

COMPARISON OF ANTIBODY RESPONSES IN MICE GENERATED BY DIFFERENT
MULTIVALENT FILOVIRUS VACCINES BASED ON RECOMBINANT GLYCOPROTEIN
SUBUNITS

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Abstract

Filoviruses cause fulminant hemorrhagic fevers with case-fatality rates up to 90%. The objective of this research is to achieve a multivalent filovirus vaccine that can be thermostabilized and used to prevent the spread of lethal filovirus disease. The central hypothesis is that since each immunogen was found to elicit a strong antibody response on its own, it should be possible to optimize the formulation dosages of each immunogen to generate a combination vaccine that induces a balanced, robust antibody response and neutralizing antibody titers against the most pathogenic filovirus species, EBOV.

Recombinant glycoprotein (GP) derived from Ebola virus (EBOV), Sudan virus (SUDV) and Marburg virus (MARV) have been expressed from stably transformed *Drosophila* S2 cells and used to formulate recombinant subunit vaccine candidates showing efficacy in rodents and non-human primates. Immunogenicity of 12 different formulations of our trivalent subunit vaccine with adjuvant was tested in Swiss Webster mice, and serum antibody concentration and neutralization of rVSV-EBOV GP was measured and compared to responses from monovalent formulations.

The 12 formulations were categorized into three sets of four groups of increasing dosage – The first set being formulations of equal antigen, the second being equal EBOV and MARV antigen with less SUDV antigen, and the third being equal amounts of Ebolavirus to Marburgvirus antigen. These formulations were determined in order to set up assays for potency testing of the vaccine and to understand antibody responses to different formulations.

When quantifying antibody concentration, the results were as expected as trivalent formulations induced high antibody concentrations for all antigens at higher dosages. Also, the

trivalent vaccine is able to produce comparable antibody concentrations to being vaccinated with monovalent formulations which suggests that trivalent formulations can still elicit antibodies that are specific for each antigen. It was observed that higher dosages of antigen elicit higher antibody concentrations against EBOV and SUDV GP, but a dose-response was not detected for antibody concentrations against MARV GP suggesting that it may be the most immunogenic antigen. Ultimately, the formulation that was able to elicit the highest, and balanced antibody concentration against EBOV, SUDV, and MARV GP was the formulation of equal antigens at the highest dosage.

Results from the neutralization tests against rVSV-EBOV GP show that higher neutralization titers are achieved with increased dosages. Neutralization titers were also increased with inclusion of SUDV GP antigen as compared to monovalent vaccination and further increases when there is less SUDV GP present compared to the EBOV and MARV antigens, suggesting SUDV GP interference. This led to highest neutralization observed from groups containing less SUDV GP as compared to EBOV and MARV GP.

From these findings, we have shown that trivalent formulations can be balanced to both optimize total antigen-binding IgG and neutralizing responses. This data will be used to formulate the next vaccines for testing in NHP's and to develop potency tests for antigen- and batch-release testing.

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Abbreviations

BDBV	Bundibugyo ebolavirus
BLA	Biologics License Application
BOMV	Bombali ebolavirus
DRC	Democratic Republic of Congo
EBOV/ZEBOV	Zaire ebolavirus
EVD	Ebola Virus Disease
FDA	Food and Drug Administration
GP	Glycoprotein
i.m.	Intramuscularly
IACUC	Institutional Animal Care and Use Committee
IND	Investigational New Drug
L	RNA-dependent RNA polymerase
LLOQ	Lower Limit of Quantification
mAb	Monoclonal Antibody
MARV	Marburg marburgvirus
MLAV	Měnglà dianlovirus
MLD	Mucin-like Domain
NHP	Non-human Primates
NP	Nucleoprotein
ns-ssRNA	Negative-Sense, Single-Stranded RNA
PFU	Plaque-Forming Units
RAVV	Ravn marburgvirus

RBD	Receptor-binding Domain
RESTV	Reston ebolavirus
rHPIV3	Recombinant Human Parainfluenza Type 3 Virus
RT	Room Temperature
rVSV	Recombinant Vesicular Stomatitis Virus
SUDV	Sudan ebolavirus
TAFV	Taï Forest ebolavirus
VP	Viral protein
WHO	World Health Organization

CHAPTER II

BACKGROUND

1.1 Filovirus

1.1.1 Virus Classification

Ebolaviruses are part of the *Filoviridae* family under the order of Mononegavirales [15, 3]. There are three confirmed genera of *Filoviridae*: Ebolavirus, Marburgvirus and Cuevavirus [15]. Figure 1 illustrates the phylogenetic relationship between the three genera and their geographical distribution of outbreaks in Africa. Within the genera of Ebolavirus there are six identified species being: Zaire ebolavirus (EBOV), Sudan ebolavirus (SUDV), Reston ebolavirus (RESTV), Tai Forest ebolavirus (TAFV), Bundibugyo ebolavirus (BDBV), and Bombali ebolavirus (BOMV) in which all but RESTV and BOMV are known to cause disease in humans [15]. In the genera of Marburgvirus there are two species, Marburg Marburgvirus (MARV) and Ravn Marburgvirus (RAVV), which were the causes of some of the earliest Filovirus outbreaks [59]. Lastly in the genera of Cuevavirus there is one species named Lloviu virus (LLOV) that is genetically distinct from the other filoviruses because it was detected in Europe rather than Africa [36]. There are other proposed new genera and species within the Filovirus family that have not been officially accepted such as Dianlovirus (species Měnglà dianlovirus (MLAV)) and filoviruses that may infect fish [21].

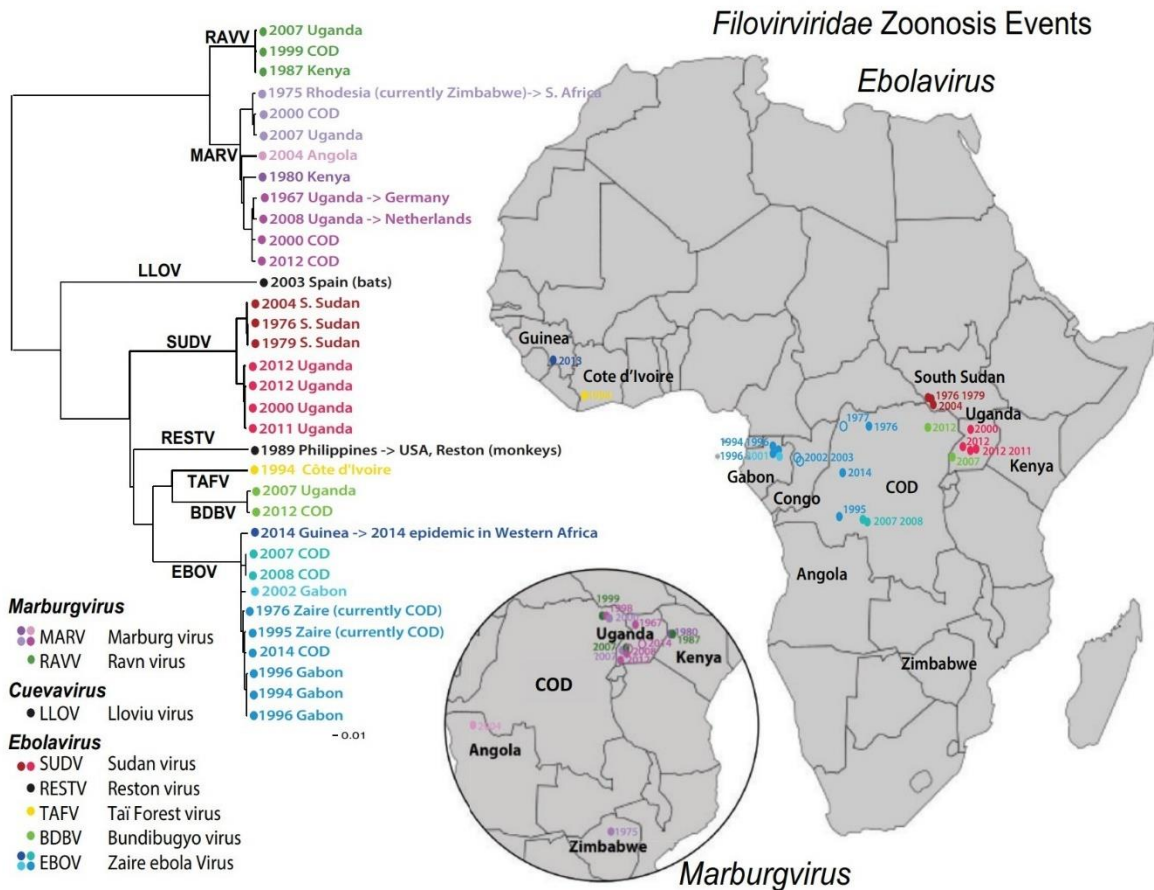


Figure 1. Filovirus outbreak phylogenetic tree and geographical distribution (Reproduced under the terms of the Creative Commons CC BY license). [59]

1.1.2 History

Filoviruses are known to cause fulminant hemorrhagic fevers in humans with case-fatality rates up to 90% [23, 25]. MARV was the first filovirus discovered in 1967 during lab outbreaks in Marburg, Germany and Yugoslavia transmitted to humans from infected monkeys imported from Uganda. This resulted in 31 cases and 7 deaths with a case-fatality rate (CFR) of ~23% [15, 5]. MARV reemerged in 1975 and has caused two large outbreaks; one in 1999 in the DRC, and one in Angola in 2005, along with continuous sporadic outbreaks [15, 19] The next novel filovirus outbreak occurred in

1976 in Zaïre (now known as the Democratic Republic of Congo (DRC)) and southern Sudan where researchers discovered a virus morphologically similar but serologically distinct from MARV [56]. The outbreak in Zaïre had a total of 318 cases and 280 deaths with a CFR of 88% and was caused by the virus named *Zaïre ebolavirus* (EBOV) which became known as the most lethal filovirus species isolated to date. The first outbreak of another novel virus that would be later named *Sudan ebolavirus* (SUDV) happened simultaneously with 284 cases, 151 deaths, and a CFR of 53% [10]. There have been other sporadic cases of SUDV throughout the years and some happening simultaneously with EBOV, but a large outbreak occurred in Gulu, Uganda in 2000 that had a total of 425 cases and 224 deaths, thus having a CFR of 53% which proved this virus to be very lethal as well [4, 38]. These viruses along with BDBV and TAFV have continued to cause sporadic outbreaks throughout the years including a devastating epidemic in West Africa in 2014 caused by EBOV. According to the world health organization (WHO) EBOV caused an outbreak totaling 28,652 suspected, probable, and confirmed cases with 11,325 deaths resulting in a ~40% CFR, being the largest filovirus outbreak in history [3]. Following that, another outbreak of EBOV was declared in 2018 and is still ongoing with the current status of 3,453 cases and 2273 deaths with a CFR of ~66%. Thankfully, the outbreak has begun to slow in terms of numbers and no new cases were reported since February 17, 2020 [13]. Filoviruses have proven to be a family of viruses that cause recurring outbreaks with high case-fatality rates in many parts of the world. Although the current outbreak in the DRC may finally have ended, it still raises concerns for future outbreaks.

1.1.3 Epidemiology and Transmission

The major pathogenic Filovirus species (MARV, EBOV, and SUDV) appear to mainly affect Africa since the MARV outbreaks in Europe actually originated from samples sent from Africa [14]. There have also been cases of RESTV from *Cynomolgus macaques* imported from the Philippines (Asia) in which people who worked closely with the monkeys developed high titers of the virus but remained asymptomatic [33]. However, better methods to measure seroprevalence are needed to assess its global distribution as well as understand the rate of unrecognized infections to truly assess the natural history of filoviruses.

It is believed that Filoviruses are zoonotic and the proposed reservoir host are (fruit) bats with an intermediate host being non-human primates (NHP), duikers, and pigs, with a possibility of natural infection in dogs based on their seropositivity without obvious EBOV-like infection [30, 3]. Bats are potential reservoir host candidates because researchers found that bats could harbor viral RNA and support Filovirus replication and circulation at high titers without becoming symptomatic, suggesting that they are the natural hosts [48]. No non-human vertebrates or arthropods have been identified to be positive for the virus at this time [14]. Transmission from reservoir host to intermediate host is thought to occur through intermediate host contact through infected bat saliva or feces [39]. Zoonotic transmission from the reservoir host or intermediate host to humans is most likely through direct contact of infected organs, blood or other bodily fluids during hunting or consumption of the infected animals. It is also suggested that through deforestation and human encroachment, bats continue to migrate to places closer to human populations, promoting spread of the virus [30].

Transmission of filoviruses from human-to-human is most commonly achieved through close contact with infected patients when virus containing blood, tissue, or other bodily fluids come into contact with the broken skin or mucous membranes of another individual or through the use of or non-sterile pre-used needles [15, 30]. Also, aerosolized transmission of the virus has shown to cause lethal disease in NHP and challenge models have been developed for various animal species [46]. Scientists were able to conclude that the virus is shed in many types of bodily fluids through culturing EBOV in breast milk, saliva, semen, and urine from infected patients as well as isolating viral RNA from sweat, stool, and tears [2]. Previous spread of the virus during outbreaks were due to cultural burial rituals without the use of proper personal protective equipment (PPE), thus contributing to transmission [30]. This is because high levels of virus are present in a variety of bodily fluids when humans die of the disease, resulting in copious amounts of virus in and on the body of a deceased to transmit the infection [41]. Tests have shown that in NHP the viral RNA is still detectible postmortem up to 3 weeks after death, and viable virus was still found up to 7 days in swab specimens and up to 3 days in tissues, suggesting that this could occur in humans or on animal carcasses as well [41]. On the other hand, in a study of asymptomatic individuals exposed to infected patients, an early and robust pro-inflammatory cytokine response was seen that may have led to inhibition of viral replication. This suggested that asymptomatic people may not be infectious and may not transmit the virus to others in contrast to symptomatic individuals [26]. Other main pathways of transmission are through nosocomial spread or via contaminated needles or due to the lack of PPE in laboratory settings [39].

1.1.4 Genome Structure and Organization

Filoviruses are a group of enveloped, filamentous, virus particles with a non-segmented, negative-sense, single-stranded RNA (ns-ssRNA) genome that is ~19kb long [14]. Their genome encodes seven structural proteins: Glycoprotein (GP), nucleoprotein (NP), viral proteins (VP) VP35, 40, 24, and 30, and RNA-dependent RNA polymerase (L). Filoviruses have only one type of surface GP within the membrane covering the outer surface of the virus which mediates receptor binding and membrane fusion for entry into host cells. NP is a protein that encapsidates the viral genome along with VP30, VP35, and L protein. VP35 is a polymerase cofactor, and VP40 is a matrix protein. These proteins (except for VP30) are found in all non-segmented, negative-sense RNA viruses. VP24 is a minor matrix protein and VP30 is a transcription activator that is unique to filoviruses. [35].

1.1.5 Replication Cycle

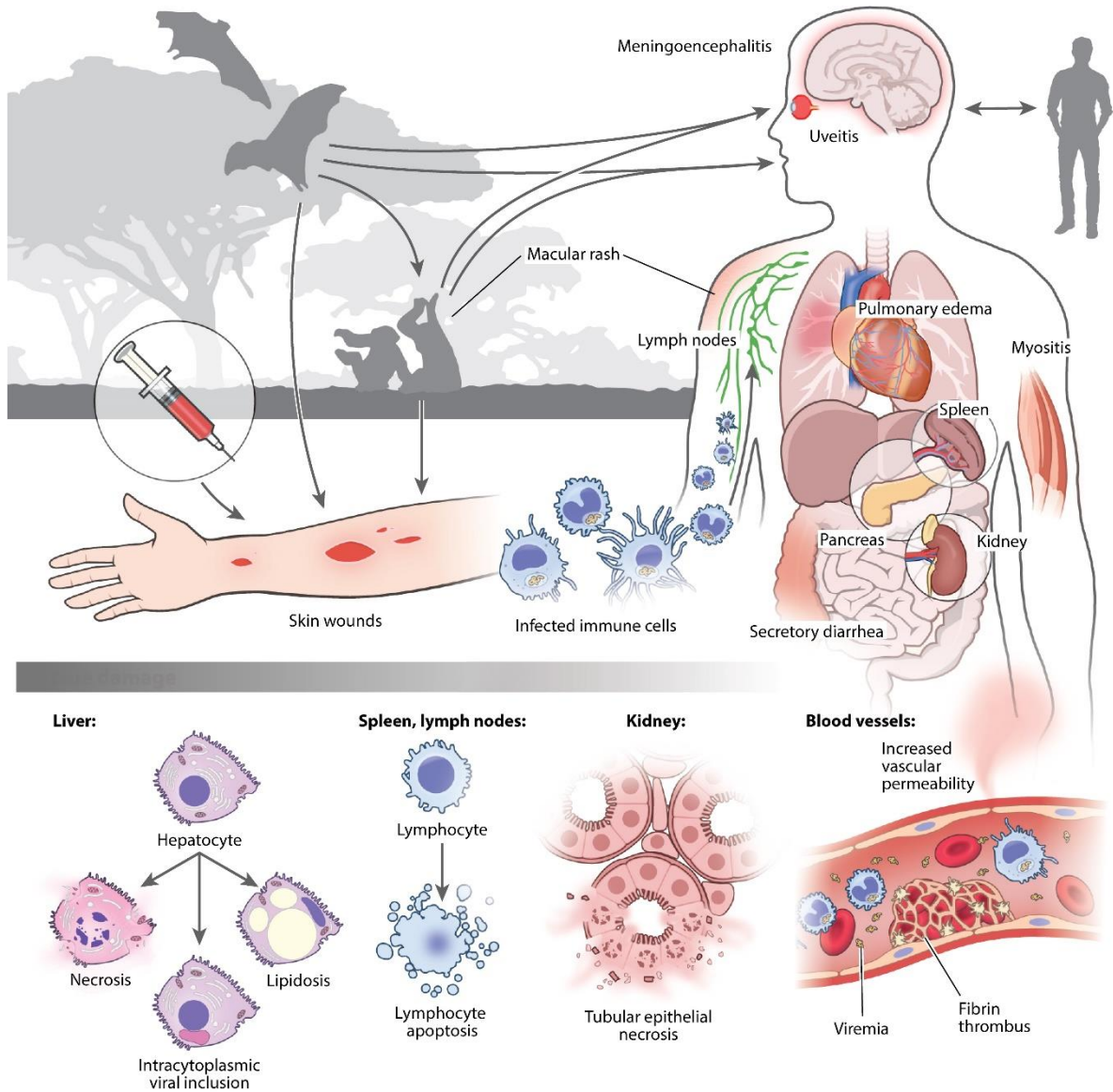
The virus enters the body through breaks in the skin or mucous membranes and targets mononuclear phagocytes such as monocytes, macrophages, dendritic cells, hepatocytes, lymphocytes, fibroblasts, and endothelial cells [30]. Filoviruses initially attach to target cells through the binding of GP. Interaction of GP1 with the host cell occurs through host cell factors such as glycosaminoglycans or C-type lectins as well as interactions between phosphatidylserine on the viral membrane and phosphatidylserine receptors on the host cell (such as T-cell immunoglobulin and mucin domain (TIM)) which will induce the cell to uptake the viral particle through micropinocytosis [21]. After binding, GP is cleaved into two parts, GP1 and GP2, and exists as covalently linked

trimers [21]. The viral particle is then transported through endosomes that provide an acidic environment which activate Cathepsin L and B to cleave GP1 receptor-binding site [21, 28, 58]. Within the endosomal membrane a protein called Neimann Pick C1 (NPC1) serves as a Filovirus receptor that redistributes cellular membranes and facilitates the fusion of GP2 to the endosomal membrane, followed by structural rearrangements of GP1 and GP2 [9, 58]. After the fusion of the viral membrane with the endosomal membrane mediated by GP2, the virus undergoes uncoating and the nucleocapsid is released into the host cytoplasm where transcription and replication occur [21]. Since Filoviruses are negative-sense (ns) ssRNA, they cannot directly produce proteins from their messenger RNA (mRNA) and need to use their ssRNA as a template along with L protein and VP35 to transcribe the viral genes into positive-sense mRNA (antigenome) that can be translated into protein or used to create more ns-ssRNA [58]. VP30 assists in viral replication as well in EBOV and LLOV but its role is not yet understood for MARV [21]. Once new viral proteins and genomes are created, assembly of the viral particle and budding can take place. GP is synthesized in the endoplasmic reticulum and is transported to the plasma membrane by the secretory vesicles of the Golgi apparatus. While in the secretory vesicles it undergoes proteolytic cleavage by furin to produce GP1 and GP2 that form a heterodimer that is disulfide-linked, resulting in the mature form of GP [22]. Movement of these vesicles from the Golgi to the plasma membrane is mediated through actin-dependent mechanisms [20]. GP also is recruited into the late endosome to join VP40 to aid in budding. Although the role of VP24 in this process is not completely understood, there are less virions released when VP24 is silenced [45, 1]. NP interacts with VP40, VP35 and VP24 to create the nucleocapsid structure which is

then transported to the plasma membrane. Once the components reach the plasma membrane, VP40 interacts with the inner leaflet and allows the membrane to encase the virion along with the surface GP on the outer leaflet to release the vesicle through budding to produce a newly formed Filovirus [45].

1.1.6 Pathogenesis and Clinical Disease

Filoviruses infect many cell types such as monocytes, macrophages, dendritic cells, and neutrophils which then disseminate throughout the body through the lymphatic system where it will further infect and replicate in new cells [39, 50]. The virus then travels through the blood to infect organs such as the liver, kidney and spleen which causes inflammation, necrosis, loss of vascular integrity, and apoptosis of the cells which in-turn produces the clinical symptoms [39]. With the increase in viremia, there is a massive increase in cytokine and chemokine responses leading to greater pathogenesis that ultimately leads to tissue damage. As a result, the disease progresses from non-specific flu-like symptoms to devastating clinical symptoms such as high temperatures, hepatic necrosis, splenomegaly, thrombocytopenia, diarrhea, and hemorrhage [8]. As the virus invades host cells it finds ways to evade the host immune system. The virus inhibits type I interferon responses through two of its structural proteins VP24 and VP35 which interrupt IFN signaling and antigen presentation, respectively [1]. The ability of filoviruses to disseminate and replicate rapidly throughout the body while evading the immune system are features that make them such lethal pathogens, urging researchers to quickly formulate prophylaxes or treatments. The pathogenesis and disease are illustrated in Figure 2.



AR Baseler L, et al. 2017.

Ann. Rev. Pathol. Mech. Dis. 12:387–418

Figure 2. Pathogenesis and disease of *ebolavirus* (Reproduced under the terms of the Copyright Clearance Center (CCC) and Annual Reviews Inc.). [1]

1.1.7 Vaccines

The high case fatality rates of EBOV have led to efforts to find and formulate a vaccine to prevent infection. There are two major categories of EBOV vaccines under development, replicating and non-replicating virally-vectored approaches. So far, only one vaccine has received approval late last year (December 2019) by the U.S. Food and Drug Administration (FDA) for prevention of EBOV. This vaccine is a replicating type named Ervebo [8]. Ervebo is a live, recombinant vaccine that uses the vesicular stomatitis virus (rVSV) backbone with its surface GP gene replaced with EBOV GP, thus expressing the EBOV GP on its surface to induce immunity to EBOV without causing disease [12]. This vaccine was used in a ring-vaccination strategy in Guinea in 2015 and was shown to have high efficacy. It is currently the only vaccine to have any clinical efficacy data [55]. Though this vaccine has been FDA-approved, tests have not yet been done to evaluate its safety and efficacy in immunocompromised populations including pregnant women and the elderly [31].

There are other replicating virus vaccines that are being developed but have not yet reached clinical trials. One vaccine is the recombinant human parainfluenza type 3 virus (rHPIV3) vaccine which is administered intranasally and has been found to protect guinea pigs and NHP's after challenge intraperitoneally. It was designed to express the EBOV GP instead of its own GP in a recombinant HPIV3 vector, or express EBOV GP and NP together [6, 5]. But since seroprevalence for HPIV is high among humans, we may have preexisting immunity to the vector and may be eliminated before providing a protective immune response. Other replicating virally-vectored vaccine candidates include Adenovirus-vectored vaccines such as chimp-adenovirus (ChAd3-EBOV), and

Ad26-EBOV boosted with Modified Vaccinia Ankara (MVA) virus. The ChAd3-vectored vaccine platform was also used to develop vaccines against other ebolaviruses and MARV, while the Ad26-EBOV and MVA combined platforms were used as an investigational prime-boost vaccine regimen designed to protect against the viruses that caused the 2014-2016 West-African outbreak as well as the 2018-2019 outbreak [12]. Another vaccine in development is a bivalent, live-attenuated or inactivated rabies strain that, similar to the previously mentioned vaccines, has its surface GP replaced with EBOV GP. This vaccine is intended to protect against both rabies virus and EBOV. So far, it has only shown to protect mice after a single vaccination [3]. The final replicating EBOV vaccine is a cytomegalovirus-based vaccine at the “proof of concept” stage of development. This vaccine would potentially disseminate from one vaccinated individual to many others through reinfection with the vector which could be used to protect great apes in the wild to prevent zoonotic transmission. This vaccine expresses the EBOV NP and is thought to induce lasting immunity in the wildlife population, but it is argued that many animals may have preexisting immunity because cytomegalovirus is ubiquitous [49].

Non-replicating virus EBOV vaccines are also in development alongside the replicating vaccines. Inactivated vaccines use formalin, heat, or radiation to inactivate the EBOV virus. These types of vaccines were shown to induce protection in mice, some protection in guinea pigs, and no protection in NHPs although NHPs produced an antibody response against the vaccine having low neutralizing titers [17]. Another type of non-replicating virus vaccine in development are DNA vaccines that include three plasmids encoding EBOV GP, SUDV GP or MARV GP, or EBOV NP. These vaccines

were shown to be immunogenic and safe during Phase I clinical trials, but still required boosting to improve overall immune response [27, 44]. Recombinant Adenovirus non-replicating vaccines are also under consideration. They were initially used to boost the DNA vaccines but have been found to protect NHPs against EBOV and SUDV after a single dose. However, concerns regarding preexisting immunity in humans also exist for Ad5 vaccines so use of Ad26 and ChAd is now preferred [40]. The final type of non-replicating vaccine are subunit vaccines. These vaccines consist of viral proteins that have been recombinantly expressed and purified individually or as virus-like particles for vaccine use. They can be produced in prokaryotic, yeast or mammalian cells and production in insect cells sometimes makes them more suitable for large-scale manufacturing and for manipulation of different antigen dosages [18, 1].

1.1.8 Treatment

Treatment of EVD is primarily through supportive care to treat the symptoms of the disease such as rehydration because of loss of fluids, correcting electrolyte imbalances, and treating secondary infections. Options for treatment are limited since there are no approved treatments for human use [34]. However, there are two monoclonal antibody therapeutics that are currently in clinical trials which are REGN-EB3 and mAb114 [29]. REGN-EB3 is a cocktail containing three monoclonal antibodies (mAb) against EBOV that was generated from a humanized mouse model that has been genetically modified to produce human mAb -- which bypasses the need to have to isolate B-cells from human survivors and recover their antibodies, which can be very limiting [29, 37]. mAb114 is a single monoclonal antibody that is generated in mammalian cells expressing heavy and light chain immunoglobulin genes recovered from

memory B cells isolated from a human survivor of the EBOV outbreak in the DRC in 1995 [29, 16]. Both of these forms of therapy outperformed previously tested treatments (ZMapp – cocktail of three modified mouse mAb, and Remdesivir – antiviral drug) in terms of treatment efficacy and patient survival, with REGN-EB3 administration proving to reduce mortality more than mAb114 [29, 37]. Both REGN-EB3 and mAb114 are now being offered to EBOV patients in the DRC for compassionate use.

1.2 Vaccine Development

1.2.1 Testing and Approval Process

To create a successfully licensed vaccine, an experimental vaccine must go through a rigorous regulatory process to ultimately assess its safety and efficacy involving a series of pre-clinical tests, clinical trials, and FDA review [7, 32]. During this process, each step performed must meet the regulatory standards of all GxP's which include Good Laboratory Practice, Good Manufacturing Practice, and Good Clinical Practice. The overview of the vaccine development process is highlighted in Figure 5.

The first step in vaccine development is pre-clinical testing, with the initial task of basic research and development. During this stage, basic research is done to better understand the pathogen, determine how the vaccine will be delivered, evaluate possible candidate immunogens, and assess potential adjuvants that need to be supplied along with the immunogen. During this time, researchers also decide on what kind of vaccine platform they would like to use. The advantage of replicating vaccines is that they are usually very immunogenic but can cause complications such as side effects and may not be safe for all types of individuals. On the other hand, non-replicating vaccines are

generally safer but usually require multiple doses and/or need to be accompanied by an adjuvant. After the basic research and drug discovery has been completed, the next step is to move on to pre-clinical studies that involve using *in vitro* assays and *in vivo* animal models. These are used to better understand the immunogenicity and safety of the vaccine candidate and to define potential correlates of protection to the disease. Usually these animal studies will involve the use of at least two different animal models, one being a small animal model, such as the mouse, to assess immunogenicity and the other being a NHP to assess response to vaccine in a model most similar to humans. During the preclinical stages of vaccine development, the product must undergo evaluation of its manufacturing process to assess if it is capable of being scaled-up to industrial levels of manufacturing. This evaluation also includes quality control of vaccine lots to assess identity, safety, purity, and potency to ensure that there is no deviation between lots and to establish a reproducible manufacturing method. Researchers will subsequently use the same or comparable vaccine lots produced under current good manufacturing practices (cGMP) for administration to humans during clinical trials.

Once the vaccine candidate completes the pre-clinical steps, and before entering the clinical trials, there must be approval from the U.S. FDA through the “Investigational New Drug Application” (IND) to allow authorization to administer a new investigational vaccine to human subjects. This application will provide information gathered from pre-clinical data and will lay out the plan for clinical evaluation including important insights into potential risks and benefits for potential participants. Once the IND is approved, the vaccine candidate can move on to clinical trials which includes three major phases. The reason for these clinical trials is to assess safety, immunogenicity, and efficacy in human

subjects. Phase I of clinical trials uses a small number (tens) of healthy individuals to primarily assess any safety issues related to dosage amount and vaccine administration route. Once proven safe, the vaccine moves to phase II trials which involves hundreds of participants to confirm safety and primarily assess immunogenicity and determine the optimal dosage that confers the best immunogenicity and safety. After a vaccine is proven to be immunogenic, Phase III trials commence which can involve thousands of participants to ultimately assess the overall clinical efficacy and safety of the vaccine through the dosage and immunization schedule determined by the previous clinical trial phases. This phase also helps determine if the vaccine can confer protection from disease and can span multiple countries, testing different types of target populations.

After successfully passing all the pre-clinical and clinical tests there needs to be submission of a Biologics License Application (BLA) which details the evaluation and performance of the vaccine candidate throughout the entire development process. There also must be meetings with regulatory agencies not only during this time, but throughout the entire development process to resolve any issues that may arise. After submission of the BLA and U.S. FDA approval, the entire vaccine development process must continue to be monitored throughout its lifetime to continuously assess safety and efficacy as well as monitor. Also, if there are any changes to the development process, it must obtain approval. There are also phase IV trials that can follow to assess the safety and efficacy over time in the context of routine use of the vaccine [32].

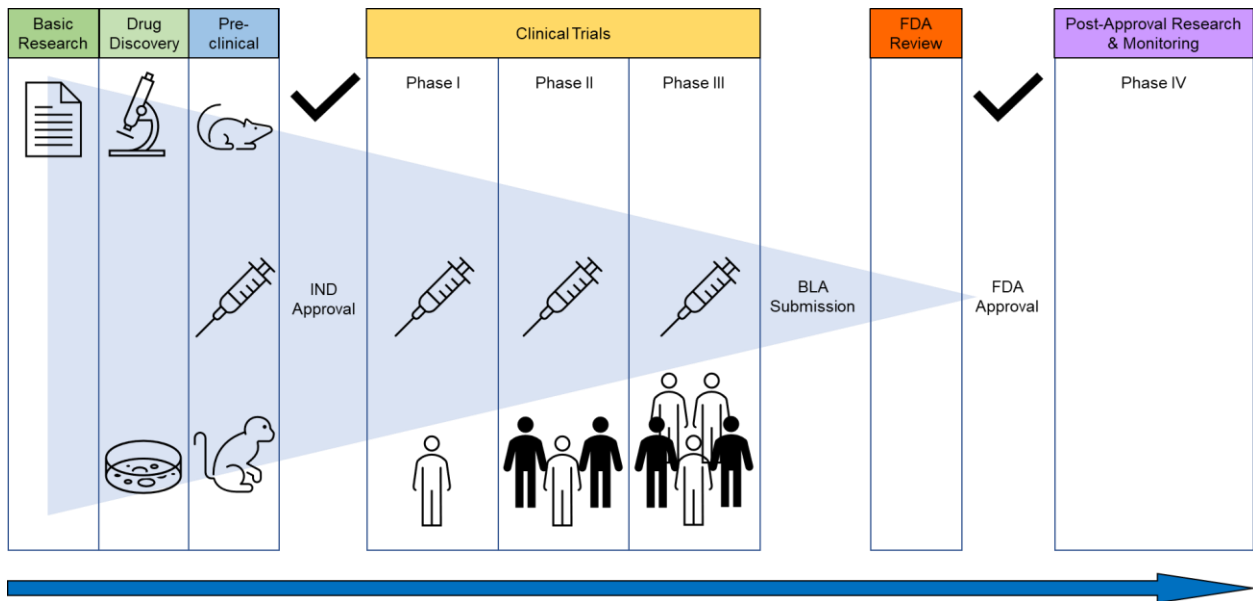


Figure 3. Overview of vaccine development and licensure.

1.2.2 Viral Subunit Vaccines

There are six main categories of licensed vaccines for humans which are live-attenuated, inactivated, toxoid, polysaccharide, conjugate, and recombinant vaccines [32]. Subunit vaccines fall under the category of recombinant vaccines which have become more available in recent years as genetic engineering advanced. Recombinant vaccines utilize recombinant DNA technology to allow multiple DNA segments from different organisms to be combined [52]. Viral subunit vaccines utilize protein subunits that are part of the target virus particle that are normally responsible for inducing protective immunity [43].

The common strategy to produce recombinant viral subunit vaccines is to generate recombinant DNA and insert it as a plasmid that can be transfected or transformed into a cell line which then produces the desired protein or antigen of choice which can be purified for vaccine use [25]. With the approach of viral subunit vaccines,

there are some advantages and disadvantages that they show in comparison to other vaccine platforms.

An advantage to subunit vaccines is that they are generally more safe in comparison to other types of vaccines because they are only composed of the purified essential immunogens necessary to stimulate an immune response without generating a reaction to the rest of the virus [32]. With that being said, subunit vaccines are safe enough to administer to immunocompromised patients whereas other vaccine platforms may not be able to because they may cause disease or immune side effects can be developed from the vaccine [53]. Since subunit vaccines do not contain live components, there is no risk for developing disease. Another advantage that subunit vaccines have is that they are easier to formulate because you can manipulate the amount of antigen dosage and are easier to scale-up and manufacture due to the *in-vitro* production method, making them generally very cost-effective. Some disadvantages of subunit vaccines are that they are not as immunogenic as other platforms but improvements to that can be made through administration of multiple dosages or the addition of an adjuvant [32, 11].

An example of a previously successful viral subunit vaccine is the widely-used recombinant hepatitis B vaccine that was efficacious enough to out-compete the previous inactivated vaccine that was derived from human plasma [32]. The switch to the viral subunit vaccine increased safety as well as made it much easier to manufacture, making the vaccine available to a larger population [51].

1.2.3 Adjuvants

Adjuvants are supplemented to formulations to aid in the immunogenicity of the vaccine by increasing potency or longevity of the antigen administered to maximize the production of host antibodies [47]. Vaccine adjuvants are often developed to serve as a delivery vehicle or immunostimulatory agent for the vaccine antigen to have prolonged release or to protect it from quick degradation in the body [32]. The safety and effectiveness profile of adjuvants are always assessed through the vaccine regulation and approval process so that it does not promote undesired immune responses [32].

The adjuvant being used in this study is CoVaccine HT™ which is a novel vaccine adjuvant that has been shown to enhance cell-mediated and humoral immunity as compared to other adjuvants [25, 24]. It is a nanoemulsion of squalane with an immunostimulatory sucrose fatty acid sulfate ester and is typically used for intramuscular administration [47].

1.2.4 Ideal Vaccine Recommendations

When developing a vaccine, there are certain characteristics that researchers should aim for to produce a safe, efficacious, ideal vaccine that can be licensed and FDA-approved. There are six main characteristics of an ideal vaccine. 1) One would be to have a protective immune response against the pathogen that the individual is being vaccinated against. Not only would an ideal vaccine develop a robust immune response, the goal is that the patient being administered the vaccine would have life-long immunity to that pathogen. 2) Another characteristic is to have a short, limited dosing regimen – ideally one dose of the vaccine should provide protective immunity. This is because the

individual should be protected in a short time from one dose versus having to return often to the health care facility to get multiple doses to achieve full vaccine protection. 3)

Another very important characteristic is to have a safe vaccine which is important because a desired vaccine is one that will not induce disease or will not produce any side effects and can be administered to more populations such as the immunocompromised.

4) Researchers should also create a vaccine that can maintain stability so that it can retain its biological activity for a long time at different temperatures. This is a goal because it is ideal to eliminate a need for a cold-chain to store and distribute the vaccine as many places do not have the resources and will also increase the cost of maintenance.

5) Another ideal characteristic is to have an ease of administration which means it can be co-administered with other vaccines in one visit. This is important because the vaccine should not have cross-reactivity occurring with other vaccines and should not require the patient to have to make multiple appointments to receive multiple vaccines. Ideally

patients should receive as many vaccines as they can in one visit. 6) The final characteristic of an ideal vaccine is that it should be easy to manufacture. During the manufacturing process the ideal vaccine can be easily scaled-up from the single laboratory level to an industry level so that production can be increased to produce large quantities of the vaccine. Ease of manufacturing can also be characterized by low-cost production – to produce the vaccine in large quantities with a low cost will be easier for will ultimately lead to a cheaper price of the vaccine for customers to want to purchase allowing wider market distribution and a positive effect on human health [32].

CHAPTER II
THESIS SCOPE

2.1 Background and Rationale

The recent Ebola outbreak in the DRC is now known as the world's second largest Ebola outbreak with more than 2000 deaths and 3000 confirmed infections since it began in 2018 [3]. The occurrence of Ebola virus outbreaks is due to the virus's ability to spread easily through contact with infected bodily fluids. Infected patients suffer a range of symptoms which could lead to the development of Ebola Virus Disease (EVD), leading to an untimely death. Other filoviruses such as SUDV and MARV raise concern as well because they are known to cause frequent outbreaks throughout history and in countries around the world. Generating an efficacious vaccine that can protect against multiple Filoviruses is necessary for the protection of the population at risk and to prevent further spread of the disease.

2.2 Long-Term Goal, Objective, and Hypothesis

The *long-term goal* of this study is to aid in the development of an efficacious filovirus vaccine that will successfully protect people who live in endemic areas as well as prevent or control filovirus outbreaks in non-endemic regions. The *specific aim* is to assess the immunogenicity (ability to evoke an effective immune response) of our lab-developed trivalent vaccine against EBOV, SUDV, and MARV, in preclinical studies. This will be done through animal testing in Swiss Webster mice. The *objective* of this research is to achieve a multivalent filovirus vaccine that can be thermostabilized. The *central hypothesis* is that since each of the immunogens can elicit a strong antibody response on their own, then it should be possible to optimize the formulation dosages of each immunogen to generate a

combination vaccine that induces a balanced, robust response of antibody titers and neutralizing titers against multiple filoviruses.

2.3 Significance

There is an ongoing Ebolavirus outbreak in the Democratic Republic of Congo (DRC), and other Filovirus outbreaks are expected to occur in the future, with the potential of creating significant losses of life and disruption to communities. However, there are only one licensed vaccine to protect the population from Ebola virus disease that could be used to further limit spread of the virus during an outbreak. The only vaccine currently approved for marketing in several countries is the rVSV-ZEBOV (Ervebo) which uses a live virus as a vector [8]. It shows protection against only a single filovirus and frequently causes side effects in vaccinated individuals [55]. Thus, generating an efficacious vaccine that is safe in the majority of the population and can protect against multiple Filoviruses causing human outbreaks would be ideal.

2.4 Innovation

The vaccine that is being tested is a lyophilized preparation, which improves its thermostability and reduces the need to maintain a strict cold-chain, making it easier to handle, transport, and store. Thermostable vaccines are especially important for potential affected areas that do not have access to reliable refrigeration. This vaccine is unlike any of its replicating competitors because it does not use a live virus vector but instead is a subunit vaccine based on recombinant filovirus surface glycoproteins which should be safer and cause less side effects.

2.5 Specific Aim 1

Specific Aim 1: To determine a trivalent filovirus subunit vaccine formulation that will generate balanced antibody titers to three filovirus immunogens.

Hypothesis: It will be possible to use a mouse model to determine a formulation with the optimal dosage levels of the three GP's that is ideal for generating balanced antibody titers.

Approach: Determining antibody concentrations will be done by first immunizing Swiss Webster mice intramuscularly (i.m.) with different EBOV, SUDV, and MARV GP dosage levels using lyophilized vaccine formulations. The mouse immunization was administered on a vaccine schedule of 3 doses at 3-week intervals, while collecting blood samples 2 weeks after each immunization. The serum will be extracted from the blood and tested for antibody titers by comparing median fluorescence intensity (MFI) using a Magpix/Luminex-based multiplex immunoassay (MIA). The antibody titers will be compared between groups.

Expected outcome: The formulation with equal and highest amounts of each immunogen will generate balanced antibody titers.

Alternative outcome(s): The immune response to one immunogen will interfere with that of another immunogen regardless of the dose. All antigen formulations will elicit an unbalanced antibody response.

2.6 Specific Aim 2

Specific Aim 2: To determine a trivalent filovirus subunit vaccine formulation that will generate balanced immunity and potent virus neutralizing titers to Ebola virus.

Hypothesis: It will be possible to use a mouse model to determine the formulation that is ideal for generating potent trivalent IgG responses while retaining high levels of virus neutralizing titers against EBOV.

Approach: The serum collected from Swiss Webster mice will be analyzed to see if the antibodies produced are able to neutralize recombinant vesicular stomatitis virus (rVSV) that have had their surface GP replaced with EBOV GP. This will be done by using conventional plaque reduction neutralization tests (PRNTs) to compare the neutralization effect of serum dilutions. Due to biosafety concerns, we will use a surrogate virus for this assay instead of the wild type virus to test the ability of the antibodies to neutralize the virus through blocking the virus entry mediated by filovirus GP. The PRNT50 neutralization titers will be compared.

Expected outcome: The formulation with the equal and highest amounts of each immunogen will generate balanced immunity while showing potent neutralizing titers against EBOV.

Alternative outcome(s): Formulations will generate balanced antibody concentrations; however, EBOV neutralization is affected by one or more of the other immunogens. Formulations may generate potent neutralizing titers that are caused by antibodies generated by the other immunogens. Surrogate virus neutralization may not be a good measure to determine proper vaccine composition if the assay result does not correlate with protection.

CHAPTER III

Antibody Concentration and Neutralization Responses to Trivalent Vaccine Formulations

3.1 Introduction

EBOV, SUDV, and MARV are known as the most pathogenic species of filoviruses that have high CFR and cause continuous outbreaks throughout history, while also leaving survivors to suffer long-term sequelae [57, 54]. While there is one licensed Ebola virus vaccine available, it has only shown to confer protection against EBOV in healthy individuals [55]. Thus, the ideal filovirus vaccine is one which generates protection against multiple pathogenic species of filovirus, while also being available to a larger proportion of the population. Our lab has developed a thermostable, lyophilized, trivalent vaccine containing the GP subunits of EBOV, SUDV, and MARV. This vaccine formulation is adjuvanted with CoVaccine HT™ to enhance the humoral and cell-mediated immunity [25]. Thermostabilization is important to supply this vaccine to remote, filovirus endemic areas in which the ability to maintain a cold chain is challenging. A thermostable formulation facilitates stockpiling of doses for future outbreaks in these regions. A multivalent filovirus vaccine is able to establish immunity to multiple filoviruses. Historically outbreaks with various filoviruses have occurred either concurrently or sequentially, thus establishing the need to create a multivalent vaccine that will provide protection from more than one species of filovirus.

3.2 Materials and Methods

Animal Use

This study was approved by the University of Hawaii Institutional Animal Care and Use Committee (IACUC) and conducted in strict accordance with guidelines established by

the National Institutes of Health and the University of Hawaii IACUC. Animals were housed and mated in 12-hour light cycles with food and water ad libitum, unless specified.

Vaccine Formulation

Recombinant antigen was prepared as described [42 – unpublished data] by Madhuri Namekar, Teri Ann Wong, and Albert To. The adjuvant used in the formulation, CoVaccine HT™ was gifted from Protherics Medicines Development (London, UK). Vaccines were lyophilized in collaboration with University of Colorado, Boulder.

Vaccine Reconstitution

Vaccine formulations are listed in Table 1. Vaccines required reconstitution before being administered. Filtered ddH₂O followed by 0.3 mg of the adjuvant used (CoVaccine HT™) was added to each bottle containing lyophilized vaccine formulation to reconstitute. The bottle was lightly shaken before administering.

Table 1. Trivalent vaccine formulations for twelve groups administered different amounts of EBOV GP, SUDV GP, MARV GP and CoVaccine HT™.

Group	Mouse Dose				Total Material need each vial (n=8)			
	E-GP [µg]	S-GP [µg]	M-GP [µg]	CoV HT [mg]	E-GP [µg]	S-GP [µg]	M-GP [µg]	CoV HT [mg]
13	0.1	0.1	0.1	0.3	1	1	1	3.0
14	0.3	0.3	0.3	0.3	3	3	3	3.0
15	1.0	1.0	1.0	0.3	10	10	10	3.0
16	3.0	3.0	3.0	0.3	30	30	30	3.0
17	0.1	0.04	0.1	0.3	1	0.4	1	3.0
18	0.3	0.12	0.3	0.3	3	1.2	3	3.0
19	1.0	0.4	1.0	0.3	10	4	10	3.0
20	3.0	1.2	3.0	0.3	3	1	3	3.0
21	0.05	0.05	0.1	0.3	0.5	0.5	1	3.0
22	0.15	0.15	0.3	0.3	1.5	1.5	3	3.0
23	0.5	0.5	1.0	0.3	5	5	10	3.0
24	1.5	1.5	3.0	0.3	15	15	30	3.0

*E – Ebola, S – Sudan, M – Marburg, CoV - CoVaccine HT™

Mouse Immunization

Swiss Webster mice were bred in house from original animals obtained from *Taconic*. Twelve groups of 4 female and 4 male Swiss Webster mice ($n = 8/\text{group}$) around 2 months of age were immunized intramuscularly (i.m.) using a 1mL syringe in each hind leg with their assigned vaccine formulation with adjuvant as seen in Table 1. First immunization was done at day 0, second immunization was done three weeks later on day 21, and third and final immunization was done three weeks after that on day 42. Blood was drawn through tail bleeds 2 weeks after the first and second immunizations; and through a cardiac puncture (terminal bleed) which was done 2 weeks after the third immunization. Blood was collected using a capillary action blood collection tube for tail bleeds, and a 3mL syringe for cardiac punctures. The immunization and blood collection schedule are illustrated in Table 2 and Figure 1.

Table 2. Swiss Webster trivalent vaccine immunization schedule.

Date	Procedure
7/24/2019	Immunize
8/7/2019	Tail Bleed
8/14/2019	Immunize
8/28/2019	Tail Bleed
9/4/2019	Immunize
9/18/2019	Bleed Out

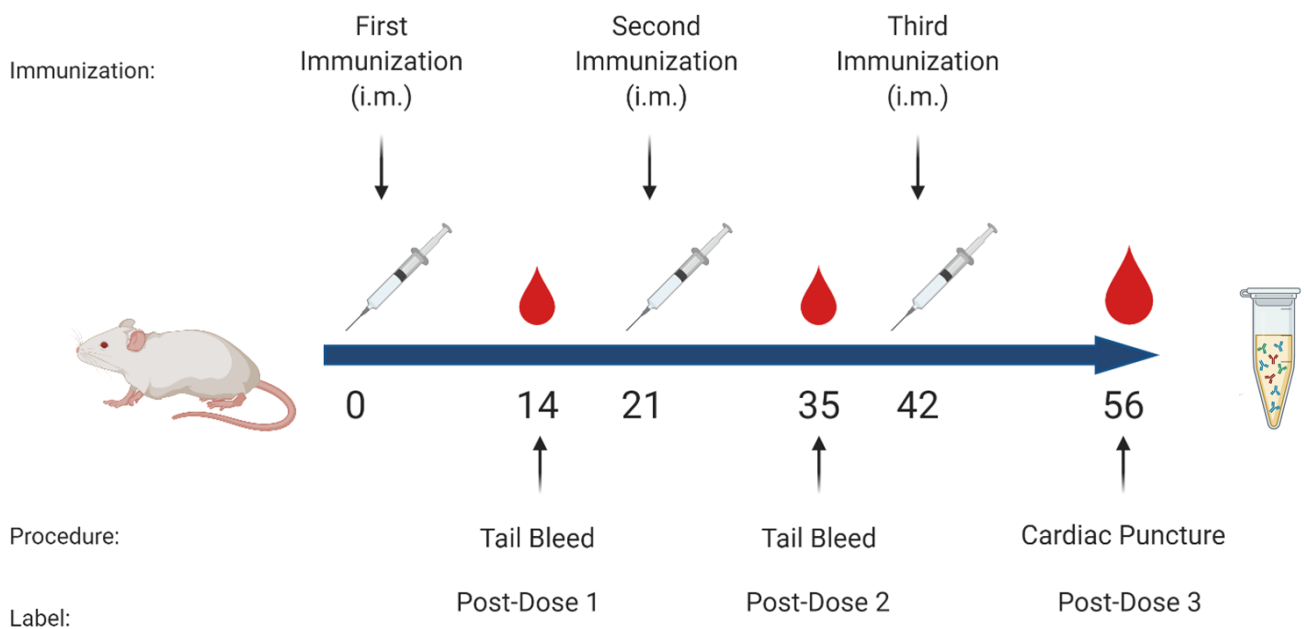


Figure 1. Swiss Webster mice immunization schedule.

Serum Isolation

Whole blood collected from mice was left at room temperature (RT) for 1 hour to allow for coagulation and then centrifuged at 1.5 rcf for 5 minutes to separate serum from the rest of the blood components. Serum was removed and transferred to new tube and stored at -80°C until use.

Measurement of Antibody Titers & Concentrations using Multiplex Immunoassay (MIA)

Antibody titers and/or concentrations in mouse sera were measured by MIA using beads from *Luminex* and coupled in-house with antigens made in-house. A bead mastermix was made that contained magnetic beads that were coupled to the following antigens: EBOV GP, SUDV GP, MARV GP, and BSA and was diluted at 1:200 with 1X PBS-1% BSA-0.02% Tween (PBS-BT). Mouse serum samples were diluted at 1:8,000 and 1:40,000 with PBS-BT and standards for the assay were diluted at a two-fold serial dilution from 8000 ng/mL to a concentration of 15.6 ng/mL. A standard was run during the assays to create a curve so that MFI's could be interpolated to report antibody concentration instead of MFI's. A 1:1 ratio of the bead mastermix and serum/antibody dilution was combined into 96-well plates and left to incubate at 37°C for 3 hours while shaking at 700 rpm. After incubation, the plate was placed on a magnet for 2 min. and supernatant was removed. The plates were taken off the magnet and were washed twice with PBS-BT and then vortexed for 30 sec. and placed onto the magnet before PBS-BT wash buffer was removed. R-PE labeled Goat anti-mouse IgG antibody (0.5 mg/ml) was diluted with PBS-BT to 1:200 and added to the wells. Plates were incubated at 37°C for 1 hour while shaking at 700 rpm. After incubation, the supernatant was removed plates were washed twice with PBS-BT according to aforementioned procedure. After washing the plates, 1X sheath fluid was added and plates were vortexed for 1 min before storing in 4°C refrigerator overnight. The next day, plates were warmed to room temperature and vortexed for 30 sec. before reading on the Magpix reader (Luminex Corp., Austin, TX).

Cell Culture

Vero cells were used for *in vitro* assays. Cell lines were maintained at 37°C, supplemented with 5% CO₂. Cell cultures were grown in DMEM-10 media (1X DMEM, 10% FBS, 1% Pen/Strep, 2% HEPES (1M)) in T75 flasks. Cell passage was conducted by removing media from the flask and cells were washed with 10 mL of PBS to inactivate trypsin inhibitors. PBS was removed, cells were washed with 1 mL of trypsin to activate cell detachment from flask wall. Cell culture was then incubated for 2 min. at 37°C, 5% CO₂. Cells were resuspended in 10 mL of DMEM-10 and mixed ~15 times by pipetting up and down. Cells were counted by doing a 1:1 dilution with Trypan blue and visualized on a hemocytometer. Cells were then passed into a new T75 flask at a 1:10 dilution with DMEM-10. The flask is returned to incubator to allow for growth of next passage.

Virus

Virus stocks of rVSV-EBOV GP were generated by Dr. Chih-Yun Lai and were stored at -80°C until use.

Measurement of Virus Neutralization by Plaque Reduction Neutralization Test (PRNT)

The PRNT assay is conducted over a 5-day period. On day 1, assay preparation, Vero cells were counted and plated at 90% confluency in 6-well culture plates to have a concentration of $\sim 2.5 \times 10^5$ cells/mL and 2 mL per well was plated. Equal volumes from each individual post-dose 3 serum samples were pooled for each group and stored at 4°C until use the following day.

On day 2, serum was incubated at 56°C for 30 minutes. After incubation, serum was diluted in six 4-fold dilutions starting at 1:10 with DMEM-2 as the diluent (1X DMEM, 2% FBS, 1% Pen/Strep, 2% HEPES (1M)) but with 2% FBS instead of 10%). The virus stock was diluted to 1200 plaque-forming units (PFU)/mL using DMEM-2, vortexing in between each dilution. This was the working concentration of virus which was used to incubate with serum (1:1) and was also used to make a virus back-titration (virus control). Virus and serum were incubated at 37°C, at 5% CO₂ for 1 hr. 6-well plates with Vero cells were labeled accordingly and ~1.3 mL of media was removed from each well, leaving 0.7 mL media. Virus-serum mixture was mixed 5 times before adding to cells (in duplicates) and was incubated for another 1 hr. at 37°C, 5% CO₂ while being agitated every 15 min. 2% agarose was prepared and 2X DMEM complete media (2X DMEM (dissolved and filtered), 4% FBS, 2% Pen/Strep, 4% HEPES, 9.8% Sodium bicarbonate) was mixed at a 1:1 ratio by gentle swirling. Two mL of complete overlay mixture (DMEM with 1% agarose) was added to each well and swirled immediately to mix the overlay with media in each well. The plates were left in the incubator at 37°C, 5% CO₂ for 63 hours.

After 63 hours (day 5), 0.05% crystal violet staining solution (0.05% crystal violet in 1% formaldehyde, 1% methanol) was added to each well for 2 hours. After staining, the crystal violet solution was removed and wells were washed twice with dH₂O. Agarose plugs were then removed using an 18-gauge needle and wells were again washed twice with dH₂O. Plates were dried at RT and plaques were visualized and counted.

Statistical Analysis

MIA and PRNT50 graphs and values were generated using Excel and GraphPad Prism 8. Statistical analysis was determined by standard one-way ANOVA.

3.3 Results

3.3.1 Aim 1

Monovalent formulations generate high concentrations of antigen-specific antibody

Understanding the immune response of the monovalent vaccine is necessary to be able to compare the antibody concentrations generated from the trivalent vaccine. We tested a total of 12 monovalent vaccine formulations containing 0.1, 0.3, 1.0, or 3.0 µg of EBOV, SUDV, or MARV GP. These relatively low doses were used to accurately compare antibody titers which cannot be done at higher doses since all readings would be at maximum, showing no significant differences. Also, specific responses could be masked by cross-reactivity of other IgG responses. Eight Swiss Webster mice per experimental group were immunized with their respective formulation (concentration of the specific GP). Serum was collected and IgG antibody concentrations were quantified as seen in Figure 2. It was observed that when vaccinated with a specific antigen, a high IgG antibody response (~1 mg/mL) above the lower limit of quantification (LLOQ) was generated towards the target antigen as compared to the control mice injected with, CoVaccine HT™ only. In Figure 2, significant dose-responses ($p < 0.05$) were observed only when sera from mice immunized with SUDV GP were assayed against EBOV GP or when sera from mice immunized with EBOV GP were assayed against SUDV GP. A significant dose-response indicates that with a change in antigen amounts, there is a change in the formed antibody response. EBOV GP

and SUDV GP produced cross-reactive antibodies towards each other while immunization with any amount of MARV GP did not produce any cross-reactive antibodies. All other conditions did not result in significant differences in antibody levels for the various antigen doses.

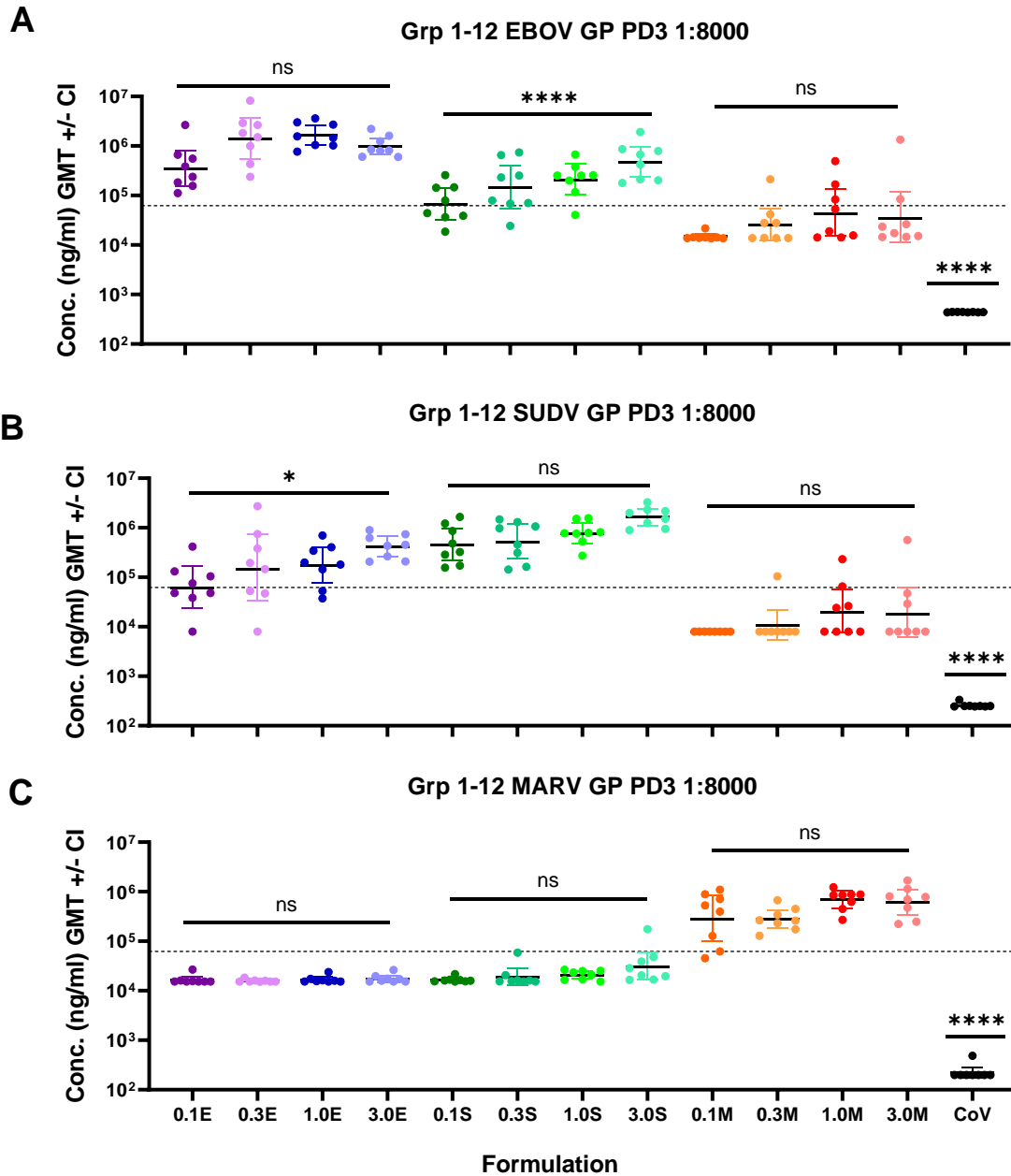


Figure 2. MIA test results of serum post-dose 3 antibody concentrations from Swiss Webster mice immunized with monovalent formulations, diluted at 1:8000 and tested against three binding antigens: A) EBOV GP, B) SUDV GP, and C) MARV GP. X-axis indicates vaccine formulation while Y-axis indicates IgG concentrations generated against the glycoprotein listed in the individual graph title. Dotted lines indicate the LLOQ.

Trivalent formulations are able to induce high antibody concentrations for all antigens.

A total of 12 trivalent vaccine formulations (3 groups of 4) containing varying amounts of EBOV, SUDV, and MARV GP were then tested. The first group was composed of equal amounts of each antigen at four different doses, the second group had similar amounts of EBOV and MARV GP but less SUDV GP because we hypothesized that SUDV GP may interfere with generation of anti-EBOV total IgG or neutralizing titers, and the third group contained reduced amounts of *Ebolavirus* antigens (half EBOV, half SUDV GP) as compared to *Marburgvirus* antigens. Eight Swiss Webster mice per group were immunized with their respective formulation. Serum was collected and IgG antibody concentrations were determined (Figure 3). All trivalent formulations induced high concentrations of antibodies reactive to all antigens (~0.1 – 1.0 mg/mL) as compared to the control animals receiving adjuvant alone. Anti-EBOV GP and anti-SUDV GP IgG concentrations were high for all formulations at higher dosages, demonstrating a dose-response that showed a significant difference between the antibody concentrations generated from the lower dosage to the higher dosage ($p < 0.05$) when analyzing these specific antibodies (Figure 3A, 3B). Although high anti-MARV GP IgG concentrations were also observed, a clear dose-response was seen only when tested against EBOV GP but not when tested against MARV GP (Figure 3C) or SUDV GP (Figure 3B). EBOV GP and SUDV GP are highly cross-reactive to each other while MARV GP does not cross-react to any other antigen. As expected, the formulation that was able to generate consistently high IgG concentrations against all three, EBOV, SUDV, and MARV GP was the formulation containing equal and highest amounts of each antigen (3.0E, 3.0S, 3.0M). The formulations that elicited antibody concentrations

under the LLOQ were the formulations that were at lowest dosage within each of the three main groups of formulations.

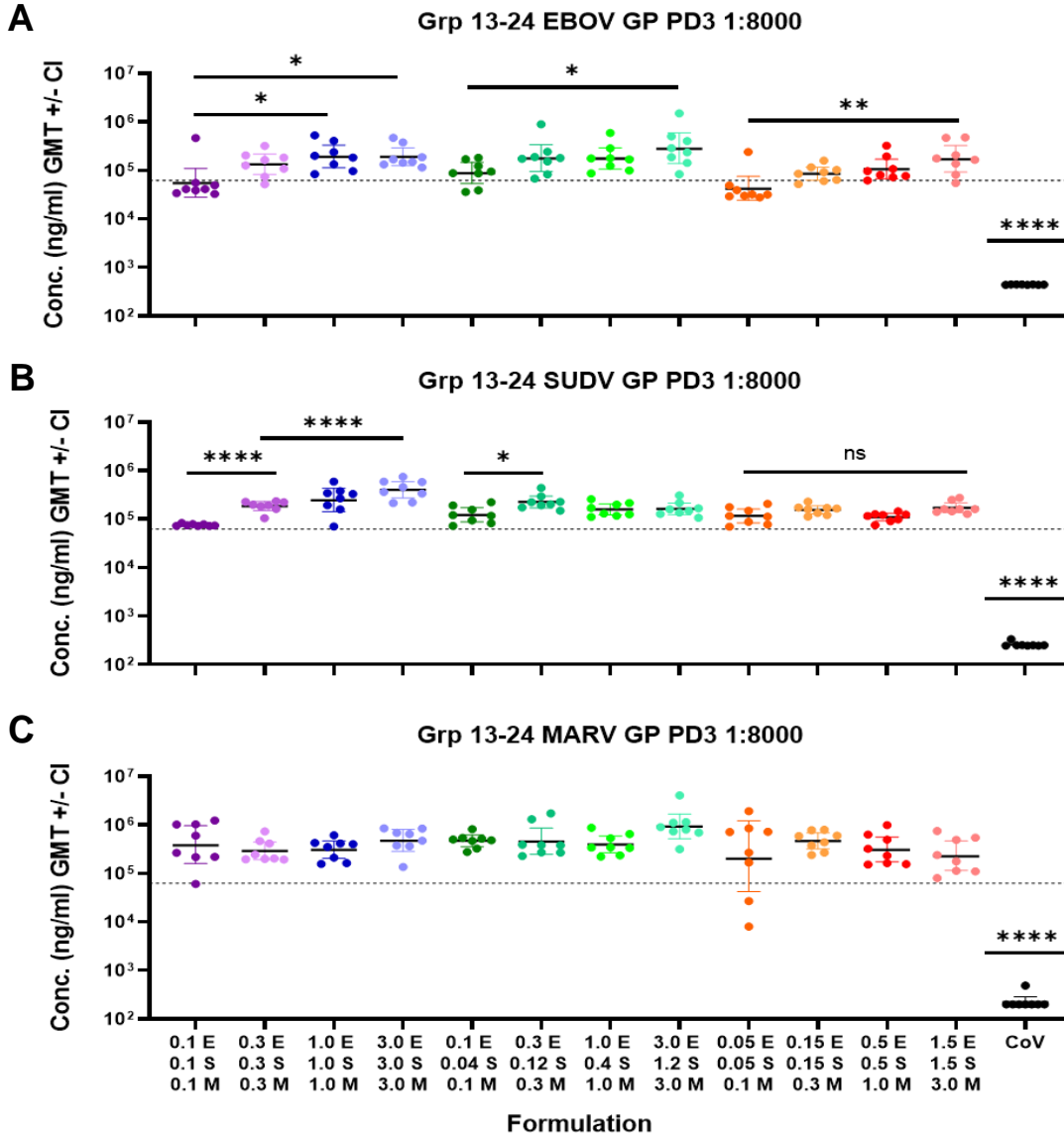


Figure 3. MIA test results of serum post-dose 3 antibody concentrations from Swiss Webster mice immunized with trivalent formulations, diluted at 1:8000 and tested against antigen A) EBOV GP, B) SUDV GP, and C) MARV GP. X-axis indicates vaccine formulation while Y-axis indicates IgG concentrations generated against the glycoprotein listed in the individual graph title. Dotted lines indicate the LLOQ.

Trivalent vaccine is able to produce comparable antibody concentrations to monovalent vaccine

Determination of IgG antibody concentrations generated from trivalent and monovalent vaccines allowed us to generate a direct graphical comparison to better understand the magnitude of the antibody response. Only the trivalent formulations containing equal amounts of each antigen were compared to the monovalent formulations (Figure 4). Overall, the trivalent vaccine generated comparable IgG antibody concentrations to the monovalent vaccine. Figure 4A illustrates a significant difference ($p < 0.05$) between the anti-EBOV GP IgG responses of the trivalent to monovalent formulations, but with increasing dosage the concentration seen with the trivalent vaccine becomes more comparable to monovalent. Anti-SUDV GP and anti-MARV-GP IgG concentrations from trivalent versus monovalent formulations were not significantly different for any formulation ($p < 0.05$).

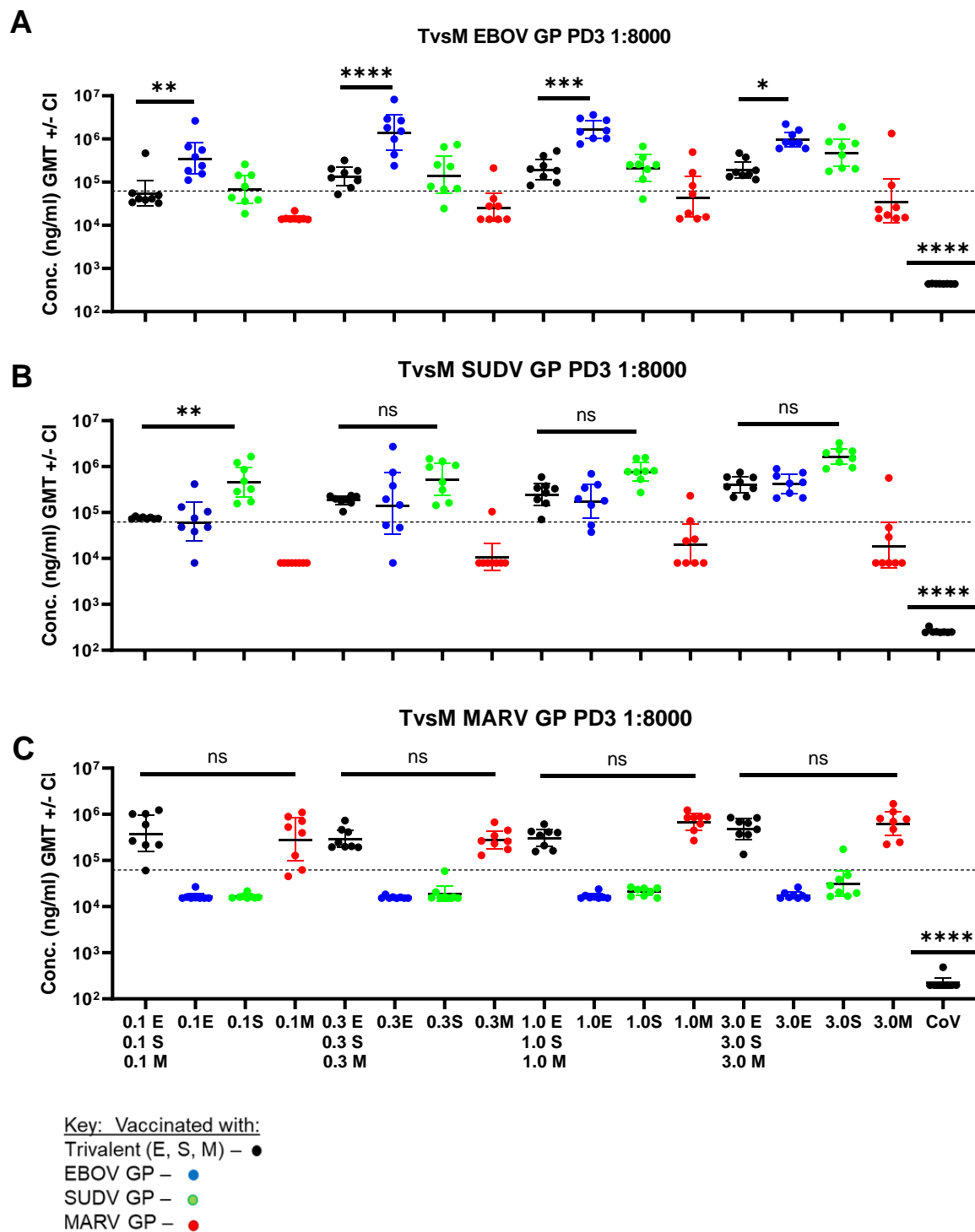


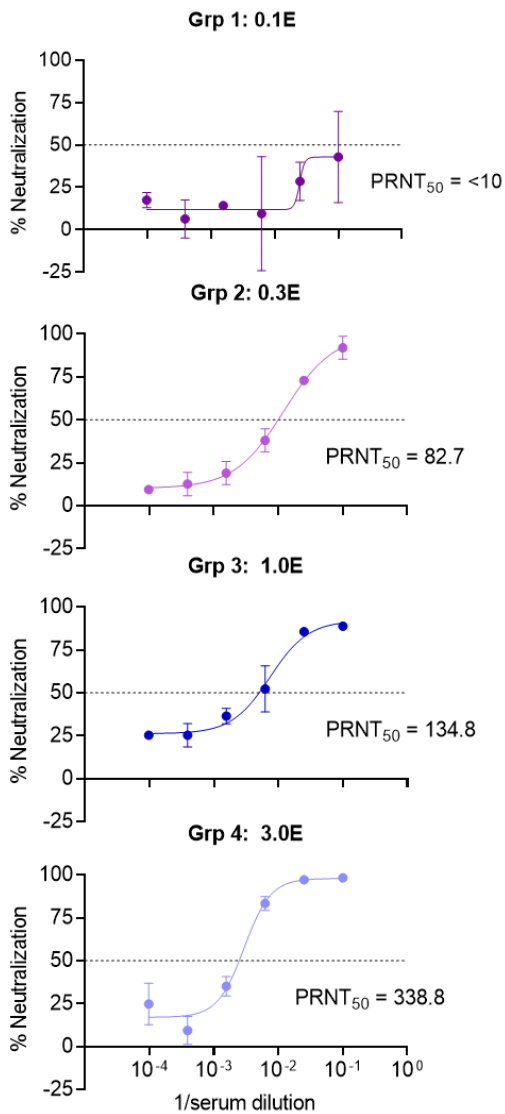
Figure 4. MIA test results of serum post-dose 3 antibody concentrations from Swiss Webster mice immunized with trivalent versus monovalent formulations, diluted at 1:8000 and tested against antigen A) EBOV GP, B) SUDV GP, and C) MARV GP. Dotted lines indicate the LLOQ.

3.3.2 Aim 2

Higher dosages of monovalent EBOV GP formulations yield higher neutralization of rVSV-EBOV.

To better understand the functionality of immune responses in the trivalent vaccines versus monovalent vaccines, it is necessary to evaluate virus neutralization titers.

Formulations for the vaccinations are as described in Aim 1. Vero cells were infected with a mixture of rVSV-EBOV GP pre-incubated with immune serum and the resulting plaques were counted to measure virus neutralization activity. A recombinant vesicular stomatitis virus expressing surface EBOV GP was used in order to conduct experiments under BSL2 conditions. This experiment focused on EBOV neutralization, as amongst the three viruses, it seems to be the most difficult to neutralize. Results of monovalent formulations only containing EBOV GP were analyzed to observe specific neutralization. Valid PRNT50 titers were obtained from all monovalent formulations containing more than 0.1 µg of EBOV GP (Figure 5). Increasing neutralization activity correlated with increasing antigen dose.

A**B**

Group/Dosage	PRNT50
Grp 1: 0.1E	<10
Grp 2: 0.3E	82.7
Grp 3: 1.0E	134.8
Grp 4: 3.0E	338.8

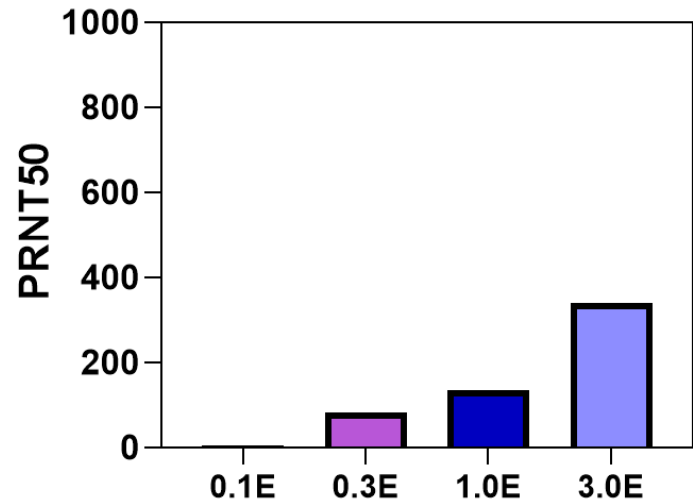
C

Figure 5. Pooled serum post-dose 3 antibody rVSV-EBOV GP neutralization data resulting Swiss Webster immunization with monovalent EBOV GP vaccine formulations. A) Neutralization curves, B) Table summarizing numerical PRNT50 values, C) Column graph displaying PRNT50 values.

Higher antigen dosage levels of trivalent formulations yield higher virus neutralization titers against rVSV-EBOV.

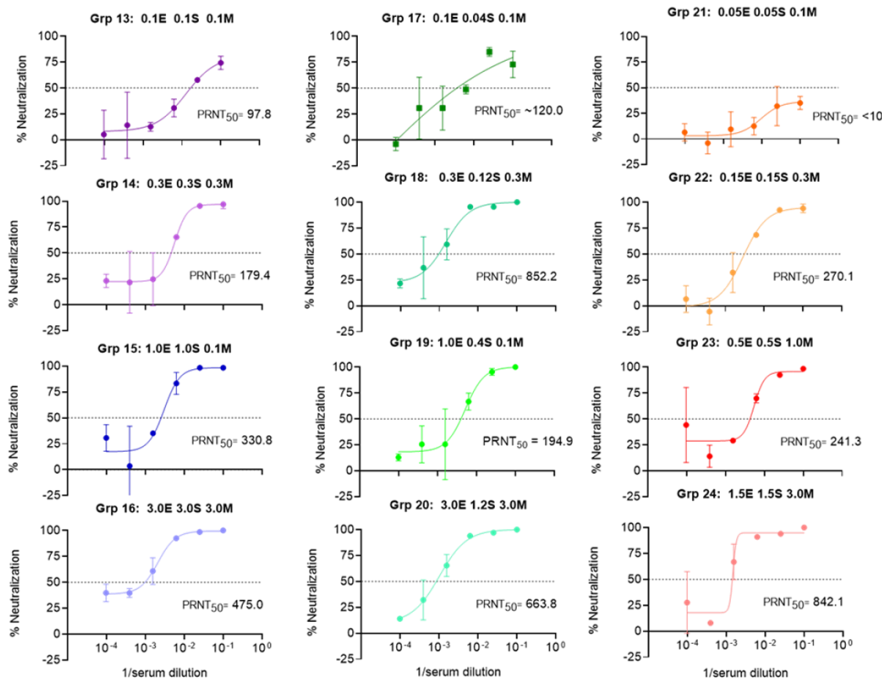
Virus neutralizing titers in groups of mice immunized with different trivalent vaccines were compared. As seen in Figure 6, eleven out of the twelve trivalent vaccine

formulations resulted in successful neutralization of the virus as measured by 50% plaque reduction neutralization titers (PRNT50) (Figure 6A). The one formulation that did not induce a detectable PRNT50 contained 0.05 µg each of EBOV and SUDV GP and 0.1 µg of MARV GP, the lowest dosage of the group of formulations containing equal *Ebolavirus* to *Marburgvirus* antigens. This trend continues also for the two next higher dose formulations of this group which overall elicited the lowest neutralization titers for rVSV-EBOV GP (Figure 6C, orange series). The exception was the highest dose formulation which resulted in a very high level of virus neutralization.

Trivalent formulations containing equal amounts of each antigen elicited higher neutralization of rVSV-EBOV GP at higher dosages (Figure 6C, purple/blue series), the only apparent dose-response observed for trivalent formulations.

Interestingly, formulations that contained equal EBOV and MARV antigen with less SUDV antigen elicited consistently higher neutralization titers as compared to the other “sets” of trivalent formulations (Figure 6C, green series).

A



B

Group/Dosage	PRNT50
Grp 13: 0.1E 0.1S 0.1M	97.8
Grp 14: 0.3E 0.3S 0.3M	179.4
Grp 15: 1.0E 1.0S 1.0M	330.8
Grp 16: 3.0E 3.0S 3.0M	475.0
Grp 17: 0.1E 0.04S 0.1M	120.0
Grp 18: 0.3E 0.12S 0.3M	640.8
Grp 19: 1.0E 0.4S 0.1M	640.7
Grp 20: 3.0E 1.2S 3.0M	663.8
Grp 21: 0.05E 0.05S 0.1M	<10
Grp 22: 0.15E 0.15S 0.3M	270.1
Grp 23: 0.5E 0.5S 1.0M	241.3
Grp 24: 1.5E 1.5S 3.0M	842.1

Key: Vaccinated with:
 Equal amounts – ●
 Less SUDV GP – ●
 Equal E to M antigen – ●

C

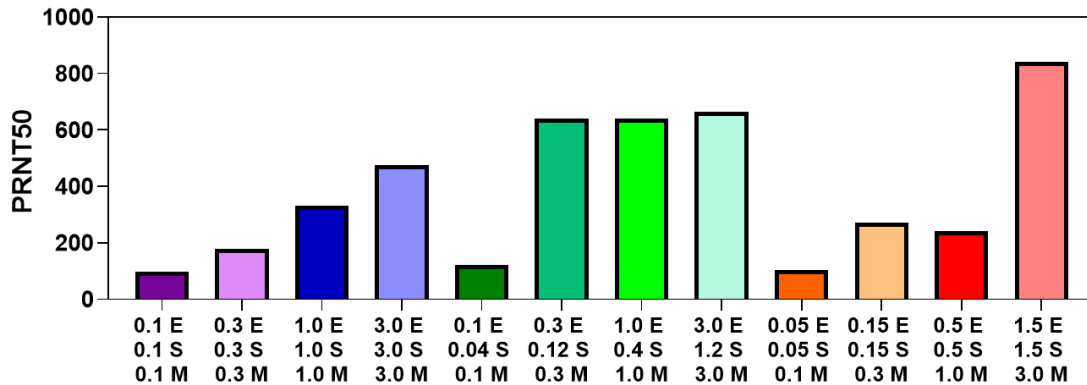


Figure 6. Pooled serum post-dose 3 rVSV-EBOV GP neutralization was observed resulting from immunization of Swiss Webster mice with trivalent vaccine formulations. A) Virus neutralization levels at various serum dilutions, B) Table summarizing numerical PRNT50 values obtained from curve-fitting, C) Column graph displaying PRNT50 values.

Trivalent vaccine formulations elicit higher neutralizing titers compared to monovalent vaccine formulations.

Formulations containing SUDV GP and MARV GP antigens along with EBOV GP yielded higher neutralization titers than monovalent EBOV GP formulations (Figure 7A, 7B).

Interestingly, the presence of low amounts of SUDV GP relative to EBOV and MARV antigens seem to induce higher neutralization titers than trivalent formulations containing equal amounts of each antigen (Figure 7A).

Possible correlation between total antigen-binding IgG concentrations versus virus neutralization titers.

Figure 7C evaluates the relationship between antibody concentration and neutralization titers. The data indicate a significant ($p < 0.05$), positive correlation between antibody concentration and neutralizing titer (R^2 of 0.61), in mice vaccinated with the various formulations.

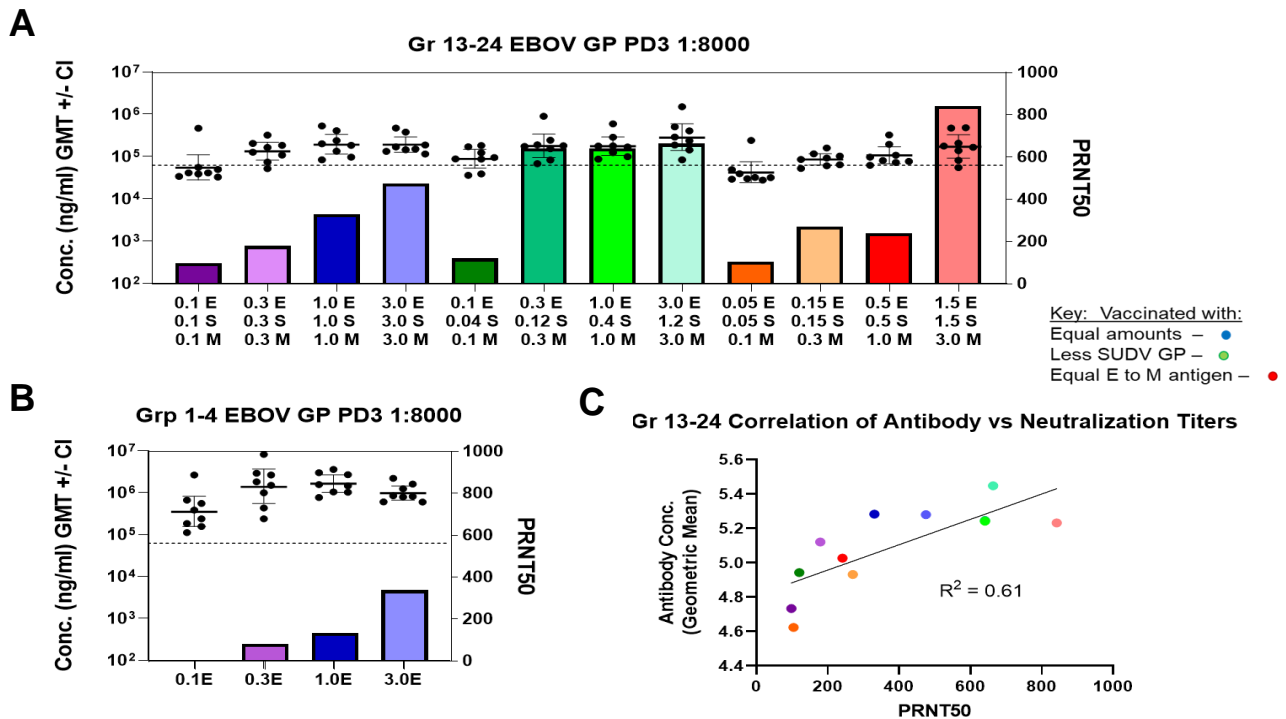


Figure 7. Comparison of virus neutralization in pooled sera (post-dose 3) of Swiss Webster mice immunized with trivalent or monovalent vaccine formulations. A) Total EBOV GP-binding IgG concentration and virus neutralizing titers (rVSV-EBOV-GP) induced by trivalent formulations, B) Total EBOV GP-binding IgG concentration and virus neutralizing titers (rVSV-EBOV-GP) induced by monovalent formulations, C) Correlation of total antigen-binding IgG versus virus neutralizing titers resulting from immunization with trivalent formulations.

3.4 Discussion

While one EBOV vaccine has recently obtained licensure, this vaccine has a number of limitations. The population that can be vaccinated is limited by safety concerns, the vaccine requires strict cold-chain parameters for transportation and storage, and its protection is limited to a single filovirus species [55]. Creating and assessing the immunogenicity of various trivalent recombinant subunit vaccine formulations are a necessary preclinical development steps to establishing its feasibility. This vaccine has the potential for use as a universal filovirus vaccine to prevent or control future outbreaks.

Specific Aim 1: To determine a trivalent filovirus subunit vaccine formulation that will generate balanced antibody titers to three filovirus immunogens.

My first aim was to determine the trivalent formulation that will generate balanced antibody titers to the three different filovirus immunogens. It was **hypothesized** that it would be possible to use a mouse model to determine a formulation with the optimal dosage levels of the three GP's generating balanced antibody titers, and it was **expected** that the formulation with equal amounts of each immunogen would generate balanced antibody titers. The results support our hypothesis that the mouse model can be used to select optimal dosage levels, and that formulations with equal antigen doses would generate a dose-dependent, balanced antibody responses to all three filovirus immunogens.

Interpretation of the antibody responses to the monovalent formulations was first completed to understand the response each antigen elicits by itself to form a basis for comparison with compare multivalent formulations. The monovalent vaccine formulations

elicited high concentrations of antibodies that were specific for the immunogen. This provided support for the immunogenicity of the three monovalent vaccines individually. This result predicted that the trivalent formulations also would be able to induce high antibody concentrations. A significant dose-response ($p < 0.05$) was also observed when these sera were tested against a virus of the same genus (i.e. between EBOV and SUDV but not MARV), suggesting that vaccination with one filovirus GP results in cross-reactive antibodies to GPs of other filovirus species. This suggested that the various antigens may have synergistic effects when given together, allowing for a multivalent formulation that could produce a balanced immune response by to antigens of the same virus family.

Analysis of antibody responses to the different trivalent formulations showed that most trivalent formulations induced high antibody concentrations for all antigens ($\sim 0.1 - 1.0$ mg/mL), at similar doses used for monovalent vaccines. Not surprisingly, the formulations that elicited antibody concentrations below the lower limit of quantification contained the lowest antigen dosage levels within each of the three main formulation groupings, suggesting that these doses were suboptimal for induction of a potent IgG response. Within each group of the trivalent formulations, dose-responses were observed for anti-EBOV and anti-SUDV IgG concentrations but no dose response was apparent for anti-MARV IgG, suggesting that MARV GP may be the most immunogenic antigen as the lowest antigen dose was able to elicit similar titers to the highest antigen dose. Thus, even lower doses may need to be applied to see a clear dose-response effect for MARV GP. EBOV GP and SUDV GP were highly cross-reactive, which would explain the interactions seen with the various antigen concentration combinations of the two antigens, while MARV GP does not cross-react and does not seem to influence the overall response to EBOV or SUDV. Ultimately, *the*

hypothesis was supported because we were able to use a mouse model to effectively determine the formulation with the optimal dosage levels to elicit balanced antibody titers as well as validate what was **expected** because most formulations generated balanced IgG antibody concentration towards EBOV, SUDV, and MARV with the formulation containing equal and highest amounts of each antigen (3.0E, 3.0S, 3.0M) showing the most potent responses to all three viruses.

To ensure that the trivalent formulation was generating a sufficient antibody response, it was compared side-by-side to the monovalent formulations of similar antigen doses. The anti-SUDV-GP and anti-MARV-GP IgG concentrations elicited from the trivalent vaccine were not significantly different from the monovalent formulations indicating that inclusion of other antigens in the formulation did not interfere with antibody production for the GP of these two viruses. However, there was a statistical difference between the EBOV IgG concentrations generated from the trivalent vaccine compared to the monovalent vaccine, especially at lower antigen dose levels. This effect seems to be alleviated by higher dosage levels of the trivalent vaccines where IgG concentrations increased and became comparable to those seen with the monovalent EBOV GP formulations. This data suggests that trivalent formulations can elicit high concentrations of antibodies (> 0.1 mg/mL) that are specific for each antigen.

Specific Aim 2: To determine a trivalent filovirus subunit vaccine formulation that will generate balanced immunity and potent virus neutralizing titers to Ebola virus.

My second aim was to determine the vaccine formulation that will maintain or improve virus neutralization of EBOV (using a surrogate rVSV-EBOV GP virus) by

assessing the neutralizing ability of the antibodies through PRNT. It was **hypothesized** that it will be possible to use a mouse model to determine the formulation that is ideal for generating potent trivalent IgG responses which would exhibit high levels of virus neutralizing titers against EBOV. It was **expected** that the formulation with equal amounts of each immunogen will generate balanced immunity while showing potent neutralizing titers against EBOV and that the presence of other antigens would not reduce virus neutralization as compared to a monovalent EBOV vaccine. The results supported the hypothesis that a formulation for generating high levels of neutralizing virus could be determined in a mouse model, but contrary to the expected outcome it was shown that EBOV-specific virus neutralization was affected by the presence of one or more of the other immunogens.

Similar to the comparison of the trivalent to monovalent formulations for antibody concentrations, comparisons of these formulations were carried out to assess virus neutralization. This study used an *in vitro* neutralization assay using rVSV-EBOV GP as a surrogate for neutralization of live EBOV, a BSL4 pathogen. Neutralization of EBOV was assessed in this study because protection against this filovirus is the most difficult to achieve. The results from the monovalent EBOV formulations followed the expected dose response and indicated that the monovalent vaccines are capable of inducing potent neutralizing concentrations on their own. We then determined whether trivalent formulations with varying amounts of antigen would result in increased, unchanged or reduced EBOV neutralization titers.

We were able to detect virus neutralizing titers for eleven of twelve trivalent formulations titers. The single formulation producing antibodies without neutralizing activity contained a low dose of EBOV and total GP antigen (0.05E 0.05S 0.1M). This

formulation also did not elicit antibody concentrations exceeding the lower limit of quantification against EBOV and SUDV GP although anti-MARV GP antibodies were detected in most animals in this group. This formulation may help to define the lower limit antigen capable of inducing potent antibody levels and neutralizing titers in mice. This is an important observation for the future stability and potency assays. It also confirms that anti-MARV GP antibodies neither cross-react nor cross-neutralize EBOV GP. The findings from the MIA that detailed the cross-reactivity of EBOV GP and SUDV GP, while MARV GP did not cross-react, is relevant to the neutralization results of animals administered 0.05E 0.05S 0.1M. This is because the serum from this group would mostly contain anti-MARV GP antibodies and therefore would not be capable of recognizing or neutralizing the rVSV-EBOV.

Other observations were made when comparing the different sets of formulations. The first observation was that a dose-response was generated when mice were immunized with increasing doses of formulations containing equal amounts of each antigen in terms of antibody production, as expected. However, it was surprising to see that formulations containing SUDV and MARV antigens in addition to EBOV GP showed higher virus neutralizing titers than formulations containing EBOV GP alone. Furthermore, equal EBOV and MARV antigen with less SUDV antigen elicited the even higher EBOV neutralization titers than when all three antigens were present in at equal amounts. This suggests that higher EBOV neutralization may be achieved through addition of another antigen, however the balance between EBOV and SUDV GP maybe important as a lower amount SUDV GP maybe preferable over equal amounts. One possible explanation for this observation is that purified SUDV GP contains a higher amount of monomer and the presence of monomeric

SUDV GP immunogenic epitopes may interfere with the immunogenicity of EBOV GP. The final observation of the group containing equal *Ebolavirus* to *Marburgvirus* antigens was that this group elicited the lowest neutralizing titers. Surprisingly, high neutralization titers were observed for the highest dose formulation. This finding may have been due to technical error and this assay will be repeated to ensure the reliability of this. *The data supported our hypothesis because we were able to determine the formulation that is ideal for generating potent trivalent IgG responses while retaining high levels of virus neutralizing titers against EBOV. However, the prior expectation was not supported, and the alternative result was supported that the formulation with equal (and highest) amounts of each immunogen did not show the most potent neutralizing titers against EBOV, but a formulation containing higher dosage of equal EBOV and MARV GP with less SUDV GP maybe superior in achieving the most potent EBOV neutralization.*

The finding that EBOV neutralization can be affected by the presence and balance of other antigens in the formulation while total antigen-binding antibody concentrations were similar to monovalent formulations, suggests that the antibodies produced from the trivalent formulations are able to more efficiently neutralize the virus. Therefore, we analyzed the correlation of anti-EBOV-GP IgG concentration to EBOV PRNT50 titers. A positive correlation was observed ($R^2 = 0.61$) suggesting, as expected, that increased antibody concentration leads to increased neutralization of EBOV. Further analysis of the phenomenon of antigen balance and its effect on virus neutralization should be conducted.

3.5 Conclusion

This study provided insight on selection of formulations that induce high GP-specific antibody concentrations, and potent EBOV neutralizing titers. This research has shown that trivalent formulations can be balanced to optimize both antigen-specific IgG and neutralizing antibody responses and contributes to the limited understanding of factors that affect the formulation of multivalent filovirus vaccines. It has provided insight into how we can select the best formulation that induces balanced immunity with high neutralizing antibody titers. A better understanding of the immune responses triggered by individual vaccine formulations in mice and will help us in formulating the next vaccines for testing in non-human primates (NHP's), including insight into the design of experiments for vaccine potency testing and dosing for future experiments.

Future directions for this research include moving forward with repeating tests on the formulations that induced high antibody and EBOV neutralizing titers, conducting studies that evaluate neutralization of rVSV-SUDV GP and rVSV-MARV GP, and evaluating the potential mechanism of protection of our recombinant subunit vaccine. These completed studies will guide future efficacy studies of trivalent formulations in NHP models. If successful, the trivalent vaccine can then move on to clinical studies in humans.

In conclusion, this research has provided useful information on establishing parameters for potency assays and formulation preparation, but more research is necessary to define immunogenicity and efficacy of the trivalent vaccine before it can be advanced into human clinical testing. The ultimate goal of this research is to aid in the development of an efficacious filovirus vaccine that will successfully protect first responders and persons living in endemic areas as well as prevent or control filovirus outbreaks in non-endemic regions.

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