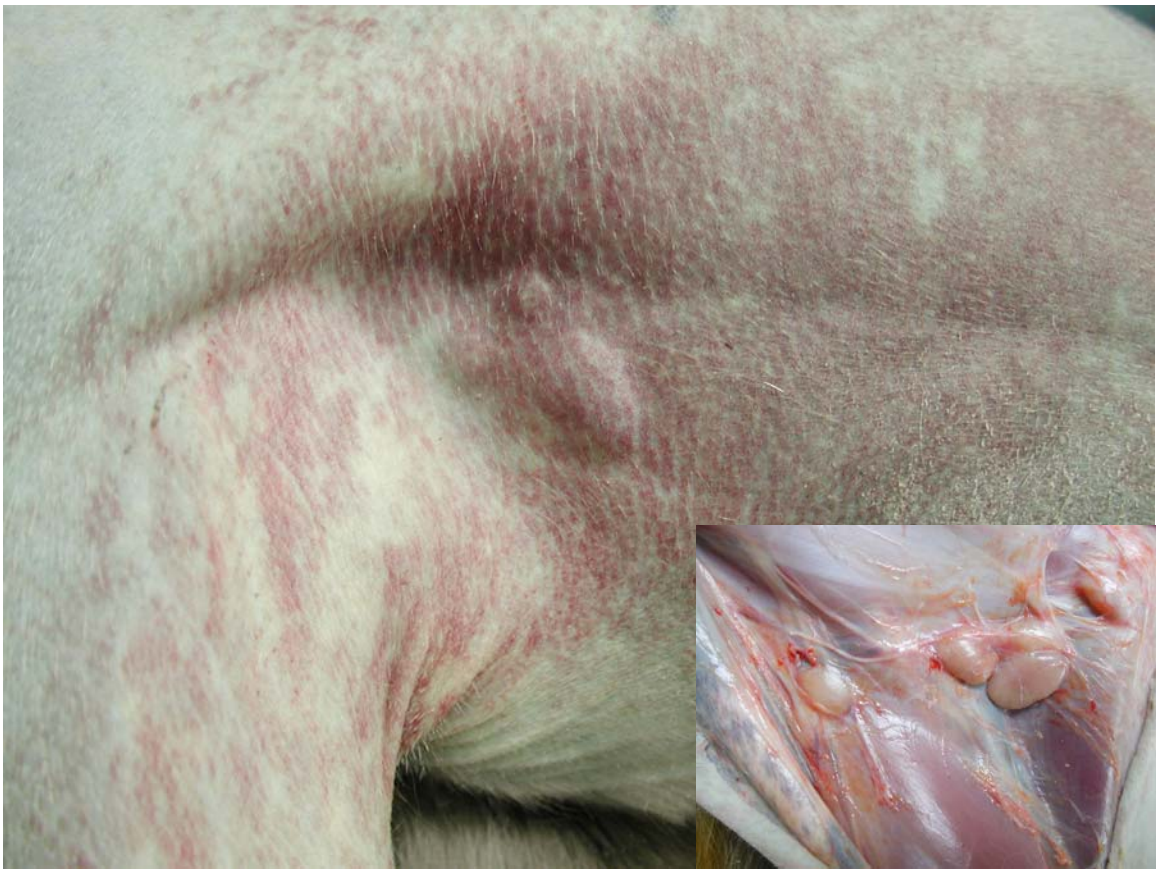

Figure 10. Representative lymphadenopathy from rhesus macaques experimentally infected with MARV-Angola. Note the significant enlargement of axillary lymph nodes observed prior to necropsy (FULL) and at necropsy (INSERT).

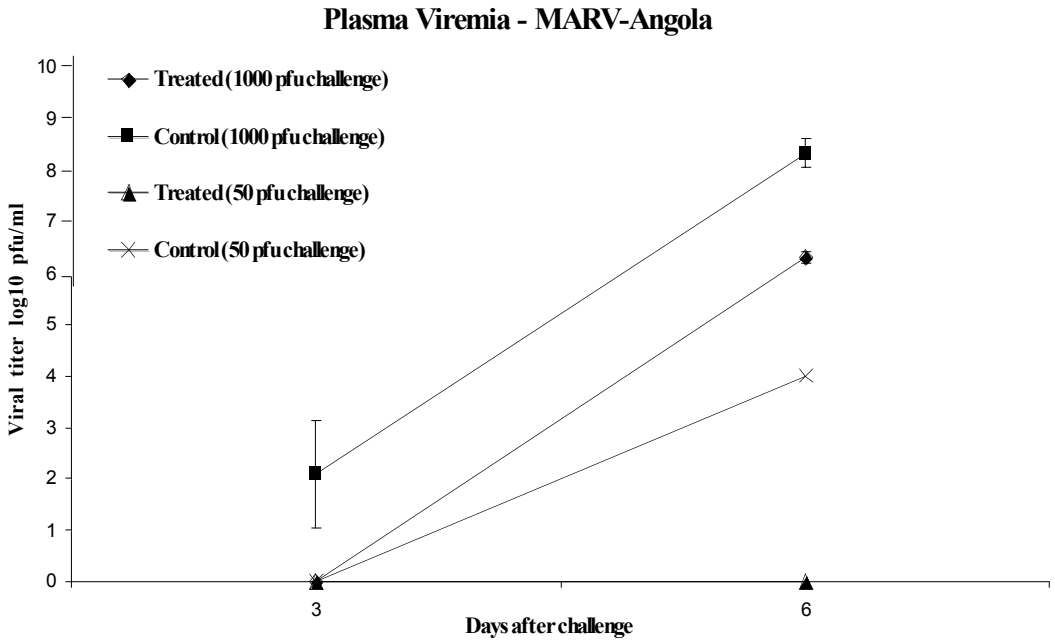

Figure 11. Development of MARV-Angola virus titers in plasma from NHPs challenged with different challenge doses. Plasma samples were collected on days 0, 3, and 6 and virus titers were evaluated by plaque assay.

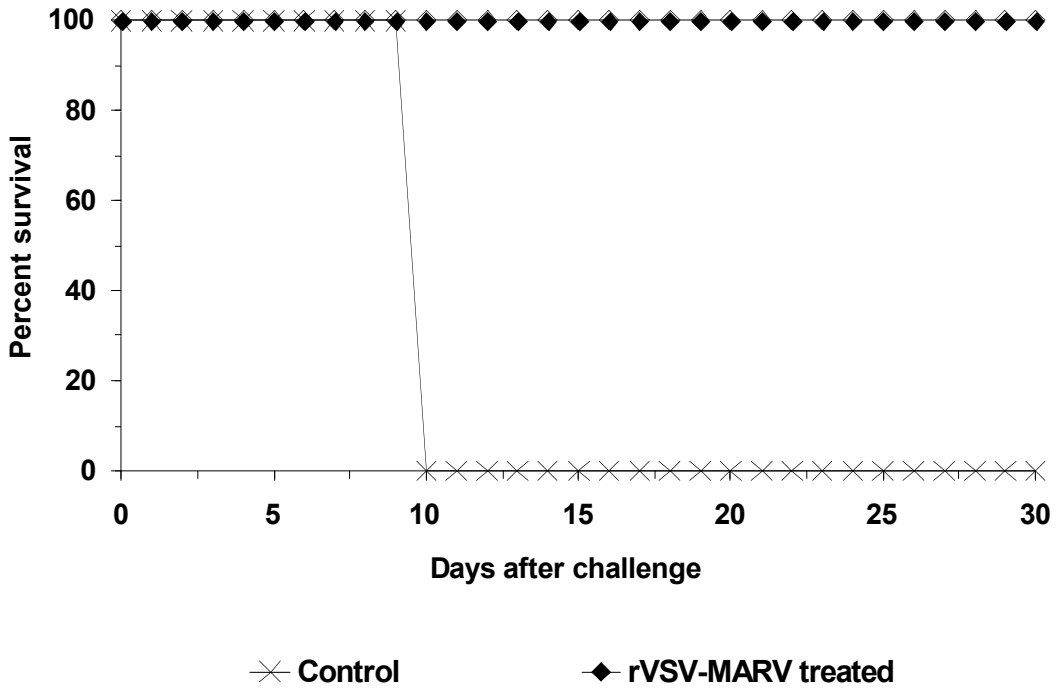

Figure 12. Kaplan-Meier mortality chart of rVSV-MARV treatment following MARV-Angola study design. ♦, animals challenged with MARV-Angola at 50 pfu and treated with rVSV-MARV; X, animals challenged with MARV-Angola 50 pfu and treated with irrelevant rVSV-EBOV.

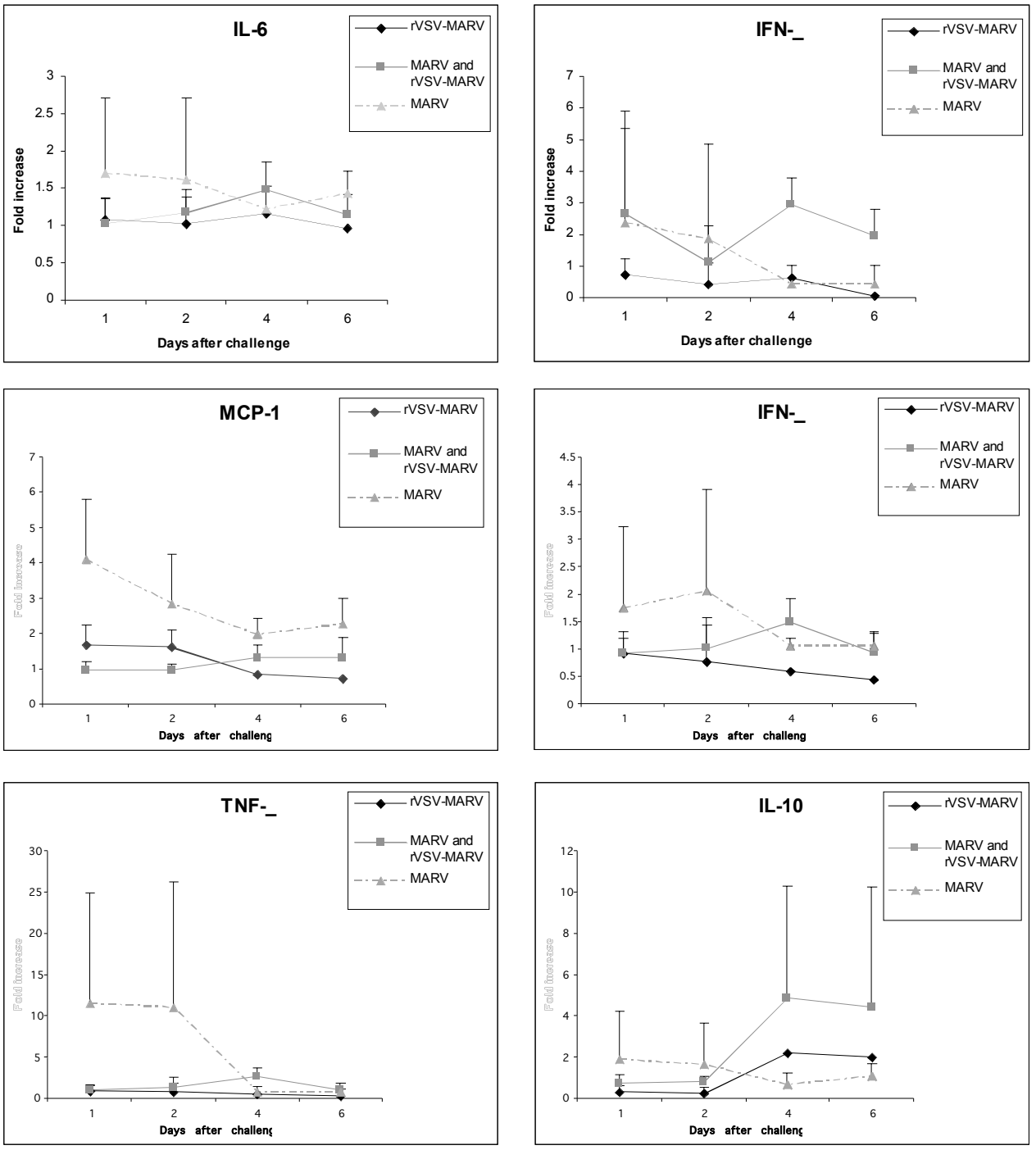

Figure 13. Cytokine analysis of plasma samples from NHPs. NHPs were challenged with either MARV-Musoke alone, rVSV-MARV alone, or MARV-Musoke with rVSV-MARV treatment 20-30 minutes after challenge.

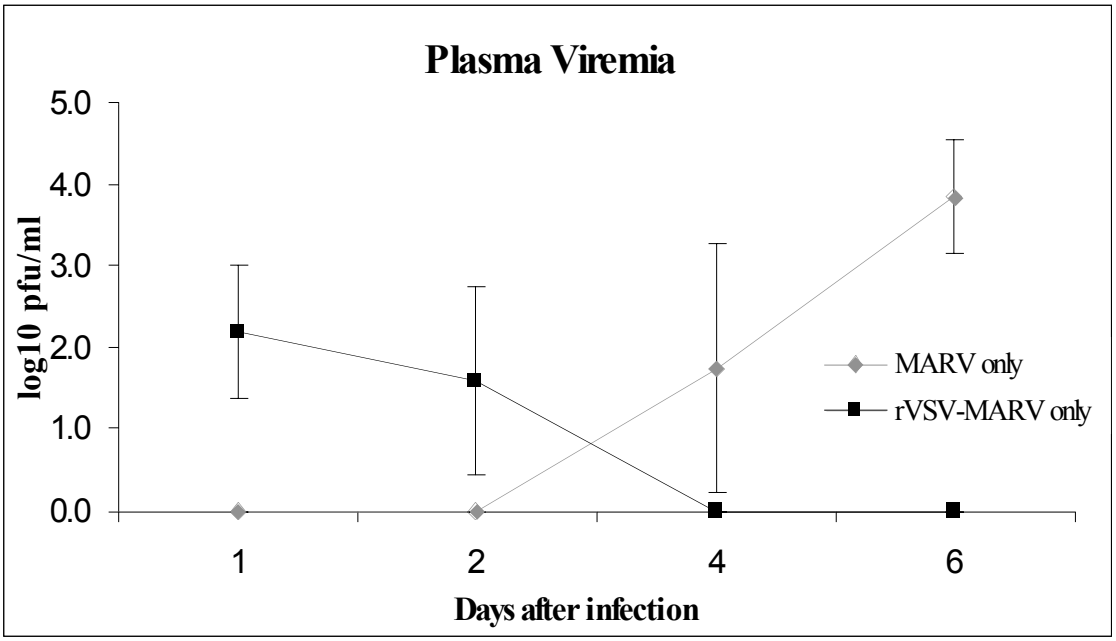

Figure 14. Temporal development of MARV and VSV-MARV virus titers in NHP plasma. Plasma samples were collected on the days outlined and virus titers were evaluated by plaque assay. Each group represents the average virus titer from 4 NHPs each challenged with either MARV alone or VSV-MARV alone. Animals were euthanized on either day 2 or day 6 after challenge.

VIII. References

Agafonova, O. A., S. A. Viazunov, et al. (1997). "[Relationship between the level of specific antibodies with disease outcome in Cercopithecus aethiops monkeys in experimental Marburg disease]." Vopr Virusol **42**(3): 109-11.

Alibek K, Handelmann. S. Biohazard: The chilling story of the largest covert biological weapons program in the world. Told from the inside man who ran it. New York, NY, Random House.

Bachmann, M. F., H. Hengartner, et al. (1995). "T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: role of antigen patterns in B cell induction?" Eur J Immunol **25**(12): 3445-51.

Bachmann, M. F. and R. M. Zinkernagel (1996). "The influence of virus structure on antibody responses and virus serotype formation." Immunol Today **17**(12): 553-8.

Baize, S., E. M. Leroy, et al. (1999). "Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients." Nat Med **5**(4): 423-6.

Baize, S., E.M., Leory, et al. (2002). "Inflammatory responses in Ebola virus-infected patients." Clin Exp Immunol **128**(1): 163-8

Basler, C.F., Mikulasova, A. et al. (2003). "The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3." J Virol **77**(14):7945-56.

Bausch, D. G., S. T. Nichol, et al. (2006). "Marburg hemorrhagic fever associated with multiple genetic lineages of virus." N Engl J Med **355**(9): 909-19.

Becker, S., and Muhlberger, E. (1999). "Co- and posttranslational modifications and functions of Marburg virus proteins. Curr. Top. Microbiol. Immunol. 235;23-34

Bi, Z. and C. S. Reiss (1995). "Inhibition of vesicular stomatitis virus infection by nitric oxide." J Virol **69**(4): 2208-13

Boontham, P., P. Chandran, et al. (2003). "Surgical sepsis: dysregulation of immune function and therapeutic implications." Surgeon **1**(4): 187-206.

Borchert, M., M. Boelaert, et al. (2000). "Viewpoint: filovirus haemorrhagic fever outbreaks: much ado about nothing?" Trop Med Int Health **5**(5): 318-24.

Borchert, M., S. Mulangu, et al. (2005). "Lessons from the outbreak of Marburg virus." N Engl J Med **353**(11): 1185.

Borio, L., T. Inglesby, et al. (2002). "Hemorrhagic fever viruses as biological weapons: medical and public health management." Jama **287**(18): 2391-405.

Bosio, C. M., M. J. Aman, et al. (2003). "Ebola and Marburg viruses replicate in monocyte-derived dendritic cells without inducing the production of cytokines and full maturation." J Infect Dis **188**(11): 1630-8.

Bowen, E. T., G. S. Platt, et al. (1980). "A comparative study of strains of Ebola virus isolated from southern Sudan and northern Zaire in 1976." J Med Virol **6**(2): 129-38.

Brandt, C. D., H. W. Kim, et al. (1969). "Infections in 18,000 infants and children in a controlled study of respiratory tract disease. I. Adenovirus pathogenicity in relation to serologic type and illness syndrome." Am J Epidemiol **90**(6): 484-500.

Bukreyev, A. A., V. E. Volchkov, et al. (1995). "The complete nucleotide sequence of the Popp (1967) strain of Marburg virus: a comparison with the Musoke (1980) strain." Arch Virol **140**(9): 1589-600.

Burioni, R., R. A. Williamson, et al. (1994). "Recombinant human Fab to glycoprotein D neutralizes infectivity and prevents cell-to-cell transmission of herpes simplex viruses 1 and 2 in vitro." Proc Natl Acad Sci U S A **91**(1): 355-9.

Burton, D. R. (2002). "Antibodies, viruses and vaccines." Nat Rev Immunol **2**(9): 706-13.

Casillas, A. M., A. M. Nyamathi, et al. (2003). "A current review of Ebola virus: pathogenesis, clinical presentation, and diagnostic assessment." Biol Res Nurs **4**(4): 268-75.

CDC, Centers for Disease Control and Prevention, I. S. f. I. (2005). Marburg hemorrhagic fever- Angola, archive no. 20051108.3269. International Society for Infectious Diseases. Brooklin, Mass.

CDC, Centers for Disease Control and Prevention. (2005) "Outbreak of Marburg virus hemorrhagic fever--Angola, October 1, 2004-March 29, 2005." MMWR Morb Mortal Wkly Rep **54**(12): 308-9.

CDC, Centers for Disease Control and Prevention, C. f. D. (1998). Infection control for viral hemorrhagic fevers in the African health care settings. CDC. Atlanta, GA.

Cerutti, A., Qiao, X. et al. (2005). "Plasmacytoid dendritic cells and the regulation of immunoglobulin heavy chain class switching." Immunol Cell Biol **83** (5):554-62

Chirmule, N., K. Propert, et al. (1999). "Immune responses to adenovirus and adeno-associated virus in humans." Gene Ther **6**(9): 1574-83.

Colebunders, R., H. Sleurs, et al. (2004). "Organisation of health care during an outbreak of Marburg haemorrhagic fever in the Democratic Republic of Congo, 1999." J Infect **48**(4): 347-53.

Daddario-Dicaprio, K. M., T. W. Geisbert, et al. (2006). "Postexposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment." Lancet **367**(9520): 1399-1404.

Davis, K. J., A. O. Anderson, et al. (1997). "Pathology of experimental Ebola virus infection in African green monkeys. Involvement of fibroblastic reticular cells." Arch Pathol Lab Med **121**(8): 805-19.

Dowling, W., Thompson, E., Bodger, C., Mellquiest, J.L., Garrison, A.R., Smith, J.M., Paragas, J., Hogan, R.J., Schmaljohn, C. (2006). "The influences of glycosylation on the antigenicity, immunogenicity, and protective efficacy of Ebola virus GP DNA vaccines." J Virol. Epub ahead of print.

Fehr, T., M. F. Bachmann, et al. (1996). "T-independent activation of B cells by vesicular stomatitis virus: no evidence for the need of a second signal." Cell Immunol **168**(2): 184-92.

Feldmann, H., Will, C., Schikore, M., Slenczka, W., Klenk, H.D. (1991). "Glycosylation and oligomerization of the spike protein of Marburg virus." Virology 182:353-356

Feldmann, H. (2006). "Marburg hemorrhagic fever--the forgotten cousin strikes." N Engl J Med **355**(9): 866-9.

Feldmann, H., H. Bugany, et al. (1996). "Filovirus-induced endothelial leakage triggered by infected monocytes/macrophages." J Virol **70**(4): 2208-14.

Feldmann, H., T. W. Geisbert, et al. (2004). Filoviridae. Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses. C. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger and L. A. Ball. London, Elsevier/Academic Press**:** 645-653.

Feldmann, H., S. Jones, et al. (2003). "Ebola virus: from discovery to vaccine." Nat Rev Immunol **3**(8): 677-85.

Feldmann, H., and Kiley, M.P. (1999). "Classification, structure, and replication of filoviruses." Curr. Top. Microbiol. Immuno. 235:1-22.

Formella, S. Jehle, C., et al. (2000). "Sequence variability of Borna disease virus: resistance to superinfection may contribute to high genome stability in persistently infected cells." J Virol **74**(17):7878-83

Fujinami, R. S. and M. B. Oldstone (1979). "Antiviral antibody reacting on the plasma membrane alters measles virus expression inside the cell." Nature **279**(5713): 529-30.

Garbutt, M., R. Liebscher, et al. (2004). "Properties of replication-competent vesicular stomatitis virus vectors expressing glycoproteins of filoviruses and arenaviruses." J Virol **78**(10): 5458-65.

Garcia-Sastre, A. (2001). "Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses". Virology **279**(2): 375-84

Gear, J. S., G. A. Cassel, et al. (1975). "Outbreake of Marburg virus disease in Johannesburg." Br Med J **4**(5995): 489-93.

Geisbert T, Jahrling. P. (2004). "Exotic emerging viral diseases: progress and challenges." Nat Med **10**(12 Suppl): S110-21.

Geisbert, T. W., L. E. Hensley, et al. (2000). "Apoptosis induced in vitro and in vivo during infection by Ebola and Marburg viruses." Lab Invest **80**(2): 171-86.

Geisbert, T. W., L. E. Hensley, et al. (2003). "Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques: evidence that dendritic cells are early and sustained targets of infection." Am J Pathol **163**(6): 2347-70.

Geisbert, T. W. and P. B. Jahrling (2003). "Towards a vaccine against Ebola virus." Expert Rev Vaccines **2**(6): 777-89.

Geisbert, T. W., P. B. Jahrling, et al. (1992). "Association of Ebola-related Reston virus particles and antigen with tissue lesions of monkeys imported to the United States." J Comp Pathol **106**(2): 137-52.

Geisbert. T.W., Jaax, N.K. (1998). "Marburg hemorrhagic fever: report of a case studied by immunohistochemistry and electron microscopy." Ultrastruct Pathol 22(1):3-17

Geisbert, T. W., S. Jones, et al. (2005). "Development of a new vaccine for the prevention of Lassa fever." PLoS Med **2**(6): e183.

Geisbert, T. W., P. Pushko, et al. (2002). "Evaluation in nonhuman primates of vaccines against Ebola virus." Emerg Infect Dis **8**(5): 503-7.

Geisbert, T. W., H. A. Young, et al. (2003). "Pathogenesis of Ebola hemorrhagic fever in primate models: evidence that hemorrhage is not a direct effect of virus-induced cytolysis of endothelial cells." Am J Pathol **163**(6): 2371-82.

Geisbert, T.W., and Jahrling, P. (1995). "Differentiation of filoviruses by electron microscopy." Virus Res. 39(2-3):129-50.

Georges AJ, L. E., Renaut AA, et al. (1999). "Ebola hemorrhagic fever outbreaks in Gabon, 1994-1997: epidemiologic and health control issues." J Infect Dis **179 Suppl**: S65-75.

Gerhard, W. (2001). "The role of the antibody response in influenza virus infection." Curr Top Microbiol Immunol **260**: 171-90.

Gessner, J. E., H. Heiken, et al. (1998). "The IgG Fc receptor family." Ann Hematol **76**(6): 231-48.

Geyer, H., Will, C., Feldmann, H., Klenk, H.D., Geyer, R. (1992). "Carbohydrate stucture of Marburg virus glycoprotein." Glycobiology 2:299-312.

Graham, M. B. and T. J. Braciale (1997). "Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice." J Exp Med **186**(12): 2063-8.

Gresser, I., M. G. Tovey, et al. (1975). "Efficacy of exogenous interferon treatment initiated after onset of multiplication of vesicular stomatitis virus in the brains of mice." J Gen Virol **27**(3): 395-8.

Gresser, I., M. G. Tovey, et al. (1976). "Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. II. Studies with herpes simplex, Moloney sarcoma, vesicular stomatitis, Newcastle disease, and influenza viruses." J Exp Med **144**(5): 1316-23.

Grignani, G. and A. Maiolo (2000). "Cytokines and hemostasis." Haematologica **85**(9): 967-72.

Harris, A. A., D. Daly-Gawenda, et al. (1991). "Vaccine choice and program participation rates when two hepatitis B vaccines are offered." J Occup Med **33**(7): 804-7.

Hart, M. K. (2003). "Vaccine research efforts for filoviruses." Int J Parasitol **33**(5-6): 583-95.

Hensley, L. E., H. A. Young, et al. (2002). "Proinflammatory response during Ebola virus infection of primate models: possible involvement of the tumor necrosis factor receptor superfamily." Immunol Lett **80**(3): 169-79.

Hevey, M., D. Negley, et al. (1997). "Antigenicity and vaccine potential of Marburg virus glycoprotein expressed by baculovirus recombinants." Virology **239**(1): 206-16.

Hevey, M., D. Negley, et al. (1998). "Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates." Virology **251**(1): 28-37.

Hevey, M., D. Negley, et al. (2003). "Characterization of monoclonal antibodies to Marburg virus (strain Musoke) glycoprotein and identification of two protective epitopes." Virology **314**(1): 350-7.

Hevey M, N. P., Pushko J, Smith J, Schmaljohn A (2001). "Determination of vaccine components required for protecting cynomolgus macaques against genotypically divergent isolates of Marburg virus, abstr." W36-4. Abstr. 20th Annu. Meet. Am. Soc. Virol., Madison, Wis.

Hirasawa, H., S. Oda, et al. (2004). "[Basic concept and definition of SIRS and sepsis--present consideration and future perspectives]." Nippon Rinsho **62**(12): 2177-83.

Huber, V. C., R. M. McKeon, et al. (2006). "Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza." Clin Vaccine Immunol **13**(9): 981-90.

Ignat'ev, G. M., M. A. Strel'tsova, et al. (1996). "[Experimental study of possible treatment of Marburg hemorrhagic fever with desferal, ribavirin, and homologous interferon]." Vopr Virusol **41**(5): 206-9.

Ignat'ev, G. M., M. A. Strel'tsova, et al. (1994). "[The immunological indices of guinea pigs modelling Marburg hemorrhagic fever]." Vopr Virusol **39**(4): 169-71.

Ignatyev, G. M., A. P. Agafonov, et al. (1996). "Inactivated Marburg virus elicits a nonprotective immune response in Rhesus monkeys." J Biotechnol **44**(1-3): 111-8.

ISID, International Society for Infectious Diseases (2005). Marburg Hemorrhagic Fever-Angola, http://www.promedmail.org Archive No. 20051108.3269).

ISID, International Society for Infectious Diseases. (2004) Ebola, l. a. d. R. S., May 22, 2004 (archive number 20040522.1377).

ISID, International Society for Infectious Diseases. (2004) Ebola, l. a. U. M., February 20, 2004 (archive number 20040440.0550).

Jaax, N. K., K. J. Davis, et al. (1996). "Lethal experimental infection of rhesus monkeys with Ebola-Zaire (Mayinga) virus by the oral and conjunctival route of exposure." Arch Pathol Lab Med **120**(2): 140-55.

Jahrling, P. B., T. W. Geisbert, et al. (1999). "Evaluation of immune globulin and recombinant interferon-alpha2b for treatment of experimental Ebola virus infections." J Infect Dis **179 Suppl 1**: S224-34.

Johnson, E. D., B. K. Johnson, et al. (1996). "Characterization of a new Marburg virus isolated from a 1987 fatal case in Kenya." Arch Virol Suppl **11**: 101-14.

Johnson, K. M., R. B. Tesh, et al. (1969). "Epidemiology of vesicular stomatitis virus: some new data and a hypothesis for transmission of the Indian serotype." J Am Vet Med Assoc **155**(12): 2133-40.

Jones, S. M., H. Feldmann, et al. (2005). "Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses." Nat Med **11**(7): 786-90.

Kahn, J. S., A. Roberts, et al. (2001). "Replication-competent or attenuated, nonpropagating vesicular stomatitis viruses expressing respiratory syncytial virus (RSV) antigens protect mice against RSV challenge." J Virol **75**(22): 11079-87.

Karpf, A.R., Lenches, E. (1997). "Superinfection exclusion of alphaviruses in three mosquito cell lines persistently infected with Sinbis virus." J Virol **71**(9):7119-23

Kayagaki, N., N. Yamaguchi, et al. (1999). "Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: A novel mechanism for the antitumor effects of type I IFNs." J Exp Med **189**(9): 1451-60.

Kennedy, J. S., S. E. Frey, et al. (2004). "Induction of human T cell-mediated immune responses after primary and secondary smallpox vaccination." J Infect Dis **190**(7): 1286-94.

Kiley MP, B. E., Eddy GA., et al (1982). "Filoviradae: a taxonomic home for marburg and ebola viruses?" Intervirology **18**((1-2)): 24-32.

Kolokol'tsov, A. A., I. A. Davidovich, et al. (2001). "The use of interferon for emergency prophylaxis of marburg hemorrhagic fever in monkeys." Bull Exp Biol Med **132**(1): 686-8.

Komatsu, T., Z. Bi, et al. (1996). "Interferon-gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons." J Neuroimmunol **68**(1-2): 101-8.

Kretzschmar, E., L. Buonocore, et al. (1997). "High-efficiency incorporation of functional influenza virus glycoproteins into recombinant vesicular stomatitis viruses." J Virol **71**(8): 5982-9.

Kruse, M., O. Rosorius, et al. (2000). "Mature dendritic cells infected with herpes simplex virus type 1 exhibit inhibited T-cell stimulatory capacity." J Virol **74**(15): 7127-36.

Kuhn, J.H., Radoshitzky, S.R., Guth, A.C., Warfield, K.L., Li, W., Vincent, M.J., Towner, J., Nichol, S.T., Bavari, S., Choe, H., Aman, M.J., Farzan, M. (2006). "Conserved receptor-binding domains of Lake Victoria marburgvirus and Zaire ebolavirus bind a common receptor." J. Biol. Chem. 281(23):15951-8.

Kuno-Sakai, H., M. Kimura, et al. (1994). "Developments in mucosal influenza virus vaccines." Vaccine **12**(14): 1303-10.

Lawson, N. D., E. A. Stillman, et al. (1995). "Recombinant vesicular stomatitis viruses from DNA." Proc Natl Acad Sci U S A **92**(10): 4477-81.

Lee, B. O., J. Rangel-Moreno, et al. (2005). "CD4 T cell-independent antibody response promotes resolution of primary influenza infection and helps to prevent reinfection." J Immunol **175**(9): 5827-38.

Lee, J. S., J. L. Groebner, et al. (2006). "Multiagent vaccines vectored by Venezuelan equine encephalitis virus replicon elicits immune responses to Marburg virus and protection against anthrax and botulinum neurotoxin in mice." Vaccine **24**(47-48): 6886-92.

Lefeuvre, A., P. Marianneau, et al. (2004). "Current Assessment of Yellow Fever and Yellow Fever Vaccine." Curr Infect Dis Rep **6**(2): 96-104.

Lefrancois, L. (1984). "Protection against lethal viral infection by neutralizing and nonneutralizing monoclonal antibodies: distinct mechanisms of action in vivo." J Virol **51**(1): 208-14.

Leirs H, M. J., Krebs et al. (1999). "Search for Ebola reservoir in Kikwit, Democratic Republic of the Congo: reflections on a vertebrate collection." J Infect Dis Suppl **179 Suppl**: S155-63.

Leroy, E.M., B., Kumulungui et al. (2005) "Fruit bats as reservoirs of Ebola virus." Nature **438**(7068): 575-6

Letchworth, G. J., L. L. Rodriguez, et al. (1999). "Vesicular stomatitis." Vet J **157**(3): 239-60.

Levi, M., E. de Jonge, et al. (2001). "Advances in the understanding of the pathogenetic pathways of disseminated intravascular coagulation result in more insight in the clinical picture and better management strategies." Semin Thromb Hemost **27**(6): 569-75.

Levine, B., J. M. Hardwick, et al. (1991). "Antibody-mediated clearance of alphavirus infection from neurons." Science **254**(5033): 856-60.

Majid, A. M., H. Ezelle, et al. (2006). "Evaluating replication-defective vesicular stomatitis virus as a vaccine vehicle." J Virol **80**(14): 6993-7008.

Martini, G. (1971). Marburg Virus Disease. Clinical Syndrome. Marburg Virus Disease. M. GA and R. Siegert. New York, Springer-Verlag**:** 1-9.

Martini, G. A., H. G. Knauff, et al. (1968). "A hitherto unknown infectious disease contracted from monkeys. "Marburg-virus" disease." Ger Med Mon **13**(10): 457-70.

Marzi, A., Gramberg, T., Simmons, G., Moller, P., Rennekamp, A.J., Krumblegel, M., Geier, M., Eisemann, J., Turza, N., Saunier, B., Steinkasserer, A., Becker, S., Bates, P., Hofmann, H., Pohlmann, S. (2004). "DC-SIGN and DC-SIGNR interacts with the glycoproteins of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus." J Virol. 78(21):12090-5.

Massoudi, M. S., L. Barker, et al. (2003). "Effectiveness of postexposure vaccination for the prevention of smallpox: results of a delphi analysis." J Infect Dis **188**(7): 973-6.

Miller J, E. S., Broad WJ (2001). Germs: Biological Weapons and America's Secret War. Waterville, ME, Simon & Schuster.

Moldoveanu, Z., M. L. Clements, et al. (1995). "Human immune responses to influenza virus vaccines administered by systemic or mucosal routes." Vaccine **13**(11): 1006-12.

Morrow CD, P. D., Ansardi DC, Moldoveanu Z, Fultz PN (1994). "New approaches for mucosal vaccines for AIDS: encapsidation and serial passages of poliovirus

replicons that express HIV-1 proteins on infection." AIDS Res Hum Retroviruses **10 Suppl**(2): S61.

Mortimer, P. P. (2003). "Can postexposure vaccination against smallpox succeed?" Clin Infect Dis **36**(5): 622-9.

Nethe, M., B. Berkhout, et al. (2005). "Retroviral superinfection resistance." Retrovirology **2**: 52.

Neuberger, M. S. and K. Rajewsky (1981). "Activation of mouse complement by monoclonal mouse antibodies." Eur J Immunol **11**(12): 1012-6.

Nimmerjahn, F. and J. V. Ravetch (2005). "Divergent immunoglobulin g subclass activity through selective Fc receptor binding." Science **310**(5753): 1510-2.

Pantaleo, G., J. F. Demarest, et al. (1995). "Effect of anti-V3 antibodies on cell-free and cell-to-cell human immunodeficiency virus transmission." Eur J Immunol **25**(1): 226-31.

Peters, C. J. and A. S. Khan (1999). "Filovirus diseases." Curr Top Microbiol Immunol **235**: 85-95.

Piedra, P. A., G. A. Poveda, et al. (1998). "Incidence and prevalence of neutralizing antibodies to the common adenoviruses in children with cystic fibrosis: implication for gene therapy with adenovirus vectors." Pediatrics **101**(6): 1013-9.

Pokhodiaev, V. A., N. I. Gonchar, et al. (1991). "[An experimental study of the contact transmission of the Marburg virus]." Vopr Virusol **36**(6): 506-8.

Publicover, J., E. Ramsburg, et al. (2005). "A single-cycle vaccine vector based on vesicular stomatitis virus can induce immune responses comparable to those generated by a replication-competent vector." J Virol **79**(21): 13231-8.

Quinones-Koch, M.I., Buonocore, L., Rose, J.K. (2002). "Role of N-linked glycans in a human immunodeficiency virus envelope glycoprotein: effects on protein function and the neutralizing antibody response. J Virol 76(9):4199-211.

Raftery, M. J., M. Schwab, et al. (2001). "Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered viral defense strategy." Immunity **15**(6): 997-1009.

Reif, J. S., P. A. Webb, et al. (1987). "Epizootic vesicular stomatitis in Colorado, 1982: infection in occupational risk groups." Am J Trop Med Hyg **36**(1): 177-82.

Riemenschneider, J., A. Garrison, et al. (2003). "Comparison of individual and combination DNA vaccines for B. anthracis, Ebola virus, Marburg virus and Venezuelan equine encephalitis virus." Vaccine **21**(25-26): 4071-80.

Rippey, J.J., Schepers, N.T., Gear, J.H. (1984). "Pathology of Marburg virus disease". S Afr Med J **66**(2):50-4

Roberts, A., L. Buonocore, et al. (1999). "Attenuated vesicular stomatitis viruses as vaccine vectors." J Virol **73**(5): 3723-32.

Rose, N. F., P. A. Marx, et al. (2001). "An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants." Cell **106**(5): 539-49.

Rupprecht, C. E. and R. V. Gibbons (2004). "Clinical practice. Prophylaxis against rabies." N Engl J Med **351**(25): 2626-35.

Rupprecht, C. E., C. A. Hanlon, et al. (2002). "Rabies re-examined." Lancet Infect Dis **2**(6): 327-43.

Ryabchikova, E., L. Kolesnikova, et al. (1996). "Ebola virus infection in guinea pigs: presumable role of granulomatous inflammation in pathogenesis." Arch Virol **141**(5): 909-21.

Samuelsson, A., C. Brostrom, et al. (1997). "Apoptosis of CD4+ and CD19+ cells during human immunodeficiency virus type 1 infection--correlation with clinical progression, viral load, and loss of humoral immunity." Virology **238**(2): 180-8.

Sanchez, A. *et al*. Filoviridae: Marburg and Ebola Viruses. in *Fields Virology* (eds. Knipe, D.M. & Howley, P.M.) 1279-1304 (Lippincott Williams & Wilkins, Philadelphia, 2001).

Sanchez, A., S. G. Trappier, et al. (1996). "The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing." Proc Natl Acad Sci U S A **93**(8): 3602-7.

Sanchez, A., S. G. Trappier, et al. (1998). "Variation in the glycoprotein and VP35 genes of Marburg virus strains." Virology **240**(1): 138-46.

Schlesinger, J. J. and S. Chapman (1995). "Neutralizing F(ab')2 fragments of protective monoclonal antibodies to yellow fever virus (YF) envelope protein fail to protect mice against lethal YF encephalitis." J Gen Virol **76 (Pt 1)**: 217-20.

Schnell, M. J., L. Buonocore, et al. (1996). "Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses are incorporated efficiently into virus particles." Proc Natl Acad Sci U S A **93**(21): 11359-65.

Schnittler, H. J. and H. Feldmann (1999). "Molecular pathogenesis of filovirus infections: role of macrophages and endothelial cells." Curr Top Microbiol Immunol **235**: 175-204.

Schroder, C. Azimzadeh, A.M., et al. (2003). "Anti-CD20 treatment depletes B-cells in blood and lymphatic tissue of cynomologous monkeys." Transpl Immunol **12**(1):19-28

Schulick, A. H., G. Vassalli, et al. (1997). "Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries. Potential for immunosuppression and vector engineering to overcome barriers of immunity." J Clin Invest **99**(2): 209-19.

Sergeev, A. N., M. Lub, et al. (1995). "[The efficacy of the emergency prophylactic and therapeutic actions of immunomodulators in experimental filovirus infections]." Antibiot Khimioter **40**(5): 24-7.

Sha, Z. and R. W. Compans (2000). "Induction of CD4(+) T-cell-independent immunoglobulin responses by inactivated influenza virus." J Virol **74**(11): 4999-5005.

Shinozawa, Y., X. Q. Xie, et al. (2004). "[Correlation between intravascular coagulation/fibrinolysis system and cytokines]." Nippon Rinsho **62**(12): 2253-61.

Siegert, R., H. L. Shu, et al. (1968). "[Isolation and identification of the "Marburg virus"]." Dtsch Med Wochenschr **93**(12): 604-12.

Simon, K., J.J. Cardomone. (1990). "Cellular mechanisms in the superinfection exclusion of vesicular stomatitis virus. Virology **177**(1): 375-9

Slenczka, W. G. (1999). "The Marburg virus outbreak of 1967 and subsequent episodes." Curr Top Microbiol Immunol **235**: 49-75.

Smith, D. H., B. K. Johnson, et al. (1982). "Marburg-virus disease in Kenya." Lancet **1**(8276): 816-20.

Stanberry, L. (2004). "Clinical trials of prophylactic and therapeutic herpes simplex virus vaccines." Herpes **11 Suppl 3**(3): 161A.

Steinhoff, U., U. Muller, et al. (1995). "Antiviral protection by vesicular stomatitis virus-specific antibodies in alpha/beta interferon receptor-deficient mice." J Virol **69**(4): 2153-8.

Steinman, R.M., Hemmi, H. (2006). "Dendritic cells: translating innate to adaptive immunity." Curr Top Microbiol Immunol **311**:17-58

Stille, W., E. Bohle, et al. (1968). "An infectious disease transmitted by Cercopithecus aethiops. ("Green monkey disease")." Ger Med Mon **13**(10): 470-8.

Stroher, U., West, E. et al. (2001). "Infection and activation of monocytes by Marburg and Ebola viruses." J Virol **75**(22): 11025-33

Sullivan, N., Geisbert, T.W., Geisbert, J., Xu, L., Yang, Z.Y., Roederer, M., Koup, R.A., Jahrling, P.B., Nabel, G.J. (2003). "Accelerated vaccination for Ebola virus hemorrhagic fever in nonhuman primates." Nature 424:681-684.

Symons, J.A., Alcami, A. et al. (1995). "Vaccinia virus encodes a soluble type I interferon receptor pf novel structure and broad species specificity." Cell **81**(4):551-60

Szomolanyi-Tsuda, E. and R. M. Welsh (1996). "T cell-independent antibody-mediated clearance of polyoma virus in T cell-deficient mice." J Exp Med **183**(2): 403-11.

Takabayashi, A., Y. Kawai, et al. (2000). "Nitric oxide induces a decrease in the mitochondrial membrane potential of peripheral blood lymphocytes, especially in natural killer cells." Antioxid Redox Signal **2**(4): 673-80.

Takada, A., Robison, C., Goto, H., Sanchez, A., Murti, G., Whitt, A., Kawaoka, Y. (1997). "A system for functional analysis of Ebola virus glycoprotein." Proc. Natl. Acad. Sci. USA. 94:14764-14769.

Takada, A., Fujioka, K. Tsuiji, M., Morikawa, A., Higashi, N., Ebihara, H., Kobasa, D., Feldmann, H., Irimura, T., Kawaoka, Y. (2004). "Human macrophage C-type lectin specific for galactose and N-acetylgalactosamine promotes filovirus entry." J Virol. 78(6):2943-7

Tesh, R. B., P. H. Peralta, et al. (1969). "Ecologic studies of vesicular stomatitis virus. I. Prevalence of infection among animals and humans living in an area of endemic VSV activity." Am J Epidemiol **90**(3): 255-61.

Tough, D.F. (2004). "Type I interferon as a link between innate and adaptive immunity through dendritic cell stimulation." Leuk Lymphoma **45**(2):257-64

Towner, J. S., M. L. Khristova, et al. (2006). "Marburgvirus genomics and association with a large hemorrhagic fever outbreak in Angola." J Virol **80**(13): 6497-516.

Van Hoeven, N.S., and Miller, A.D. (2005). "Use of different but overlapping determinants in a retrovirus receptor accounts for non-reciprocal interference between xenotropic and polytropic murine leukemia viruses." Retrovirology **2**(1): 76

Villinger, F., P.E., Rollin et al. (1999). "Markedly elevated levels of interferon (IFN)-gamma, IFN-alpha, interleukin (IL)-2, IL-10, and tumor necrosis factor-alpha associated with fatal Ebola virus infection." J Infect Dis **179 Suppl 1**: S188-91

Wagner R, R. J. (2001). Rhabdoviridae. Philadephia, Lippincott Williams & Wilkens.

Wagner, R. R. and J. K. Rose (1996). Rhabdoviridae: The Viruses and their Replication. Fields Virology. D. M. Knipe and P. M. Howley. Philadelphia, Lippincott Williams & Wilkins**:** 1121-1135.

Wang, D., A. L. Schmaljohn, et al. (2006). "De novo syntheses of Marburg virus antigens from adenovirus vectors induce potent humoral and cellular immune responses." Vaccine **24**(15): 2975-86.

Warfield, K. L., D. L. Swenson, et al. (2004). "Marburg virus-like particles protect guinea pigs from lethal Marburg virus infection." Vaccine **22**(25-26): 3495-502.

WHO, World Health Organization, (1967). Wkly Epidemiol Rec **42**: 479- 480.

WHO, World Health Organization (1978a). "Ebola Hemorrhagic fever in Sudan, 1976. Report of an International study team." Bull. World Health Organ. **56**: 247-270.

WHO, World Health Organization (1978b). "Ebola Hemorrhagic Fever in Zaire, 1976. Report of an International Commision." Bull. World Health Organ. **56**: 271-293.

WHO, World Health Organization (1999). "Marburg fever, Democratic Republic of the Congo." Wkly Epidemiol Rec **74**(19): 145.

WHO, World Health Organization (2005). "Marburg haemorrhagic fever, Angola." Wkly Epidemiol Rec **80**(18): 158.

Wilde, H., P. Khawplod, et al. (2005). "Rabies control in South and Southeast Asia." Vaccine **23**(17-18): 2284-9.

Wilson, J. A., M. Hevey, et al. (2000). "Epitopes involved in antibody-mediated protection from Ebola virus." Science **287**(5458): 1664-6.

Xiong, C., R. Levis, et al. (1989). "Sindbis virus: an efficient, broad host range vector for gene expression in animal cells." Science **243**(4895): 1188-91.

Yu, A. S., R. C. Cheung, et al. (2004). "Hepatitis B vaccines." Clin Liver Dis **8**(2): 283-300.

Zaki, S. R. and C. S. Goldsmith (1999). "Pathologic features of filovirus infections in humans." Curr Top Microbiol Immunol **235**: 97-116.