THE IMMUNE RESPONSE TO RECOMBINANT SUBUNIT VACCINES

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTORATE OF PHILOSOPHY IN

BIOMEDICAL SCIENCES (TROPICAL MEDICINE)

December 2019

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Acknowledgements

I would like to thank Dr. Axel Lehrer, for accepting me into his lab and guiding me in my journey as a graduate student. It is thanks to his guidance that I was able to grow and succeed as a scientist.

I would also like to thank my committee members, Dr. George Hui, Dr. William Gosnell, Dr. Alan Katz, and Dr. F. DeWolfe Miller, for taking time out of their busy schedules to assist with my research and share with me their advice and wisdom.

I am very grateful to Ms. Teri Ann Wong, for all that she's done for me in and out of the lab, and for teaching me so much with so much patience. As well as Dr. Alexandra Gurary, Dr. Michael Lieberman, Dr. Eileen Nakano and all of the faculty members of Tropical medicine for their contributions to my project and my education.

Lastly I am thankful to my family and friends, who have supported and believed in me throughout this entire journey.

Abstract

Zika Virus (ZIKV) is a positive sense, single stranded RNA virus that for many years was not associated with severe clinical symptoms. However, it became a public health threat following an epidemic in French Polynesia 2013–2014 that resulted in neurological complications associated with infection. Ebola virus (EBOV) on the other hand is a negative sense, single stranded RNA virus with case fatality rates in outbreaks reaching 90%. Between 2013 and 2016 an outbreak of an unprecedented scale occurred in West Africa with almost 30,000 people infected and over 11,000 fatalities were reported. There are currently no vaccines or therapeutics approved for ZIKV while the first EBOV vaccine, Ervebo (rVSVΔG-ZEBOV-GP), was approved in November 2019 by the European Commission. Another candidate vaccine against EBOV combining two different virally vectored approaches is in advanced clinical trials. Several different vaccine platforms are being used to develop additional strategies to prevent infections with these viruses, mostly based on recombinant viral vectors. However, virally vectored vaccines have shown significant safety risks, particularly in immunocompromised populations.

Recombinant subunit vaccines have recently been put into use against several viral infections, such as Engerix-B and Recombivax HB against Hepatitis B virus, FluBlok against seasonal influenza, and GARDASIL and CERVARIX against human papilloma virus. Vaccines made using this versatile platform have high safety profiles and are relatively easy to manufacture and scale up. Using a recombinant subunit platform

consisting of antigens produced in *Drosophila melanogaster* S2 cells, we have developed vaccine candidates for ZIKV and EBOV. The efficacy of our recombinant subunits against EBOV has been evaluated in a guinea pig and non-human primate (NHP) model, while the efficacy of our ZIKV vaccine has been evaluated in immunocompetent mice and NHPs.

In our ZIKV NHP model, high neutralizing antibody titers were seen in all protected cynomolgus macaques, and passive transfer demonstrated that plasma from these NHPs was sufficient to protect against viremia in mice subsequently infected with ZIKV. Taken together these data demonstrate the immunogenicity and protective efficacy of the recombinant subunit vaccine candidate against ZIKV in NHPs and highlights the importance of neutralizing antibodies in protection against ZIKV infection, validating their potential to serve as a correlate of protection.

Vaccine candidates containing the EBOV glycoprotein with or without matrix proteins Viral Protein 24 and Viral Protein 40, formulated with several different adjuvants were tested in mice, guinea pigs and NHPs for immunogenicity and efficacy against lethal EBOV challenge. We also evaluated bi- and trivalent formulations in guinea pigs and NHPs in an effort to develop multivalent filovirus vaccines. The results demonstrated that the monovalent vaccine candidates engendered high titers of antigen-specific antibodies in immunized animals, and two of these vaccine candidates afforded complete or nearly complete protection against lethal challenge. All vaccine candidates were able to elicit virus-specific anti-GP IgG titers in all species tested, but high antibody

titers were also seen in animals not protected from viral challenge. Cell-mediated immunity was analyzed in samples taken from vaccinated NHPs with the goal of discovering responses that correlated with protection, and exploring cross-reactive responses to direct development of future formulations. While we were able to find vaccine specific cell-mediated immune responses, we so far were unable to correlate presence or absence or magnitude of these responses with vaccine efficacy.

Acknowledgements ii
Abstract iii
List of Tables vii
List of Figures viii
Chapter 2 viii
List of Abbreviations x
Chapter 1: Background 1
Recombinant Subunit Vaccines 1
Filovirus Vaccines and Correlates of Protection 3
Flaviviruses and Zika Virus Vaccines 8
Dissertation Scope 12
Specific Aims 14
Aim 1. Develop an insect cell derived recombinant subunit Zika virus vaccine and determine its efficacy and establish a correlate of protection in cynomolgus macaques. 14
Aim 2. Determine cellular and humoral correlates of protection in animal models vaccinated with a recombinant subunit Ebola virus vaccine 15
Aim 3. Establish cross-reactivity and cross-protective potential of a recombinant subunit based Ebola virus vaccine in cynomolgus macaques against other members of the filovirus family (SUDV and MARV). 16
Chapter 2: Efficacy and Immune Response of a Recombinant Subunit Vaccine in Nonhuman Primates against ZIKV 19
Methods 19
Results 23
Discussion 32
Chapter 3: Efficacy and Immune Response of a Recombinant Subunit Vaccine against EBOV in Guinea Pigs and Nonhuman Primates 38
Methods 38
Results 45
Discussion 61
Chapter 4: Exploring the Immune Response to a Multivalent Filovirus Vaccine and Analysis of Filovirus Cross-Reactivity 66
Methods 66
Results 69
Discussion 86
Chapter 5: Summary and Future Directions 91
References 96

List of Tables

Chapter 2

TABLE 1: SERUM $PRNT_{50}$ TITERS AT DAY 35 OF ALL ANIMALS RECEIVING ZIKV E WITH COVACCINE HT AGAINST ZIKV, DENV2, AND WNV 26

TABLE 2: SERUM $PRNT_{50}$ TITERS AT DAY 35 OF ALL ANIMALS RECEIVING ZIKV E WITH ALUM AGAINST ZIKV, DENV2, AND WNV 27

TABLE 3: VACCINE FORMULATIONS, SURVIVAL, AND TIME POINTS ANALYZED 56

TABLE 4: VACCINE FORMULATIONS, DOSING, SURVIVAL, AND TIME POINTS ANALYZED 78

List of Figures

Chapter 2

FIGURE 1: IMMUNOGENICITY OF RECOMBINANT ZIKV E PROTEIN IN CYNOMOLGUS MACAQUES. 25

FIGURE 2: VIREMIA IN VACCINATED AND CONTROL CYNOMOLGUS MACAQUES CHALLENGED WITH ZIKV. 28

FIGURE 3: PASSIVE PROTECTION IN BALB/C MICE USING ZIKV VACCINATED NHP PLASMA. 30

Chapter 3

FIGURE 4: GEOMETRIC MEAN TITERS OF ANTI-EBOV GP DETERMINED USING ELISA IN GUINEA PIG SERUM 47

FIGURE 5: SURVIVAL CURVE OF GUINEA PIGS RECEIVING EBOV VACCINE FORMULATIONS WITH DIFFERENT ADJUVANTS. 49

FIGURE 6: GEOMETRIC MEAN TITERS OF ANTI-GP DETERMINED USING ELISA IN GUINEA PIG SERUM STRATIFIED BY SURVIVAL STATUS 50

FIGURE 7: SURVIVAL CURVE OF NHPS RECEIVING EBOV VACCINE FORMULATIONS 53

FIGURE 8: EBOV GP SPECIFIC IGG MFI OF SERUM FROM NHPS THAT RECEIVED DIFFERENT EBOV VACCINE FORMULATIONS 54

FIGURE 9: INDIVIDUAL TNF-α PRODUCTION OF NHP PBMCS AFTER STIMULATION WITH A COMBINATION OF GP ANTIGEN AND PEPTIDE POOL. 57

FIGURE 10: GROUPED TNF-α PRODUCTION OF NHP PBMCS AFTER STIMULATION WITH A COMBINATION OF GP ANTIGEN AND PEPTIDE POOL. 58

FIGURE 11: ELISPOT DATA SHOWING IFN-γ SECRETION IN PBMCS FROM NHPS VACCINATED WITH AN EBOV VACCINE 60

Chapter 4

FIGURE 12: KAPLAN-MEIER SURVIVAL CURVES OF GUINEA PIGS RECEIVING MONOVALENT AND BIVALENT VACCINE FORMULATIONS WITH COVACCINE HT.....71

FIGURE 13: EBOV, SUDV AND MARV GP SPECIFIC IGG OF SERUM FROM NHPS THAT RECEIVED 2 OR 3 DOSES OF EBOV GP WITH COVACCINE HT. 74

FIGURE 14: FILOVIRUS SPECIFIC IGG MFI OF SERUM FROM NHPS THAT RECEIVED 2 DOSES OF EBOV GP, SUDV GP AND MARV GP WITH COVACCINE HT. 76

FIGURE 15: KAPLAN-MEIER SURVIVAL CURVES OF NHPS RECEIVING MONOVALENT AND TRIVALENT VACCINE FORMULATIONS FOLLOWING CHALLENGE WITH EBOV. 77

FIGURE 16: ELISPOT DATA SHOWING IFN-γ PRODUCED IN VITRO BY ANTIGEN-STIMULATED PBMC FROM NHPS VACCINATED WITH MONOVALENT OR TRIVALENT FILOVIRUS VACCINES. 80

FIGURE 17: ELISPOT DATA SHOWING IL-4 PRODUCED IN VITRO BY ANTIGEN-STIMULATED PBMC FROM NHPS VACCINATED WITH MONOVALENT OR TRIVALENT FILOVIRUS VACCINES. 81

FIGURE 18: TNF-α PRODUCTION OF NHP PBMCS AFTER STIMULATION. 83

FIGURE 19: ELISPOT DATA SHOWING IFN-γ PRODUCTION IN VITRO BY ANTIGEN-STIMULATED PBMC FROM NHPS VACCINATED WITH THREE DOSES OF EBOV GP WITH COVACCINE HT 85

List of Abbreviations

Antibody-Dependent Enhancement (ADE)

Baculovirus Expression Vector (BEV)

Bundibugyo Ebolavirus (BDBV)

Dengue hemorrhagic fever (DHF)

Dengue shock syndrome (DSS)

Drosophila Schneider cell expression system (DES or S2)

Dengue Virus (DENV)

Dimethylsulfoxide (DMSO)

Ebola Virus Disease (EVD)

Envelope protein (E)

Glycoprotein (GP)

Guinea Pig Adapted (GPA)

Human Papilloma Virus (HPV)

Immunoaffinity Chromatography (IAC)

Interferon γ (IFN-γ)

Japanese encephalitis virus (JEV)

Marburg marburgvirus (MARV)

Median fluorescence intensity (MFI)

Multiplex Immunoassay (MIA)

Nonhuman Primate (NHP)

Peripheral Blood Mononuclear Cell (PBMC)

Plaque Forming Unit (PFU)

Plaque reduction neutralization test (PRNT)

Pokeweed Mitogen (PWM)

Premembrane (prM)

Purified Inactivated Virus (PIV)

Recombinant Vesicular Stomatitis virus (rVSV)

Reston Ebolavirus (RESTV)

Simian Immunodeficiency Virus (SIV)

Spot forming cell (SFC)

Subviral Particles (SVP)

Sudan Ebolavirus (SUDV)

Tai Forest ebolavirus (TAFV)

Tumor Necrosis Factor α (TNF-α)

Vesicular Stomatitis Virus (VSV)

Viral Protein 24 (VP24)

Viral Protein 40 (VP40)

West Nile Virus (WNV)

Yellow fever virus (YFV)

Zaire Ebolavirus (EBOV)

Zika Virus (ZIKV)

Chapter 1: Background

Recombinant Subunit Vaccines

The goal of vaccination is to induce a protective immune response against a particular pathogen without causing disease [1]. This can be achieved through a variety of strategies, beginning with the earliest attempts using attenuation and inactivation, which were done by reducing the virulence of the pathogen while maintaining its immunogenicity or by using killed or inactivated pathogens [2]. These strategies were followed by more modern approaches that did not rely on whole pathogens, using instead specific antigenic components, such as protein purified from cultures of the pathogen or recombinantly produced protein, to elicit a protective response [1]. Recombinant subunit vaccines use purified antigens to elicit a targeted immune response to the desired pathogen [3]. This strategy avoids the health risks of attenuated and inactivated vaccines, such as reversion or virulence [3]. There are various ways to produce these recombinant proteins, including prokaryotic expression systems such as bacteria, and eukaryotic systems that include yeast, insect and mammalian cell lines. Each of these systems comes with their own advantages and drawbacks. In the U.S. there are currently five licensed recombinant subunit vaccines against three viral pathogens; Engerix-B and Recombivax HB are Hepatitis B virus vaccines that utilize *Saccharomyces cerevisiae* derived surface antigen that use aluminum hydroxide as an adjuvant, FluBlok, which is a trivalent seasonal influenza virus vaccine that uses baculovirus-insect cell derived proteins, and GARDASIL and CERVARIX which are human papilloma virus vaccines that use *S. cerevisiae* or baculovirus infected

Trichoplusia ni cell derived proteins, respectively [3-5]. Recombinant subunit vaccines have the advantage of a better safety profile and a reduced manufacturing cost compared to traditional vaccines but may suffer from low immunogenicity when not properly adjuvanted [3, 5]. The use of insect cells for antigen production is an attractive system due to their ability to produce proteins with post-translational modifications as well as being well suited for rapid large scale production [6, 7].There are currently two commonly used insect cell expression systems, the Baculovirus expression vector (BEV) and the *Drosophila* Schneider cell expression system (DES or S2). The BEV system is based on infecting insect cells with recombinant Baculovirus, resulting in transient expression of the desired protein by host cells in suspension culture. While this system does offer the advantage of post translation modifications not seen in bacterial expression systems and high levels of protein expression, it suffers from difficulties in the expression of secretory and membrane-associated proteins due to the effects of Baculoviruses on cell secretory pathways, as well as an inability to establish stably transfected cell lines [8, 9]. Despite these drawbacks, Baculovirus expression systems have been used in the production of antigens for two FDA approved vaccines, the Human Papilloma Virus CERVARIX vaccine (developed by GSK) and the Influenza virus vaccine, Flublok from Sanofi Pasteur, as well as several veterinary vaccines [5, 9, 10]. The second type of insect cell expression system, the S2 cell expression system is based on a cell line established in the 1970's using embryonic *Drosophila melanogaster* cells [11]. These cell lines can be transfected with a vector in which the gene of interest has been inserted leading to integration into the host cell DNA and resulting in stably transformed cell lines after selection [9]. This S2 cell expression system is currently

being used to develop recombinant subunit antigens for several different viral pathogens including several members of the Flavivirus family, Human immunodeficiency virus, Rotavirus, and members of the Filovirus family [9, 10, 12-15]. Although the antigens can be highly immunogenic, vaccines developed using highly purified recombinant protein antigens typically require proper adjuvantation to achieve protective efficacy [12, 16]. Various adjuvants have been tested with this vaccine platform including Alum, ASO_4, an Alum based adjuvant with a TLR-4 agonist (monophosphoryl lipid A), the saponin based adjuvants QS-21 and GPI-0100, and the emulsion based adjuvant CoVaccine HT [5, 10, 16, 17].

Filovirus Vaccines and Correlates of Protection

Filoviruses are a family of single stranded negative sense RNA viruses within the order *Mononegavirales*. The family is comprised of three genera; *Ebolavirus*, *Marburgvirus*, and *Cuevavirus*. While the genus *Marburgvirus* contains a single species, *Marburg marburgvirus* (MARV), the genus *Ebolavirus* contains five confirmed species: *Zaire ebolavirus* (EBOV), *Sudan ebolavirus* (SUDV), *Bundibugyo ebolavirus* (BDBV), *Reston ebolavirus* (RESTV) and *Tai Forest ebolavirus* (TAFV) [18]. Marburg virus was first identified in 1967 when hemorrhagic fever from a laboratory acquired infection occurred in Marburg, Germany, in people processing tissue from African green monkeys [18]. Two Ebolaviruses were identified in 1976, when outbreaks of unknown hemorrhagic fevers occurred simultaneously in the former Zaire (now Democratic Republic of the Congo) and in Sudan (now South Sudan), resulting in 318 and 284 cases, respectively [18-20]. Since their discovery, filoviruses have caused viral hemorrhagic fever disease in humans for which there are no FDA approved vaccines or therapeutics, although

several Ebola vaccine candidates are in or have concluded phase 2/3 clinical trials [21] including the heterologous Ad26.ZEBOV prime MVA-BN-Filo boost vaccine trial NCT02509494 [22], the ChAd3l-EBO Z and VSVΔG/EBOVGP vaccine trial, PREVAIL I NCT02344407 [23] , the STRIVE trial NCT02378753 for VSVΔG/EBOVGP [24] and the Ebola ça suffit trial for VSVΔG/EBOVGP, PACTR201503001057193 [25]. Despite the efficacy and clinical advancement of several EBOV vaccine candidates, several obstacles remain. While the recombinant Vesicular Stomatitis Virus (rVSV) vectored VSVΔG/EBOVGP has been reported to be highly efficacious in the prevention of EVD [25] a high number of adverse events have also been reported with this vaccine which could hamper its implementation during an outbreak [25, 26] or for mass vaccination campaigns. Other challenges include vaccine stability, durability of immunity, concerns about pre-existing immunity and cost of production [21] and distribution.

Infection with a filovirus occurs through contact with infected bodily fluids or tissue from an infected human or animal [27]. The Egyptian fruit bat (*Rousettus aegyptiacus*) has been confirmed to be an animal reservoir of MARV [28]. The hunt for the reservoir for ebolaviruses is ongoing, with bats being suspected, but not confirmed, and several other mammals within the geographic range also being investigated as possible reservoirs [27]. It is, however, known that nonhuman primates in Africa are susceptible to infection with ebolaviruses and may subsequently transmit them to humans [29]. Following transmission, the virus infects dendritic cells and travels to the lymph nodes, where it replicates and disseminates. The viruses then infect multiple different cell types including hepatocytes, splenocytes, and endothelial cells, resulting in widespread tissue

damage [27]. 2013 saw the beginning of a large widespread EBOV outbreak in West Africa that would not end until 2016 and resulted in 28,646 cases and 11,323 fatalities [30].

Due to the existence of multiple hemorrhagic fever causing viruses within the *Filoviridae* family, efforts have been made to develop multivalent or panfilovirus vaccines that are capable of protecting against two or more filoviruses. These vaccines generally include components to protect against one or more species of the *ebolavirus* genus as well as the more distantly related *marburgvirus*. The differences in nucleotide and amino acid sequence between EBOV species range from 32-41% while the differences between EBOV and MARV are 55% [18]. Cross-reactivity and cross-protection between some ebolaviruses have been seen in the rVSV vectored vaccination platform in mice but not guinea pigs. Mice vaccinated with rVSV expressing TAFV, EBOV, or RESTV glycoproteins (GP) were protected against lethal challenge with EBOV, while those that received vaccines expressing SUDV GP succumbed to challenge. No cross-protection, however, was seen in guinea pigs [31]. In contrast, when guinea pigs were infected with wild-type virus cross-protection was seen, suggesting the role other viral immunogens may play [31]. Another study in mice showed that after being given two doses of a monovalent rVSV vaccine expressing either MARV, SUDV, or EBOV GP, animals that received the EBOV construct developed cell mediated cross-reactive responses against SUDV but not against MARV [32]. In this study none of the other monovalent vaccines were able to elicit a cross-reactive cell mediated response. The EBOV vaccinated mice also showed a humoral cross-reactive response to SUDV, for which the inverse was also seen, with mice vaccinated with SUDV showing a cross-reactive humoral response

against EBOV. None of the mice showed a cross-reactive humoral response to MARV [32]. This is similar to what was seen in studies using an adenovirus-vectored vaccine in mice, which demonstrated cross-reactive humoral responses to SUDV and TAFV when vaccinated with an adenovirus vectored EBOV GP [33].

Although there are no vaccines or therapeutics for filoviruses currently approved by the FDA there are multiple vaccine candidates for EBOV that are in phase 2/3 clinical trials, as well as one, Ervebo, that was approved by the European Medicines Agency in November 2019 [21, 34]. The most promising of these is the rVSV-ZEBOV vaccine which has been shown to be highly efficacious in the prevention of Ebola virus disease (EVD) [25]. Despite its reported efficacy, as a replication competent virally vectored vaccine it has been associated with adverse events and may not be suitable for all individuals [25, 26]. The chAd3-ZEBOV is currently in phase 2 clinical trials where it is being tested alongside the rVSV-ZEBOV vaccine candidate [35]. It is a replication incompetent chimpanzee Adenovirus vectored vaccine, which has also been associated with adverse events [36, 37] as it requires very large doses of the viral vector, and based on NHP studies may not be able to elicit durable protection without the addition of a heterologous boost with MVA expressing filovirus GPs [38-40]. The durability of protection in cynomolgus macaques using the heterologous prime boost strategy (ChAd3/MVA) was analyzed to determine potential correlates and mechanisms of protection. Results demonstrated that while short term protection was associated with antibody titers, durability was seen to be correlated to the quality of the CD8+ T-cell response. With protection 10 months post vaccination being associated with TNF-α and

IFN-γ co-producing CD8+ T-cells [40].

Although definitive correlates of protection following EBOV vaccination have not yet been clearly elucidated there has been much work done on answering this question with occasionally contradictory results [41, 42]. Experiments have demonstrated that a correlate is also potentially dependent on the vaccine platform, and that both the humoral and cell mediated response are potentially correlated with protection. Guinea pigs vaccinated with various paramyxovirus vectored EBOV GP vaccines displayed widely different antibody repertoires yet were almost all protected from lethal challenge [43]. Studies done in mice, guinea pigs and nonhuman primates (NHPs) using a human adenovirus vectored vaccine showed that high levels EBOV GP specific IgG titers were correlated with survival in all three animal models, with cell mediated immunity playing a supporting role in NHPs as demonstrated by a higher number of IFN-y secreting cells in survivors versus non-survivors [41]. This, however, was not supported by an earlier study done in nonhuman primates using the same vaccine platform that demonstrated that CD8+ T-cells played an essential role in protection. The NHPs in this study that were vaccinated and subsequently had their CD8+ cells depleted were unable to survive EBOV challenge despite having high antibody titers, while passive transfer of high EBOV GP IgG titer serum did not confer protection to serum recipients [42]. This does not, however, mean that IgG may not be used as a surrogate marker for protection as it could be an indicator of the development of a protective humoral and cell mediated response [32, 44]. Studies done using the rVSV vectored vaccine with EBOV GP in rhesus macaques with simian immunodeficiency virus (SIV) were done to demonstrate

the efficacy of the vaccine in an immunocompromised model as well as to show the contribution of CD4+ T-cells to protection, as these animals were CD4+ deficient to varying degrees due to the SIV infection. These experiments showed that although the vaccinated animals with SIV did not develop a robust humoral response, four of the six vaccinated animals survived viral challenge. Those that did succumb to EBOV infection were those with the lowest CD4+ cell counts, suggesting the importance of their role in protection [45].

Flaviviruses and Zika Virus Vaccines

Flaviviruses are a genus of positive sense RNA viruses in the family *Flaviviridae*. Most Flaviviruses are arthropod borne and have known tick or mosquito vectors. Many of these viruses are significant human pathogens, including West Nile (WNV), Dengue (DENV), Zika virus (ZIKV), Yellow fever virus (YFV) and Japanese encephalitis virus (JEV). With the exception of YFV,JEV, and to a certain degree dengue virus (since the introduction of Dengvaxia), there are no approved human vaccines for mosquito-borne flaviviruses [18]. Multiple vaccine strategies have been used to develop interventions for those mosquito-borne flaviviruses that are currently without effective prophylactic measures, including recombinant protein vaccines which generally contain the Envelope (E) protein due to its role as the primary target for neutralizing antibodies [46]. For four flaviviruses in particular, DENV, WNV, tick-borne encephalitis virus (TBEV) and ZIKV, vaccines based on S2 cell expressed recombinant proteins are in development. Dengue virus exists as four distinct serotypes all of which are the causative agents of Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS), which affects 200,000 to 500,000 people per year [47] and constitute the most severe forms of dengue disease

burden. Infection with one serotype results in immunity against reinfection with that same serotype, but offers no protection against the other three and may in fact enhance infection with a different serotype [47, 48]. Thus a successful vaccine against DENV must be able to offer protection against all four serotypes. This makes it a particularly suitable candidate for a recombinant protein vaccine as this platform easily allows for adjustments in antigen dosing to balance the response against each serotype while maintaining a high safety profile [12]. Studies done in mice and NHPs have shown that insect cell derived recombinant protein based vaccines can elicit high neutralizing antibody titers against all four serotypes when properly formulated as well as prevent viremia in an NHP challenge model [12, 13, 49, 50]. West Nile virus was first introduced to North America in 1999 when a large outbreak occurred in New York [51]. Although most infections are asymptomatic ~1% of infected people develop neuroinvasive disease that may result in death or permanent damage [51]. While veterinary vaccines exist for use in horses, there is currently no vaccine available for human use [51]. Development of a WNV virus vaccine based on recombinant E protein is underway. This protein has been found to be safe and immunogenic in mice and immunogenic and protective in hamsters, in which WNV is lethal, as well as capable of preventing viremia in NHPs [14, 15, 52].

Zika virus, while only recently considered to be a human pathogen of interest very quickly became a concern when a large outbreak in Brazil in 2015 resulted in the discovery of an association between maternal infection with Zika virus and microcephaly in their infants [53]. Currently no vaccine exists against Zika virus, but vaccine candidates using a variety of different platforms are currently being explored. Studies

using a Zika purified inactivated virus (PIV) based on the Puerto Rico strain have been shown to successfully protect against challenge with both homologous and non-homologous strains in murine and NHP models [54]. The PIV vaccine also provided protection in BALB/c mice and Rhesus macaques in passive transfer studies, highlighting the importance of the humoral immunity [55]. A DNA vaccine platform using ZIKV prM-E genes has also been tested in mice and NHPs [54-56]. One vaccine candidate used constructs made using prM-E sequences from the French Polynesian strain to create subviral particles (SVP) that were not adjuvanted. Portions of the ZIKV prM or E genes were exchanged with analogous portions of JEV to improve expression and secretion, and the vaccine was administered using electroporation. Immunization of C57BL/6J and BALB/c mice with two of the constructs showed ZIKV specific neutralizing antibody titers. In Rhesus macaques that received two doses, 94% had no detectable viremia after challenge with ZIKV Puerto Rican strain (PRVABC-59) [56]. Other DNA vaccines have used the prM-E sequence from the Brazilian strain (BeH815744) and have shown protection against homologous strains in monkeys after two doses [55] and against both homologous and non-homologous strains in BALB/c, SJL and C57BL/6J mice [54]. A third vaccine platform using a rhesus Adenovirus vector has been shown to elicit antibody responses against a wide range of ZIKV envelope protein epitopes and protect against challenge with a homologous strain [55]. Unlike filoviruses, where the mechanisms and correlates of vaccine protection remain undefined, antibodies and in particular neutralizing antibodies have been demonstrated to be the key players in protecting against flavivirus infection [46]. ZIKV is no exception, with studies demonstrating the correlation between neutralizing antibodies and

protection in vaccinated NHPs [57] as well as showing that protection in mice and NHPs can be achieved by passive transfer of IgG from immunized animals [55].

The goals of this project are to assess insect cell produced antigens as vaccine candidates against both EBOV and ZIKV. In addition, it is aimed at determining mechanisms of protection for each vaccine candidate to establish correlates that can be used to predict vaccine efficacy in humans. We also want to establish the cross-reactivity of our EBOV vaccine candidate to other members of the filovirus family to guide the formulation of a multivalent filovirus vaccine. We hypothesize that recombinant subunit protein vaccines containing antigens produced in insects will offer protection against our target viruses, ZIKV and EBOV. For EBOV we hope to demonstrate this protection in both the guinea pig and nonhuman primate models, and hypothesize that protection will be mediated by both cellular and humoral immune responses. For our ZIKV vaccine candidate we hope to provide protection in nonhuman primates, and hypothesize that protection will be contingent on the development of neutralizing antibodies.

Dissertation Scope

Insect cell derived proteins offer a unique approach to the development of recombinant subunit vaccines. These proteins are produced using stably transformed *Drosophila melanogaster* S2 cells which express and secrete proteins, generating native protein structure and proper folding, and allow for high volume, low cost protein production [58]. Insect cells can be used to produce viral antigens that are purified using immunoaffinity chromatography (IAC) yielding highly purified products that can be used in the production of vaccines. Because there is no infectious material present in these vaccines, they show a high safety profile and can also be highly immunogenic when properly adjuvanted. It is a highly attractive option for the development of vaccines against pathogens where the safety of a traditional approach such as attenuation is a concern, and where a properly folded and glycosylated antigen is necessary to induce protection. The emergence of Zika virus as an infectious agent capable of causing severe neurological defects in developing fetuses [53], as well as one capable of sexual transmission as well as establishing persistent presence in the testes [59] make it an ideal candidate for the development of a recombinant subunit vaccine, as the population in which a live attenuated vaccine might be contraindicated due to risk (pregnant women) would also be the most at risk for infection. Filoviruses, which are highly pathogenic viruses that cause hemorrhagic fever, are also ideal candidates for this

vaccine platform due to the safety risks and logistical challenges that live attenuated or inactivated platforms carry. Filovirus vaccines developed using insect cell derived recombinant subunits would result in a non-replicating, safe, and efficacious filovirus vaccine that would be economical to manufacture and possibly allow for thermostabilization, reducing logistical burden in vaccine storage and distribution. To develop a ZIKV or filovirus vaccine using this strategy would, however, require establishing assays to explore vaccine correlates of protection as both vaccines may need to be licensed using the FDA animal rule [44]. In the case of filoviruses these established assays could then be used to determine to what extent cross-reactive responses from a monovalent vaccine would contribute to protection in a multivalent vaccine candidate, and help guide its formulation.

The objective is to develop a vaccine platform that can be used to protect against multiple infectious agents, either in a monovalent formulation (ZIKV or EBOV) or as a multivalent (filovirus) vaccine, as well as to establish assays that will allow us to correlate immune responses with vaccine efficacy. While work has been done in determining what constitutes a protective response in both flavivirus [46] and filovirus [32, 41, 44] vaccines, none of this work has been done in the context of a recombinant subunit vaccine platform. Evidence exists that what constitutes a protective response is very likely to be vaccine dependent [43], highlighting the importance of determining what response is necessary for protection using this particular vaccine platform. This becomes particularly important with the added complication of formulating a vaccine against multiple targets, which can result in immune interference and reduced efficacy when one component is a stronger immunostimulant than the others, interfering with the

ability to elicit a strong immune response to the other components [60-62]. This has been documented with several viruses, such as HPV [60], Hepatitis A and B [61], and DENV [62].

Specific Aims

Aim 1. Develop an insect cell derived recombinant subunit Zika virus vaccine and determine its efficacy and establish a correlate of protection in cynomolgus macaques.

We hypothesize that a recombinant subunit based vaccine containing the Zika Virus E protein when properly adjuvanted will be protective against viral challenge in cynomolgus macaques and that the humoral immune response will largely determine the level of protection in vaccinated animals.

Experimental approach: Immunize cynomolgus macaques with vaccines formulated with ZIKV E, using two different adjuvants, Alum and CoVaccine HT, and challenge them with ZIKV. Serum/plasma and PBMC samples will be collected during the vaccination period to examine the humoral and cell mediated response to vaccination and correlate to challenge outcome. Humoral response will be measured to determine IgG binding titers, and virus neutralizing antibody response.

Expected Results, Interpretation and Pitfalls: We expect humoral immunity to play a major role in preventing viremia after challenge and the quantity and quality of the

antibody response to be closely correlated with protection. The humoral response that we primarily expect to find protective will be the development of neutralizing antibodies against ZIKV E protein, which is the antigen in our vaccine candidate, as well as the primary target of the antibody response in a natural infection. The role of the cell-mediated immune response is unclear and it is possible that we will not find any cytokine output from T-cells following antigenic stimulation.

Aim 2. Determine cellular and humoral correlates of protection in animal models vaccinated with a recombinant subunit Ebola virus vaccine

We hypothesize that a recombinant subunit vaccine comprised of one of multiple viral proteins will be efficacious in multiple animal models, and that a protective immune response will be comprised of both humoral and cell mediated responses following vaccination.

Experimental Approach: A guinea pig and a cynomolgus macaque model will be used to assess the protective efficacy of different EBOV vaccine formulations using different viral proteins and adjuvant. Vaccination will be followed by viral challenge. Serum/plasma and PBMC samples will be collected throughout the vaccination period to determine the roles of both the cell mediated and humoral response in protection. Antigen-binding IgG will be measured using ELISA and bead-based immunoassays. The cell mediated immune response will be determined by *in vitro* stimulation of PBMCs with homologous whole antigen and peptide pools derived from the antigen and measuring cytokine output using flow cytometry and ELISpot assays. We will also use

flow cytometry to determine the T-cell memory populations throughout the vaccination period. Results of the humoral and cell mediated immunity assays will be compared to animal survival post-challenge to determine what components comprise a protective response.

Expected Results, Interpretation and Pitfalls: We expect that a strong humoral response will be associated with survival, although we do expect to see cell mediated responses, especially from CD4+ T-cells. If it is not apparent that either the humoral or cell mediated response are strongly linked with survival, other aspects of the response, such as kinetics or antigen-specificity will need to be analyzed to determine if any response parameter can be correlated with protection. This can include investigating different cytokines or memory markers using ELISpot and flow cytometry or delving further into the qualities of the antibodies developed in response to vaccination.

Aim 3. Establish cross-reactivity and cross-protective potential of a recombinant subunit based Ebola virus vaccine in cynomolgus macaques against other members of the filovirus family (SUDV and MARV).

While prior work has focused on exploring the immune response to homologous viral antigen, the same type of assays can also be used to determine the cross-reactive immune response to other members of the filovirus family. We hypothesize that EBOV vaccine formulations will result in some cross-reactive responses in both cell mediated and humoral immunity, primarily to more closely related filovirus species such as SUDV. Cross-reactive responses to MARV are expected to be lower due to the lower homology

between EBOV and MARV.

Experimental Approach: The cross-reactive humoral response will be assessed in NHPs vaccinated with our EBOV vaccine candidates. These include animals that have received two or three doses of EBOV GP with varying amounts of CoVaccine HT as well as animals that have received a trivalent formulation. Serum and PBMCs will be collected throughout the vaccination period. The animals will be challenged with EBOV-Kikwit 28 days after the final immunization to assess efficacy. Cross-species immune responses will be assessed using the previously developed humoral and cell mediated response assays. For the humoral response, bead based immunoassays will be used to determine the presence of binding titers against the GP of other filoviruses such as SUDV and MARV. Cross-reactive cell mediated immune responses will be assessed by stimulating PBMCs with SUDV and MARV whole antigen and peptide pools and examining T-cell cytokine secretion using flow cytometry. ELISpot assays will be performed to detect IL-4 or IFN-γ secretion.

Expected Results, Interpretation and Pitfalls: We expect antigen-binding titers to be present in animals vaccinated with both the EBOV GP only and mixed GP vaccine candidates. This would demonstrate the ability of our vaccine to elicit antibodies against conserved epitopes between filovirus species. Cross-binding titers will likely be higher against SUDV GP than MARV GP due to closer amino acid homology between EBOV and SUDV. Stimulation with SUDV and MARV GP antigen and peptide pools is expected to result in the PBMC production of cytokines, particularly TNF-α, IL-4 and IFN-γ. While a lack of cytokine secretion would not necessarily indicate a lack of cross-

protective potential, a cross-reactive cell mediated response in conjunction with a humoral response could give insight into possible mechanisms of protection. However, if there are no binding antibody titers this would tell us that our current vaccine formulations, containing 25μg EBOV GP with CoVaccine HT or 25μg each of EBOV, SUDV, and MARV GP with CoVaccine HT, would likely have no efficacy against other filovirus species and reformulation would be required for a multivalent vaccine.

Chapter 2: Efficacy and Immune Response of a Recombinant Subunit Vaccine in Nonhuman Primates against ZIKV

Methods

Virus stock and cell culture

ZIKV, Puerto Rican Strain PRVABC59 stock, Dengue virus type 2 (DENV2) (Dakara strain) and West Nile Virus (WNV) (NY 99 Crow strain) were grown in Vero cells as previously described [63-65].

Cynomolgus macaque (*Macaca fascicularis*) vaccination, challenge, and blood collection

Expression and purification of the ZIKV E protein was done as previously published [63]. Two separate cynomolgus macaque studies were done: An initial immunogenicity study in which 25 μg of ZIKV E was adjuvanted with 10mg Co-Vaccine HT™ (Protherics Medicines Development Ltd, London, United Kingdom) and a follow up study where 50 μg ZIKV E protein was adjuvanted with Alum (2% Alhydrogel adjuvant, Invivogen, San Diego, CA). Both studies used eight female cynomolgus macaques that were 9 years of age and weighed between 3-5kg. All cynomolgus macaques were vaccinated intramuscularly (IM) in the leg at days 0 and 21 (vaccine group, n=4 for each study). Control animals in the first study received 25μg each of Sudan Virus, Ebola Virus, and Marburg Virus glycoproteins (control group, n=4) at day 0 and PBS at day 21. In the second study control animals received 25μg of either SUDV GP or MARV GP

adjuvanted with 10mg Co-Vaccine HT at 0, 21 and 42 days. The control animals used in the second study were concurrently part of an unrelated immunogenicity study. Pre-challenge serum and plasma samples were collected at days 0, 14, 21, 35, and 49. Challenge for both groups was performed on day 49 subcutaneously in the hind thigh with 10^4 TCID50 of ZIKV Puerto Rican strain PRVABC59 in a volume of 1 ml PBS. Blood samples were taken daily for the following 7 days, then weekly until day 77. Plasma and/or serum samples were collected and stored at -80C.

Viremia assays

Viremia was assessed either by quantitative RT PCR or plaque assay. For the RT PCR, RNA was isolated from 200µl plasma using the QIAamp MinElute Virus spin kit (Qiagen, Frederick, MD). Extracted RNA was used for amplification using the SensiFAST Probe Lo-ROX One-Step Kit (Bioline BIO-78005, Taunton, MA) on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). Primers and probe were designed to amplify a conserved region of the capsid gene from ZIKV BeH815744, as follows: Fwd: GGAAAAAAGAGGCTATGGAAATAATAAAG; Rev: CTCCTTCCTAGCATTGATTATTCTCA;
Probe: AGTTCAAGAAAGATCTGGCTG. Primers and probe were used at a final concentration of 2µM, and the following program was run: 48°C for 30 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 1 min at 60°C. Assay sensitivity was 50 copies/ml. Plaque assays were performed using a previously reported protocol, limit of detection was 50 pfu/ml (27).

Plaque reduction neutralization test (PRNT)

Sera from individual animals were heat-inactivated by incubation at 56°C for 30 minutes. For the DENV and WNV PRNTS A series of 3-6 two or four-fold dilutions, starting at 1:20 of each sample was prepared using M199 medium, and incubated for 30 minutes at room temperature with media containing previously titrated virus in a 1:1 (v/v) ratio to generate approximately 50 pfu of virus per well. The antibody-virus complex was then added to Vero cells in duplicate wells of a 6-well plate, and incubated at 37 °C for 1 hour. Cells were overlaid with M199 containing 1% agarose and incubated for either 48 hours (WNV) or 72 hours (DENV). A second overlay of 1% agarose in Dulbecco's phosphate buffered saline (DPBS) containing 1.5% (WNV) or 2% (DENV) neutral red (0.33% solution, Sigma, St. Louis, MO) was added, and plaques were counted 24-48 hours later. For the ZIKV PRNTS a series of 3-6 four-fold dilutions, starting at 1:40 of each sample was prepared using M199 medium and incubated at 4°C overnight with media containing previously titrated virus in a 1:1 (v/v) ratio to generate approximately 50 pfu of virus per well. The antibody complex was then added to Vero cells in duplicate wells of a 6-well plate and incubated at 37 °C for 1 hour. Cells were overlaid with M199 containing 1% agarose and incubated for 24 hours. A second overlay of 1% agarose in M199 containing 1.5% neutral red was added, and plaques were counted 24 hours later. $PRNT_{50}$ values, the serum dilutions yielding 50% virus neutralization, were generated using a variable-slope sigmoidal dose response computer model (Prism, Graphpad Software, San Diego, CA). PRNT data shown for all

viruses are from serum samples collected at day 35 (14 days after the booster immunization).

Coupling of microspheres with recombinant antigens and microsphere immunoassay (MIA)

The coupling of microspheres with E proteins of ZIKV, DENV2, DENV3 and WNV, the MIA, and the derivation of EC_{50} antibody titers were performed as described previously [63].

Passive transfer studies in BALB/c mice

BALB/c mice were bred in colonies at JABSOM from original stocks obtained from Taconic Biosciences, Inc. (Hudson, NY). One hundred microliters of plasma taken from cynomolgus macaques two weeks after the second immunization with CoVaccine HT adjuvanted ZIKV E (day 35) were injected intraperitoneally (IP) into 3 groups of 6 male and 6 female, 6-7 month old mice one day prior to challenge with 100pfu of live ZIKV via the tail vein. Serum collection by tail vein bleeds was performed 6 hours after the serum transfer, and blood collection by cardiac puncture was performed at day 3 after challenge for six of the animals and 2 weeks after challenge for the remaining animals. ZIKV E-specific IgG antibody titers respective to both animal species, assayed by MIA, and ZIKV neutralizing titers were determined on serum samples.

Statistical analysis

Determination of statistical significance in the number of viremic days between vaccinee and control groups in the NHP challenge experiment was done using the Fisher exact

probability test. Differences in viremia and IgG titers between groups of mice in the passive transfer experiment was assessed for statistical significance using an unpaired t-test (Prism, Graphpad Software, San Diego, CA). $P<0.05$ was considered significant.

Results

The recombinant protein vaccine platform has been shown to be efficacious against a number of viral pathogens including flaviviruses. A vaccine using this platform was developed against ZIKV with efficacy assessed first in a mouse model, and later in a nonhuman primate model to recapitulate infection in humans. This vaccine utilized the E protein of the virus using two different adjuvants, Alum and CoVaccine HT. Our E protein was comprised of the envelope ectodomain and was expressed using stably transformed *Drosophila* S2 cells. It was then purified using immunoaffinity chromatography. Initial studies in mice were done to assess immunogenicity of the vaccine candidate. These experiments were done in Swiss Webster mice where animals received ZIKV E with Alum, CoVaccine HT or alone and indicated that two doses of the vaccines with ZIKV E and adjuvant were enough to elicit a robust ZIKV E antibody response. A second experiment using three different mouse strains, Swiss Webster, BALB/c and C57BL/6, demonstrated that there was no difference in the neutralizing antibodies elicited between the mouse strains, although slight differences were seen in the overall binding titers with Swiss Webster mice having lower titers than BALB/c and C57BL/6 mice. To determine protective efficacy, an immunocompetent mouse viremia model was developed using BALB/c mice, where it was shown that intravenous infection was able to consistently cause viremia in the mice at day three post infection. Using this model, vaccines with both Alum and CoVaccine HT were able

to offer full protection against infection after two doses, and partial protection in mice receiving one dose. Protection was also seen in a passive transfer experiment in mice that received high ZIKV GP antibody titer sera from vaccinated mice confirming the results seen in directly challenged animals and demonstrating the importance of antibodies for control of viremia. Protection was assessed by seroconversion following viral challenge of sera recipients [16]. Due to the limitations of the mouse model, and in an effort to more closely simulate infection in humans, the vaccine candidate was also tested in cynomolgus macaques. Two different studies were performed in NHPs using two different adjuvants. One study used eight animals, of which four received the vaccine candidate adjuvanted with CoVaccine HT and the other four were control animals while in the second study four of the eight animals received a formulation with Alum and the remaining four were control animals.

The immunogenicity of the tested candidate vaccine formulation in NHPs was assessed by measuring ZIKV E protein specific IgG levels in the serum of vaccinated and control NHPs using a bead based multiplex immunoassay (MIA) which uses beads coated with a target antigen to detect antibodies against that target (**Figure 1**).

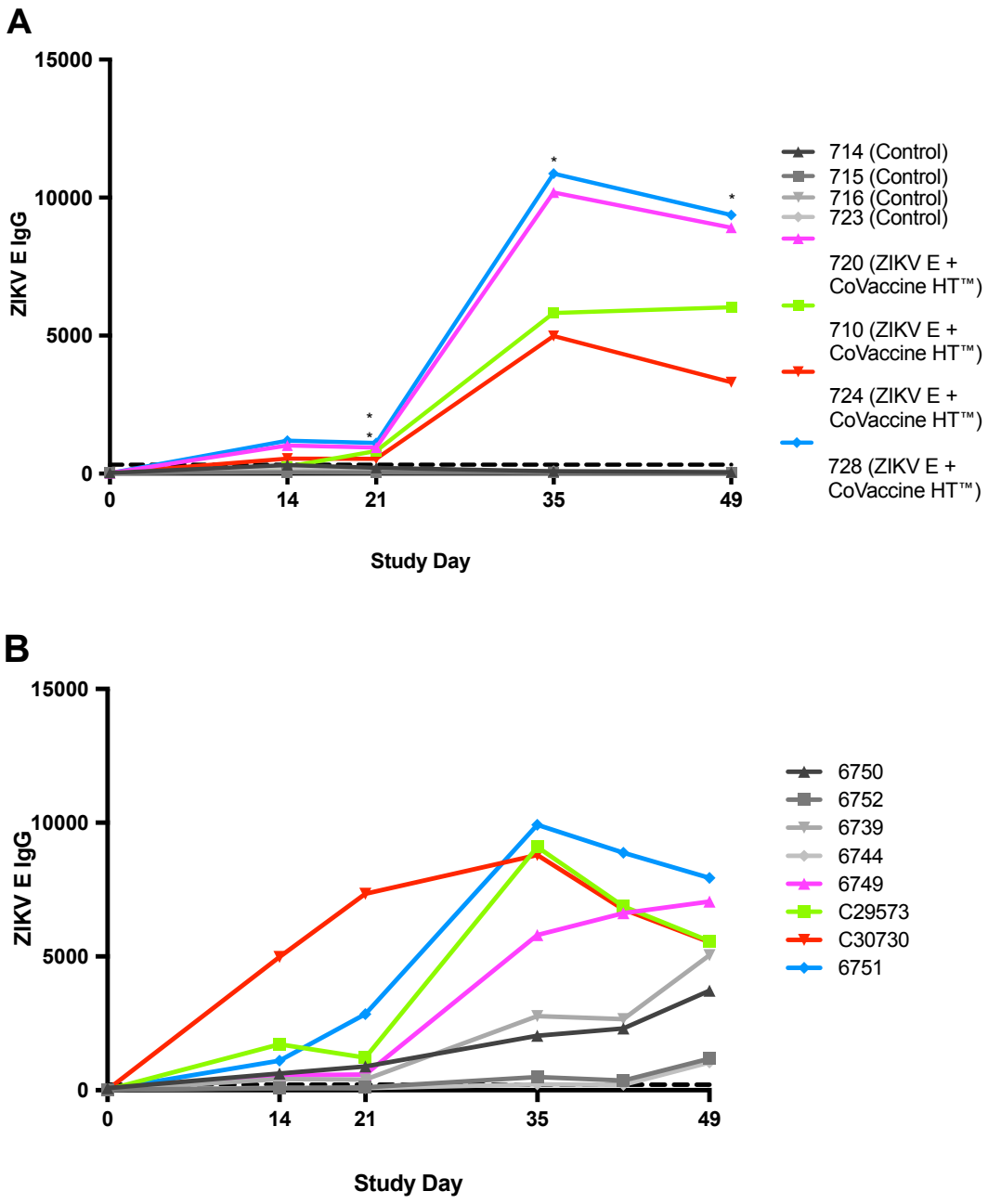


Figure 1: Immunogenicity of recombinant ZIKV E protein in cynomolgus macaques. Animals were given A) 25 μg ZIKV E protein with CoVaccine HT as an adjuvant at day 0 and 21 with the control group receiving unrelated glycoproteins at day 0 and PBS at day 21 or B) 50 μg ZIKV E protein with Alum and control animals receiving three doses of SUDV, EBOV, and MARV GP with CoVaccine HT. Blood was collected at various points

throughout the vaccination. Geometric means (GMT) with 95% CI of ZIKV E specific IgG MFI of serum from the vaccinated and control groups are depicted. The negative cutoff was calculated by taking the mean value of negative control samples and adding 3 standard deviations and indicated by the dotted line

Animals in both vaccine groups showed a ZIKV E specific IgG response by day 14 post immunization, which continued to increase following the second vaccine dose at day 21. Both groups, with the exception of one animal in the vaccine recipients ZIKV E with Alum, show similar ZIKV E specific IgG titers at the time of challenge (D49). These results demonstrate the ability both ZIKV vaccine candidate formulations to elicit ZIKV E binding antibodies. The control animals in the second study also show an increase in ZIKV E IgG titers which may be reflective of impurities present in the protein preparations used for their vaccination, which were expressed and purified using the same methods as for ZIKV E. High neutralizing antibody titers against ZIKV were found in three of the four vaccinated animals at day 35 in the group receiving ZIKV E with CoVaccine HT. One animal (724) showed a low level of neutralizing antibody titers despite having a high level of antigen binding antibodies (determined by MIA). All four animals in the second study that received ZIKV E with Alum developed high neutralizing antibody titer. None of the control animals in either group developed any neutralizing antibody titers against ZIKV, WNV, or ZIKV (**Tables 1 and 2**).

Table 1: Serum $PRNT_{50}$ titers at day 35 of all animals receiving ZIKV with CoVaccine HT against ZIKV, DENV2, and WNV

	Controls				**Vacinees (ZIKV-E +CoVaccine HT)**			
Animal ID	**714**	**715**	**716**	**723**	**710**	**720**	**724**	**728**
ZIKV $PRNT_{50}$	<40	<40	<40	<40	5242	40446	48	4866
DENV2 $PRNT_{50}$	<20	<20	<20	<20	134	1186	<20	118
WNV $PRNT_{50}$	<20	<20	<20	<20	<20	392	<20	30

Table 2: Serum $PRNT_{50}$ titers at day 35 of all animals receiving ZIKV E with Alum against ZIKV, DENV2, and WNV

	Controls				**Vaccinees (ZIKV-E + Alum)**			
Animal ID	**6750**	**6752**	**6739**	**6744**	**6749**	**C29573**	**C30730**	**6751**
ZIKV $PRNT_{50}$	<40	<40	<40	<40	4558	4632	2420	2406
DENV2 $PRNT_{50}$	<20	<20	<20	<20	65	106	71	201
WNV $PRNT_{50}$	<20	<20	<20	<20	24	<20	59	24

Neutralizing antibody assays showed variability in cross-reactivity to other flaviviruses between vaccinated animals. Of the four vaccinated animals in the first study, 728 and 720 had detectable neutralizing antibodies to DENV2 and WNV, with a $PRNT_{50}$ of 120 and 1187, and 92 and 103, respectively, at day 35 (**Table 1**). Vaccine recipients in the second study (Table 2) developed more consistent, but lower, cross-neutralizing antibody responses. All four vaccinated animals had a DENV cross-neutralizing response with a $PRNT_{50}$ of 65-207 and three of the four vaccinated animals had WNV cross-neutralizing antibodies with a $PRNT_{50}$ between 24 and 104.

After challenge with ZIKV on day 49, virus RNA was detectable in the control animals from both studies starting at day 2 post challenge with peak virus RNAemia being seen between days 3 and 4 (**Figure 2**).

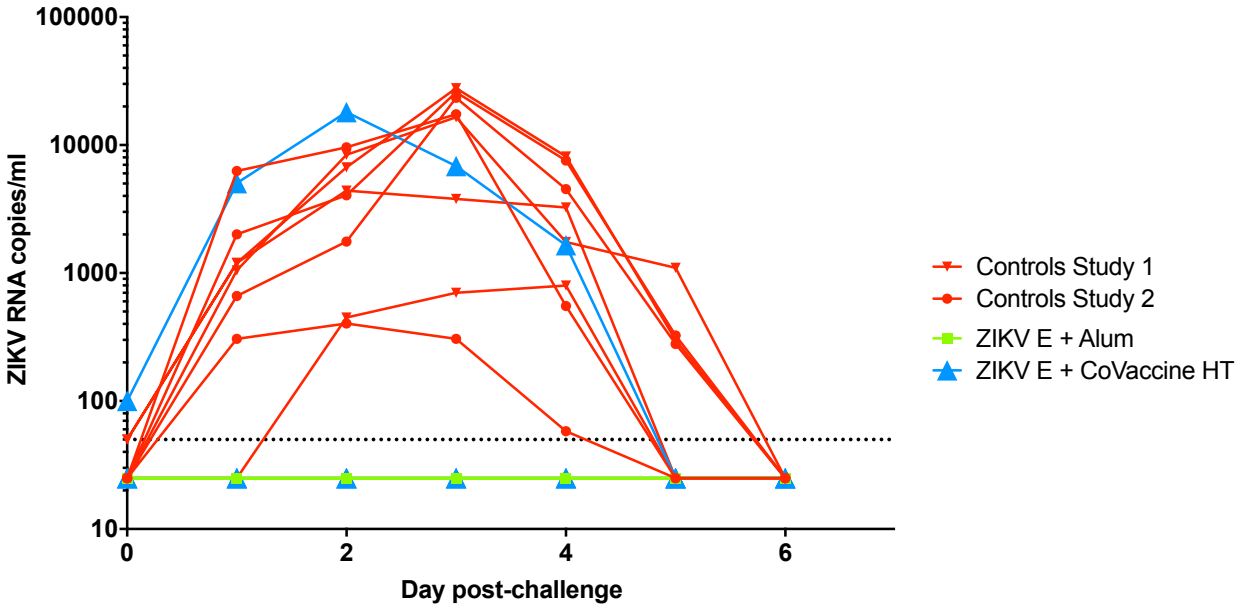


Figure 2: Viremia in vaccinated and control cynomolgus macaques challenged with ZIKV. Data from both experiments is combined in the figure. Animals were challenged at day 49 of both experiments. Blood was taken daily for 7 days post challenge, and viremia was measured using quantitative RT-PCR. Limit of detection was 50 copies/ml of plasma, shown by dotted line. Vaccinated animals had a significantly reduced number of viremic days compared to control animals ($p<0.0002$)

In the ZIKV E with CoVaccine HT vaccine group, three of the four animals had undetectable viremia on all days, while animal 724, which had low neutralizing antibody titers prior to challenge showed viral RNA levels similar to the control animals. In the group that received ZIKV E with Alum, all four animals were completely protected against viral challenge. By day 7, none of the animals had detectable levels of virus

RNA in serum. These results demonstrate that infection of the animals was successful and that both ZIKV vaccine candidate formulations had the ability to elicit protection in three of the four vaccine recipients (ZIKV E with CoVaccine HT) or completely protect (ZIKV E with Alum).

Although the PRNTs show that the antibodies elicited in the vaccinated NHPs were capable of neutralizing virus *in vitro*, we wanted to determine if these antibodies were sufficient to neutralize virus *in vivo* and protect against ZIKV challenge. Plasma from three individual NHPs from the ZIKV E with CoVaccine HT group was injected IP into groups of 12 mice: an unvaccinated control animal with no ZIKV neutralizing activity (714), an animal with low neutralizing titers (724) and one with high neutralizing titers (720). Blood was collected from mice 6 hours post-transfer to determine the levels of NHP and mouse anti-ZIKV E IgG using MIA (**Figure 3**).

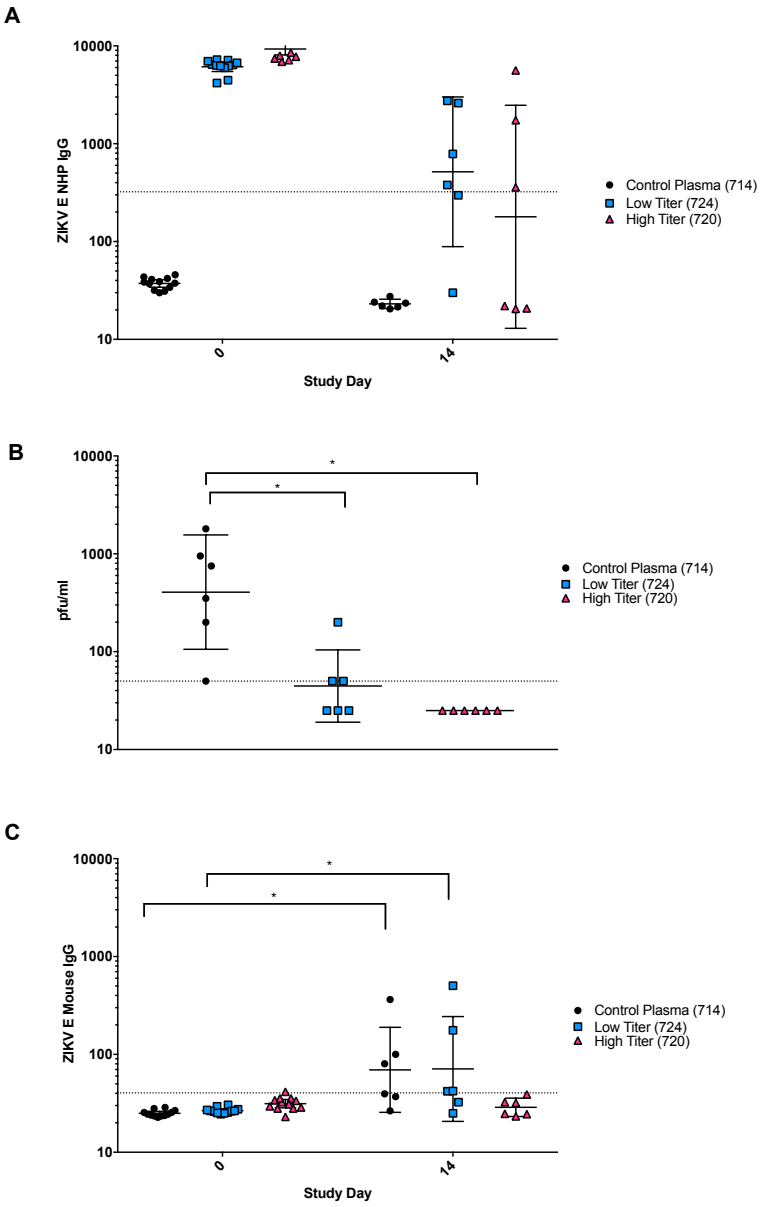


Figure 3: Passive Protection in BALB/c mice using NHP plasma. A) Cynomolgus ZIKV E specific IgG titers expressed as MFI from serum at day 0 (6 hours after passive transfer of plasma) and day 14 after challenge. The negative cutoff was calculated by taking the mean value of negative control samples and adding 3 standard deviations and is shown by the dotted line. B) Viremia from six individual mice from each group was determined using a standard plaque assay on Vero cells. Limit of detection is 50 pfu/mL, shown by dotted line. Data points below the level of detection are depicted as 25. Significant differences between the mice receiving plasma with no, low, or high neutralizing titers were calculated using unpaired t-tests (*p-value<0.05). C) Mouse ZIKV E specific IgG titers expressed as MFI. Values are shown as GMT with 95% CI.

The negative cutoff was calculated by taking the mean value of negative control samples and adding 3 standard deviations and is shown by the dotted line

Mice were challenged the following day. On day 0, after passive transfer but before viral challenge, titers of ZIKV E specific NHP IgG were similar for the low and high titer mouse groups, and undetectable in all mice receiving the naïve plasma (**Figure 3A**). By day 14 levels of NHP IgG had dropped for low and high titer recipients and remained undetectable in mice that received naïve plasma. Despite the similar levels of binding antibodies present in the low and high titer mouse groups, three of the six mice that received low titer NHP serum developed viremia after challenge, demonstrating only partial protection, while the mice that received the high titer plasma were completely protected (**Figure 3B**). These results were supported by the mouse specific IgG titers from mouse sera (**Figure 3C**). At day 0, none of the mice showed any murine IgG titers against ZIKV E, however, by day 14 post challenge, mice in the naïve and some animals in the low titer group had increased levels of ZIKV E-reactive murine IgG indicating successful virus replication in these groups. Mouse IgG titers against the E protein, which were only seen in mice of the naïve and low titer groups, suggest that viremia in the high titer group caused by the i.v. infection route was transient and did not last long enough for IgG antibodies to develop. This result suggests that the serum had sufficient neutralizing antibodies to confer protection from viremia.

Cell-mediated immune responses were analyzed by flow cytometry using PBMCs and splenocytes collected post challenge from the animals that received ZIKV E with CoVaccine HT. Splenocytes and PBMCs were stimulated with whole antigen produced

in S2 cells as well as 15mer peptides that spanned the full length of flavivirus E and NS1 proteins. The stimulants used were ZIKV E and NS1, DENV2 E and NS1 and WNV E and NS1 antigen and peptides to determine if there were any ZIKV E specific and cross flavivirus cellular immune responses. Several cytokines and markers of cell mediated responses were analyzed in these experiments including IL-6, IL-4, TNF-a, IFN-g, IL-10 and CD107a. Analyzing both the CD4+ and CD8+ compartments, we were not able to observe specific responses from either CD4+ or CD8+ cells.

Discussion

Many strategies have been used in the development of efficacious candidate flavivirus vaccines, including recombinant subunit platforms [12, 13, 15, 49, 66, 67]. Vaccines against DENV and WNV using the S2 cell based recombinant subunit platform have already successfully undergone phase I clinical trials (NCT00936429, NCT01477580 and NCT00707642). The tetravalent recombinant subunit DENV vaccine has proven to be safe and immunogenic, and protected NHPs from viremia [12, 13, 49]. This report, however, provides the first documentation of the development of a recombinant subunit vaccine that affords effective protection against ZIKV infection in NHPs. It has been well established that humoral immunity plays a major role in protection against flavivirus infection, with the E protein as the major antigenic target [46, 66, 68, 69]. Using an embryonic *Drosophila melanogaster* Schneider S2 cell-based expression system to produce ZIKV E protein, we have developed a vaccine candidate using either CoVaccine HT or Alum as adjuvant. Candidates are administered in only two doses spaced three weeks apart, making this an attractive clinical option for rapid

development of protective efficacy. These vaccine candidates were previously tested in a mouse model, using immunocompetent Swiss Webster, BALB/c, and C57BL/6 mouse strains [63]. Our studies showed that both vaccine candidates were able to elicit robust antigen binding and neutralizing antibody titers after two doses, and we demonstrated the ability of our vaccine to protect against viral replication after challenge with ZIKV [63]. We have chosen to test our vaccine candidate using a cynomolgus macaque model, in which it was previously demonstrated that ZIKV PRVABC59 replicates robustly and with very similar kinetics and duration to what is seen in rhesus macaques, albeit with slightly lower peak viral titers [70]. In this model the recombinant subunit vaccine candidates were found to elicit binding IgG antibody titers against ZIKV E protein by day 14 after the first vaccine dose which increased further after a second vaccination on day 21, a response that lasted until ZIKV challenge at day 49 (**figure 1**), demonstrating the immunogenicity of the vaccine candidate. This rapid response makes it an ideal candidate for an outbreak setting where a strong response elicited quickly and with few doses is necessary. ZIKV neutralizing antibody titers were also found to be high for three of the four vaccinated animals in the ZIKV E with CoVaccine HT group and all vaccinated animals in the ZIKV E with Alum group (**tables 1 and 2**). Animal 724 showed lower neutralizing antibody titers compared to other animals, despite the presence of comparable antigen binding IgG titers. Viral RNA after ZIKV challenge in all animals with high $PRNT_{50}$ titers was undetectable (Figure 3) throughout the challenge period demonstrating a correlation between the neutralizing antibodies elicited by vaccination and protection against peripheral viral infection. The animal that had low $PRNT_{50}$ titers developed viremia similar to the controls, and the viral kinetics agreed

with what has been seen in cynomolgus macaques in previous experiments [70]. These data highlight the importance of sufficient virus neutralizing antibody titers which have been suggested as a correlate for efficacy in flavivirus vaccine development [71, 72] and which have been shown to prevent ZIKV transmission to fetuses in a pregnant mouse model [72]. The high levels of ZIKV E binding IgG but low $PRNT_{50}$ titer in animal 724, which showed breakthrough viremia, correspond to the development of antibodies that are capable of binding ZIKV E but are incapable of virus neutralization; therefore, these data support the use of neutralizing antibodies as a correlate of protection. Other ZIKV vaccine studies conducted in rhesus macaques have shown similar results with viremia being seen in animals with comparatively low neutralizing titers after receiving a DNA vaccine expressing ZIKV prM and E protein or an mRNA vaccine encoding ZIKV prM and E protein, while other animals with higher neutralizing titers were protected [56, 73]. Using the neutralizing antibody results from the DNA vaccine studies and probability analysis the group concluded that an EC_{50} microneutralization titer of 100 or greater would be sufficient to protect 70% of NHPs against infection [56]. Based on the results from our own vaccine candidate in NHPs, we know that a $PRNT_{50}$ of 48 or lower will likely not be protective and $PRNT_{50}$ values greater than 4,900 will protect against viral challenge. A comprehensive overview of these vaccine platforms and their performance in different animal models is covered in the recent review by Poland, et al. [74]. These vaccines in development include a purified inactivated vaccine (PIV) that was able to confer complete protection in mice and rhesus macaques. DNA and RNA based vaccines that encode the prM and E proteins are also under development and have shown efficacy in both the mouse and the NHP model, as well as a rhesus

adenovirus vectored vaccine that was also efficacious in both mice and nonhuman primates [74].

Interestingly, our recombinant subunit ZIKV vaccine elicits antibodies that in some animals are capable of cross-neutralizing DENV and WNV viruses (tables 1 and 2). Despite the presence of cross-binding antibodies in all animals (data not shown), only 6 of the 8 vaccinated animals had antibodies cross-neutralizing DENV2 or WNV. Of course, the question whether animal 724 might have had antibodies from a previous flavivirus infection resulting in an *in vivo* manifestation of antibody-dependent enhancement of infection (ADE) presented itself. However, this animal at beginning of the study had no neutralizing antibodies for any of the tested flaviviruses (data not shown), and despite a high titer of ZIKV E binding antibodies present before challenge, it had the lowest virus neutralizing titers in the vaccinated group. ADE is a concern in the field of ZIKV vaccine development due to its similarity to DENV and experiments done in immunocompromised and wild type mice have yielded inconsistent results [68, 75, 76]. Studies using rhesus macaques have shown that preexisting immunity to DENV does not enhance the pathogenesis of ZIKV infection [77], however preexisting immunity to ZIKV may result in ADE in a subsequent DENV infection [78]. Many questions remain, and further studies are required in this area.

A passive transfer experiment shed further light into the importance of neutralizing antibodies. Mice receiving plasma from the control NHP had no detectable cynomolgus IgG binding ZIKV E while the mice that received low and high titer plasma had high pre-

challenge titers demonstrating a successful transfer. Mice that received plasma from the unvaccinated control macaque uniformly showed viremia after challenge, while three of the six mice receiving the low titer plasma and none of the animals that received the high titer plasma developed viremia. Mouse IgG titers rose only in sera of mice that received control and low titer plasma. The development of IgG titers in the low and control recipient mice is consistent with the viremia seen in these groups, while the lack thereof in the group that received high titer plasma can be explained by a lack of viral replication in these animals. These data correlate well with other studies that have demonstrated that protection against several different flaviviruses in mouse models can be achieved through antibodies alone [68, 69, 79-83]. In the case of our passively protected mice, lack of viremia in the high titer antibody recipients and a failure to develop ZIKV E-specific IgG suggest that protection against viral replication was achieved, and demonstrate the ability of our vaccine candidate to raise a completely protective humoral immune response. Flow cytometry was used to detect cell mediated immune responses using PBMCs and splenocytes taken from NHPs post-challenge. Any responses detected in these cells would have been reflective not only of vaccine induced responses but of a response formed as a result of viral challenge. This is particularly true of those cells taken from the control animals and the vaccinated animal 724, as they all had active viral replication. The results of our flow cytometry experiments showed no specific T-cell responses after stimulation with either flavivirus whole antigen or peptides (data not shown), which is consistent with the mechanism of protection being antibody driven. Taken together these results suggest that recombinant

ZIKV subunits could be a safe and efficacious option for the prevention of ZIKV infection in humans.

Chapter 3: Efficacy and Immune Response of a Recombinant Subunit Vaccine against EBOV in Guinea Pigs and Nonhuman Primates

Methods

Protein expression and purification

Expression and purification of recombinant subunit proteins was conducted as described previously [10]. In addition to single-step purification yielding a >90% pure EBOV GP preparation, the same material was subjected to size-exclusion chromatography using a Superdex 200 column (GE Healthcare, Piscataway, NJ) equilibrated in phosphate-buffered saline (PBS) to separate trimer and high molecular weight (HMW) fractions generating three distinct protein lots: HMW, trimer and a mixed population collected between the two. Monomeric GP was discarded and not used for animal studies as it is presumed to not be in the proper conformation.

Ethics and Biosafety

All work with animals was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. All guinea pig procedures were reviewed and approved by the appropriate Institutional Animal Care and Use Committees at Lovelace Biomedical and Environmental Research Institute (LBERI) (Albuquerque, NM) and the Rocky Mountain Laboratories (RML) (Hamilton, MT), DIR,

NIAID, NIH. The NHP vaccination portion of the study was conducted at BIOQUAL (Rockville, MD) and was approved by BIOQUAL, Laboratory Animal Care and Use Committees.

Challenge viruses

Gpa-EBOV [84] was propagated and virus titers determined on Vero E6 cells and used for the challenge part of this study. Gpa-EBOV was obtained by passing EBOV isolate (strain Mayinga) through strain 13 guinea pigs until uniform lethality was achieved [84]. NHP challenge virus was EBOV isolate 199510621 (strain Kikwit) that originated from a 65-year-old female patient who had died on 5 May 1995. The study challenge material was from the second Vero E6 passage of EBOV isolate 199510621. Briefly, the first passage at the University of Texas Medical Branch at Galveston (UTMB) consisted of inoculating CDC 807223 (passage 1 of EBOV isolate 199510621) at a multiplicity of infection (MOI) of 0.001 onto Vero E6 cells. The cell supernatants were subsequently harvested at 10 days postinfection and put in vials in 1-ml aliquots. Deep sequencing indicated the EBOV was greater than 98% 7U (consecutive stretch of 7 uridines). No detectable mycoplasma or endotoxin levels were measured at <0.5 endotoxin units (EU)/ml.

Guinea pig vaccination and challenge

For the immunogenicity studies, Hartley guinea pigs were immunized using three different adjuvants with different modes of action; 1, an emulsion-based adjuvant, CoVaccine HT (an emulsion of squalane with immunostimulatory sucrose fatty acid

sulphate esters and an adjuvant of Protherics Medicines Development, London, United Kingdom) [85] was used at a dose of 1 mg; 2, a saponin-based, immunomodulatory adjuvant, GPI-0100 (Hawaii Biotech, Inc., Honolulu, HI) [86, 87] was used at a dose of 100µg; 3, Alhydrogel® 85 ("Alum"; Brenntag, Reading, PA) was used at 1mg AL(OH)3 per dose. Groups of 8 or 16 male and female Hartley guinea pigs (>5 weeks old) were obtained from Charles River Laboratories, acclimated for 14 days and vaccinated intra-muscularly (i.m.) three times in the hind legs with individual subunit proteins at the indicated dose and formulated with one of the selected adjuvants at 3-week intervals. Vaccine formulations were prepared fresh for each vaccination from frozen antigen stocks, adjuvant stock solutions and sterile PBS at PanThera Biopharma, where all formulations were made and immunoassays for the GP studies were done, to reach the desired dose within a final volume of 0.2 mL and were sent refrigerated to the facility for immunizations within 3-4 days from preparation. Negative control groups received equivalent doses of GPI-0100 adjuvant in PBS only (also prepared fresh for each administration). Pre-challenge serum samples were collected under anesthesia on study days 0, 20, 38/39 and 56 for study 1 or days 0, 21, 42 and 63 for study 2 to allow serological analysis of the vaccine induced responses. Groups of 8-16 previously immunized Hartley guinea pigs were transferred into the ABSL4 facility at RML, acclimated to the new environment and challenged intraperitoneally (i.p.) with 1,000 LD_{50} (10 focus-forming units) of guinea pig adapted (gpa)-EBOV. Animals were observed daily for signs of morbidity and mortality. Surviving animals were euthanized 28 days after challenge. Serum samples were collected from each animal at their respective final study day.

Nonhuman primate vaccination and challenge

16 healthy, filovirus-naive, adult (~3 to 9.5 kg) cynomolgus macaques (*Macaca fascicularis*) were randomized into 2 groups of 6 experimental animals and 2 control animals each. At Bioqual, the experimental animals were vaccinated by intramuscular injection with 25μg EBOV GP, 25μg EBOV VP24, and 5μg VP40 with CoVaccine HT; 25μg EBOV GP with CoVaccine HT; or PBS. The macaques were transferred to UTMB and challenged 4 weeks after vaccination by intramuscular injection with 1,000 PFU of EBOV strain Kikwit. All the macaques were given physical examinations, and blood was collected before vaccination; at days 7, 14, 21, 35, 42, 49 and 56 throughout vaccination; at the time of challenge; and on days 3, 6, 10, 14, 22, and 28 after challenge. The macaques were monitored daily and scored for disease progression with an internal filovirus scoring protocol approved by the UTMB Institutional Animal Care and Use Committee (IACUC) in accordance with state and federal statutes and regulations relating to experiments involving animals and by the UTMB Institutional Biosafety Committee. The scoring changes measured from baseline included posture/activity level; attitude/behavior; food and water intake; weight; respiration; and disease manifestations, such as visible rash, hemorrhage, ecchymosis, or flushed skin, with increased scores resulting in euthanasia.

Analysis of antibody responses by ELISA

Individual guinea pig serum samples were titrated for IgG specific to the recombinant EBOV GP and VP40 by standard ELISA technique using plates coated with purified

recombinant antigen (using 75μl of 1μg/ml stocks for coating). The titers presented are defined as the dilution of antiserum yielding 50% maximum absorbance values (EC_{50}) and were determined using a sigmoidal dose response curve fitting algorithm (Prism, Graphpad Software, San Diego, CA).

Microsphere Immunoassasy

Microspheres coupled with EBOV GP, EBOV VP24, EBOV VP40, SUDV, GP, or MARV GP, the untransformed S2 supernatant, bovine serum albumin (BSA), and PBS were pooled in PBS-1% BSA (PBS-BSA) at a dilution of 1:200. Fifty microliters of the coupled microsphere immunoassay (MIA) suspension were added to each well of black-sided 96-well plates. Serum samples were diluted 1:1000 in PBS-BSA, and 50μl were added to the microspheres in duplicate and incubated for 30 min on a plate shaker set at 700 rpm in the dark at room temperature. The plates were then washed twice with 200μl of PBS-BSA using a magnetic plate separator (Millipore Corp., Billerica, MA). Fifty microliters of red phycoerythrin (R-PE)-conjugated *F*(ab')2 fragment goat anti-human IgG specific to the Fc fragment (Jackson ImmunoResearch, Inc., West Grove, PA) were added at 2μg/ml to the wells and incubated for another 45 min. The plates were washed twice, as described above, and microspheres were then resuspended in 100μl of sheath fluid and analyzed on a Luminex 100 apparatus (Luminex Corporation, Austin, TX). Data acquisition detecting the MFI was set to a minimum of 50 beads per spectral region. Antigen-coupled beads were recognized and quantified based on their spectral signature and signal intensity, respectively.

Flow Cytometry and ELISpot Assays

Analysis of peripheral blood mononuclear cells using ELISpot and flow cytometry: peripheral blood mononuclear cells (PBMC) were isolated from cynomolgus macaque whole blood samples by separation over Ficoll, frozen in complete RPMI (10% FBS) with 10% DMSO and stored in LN2 until analysis. To analyze, cells were quickly thawed in a 37C water bath and resuspended in pre-warmed RPMI 1640 media with 10% fetal bovine serum (FBS) (Fisher Scientific, Hampton, NH) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). PBMCs were stimulated with Lectin from *Phytolacca Americana,* pokeweed mitogen (PWM), (Sigma-Aldrich, St. Louis, MO) at a final concentration of 5μg/ml (flow cytometry) or 10μg/ml (ELISpot); EBOV whole GP at 10μg/ml (flow cytometry) or 20μg/ml (ELISpot) plus EBOV GP peptide pool at 5μg/ml (flow cytometry) or 10μg/ml (ELISpot); or left unstimulated. For analysis by flow cytometry, after two hours in the presence of stimulants at 37C, Brefeldin-A and Monensin (BD Biosciences, Franklin Lake, NJ) were added at the manufacturer's recommended concentration and cells were incubated at 37° C for a further 12 hours. Cells were stained with a mixture of antibodies against lineage markers; CD3 Alexa Fluor 700, CD4 PerCP-Cy5.5 (BD Biosciences), CD8 PE-Cy7 (BioLegend, San Diego, CA), and memory markers CCR7 BV711, CD27 BV510 (Biolegend) and CD45RA APC-H7 (BD Biosciences), then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) followed by intracellular staining with antibodies against cytokines intracellular markers IL-4 APC (eBioscience Inc, San Diego, CA), IL-10 PeDazzle 594 (BioLegend) TNF-α BV605, and IFN-γ BV421 (BioLegend). The viability dye FVS575V

(BD Biosciences) was included to allow discrimination between live and dead cells. Samples were acquired on an Attune NxT Flow Cytometer (ThermoFisher Scientific, Waltham, MA) and analyzed using FlowJo (BD, Ashton, OH). Cytokine-positive cells are expressed as a percentage within CD3+CD4+ T cell subsets. For analysis by ELISpot, primate IFN-γ and human IL-4 kits were used (R&D systems, Minneapolis, MN). Cells were plated at 250,000 cells per well in an NHP IFN-γ or human IL-4 capture antibody coated 96 well plate and incubated for 48 hours. IFN-γ and IL-4 secreting cells were detected as per the manufacturer's protocol.

Preparation of EBOV peptide pool

EBOV GP peptide pool (LifeTein, Somerset, NJ) was prepared from a peptide library composed of 121 15mer peptides with an 11 amino acid overlap spanning the length of protein. The peptides were individually suspended in dimethylsulfoxide (DMSO) at a concentration of 1mg/ml and equal amounts of each peptide were pooled. Aliquots of the peptide pools were stored at -80C until use. To stimulate cells, aliquots were thawed and a working stock was made by adding PBS to bring the pooled concentration to 100μg/ml. Peptide pools were used at a final concentration of 5μg/ml of the combined 121 peptide pool.

Statistical analysis

Determination of significant differences in EBOV GP-specific IgG titers between animal groups vaccinated with different vaccine formulations was done using an unpaired t-test

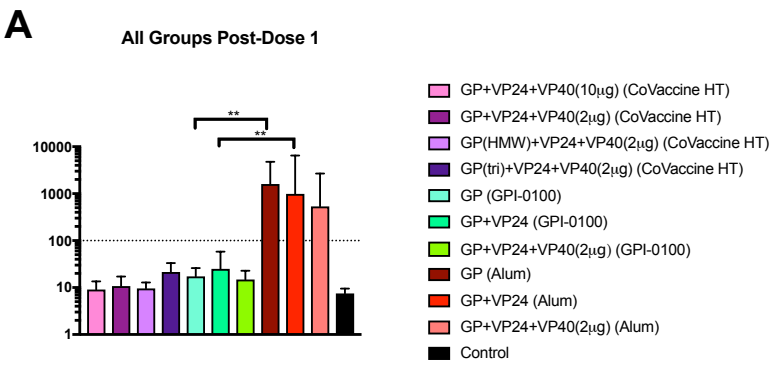
(Prism, Graphpad Software, San Diego, CA). Significant differences in survival between immunized (or non-immunized control) groups subsequently challenged were determined by the Fisher exact probability test (GraphPad Prism). Kaplan-Meier survival curves were compared using the log-rank (Mantel-Cox) test for significant differences (Graph Pad Prism). For all tests $p < 0.05$ was considered significant.

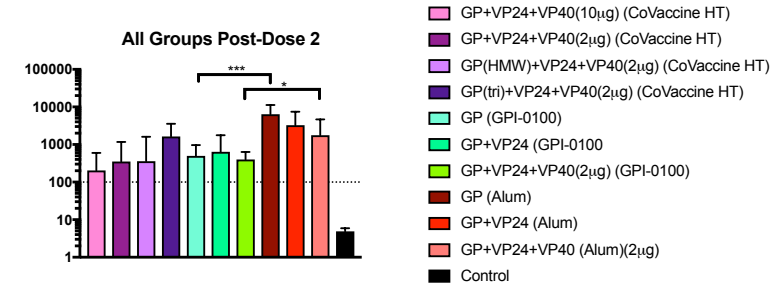
Results

Guinea Pigs

We have used multiple animal models to determine the immunogenicity and efficacy of our vaccine candidate, including guinea pigs and NHPs (cynomolgus macaques). Experimental groups of 7-8 Hartley guinea pigs received three immunizations in 3-week intervals. Formulations were made using three different adjuvants, Alum, GPI-0100 or CoVaccine HT with either EBOV Glycoprotein (GP) alone or in conjunction with VP24 and/or VP40. Formulations containing CoVaccine HT were designed to allow comparison of multi-antigen formulations and test a reduced amount of VP40 versus equal amounts of all three antigens. Vaccines were formulated that contained either the trimeric GP or high molecular weight GP fractions prepared by an additional size-exclusion chromatography step to further understand the importance of oligomerization state of GP in immunogenicity and efficacy. In the GPI-0100 and Alum containing groups, animals were given vaccines with either 10 μg of GP only, 10μg each of GP and VP24, or 10μg each of GP and VP24 with 2μg of VP40. An assessment of anti-GP IgG titers using ELISA (**figure 4**) showed that after the first dose, titers in all groups receiving the Alum adjuvanted vaccines were higher than in other adjuvant groups. In

groups that received GP only or GP with VP24 they were significantly higher than the corresponding GPI-0100 groups, showing the potent response induced by Alum compared to GPI-0100, particularly in the groups receiving fewer antigens. This difference remained consistent after dose 2, with titers in the Alum groups being higher than for other adjuvant groups. After the third dose, titers were similar for all adjuvants, although some differences between groups were still seen. Animals in the GPI-100 group that received all three antigens had significantly lower titers than the corresponding Alum and CoVaccine HT groups. Formulations containing GPI-0100 that used GP only or GP with VP24 also had significantly lower titers than corresponding formulations containing Alum. Control animals that received GPI-0100 alone did not develop titers above the cut-off at any point.





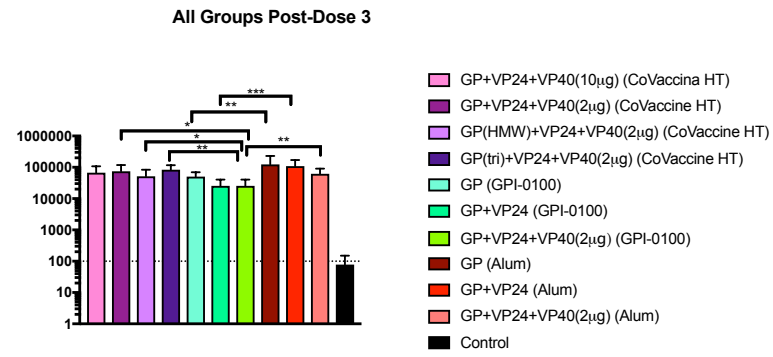


Figure 4: Geometric Mean titers of anti-EBOV GP determined using ELISA in Guinea Pig serum following the a) 1st b) 2nd and c) 3rd vaccine dose for each adjuvant and antigen formulation. Blood was taken 3 weeks following each vaccination. Statistical significance was determine using an unpaired t-test. (*p<0.05, **p<0.01, ***p<0.001)

Four weeks after the 3rd vaccine dose, animals in all groups were challenged with 1000*pfu* of guinea pig adapted (gpa) EBOV. Amongst the groups that received formulations adjuvanted with CoVaccine HT, all formulations that were antigen balanced (contained reduced amounts of VP40) protected all animals against viral challenge (**figure 5**). In the group that received the formulation containing equal amounts of all three antigens adjuvanted with CoVaccine HT the survival was 75% (6/8). Animals in groups that received formulations with GPI-0100 showed varying levels of protection, although no group was fully protected using this adjuvant. The highest level of protection was seen in animals that received GPI-0100 with GP alone where 50% (4/8) of the animals survived challenge. The lowest protection was seen in the group receiving all three antigens, with only 25% (2/8) of survival, while GP with VP24 showed 37.5% (3/8) protection against challenge. All animals that succumbed to infection did so between days 8-12 post challenge showing no significant increase in survival time over controls that succumbed between days 7-10. Alum was shown to be the most potent adjuvant for these vaccines in guinea pigs, as all the animals in groups receiving formulations with Alum survived challenge. This is consistent with the rapid and robust antibody responses observed.

To determine if the levels of anti-GP antibody titers were correlated to survival post challenge (**figure 6**), IgG titers between survivors and non-survivors were compared. Titers from groups that received Alum adjuvanted vaccines were excluded from this analysis due to the very high titers elicited by these formulations and due to an absence of non-survivors in these groups.

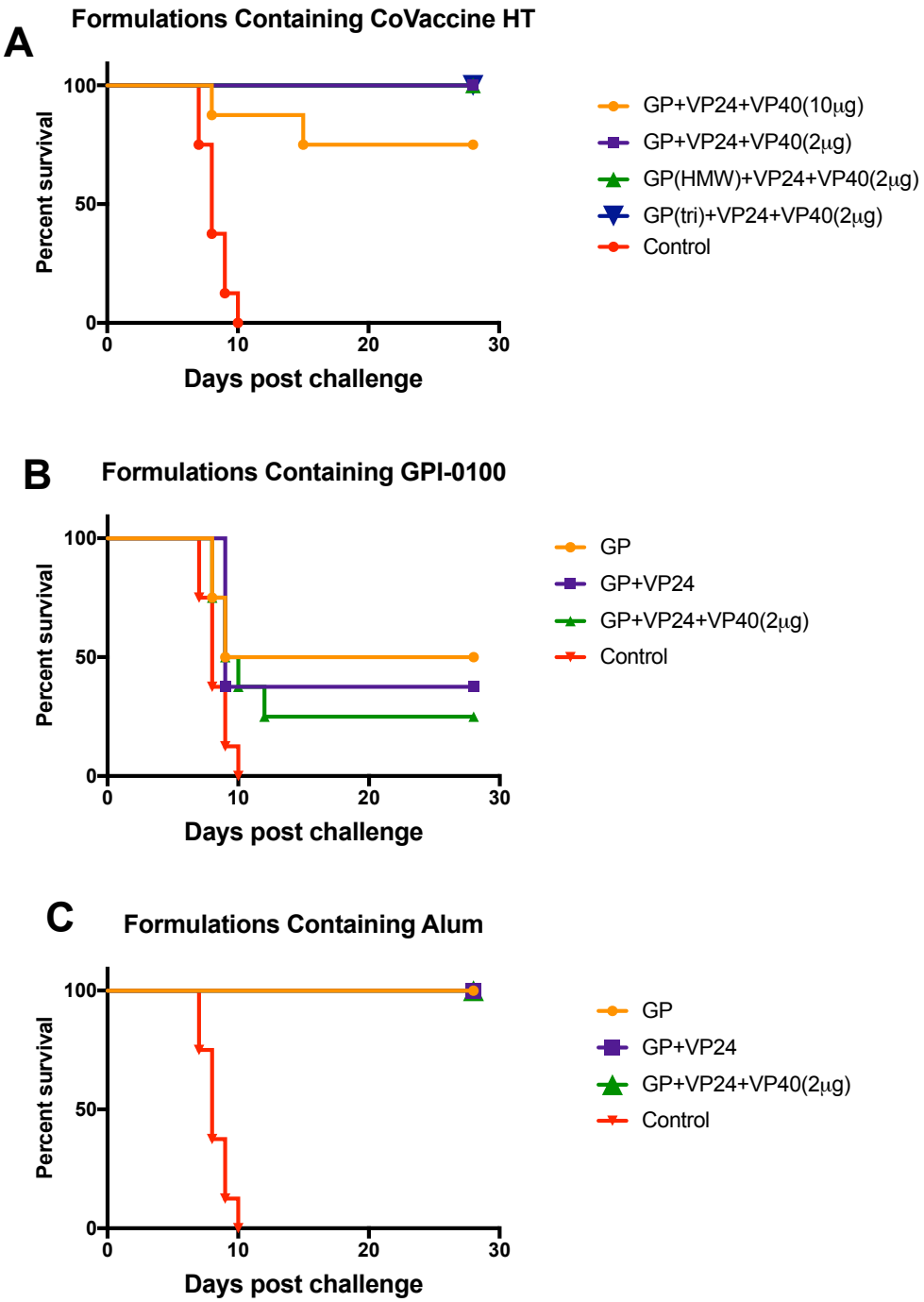


Figure 5: Survival curve of guinea pigs receiving EBOV vaccine formulations with a) CoVaccine HT, b) GPI-0100, and c) Alum following lethal EBOV challenge. Controls received GPI-0100 without antigen

Significant differences were found between the titers of survivors and non-survivors after dose two. These differences were even more marked post-challenge.

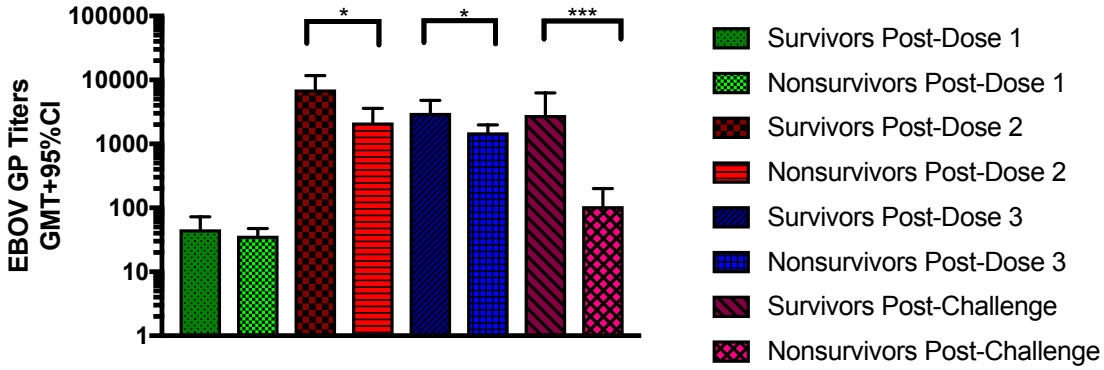


Figure 6: Geometric Mean titers of anti-GP determined using ELISA in guinea pig serum stratified by survival status by time point. Anti-GP titers from the animals that received formulations with Alum were not included due to their unusually strong antibody response. Blood was taken 3 weeks following each vaccination. Statistical significance was determined using an unpaired t-test. (*$p<0.05$, **$p<0.01$, ***$p<0.001$)

ELISA titers following the initial vaccine dose showed the highest anti-GP titers for all animals receiving formulations with Alum as an adjuvant. This was not unexpected considering Alum's ability to elicit strong humoral responses [88, 89]. Titers in the groups containing GPI-0100 and CoVaccine HT rose above the cut-off by the second dose and for the animals receiving formulations with CoVaccine HT were not significantly different from the Alum adjuvanted animals after the third dose. No significant differences were found between animals receiving formulations containing the high molecular weight, trimeric or IAC purified GP.

Protection within the different adjuvant groups was varied, with all animals in the CoVaccine HT adjuvant groups being protected against EBOV challenge with the

exception of animals that received equal amounts of all three antigens. Previous work has shown that VP40 is able to elicit extremely high, but not necessarily protective antibody responses in mice [10]. Other studies have found that VP40 plays a protective role, but that this role is based on its ability to elicit a cell mediated response [90, 91]. It is possible that in this vaccine platform the production of VP40 antibodies interferes with the development of a protective anti-GP response and highlights the importance of proper antigen formulation to produce a balanced response. Amongst the animals receiving vaccine formulations with GPI-0100, low levels of protection were seen with only 25-50% of the animals surviving challenge, despite previous promising results with this adjuvant in mice [10, 92]. The observed lower efficacy corresponds to the significantly lower levels of GP antibody titers that were seen in all these animals. In the animals receiving Alum adjuvanted formulations full protection was seen with all antigen combinations.

Non-human Primates

While EBOV infection in guinea pigs results in some of the same coagulation disorders seen in primates, it is not accompanied by visible hemorrhaging or bystander lymphocyte apoptosis, both of which are features of the disease in primates [93]. Guinea pigs must also be infected with gpa-EBOV rather than wild type virus for there to be any virulence. Due to these differences, while the success of our vaccine candidate in this model was promising, further testing needed to be done in an animal model that more closely mimicked human disease [93]. For this purpose cynomolgus and rhesus macaques are the optimal choice, as the clinical and histopathological features seen

closely resemble the human disease [93]. Currently our filovirus vaccine is being tested in cynomolgus macaques. Several studies have been conducted testing various formulations in an effort to optimize a monovalent EBOV vaccine as well as to develop a trivalent formulation that is efficacious against SUDV, EBOV and MARV. In the monovalent vaccine studies, the formulations tested have either included EBOV GP with VP24 and VP40, or GP alone with CoVaccine HT. One such study contained six animals per group which were given vaccine formulations containing either EBOV GP with CoVaccine HT or GP, VP24 and VP40 with CoVaccine HT. Three immunizations were given three weeks apart. Four weeks following the final vaccination, the NHPs were challenged with EBOV. Throughout the vaccination period, serum and peripheral blood mononuclear cells (PBMCs) were collected from the animals at day 0, post dose 1, post dose 2, and one week post dose 3. Anti-GP IgG antibody titers in the serum were determined using a bead based assay (Luminex). This assay showed the development of GP specific IgG titers following the initial dose, which continue to rise following the second and third dose and remained consistent until the time of challenge (**Figure 8**). This is consistent with the survival outcome of challenge with 5 out of the 6 animals in each vaccination group surviving challenge (**figure 7**).

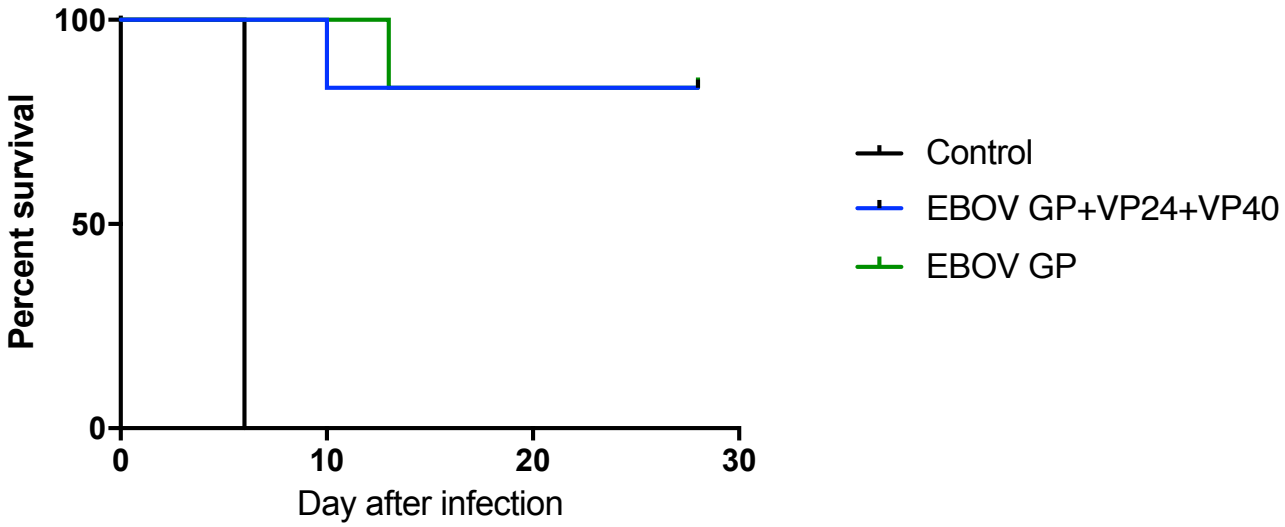


Figure 7: Survival curve of NHPs receiving EBOV vaccine formulations with EBOV GP+VP24+VP40 or EBOV GP with CoVaccine HT

In the animals that received all three antigens with CoVaccine HT the animal that did not survive challenge had a slower IgG response to vaccination, with lower titers than the other vaccinated animals until after the third vaccine dose, but this was also seen in animals that were protected from challenge (**Figure 8A**).

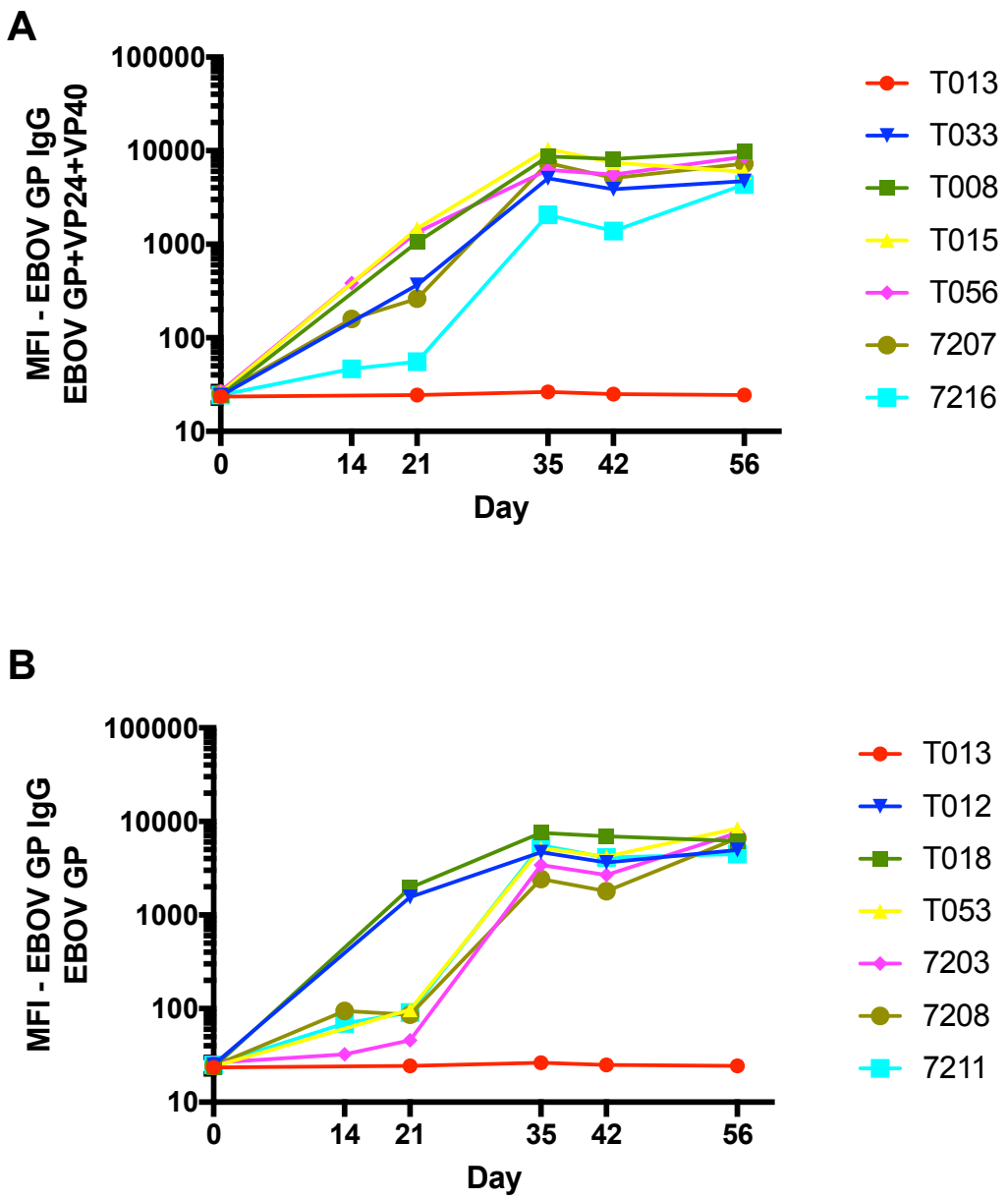


Figure 8: EBOV GP specific IgG MFI of serum from NHP's that received A) all three antigens and CoVaccine HT or B) GP only with CoVaccine HT. All animals in both groups developed an IgG response following the initial vaccine dose that continued to rise throughout the vaccination period

Flow cytometry and ELISpot assays have been performed on PBMC's from animals that have received all three antigens with CoVaccine HT with samples collected at day 0 and

post dose 3 to explore the cell mediated immune response in vaccinated animals (**Table 3)**. Flow cytometry assays have focused on exploring the presence of memory T-cell subsets within the PBMC population at various points throughout the vaccination schedule, as well as looking at the cytokine secretion profile of CD4+ and CD8+ T cells following stimulation with homologous antigen and peptide pools (15mers spanning the full length of GP. Understanding which cell type is secreting each cytokine can tell us if a protective response involves mainly CD4+ or CD8+ compartments. PBMCs are also being stimulated with homologous antigen and peptide pool for the ELISpot assays where the secretion of IFN-γ and IL-4 have been analyzed, to determine if the observed responses are primarily a Th1 or Th2 response and to establish a method that could be used for later clinical evaluation.

Flow cytometry analysis has shown that CD4+ T cells from vaccinated animals after the third vaccine dose secrete TNF-α after stimulation with a combination of GP antigen and peptide, while those from the control animal do not (**Figure 9**). While the secretion of TNF-α from cells is variable between animals, as a group, the GP stimulated CD4+ cells from these animals have significantly higher TNF-α secretion than what is seen in unstimulated cells (**Figure 10**, p=0.04). While there were multiple cytokines analyzed, including IFN-γ, IL-4, and IL-10, TNF-α was the only cytokine detected by flow cytometry as a response to GP stimulation. Many of the animals showed high levels of IL-4 production, but this was largely seen as background and not as a response to stimulation.

Table 3: Vaccine formulations, survival (to day 28), and time points analyzed

Animal ID	Vaccine Formulation	Survived	Time Points
T013	Control	No	D0, PD3
T033	GP+VP24+VP40 w/adjuvant	Yes	D0, PD3
T008	GP+VP24+VP40 w/adjuvant	Yes	D0, PD3
T015	GP+VP24+VP40 w/adjuvant	Yes	D0, PD3
T056	GP+VP24+VP40 w/adjuvant	Yes	D0, PD3
7207	GP+VP24+VP40 w/adjuvant	Yes	D0, PD3
7216	GP+VP24+VP40 w/adjuvant	No	D0, PD3

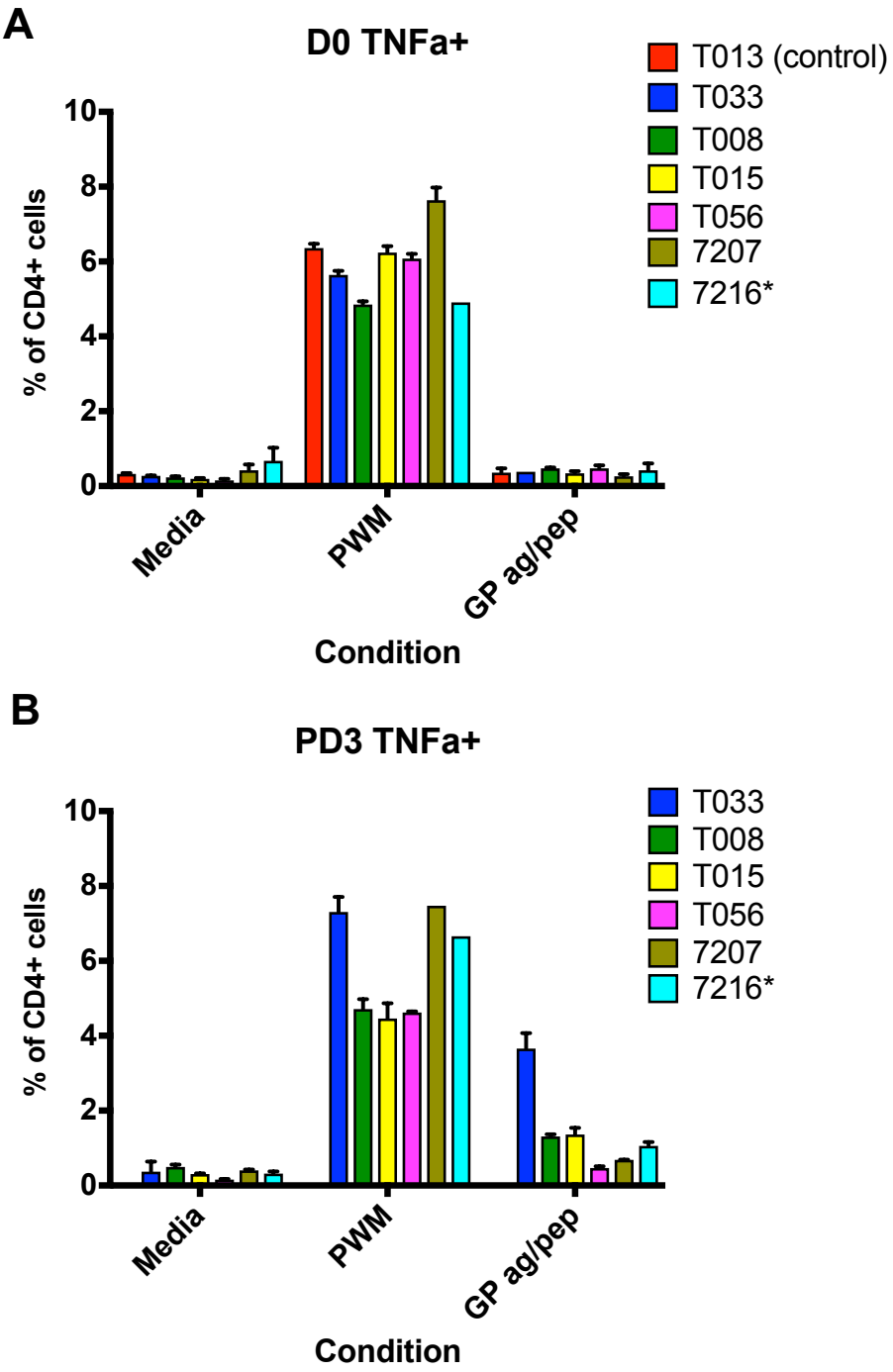


Figure 9: Individual TNF-α production of CD3+CD4+ cells after stimulation with a combination of GP antigen and peptide pool. A) No TNF-α production is seen after GP stimulation in any of the animals prior to immunization. However, after 3 doses B) most of the immunized animals show more TNF-α production compared to unstimulated cells or cells from the same animals prior to vaccination (* denotes vaccine recipient that succumbed to challenge)

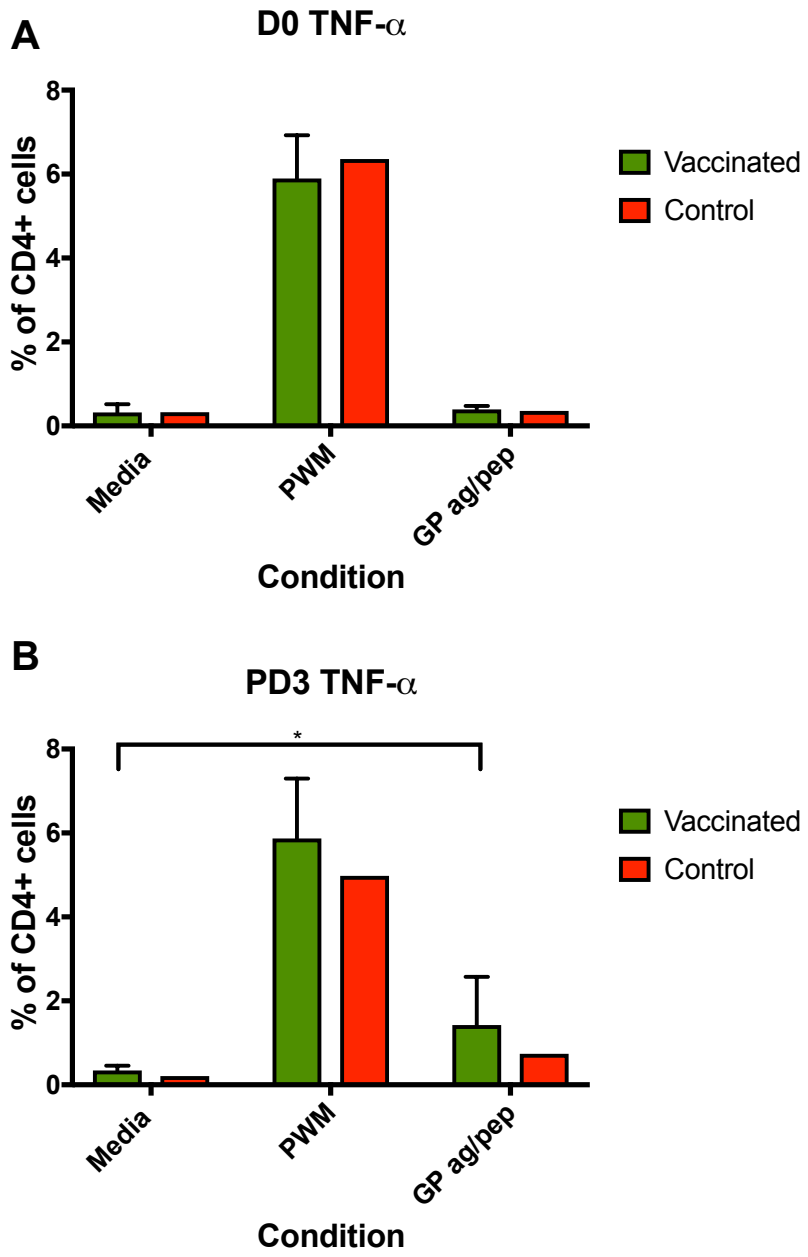


Figure 10: Grouped TNF-α production of CD3+CD4+ cells after stimulation with a combination of GP antigen and peptide pool. A) No TNF-α production is seen after GP stimulation in any of the animals prior to immunization. However, after 3 doses B) immunized animals show significantly more TNF-α production compared to unvaccinated animals

ELISpot data also shows a cell mediated immune response to vaccination, with PBMCs

secreting both IFN-γ and IL-4 after stimulation with whole antigen and peptide pools. IFN-γ secretion occurs only after antigenic stimulation, while there is a notable amount of background IL-4 production. Due to the whole PBMC population being used for the ELISpot assay, it is unclear which cell types are the ones secreting these cytokines. IFN-γ however has not yet been detected from either CD4+ or CD8+ cells using flow cytometry. One explanation could be that secretion of this cytokine originates from cells other than T cells (**Figure 11).** Timing may also play a role in the detecting of IFN-γ in the ELISpot assay but not using flow cytometry. In the ELISpot assay cells are incubated in the presence of stimulants for 48 hours and the assay is reflective of total IFN-γ throughout that entire time period. In the flow cytometry assays the cells are incubated with stimulants for 12 hours which may reduce the amount of certain cytokines seen using this assay. Taken together these assays show that the vaccine candidate elicits a GP-specific cell mediated response, primarily from CD4+ T cells, with no cytokine production seen from the CD8+ T cell population following stimulation.

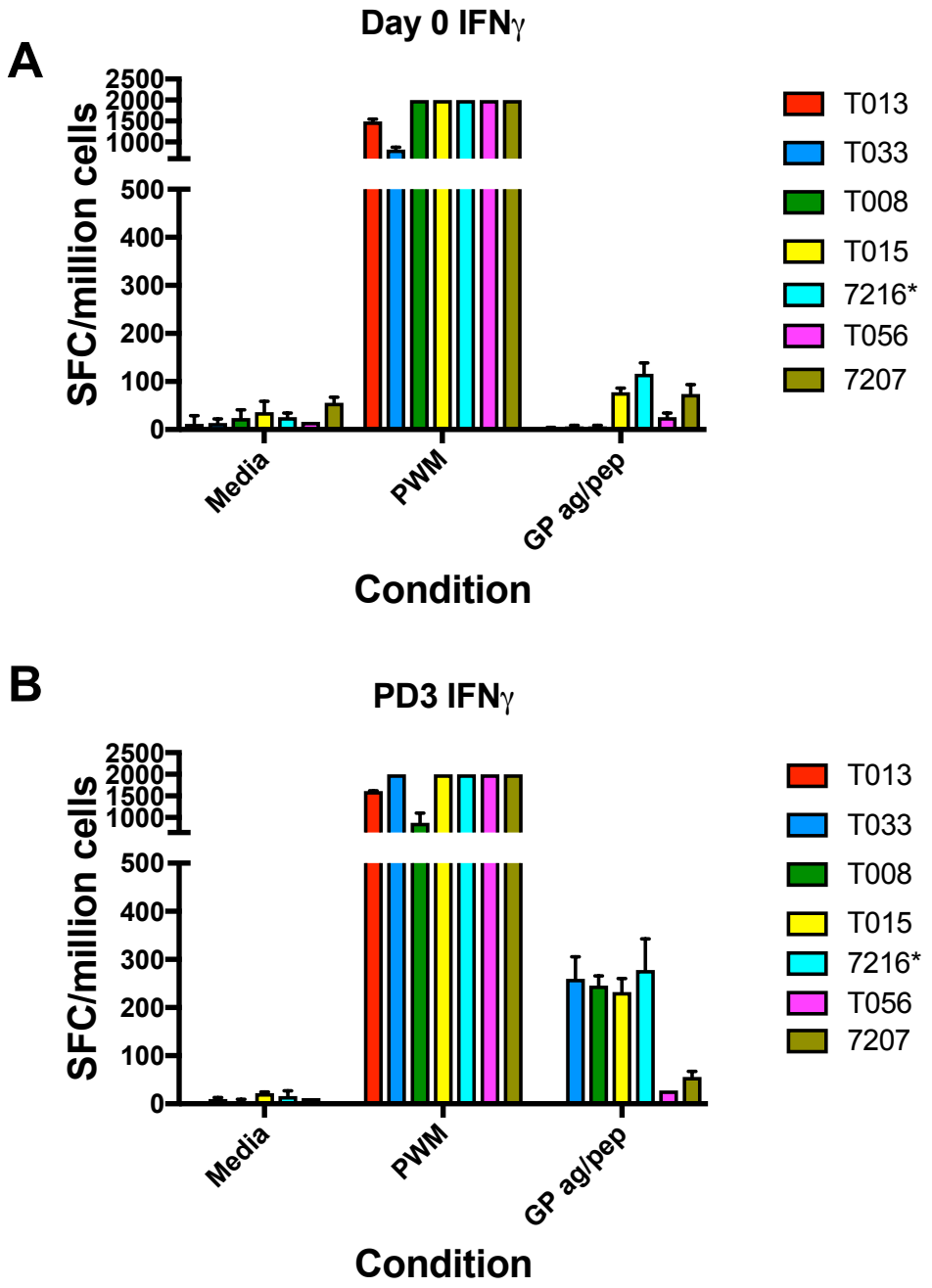


Figure 11: ELISpot data showing IFN-γ in animals vaccinated with EBOV GP, VP24 and VP40 with CoVaccine HT versus the control animal on day 0 and Day 49 (1 week post dose 3). Neither the vaccinated nor the control produce IFN-γ in response to stimulation at day 0 (A). Following the 3rd vaccination dose nearly all vaccinated animals respond to antigen+peptide stimulation by producing IFN-γ (B) (* denotes vaccine recipient that succumbed to challenge)

Discussion

Three different clinically relevant adjuvants were selected for use in guinea pigs to determine which offered the best immunogenicity and protection. All three selected adjuvants have previously been shown to be safe, immunogenic and efficacious in subunit vaccines [16, 92, 94]. ELISA titers following the initial dose showed the highest anti-GP titers for all guinea pigs receiving formulations with Alum as an adjuvant, which was not unexpected considering Alum's ability to elicit strong humoral responses [88, 89]. Titers in the groups containing GPI-0100 and CoVaccine HT rose above the cut-off by the second dose and for the animals receiving formulations with CoVaccine HT were not significantly different from the Alum adjuvanted animals after the third dose. The lack of differences in the antibody response seen between the animals that received the high molecular weight, trimeric or IAC GP suggests that the quality of the immune response elicited by these is not affected by which oligomeric species is used for vaccination.

Protection of guinea pigs within the different adjuvant groups was varied, with all animals in the CoVaccine HT adjuvant groups being protected against viral challenge with the exception of animals that received equal amounts of all three antigens. Previous work has shown that VP40 is able to elicit extremely high, but not necessarily protective antibody responses in mice [10]. Other studies have found that VP40 plays a protective role, but that this role is based on its ability to elicit a cell mediated response [90, 91]. It is possible that in this vaccine platform the production of VP40 antibodies interferes with the development of a protective anti-GP response and highlights the

importance of proper antigen formulation to produce a balanced response. Amongst the animals receiving vaccine formulations with GPI-0100, low levels of protection were seen with only 25-50% of the animals surviving challenge, despite previous promising results with this adjuvant in mice [10, 92]. The observed lower efficacy corresponds to the significantly lower levels of GP antibody titers that were seen in all of these animals. Alum is shown to induce rapid and robust antibody responses in guinea pigs with all vaccine formulations, corresponding to the full protection seen with all antigen combinations.

Previous efforts to develop subunit-based filovirus vaccines have been met with mixed results. Previous research using GP fused to the Fc portion of human IgG1 showed that properly adjuvanted GP-Fc was fully protective in guinea pigs [88]. In contrast to our own findings, this manuscript reports that Alum as an adjuvant did not fully protect when paired with their GP construct. Baculovirus expressed GP protein, also tested in guinea pigs, has been shown to induce both a humoral and cell mediated immune response but was also not able to fully protect against challenge [95]. However, an adjuvant was not included in the vaccine formulation used in this study. Our studies in guinea pigs and NHPs as well as studies done by others have shown high levels of antibody production in response to vaccination in small animal as well as NHP models [10, 88, 95, 96], which have not consistently correlated to protection. Analysis of EBOV GP specific antibody titers in guinea pigs demonstrated a correlation of anti-GP antibody levels with protection against challenge, as animals surviving viral challenge showed significantly higher IgG titers than those that did not (post-dose 2 through post-challenge time

points). This same correlation, however, was not seen in NHPs. This points to the important role that humoral immunity plays in protection against EBOV infection, but does not diminish the potential importance of cell mediated immunity, especially the cell mediated responses elicited by VP24 and VP40, which have previously been documented in other animal species by our group as well as others [10, 90, 91].

Protection against infection using EBOV vaccination has been shown using a variety of different vaccine platforms in small animals and NHPs, however, definitive correlates of protection have been elusive. Early experiments in nonhuman primates using an EBOV GP DNA vaccine with an adenovirus boost, showed that after depletion of CD4+ T cells, PBMC's collected from vaccinated NHPs had reduced antigen-induced lymphocyte proliferation, while CD8+ T cell depletion had no effect [97]. A follow up paper using the same strategy resulted in conflicting results, with CD8+ T cell TNF-α production being correlated with protection [98]. Analysis of immune responses to the rVSV-ZEBOV vaccine in humans in the plasma of vaccine recipients shows that cytokine and chemokine secretion from monocytes plays a large role in clinical outcome after vaccination in terms of adverse events and immunogenicity [36]. In particular TNF-α was associated with the development of myalgia in vaccine recipients, while IL-10 expression was associated with the reverse [36]. Another cytokine of interest is IFN-γ, with its production, in conjunction with other cytokines being associated with viral vaccine immune responses [99, 100]. Detection of IFN-γ from PBMCs taken from our vaccinated NHPs using ELISpot assays but not flow cytometry suggest that this cytokine is not being produced by either CD4+ or CD8+ T cells, but rather implicates a

different cell type.

In the non-replicating ChAd3/MVA prime boost vaccine strategy, an association was shown between polyfunctional CD8+ T cells expressing TNF-α, IFN-γ and IL-2, GP specific CD4+ T cells, and protection in NHPs [40]. In a durability study, analysis of the cell mediated immune response showed that effector CD8+ T cells co-producing TNF-α and IFN-γ as well as polyfunctional CD8+ T cells producing those two cytokines along with IL-2 were associated with full protection in NHPs [40]. It is also clear that with the adenovirus vectored vaccine the humoral response is not nearly as important as the CD8+ T cell response, as demonstrated by passive transfer and depletion experiments [42]. This is in contrast to the rVSV vaccine where it was demonstrated in CD4+ T cell depleted cynomolgus macaques that a robust antibody response was necessary for protection using this vaccine [101]. Our vaccine candidate has not been shown to elicit any CD8+ T cell responses, which is not surprising given the difficulty in generating a strong CD8+ T cell response with a recombinant subunit vaccine platform. Given that our vaccine candidate is fully protective despite the absence of a detectable CD8+ T cell response, it is likely that in our vaccine platform cytotoxic T cell responses are not responsible for protection, but does not preclude the importance of other cell types. In particular, antigen specific CD4+ T cells are seen in vaccinated animals and respond to antigen stimulation by secreting TNF-α, although this response has not thus far been shown to be correlated with protection. This could be due to the low number of unprotected animals that have been analyzed.

Many studies have shown that antibody response is a strong predictor of protection in

multiple vaccine platforms [41, 96]. Our vaccine is capable of inducing a robust EBOV GP IgG response in both guinea pigs and NHPs, however, high titers of binding EBOV GP IgG are seen even in NHPs that succumb to infection, demonstrating that this alone cannot be used as a correlate of protection. It is possible that antibody function and not just quantity will help define a protective response for this vaccine platform. We have also determined that our vaccine candidate is capable of eliciting a measurable cell mediated immune response in NHP PBMCs as shown by the production of TNF-α by CD4+ T cells and the IFN-γ seen in the ELISpot assays. No response has yet been identified from CD8+ T cells using our vaccine candidate. Unfortunately, we have been unable to correlate the cell-mediated immune responses documented in our assays with protection. Based on the limited data collected from our experiments as well as on many other studies published on various vaccine platforms, it is likely that correlates and mechanisms of protection for EBOV vaccines are complex, and that further analysis using more vaccinated animals is necessary to determine what is protective following vaccination with a recombinant subunit EBOV vaccine [102].

Chapter 4: Exploring the Immune Response to a Multivalent Filovirus Vaccine and Analysis of Filovirus Cross-Reactivity

Methods

Ethics and Biosafety

All work with animals was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. For institution details please refer to the materials and methods section in Chapter 3.

Challenge viruses

Guinea pig-adapted (gpa-) EBOV [84] was propagated and titered on Vero E6 cells and used for the challenge part of this study. Please see Chapter 3, materials and methods for more details.

Guinea pig vaccination and challenge: Multivalent formulations

For the multivalent vaccine efficacy studies, Hartley guinea pigs were immunized using three different adjuvants with different modes of action; 1, an emulsion-based adjuvant, CoVaccine HT^{TM} (an emulsion of squalane with immunostimulatory sucrose fatty acid

sulphate esters and an adjuvant of BTG International Ltd, London, United Kingdom) [85] was used at a dose of 1 mg; 2, a saponin-based, immunomodulatory adjuvant, GPI-0100 (Hawaii Biotech, Inc., Honolulu, HI) [86, 87] was used at a dose of 100μg; 3, Alhydrogel® 85 ("Alum"; Brenntag, Reading, PA) was used at 1mg per dose. Groups of 8 or 16 male and female Hartley guinea pigs (>5 weeks old) were obtained from Charles River Laboratories, acclimated for 14 days and vaccinated intra-muscularly (i.m.) three times in the hind legs with individual subunit proteins at the indicated dose and formulated with one of the selected adjuvants at 3-week intervals. Please see Chapter 3, materials and methods for details.

Nonhuman primate vaccination and challenge

In one study 10 healthy, filovirus-naive, adult (~3 to 9.5 kg) cynomolgus macaques (*Macaca fascicularis*) were randomized into 2 groups of 4 experimental animals and 2 control animals each. At Bioqual, the experimental animals were vaccinated by intramuscular injection with 25μg EBOV GP, 25μg SUDV GP, and 25μg MARV GP with CoVaccine HT; 25μg EBOV GP with CoVaccine HT; or CoVaccine HT alone. Two vaccine doses were given, three weeks apart. In the second study 16 healthy, filovirus-naive, adult (~3 to 9.5 kg) cynomolgus macaques (*Macaca fascicularis*) were randomized into 2 groups of 6 experimental animals and 2 control animals each. At Bioqual, the experimental animals were vaccinated by intramuscular injection with 25μg EBOV GP with CoVaccine HT; or CoVaccine HT alone. Three doses were given three weeks apart. Six of the macaques were transferred to UTMB and challenged 4 weeks after vaccination by intramuscular injection with 1,000 PFU of EBOV strain Kikwit. The

remaining six of the macaques were held at Bioqual for 13 months before being transferred to UTMB and challenged by intramuscular injection with 1,000 PFU of EBOV strain Kikwit on week 62. All the macaques were given physical examinations, and blood was collected before vaccination; at days 7, 14, 21, 35, 42, 49 and 56 throughout vaccination; at the time of challenge; and on days 3, 6, 10, 14, 22, and 28 after challenge. The macaques were monitored daily and scored for disease progression with an internal filovirus scoring protocol approved by the UTMB Institutional Animal Care and Use Committee (IACUC) in accordance with state and federal statutes and regulations relating to experiments involving animals and by the UTMB Institutional Biosafety Committee. The scoring changes measured from baseline included posture/activity level; attitude/behavior; food and water intake; weight; respiration; and disease manifestations, such as visible rash, hemorrhage, ecchymosis, or flushed skin, with increased scores resulting in euthanasia.

Microsphere Immunoassay

Microspheres coupled with EBOV GP, EBOV VP24, EBOV VP40, SUDV, GP, or MARV GP, the untransformed S2 supernatant, bovine serum albumin (BSA), and PBS were pooled in PBS-1% BSA (PBS-BSA) at a dilution of 1:200. Fifty microliters of the coupled microsphere immunoassay (MIA) suspension was added to each well of black-sided 96-well plates. Serum samples were diluted 1:1000 in PBS-BSA, and 50μl was added to the microspheres in duplicate and incubated for 30 min on a plate shaker set at 700 rpm in the dark at room temperature. The plates were then washed twice with 200μl of PBS-BSA using a magnetic plate separator (Millipore Corp., Billerica, MA). Fifty microliters of

red phycoerythrin (R-PE)-conjugated *F*(ab=)2 fragment goat anti-human IgG specific to the Fc fragment (Jackson ImmunoResearch, Inc., West Grove, PA) was added at 2 μg/ml to the wells and incubated another 45 min. The plates were washed twice, as described above, and microspheres were then resuspended in 100μl of sheath fluid and analyzed on a Luminex 200 apparatus (Luminex Corporation, Austin, TX). Data acquisition detecting the MFI was set to 50 beads per spectral region. Antigen-coupled beads were recognized and quantified based on their spectral signature and signal intensity, respectively.

Peptide Pool Preparation

EBOV GP peptide pool (LifeTein, Somerest, NJ) was prepared as specified in the Chapter 3 materials and methods section.

Flow Cytometry and ELISpot Assays

Cell stimulations, flow cytometry assays, and ELISpot assays were performed as described in the materials and methods section of Chapter 3.

Results

Guinea Pigs

Multivalent formulations have been tested in multiple animal models, using EBOV, SUDV and MARV GP as antigens in conjunction with CoVaccine HT as an adjuvant. In guinea pigs, bivalent formulations using either MARV or SUDV GP in combination with

EBOV GP have been tested, while in cynomolgus macaques, trivalent formulations containing GP from EBOV, SUDV, and MARV have been evaluated alongside a monovalent EBOV formula. The development of a successful multivalent vaccine is dependent on a thorough understanding of the cross-reactivity resulting from a monovalent formulation. This allows for proper balancing and avoids immunodominance or immune interference of a single component. Evidence of immunodominance/immune interference has been seen in guinea pigs that received formulations containing either SUDV or MARV GP along with the EBOV antigens (**figure 12).**

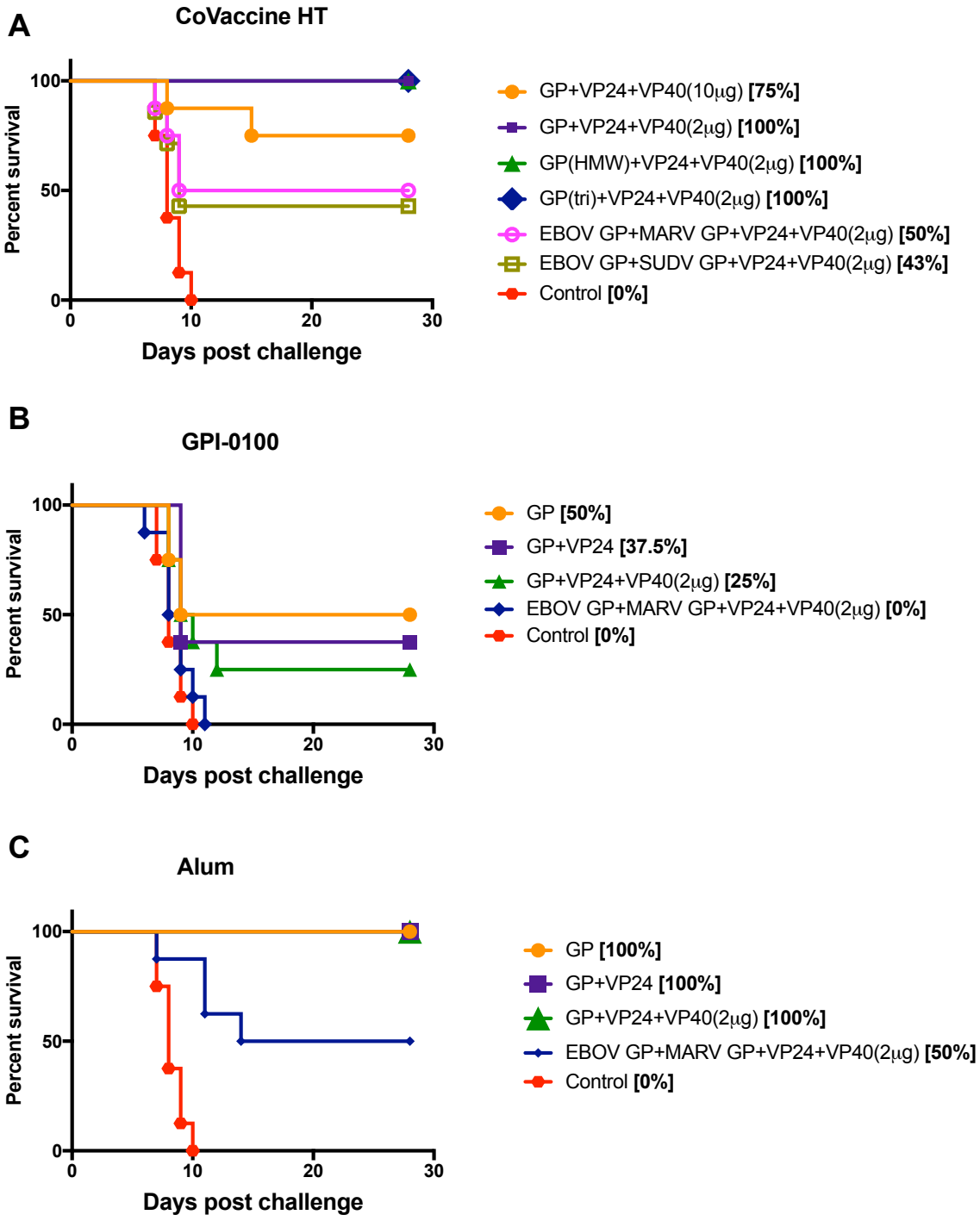


Figure 12: Kaplan-Meier survival curves of animals receiving monovalent and bivalent vaccine formulations with CoVaccine HT (A), GPI-0100 (B), and Alum (C) following lethal challenge with gpa-EBOV. Control animals received GPI-0100 without antigen. The same control group is shown in panels A-C

Three groups of eight animals received three doses of formulations that contained either SUDV or MARV GP alongside EBOV GP in the different adjuvant groups, with vaccinations for all formulations being given three weeks apart. Four weeks after the 3rd vaccine dose, animals in all groups were challenged with 1000*pfu* of guinea pig adapted EBOV. In all groups where GPs from two different filoviruses were used, survival was reduced when compared to groups that received formulations containing only EBOV GP. In the groups that received CoVaccine HT adjuvanted formulations, animals that received SUDV GP, or MARV GP, with EBOV GP, VP24, and 2ug VP40 survival was 43% and 50% respectively, compared to the 100% survival seen in the group that received only EBOV antigens. In the GPI-0100 groups, animals that received MARV, and EBOV GP with EBOV VP24, and 2ug VP40 had no survivors following challenge, while the group that received only EBOV antigens had 25% survival. Even in the Alum groups, where all monovalent vaccine formulations were completely protective due to the strong immune response induced by Alum, there was reduced efficacy when additional filovirus components were added. In this adjuvant group the addition of MARV GP reduced survival to 50%.

Non-human Primates

Despite the limited progress achieved in the guinea pig experiments paired with the limited predictive potential of guinea-pig efficacy testing, the development of a uniformly protective EBOV vaccine meant the next step was to test a multivalent filovirus vaccine

in NHPs. Part of this development is understanding the cross-reactivity of the monovalent EBOV vaccine. To do this, three NHP studies were used to assess humoral and cell-mediated cross-reactive immunity, including animals from a study using a trivalent (EBOV, SUDV, and MARV GP) formulation that could be compared to animals that received a monovalent formulation. The first study was used to determine cell mediated cross reactivity to other filovirus GPs in animals that received three doses every three weeks of EBOV GP alone with CoVaccine HT. These animals were challenged with EBOV four weeks after the final vaccination. The second NHP study included a total of twelve vaccinated animals, all of which also received three doses of EBOV GP alone with CoVaccine HT, three weeks apart. Six of the animals were challenged four weeks after the final dose, alongside two control animals, while the remaining six animals along with two control animals were challenged thirteen months after the final dose to determine durability of protection. The third study used two groups of four animals each that received two doses of either EBOV GP alone with CoVaccine HT or MARV, SUDV and EBOV GP with CoVaccine HT, with two control animals. The time between the final vaccine dose and the time to EBOV challenge in this study was seven instead of four weeks, which may have affected the efficacy level for these vaccine formulations.

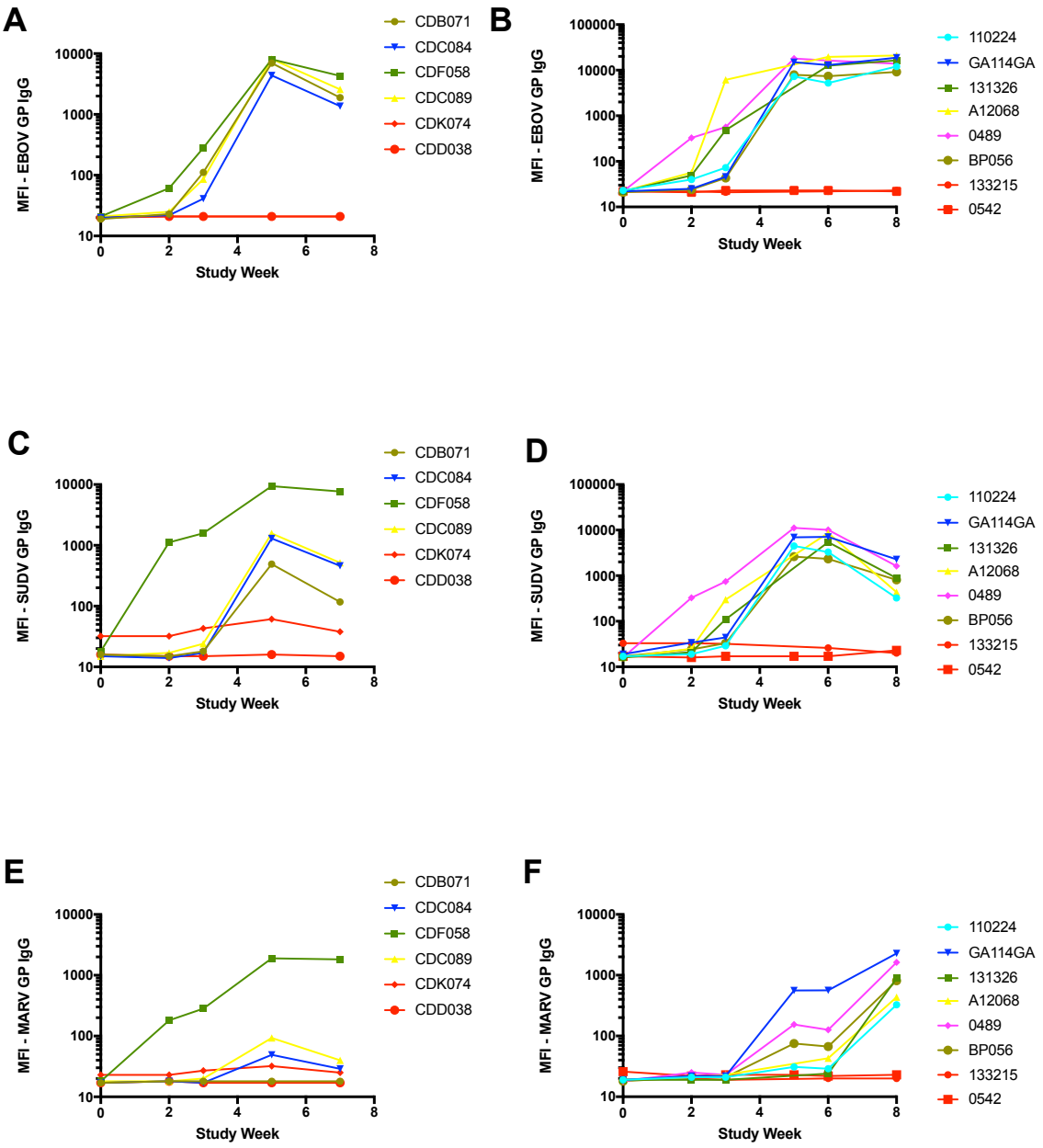


Figure 13: EBOV specific IgG MFI of serum from animals that received A) 2 doses of EBOV GP with CoVaccine HT or B) 3 doses of EBOV GP with CoVaccine HT; SUDV GP specific IgG MFI of serum from animals that received C) 2 doses of EBOV GP with CoVaccine HT or D) 3 doses of EBOV GP with CoVaccine HT; and MARV GP specific IgG MFI of serum from animals that received E) 2 doses of EBOV GP with CoVaccine HT or F) 3 doses of EBOV GP with CoVaccine HT. All animals in both groups developed an IgG response following the initial vaccine dose that continued to rise throughout the vaccination period, but animals receiving the 2 dose regimen saw a drop in titers by week 8. Cross-reactive titers were seen against SUDV GP and followed the

same pattern as EBOV GP specific titers, while much less cross-reactivity was seen against MARV GP

For all studies, serum and PBMCs were collected throughout the vaccination period and were used to analyze the cell mediated and humoral response to vaccination using bead based Luminex assays to detect the anti-GP IgG response, and flow cytometry and ELISpot to detect the filovirus GP specific cell mediated response. Luminex assays performed on sera collected from animals that received either two or three doses of EBOV GP only with CoVaccine HT (**figure 13**) showed that these animals developed high anti-EBOV GP IgG titers, as expected. In both dosing regimens, by week 5, which is after the 2nd dose, all animals had developed similar anti-EBOV GP IgG titers (**A, B**), however, in the animals that only received two doses these titers began to wane by week 6. In animals that received 3 doses the titers are stabilized and remain high to week 8. Cross-reactive anti-SUDV GP titers (**C, D**) are seen in both dosing regimens and follow the same trend as the EBOV GP titers, with a drop being seen in animals that only received two doses. Far less cross-reactivity is seen to MARV in animals receiving only two doses in most animals (**E**), only one animal showed high titers of cross-reactive anti-MARV GP IgG. However, in animals that received three doses, cross-reactive MARV titers increase after the 3rd dose in all animals (**F**). The antibody titers against all three filovirus GPs (EBOV, SUDV, and MARV) were also analyzed in animals that received trivalent formulations. All four animals developed very similar titers against each of the three filovirus GPs. Because these animals only received two doses of the trivalent vaccine, titers showed a similar trend of beginning to decline two to three weeks after the 2nd dose (figure 14).

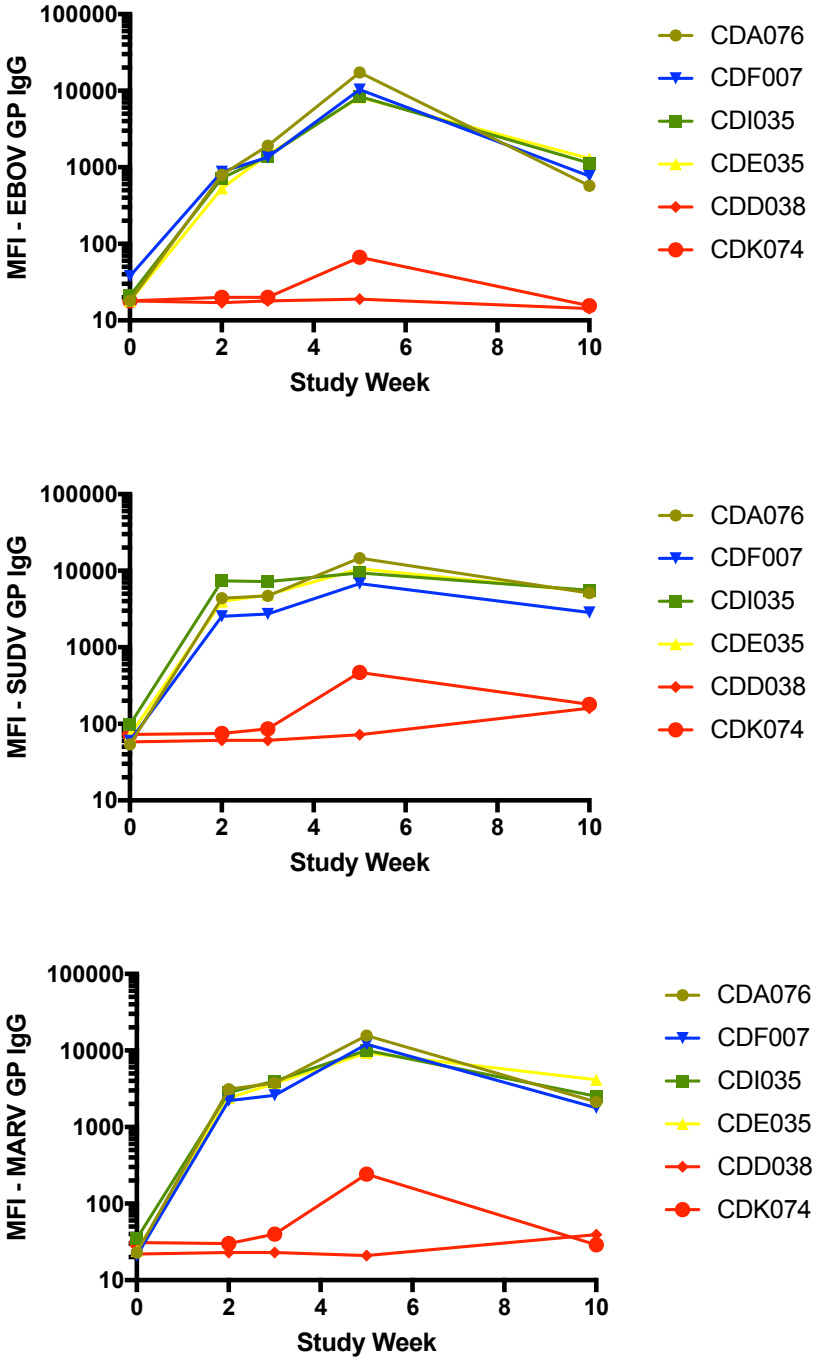


Figure 14: Filovirus specific IgG MFI of serum from NHPs that received 2 doses of EBOV GP, SUDV GP and MARV GP with CoVaccine HT. Titers were determined for A) EBOV GP specific IgG, B) SUDV GP specific IgG, and C) MARV GP specific IgG titers

In animals that received two doses of either the monovalent or trivalent vaccine formulations, a difference in efficacy was seen, with animals that received the monovalent formulation having 75% survival following challenge, while none of the trivalent recipients survived, although an extension to time of death was seen in the nonsurvivors in both vaccination groups, compared to control animals (**figure 15**).

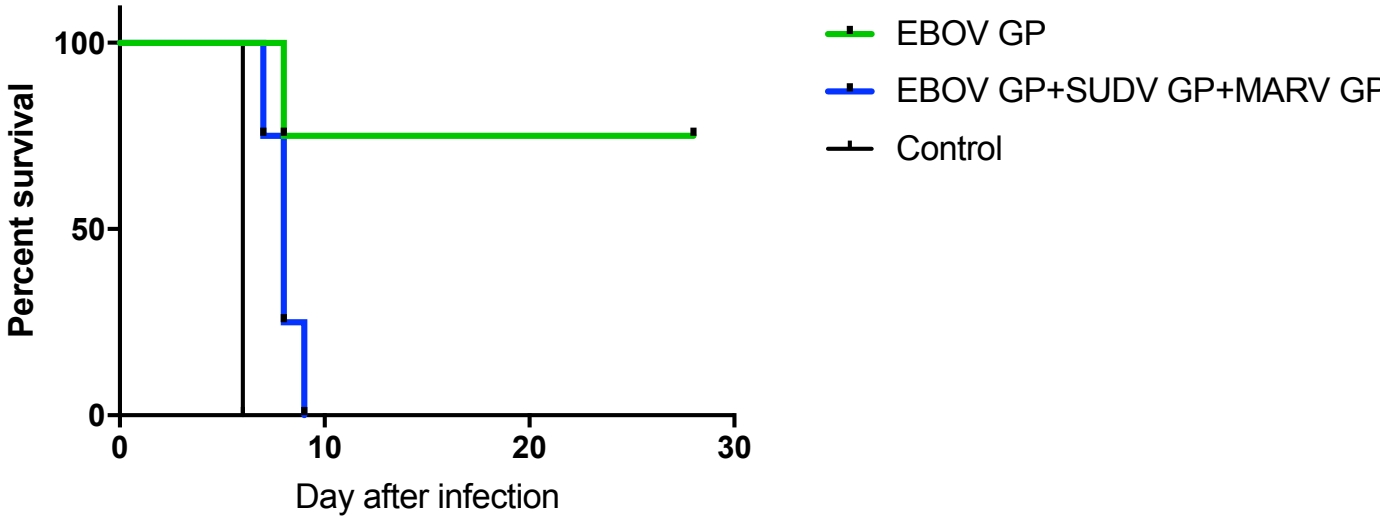


Figure 15: Kaplan-Meier survival curves of NHPs receiving monovalent and trivalent vaccine formulations following challenge with EBOV

Despite the high IgG titers against all filovirus GPs seen in the animals that received two doses of the trivalent formulation, none of the animals in this group survived challenge, and it is possible that the additional GP components and the immune response to these components interfered with a protective response. Flow cytometry and ELISpot assays have been performed on PBMCs collected from animals that have received either GP alone with CoVaccine HT in two or three doses, as well as on PBMC's from animals that received two doses of the trivalent formulations. The time points analyzed for these

animals have been the day 0 and post dose 3 time points, to explore the cell mediated immune response in vaccinated animals (**Table 4)**.

Table 4: Vaccine formulations, Dosing, survival (to day 28), and time points analyzed

Animal ID	Vaccine Formulation	# of Doses	Survived	Time Points
T013	Control	NA	No	D0, PD2
CDK074	Control	NA	No	D0, PD2
T018	EBOV GP w/adjuvant	3	Yes	D0, PD3
T053	EBOV GP w/adjuvant	3	Yes	D0, PD3
CDC084	EBOV GP w/adjuvant	2	Yes	D0, PD2
CDA076	EBOV GP+SUDV GP+MARV GP w/adjuvant	2	No	D0, PD2

Flow cytometry assays have focused on exploring the cross-reactivity of the PBMC population at various points throughout the vaccination schedule, by looking at the cytokine secretion profile of CD4+ and CD8+ T-cells following stimulation with homologous antigen and peptide pool (15mers spanning the full length of either GP) as well as stimulation with heterologous antigens such as SUDV and MARV GP. PBMCs were also stimulated with homologous antigen and peptide pool and heterologous antigens for the ELISpot assays where the secretion of IFN-γ and IL-4 have been

analyzed. This allows us to determine if homologous or heterologous antigen stimulation of PBMC's from animals that have received the monovalent EBOV vaccine are the same or similar to responses seen in cells from animals vaccinated with closely related filoviruses. In animals receiving the trivalent formulation this will help us determine if responses to each of the antigenic components of the vaccine are equal or if certain antigens elicit weaker or stronger responses.

Flow cytometry analysis of PBMC's from animals that received two doses of either the monovalent or trivalent formulation did not yield evidence of cytokine production, however, ELISpot analysis did show cellular responses in vaccinated animals (**figure 16, figure 17**). At day 0, none of the animals showed any IFN-γ secretion in response to stimulation with EBOV GP antigen/peptide stimulation, but cells from the control animal secreted IFN-γ in response to stimulation with MARV GP, and to a lesser extent SUDV GP. One vaccinated animal also showed a small number of cells secreting IFN-γ in response to MARV GP at D0. None of the cells were seen to secrete this cytokine in the absence of stimulant showing a potential innate immune stimulatory effect of these GP preparations. Post dose 2, cells from both vaccinated animals secreted IFN-γ when stimulated with EBOV GP antigen/peptide, while all three animals secreted it in response to SUDV and MARV GP antigen stimulation. As seen at day 0, the response to MARV GP antigen is more potent than the response to either EBOV or SUDV GP antigen. The responses seen to MARV and SUDV GP stimulation are interesting, as only one of the animals (CDA076) received a formulation containing these antigens. While the response to these antigens is strongest in that animal, the responses seen at

day 0 and from animals receiving either no vaccine, or a monovalent vaccine suggest that this antigen can act as a nonspecific inducer of IFN-γ secretion.

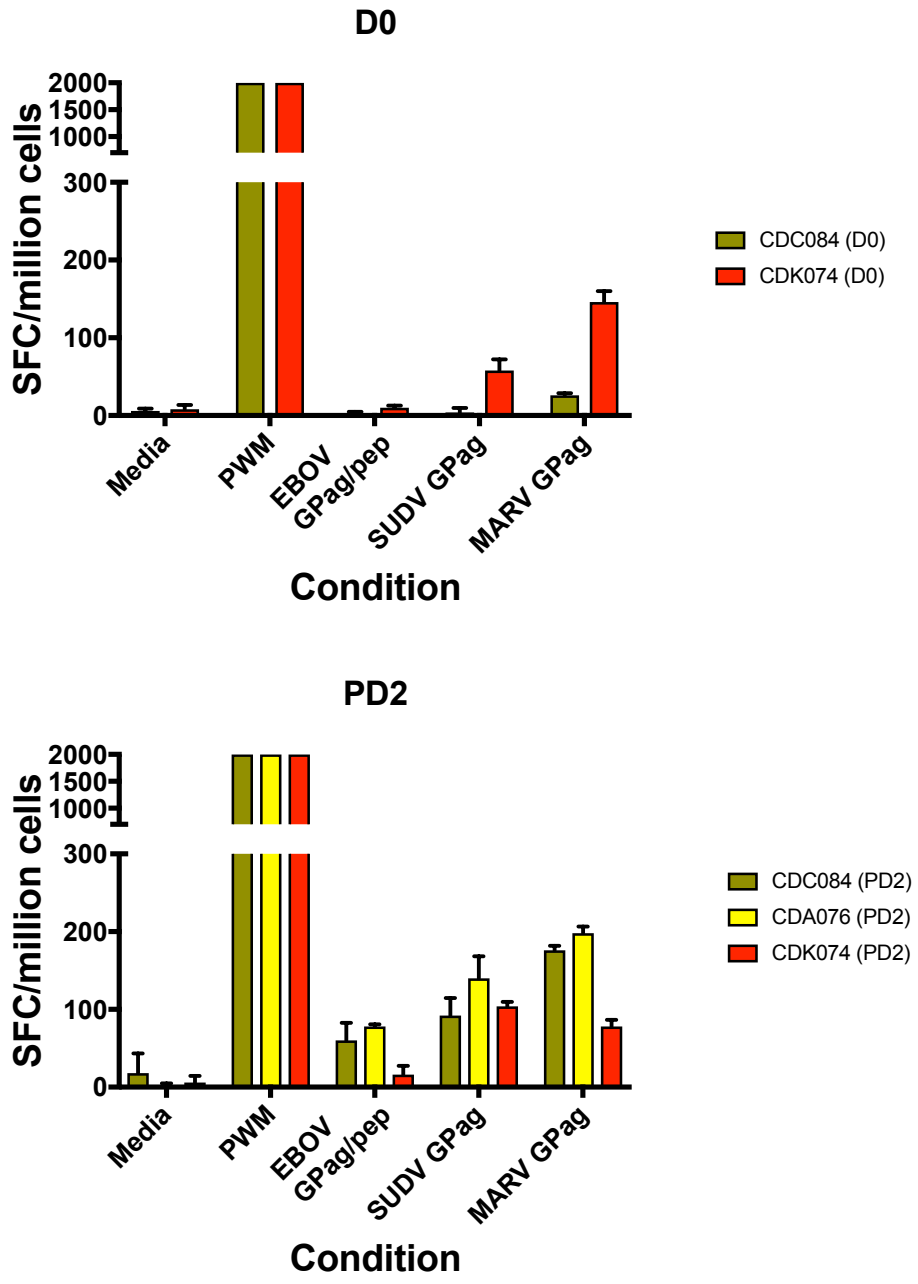


Figure 16: ELISpot data showing IFN-γ secretion from PBMCs collected from NHPs vaccinated with EBOV GP (CDC084) with CoVaccine HT, or EBOV GP, SUDV GP and MARV GP with CoVaccine HT (CDA076), versus the control animal (CDK074) on day 0 and post-dose 2

Il-4 secretion displays a different pattern in response to antigen stimulation (**figure 17**).

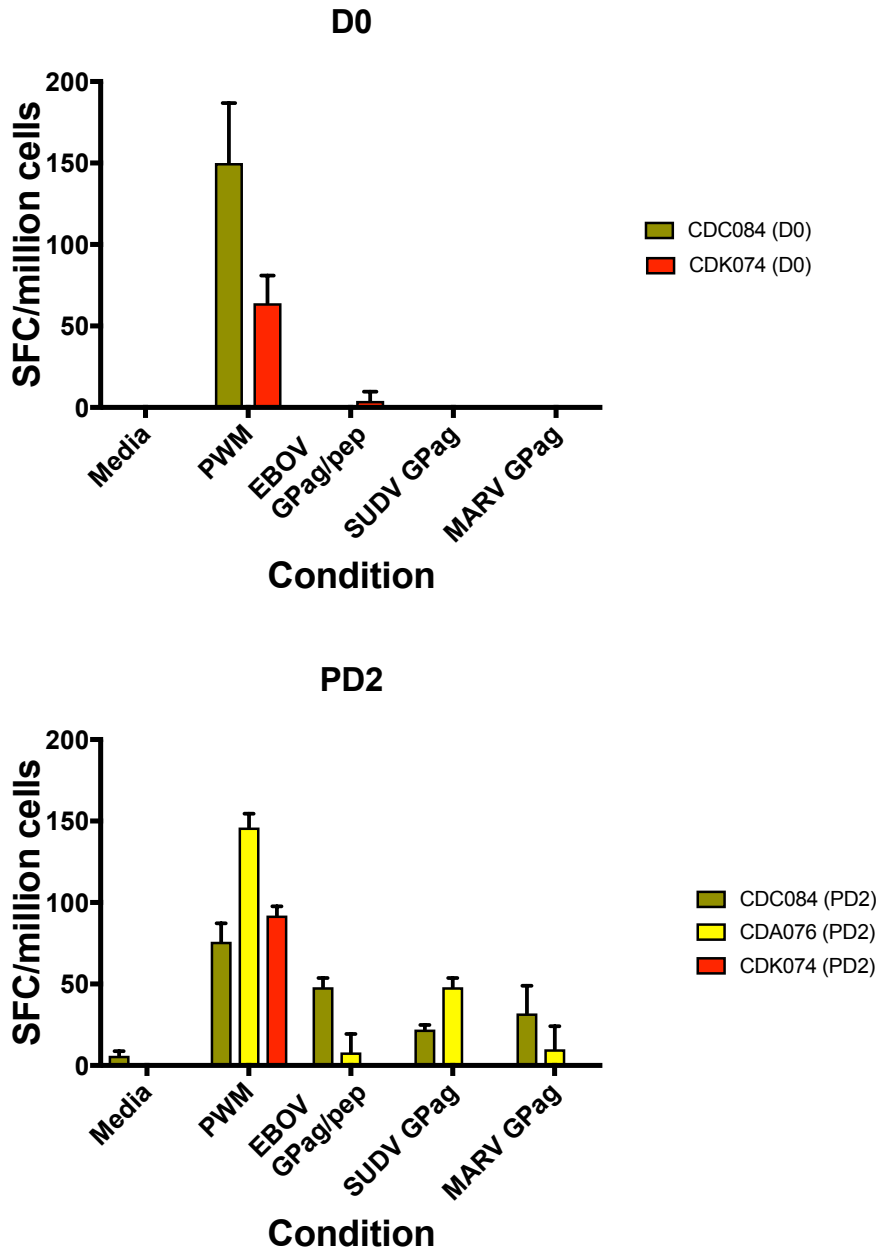


Figure 17: ELISpot data showing IL-4 secreted from PBMCs collected from NHPs vaccinated with EBOV GP with CoVaccine HT (CDC084) or EBOV GP, SUDV GP and MARV GP with CoVaccine HT (CDA076) versus the control animal (CDK074) on day 0 and post-dose 2

No IL-4 secretion is seen in cells from vaccinated or control animals at day 0, while at post dose 2 only the vaccinated animals secrete IL-4 in response to any filovirus GP stimulation. It was also seen that upon stimulation with EBOV GP antigen/peptide, PBMC's from the animal that received the trivalent formulation (CDA076) had a much lower frequency of IL-4 responses than cells from the animal that received the monovalent EBOV GP vaccine. When stimulated with SUDV or MARV GP antigens, cells from both the trivalent vaccine recipient and the monovalent vaccine recipient secreted IL-4, suggesting that this cytokine is secreted in response to not only homologous stimulation but also in response to stimulation with closely related filovirus antigens. Cells stimulated with SUDV and MARV GP antigens from animals that received three doses of a monovalent (EBOV GP only with CoVaccine HT) vaccine also showed cross-reactive responses when analyzed using flow cytometry (**figure 18**).

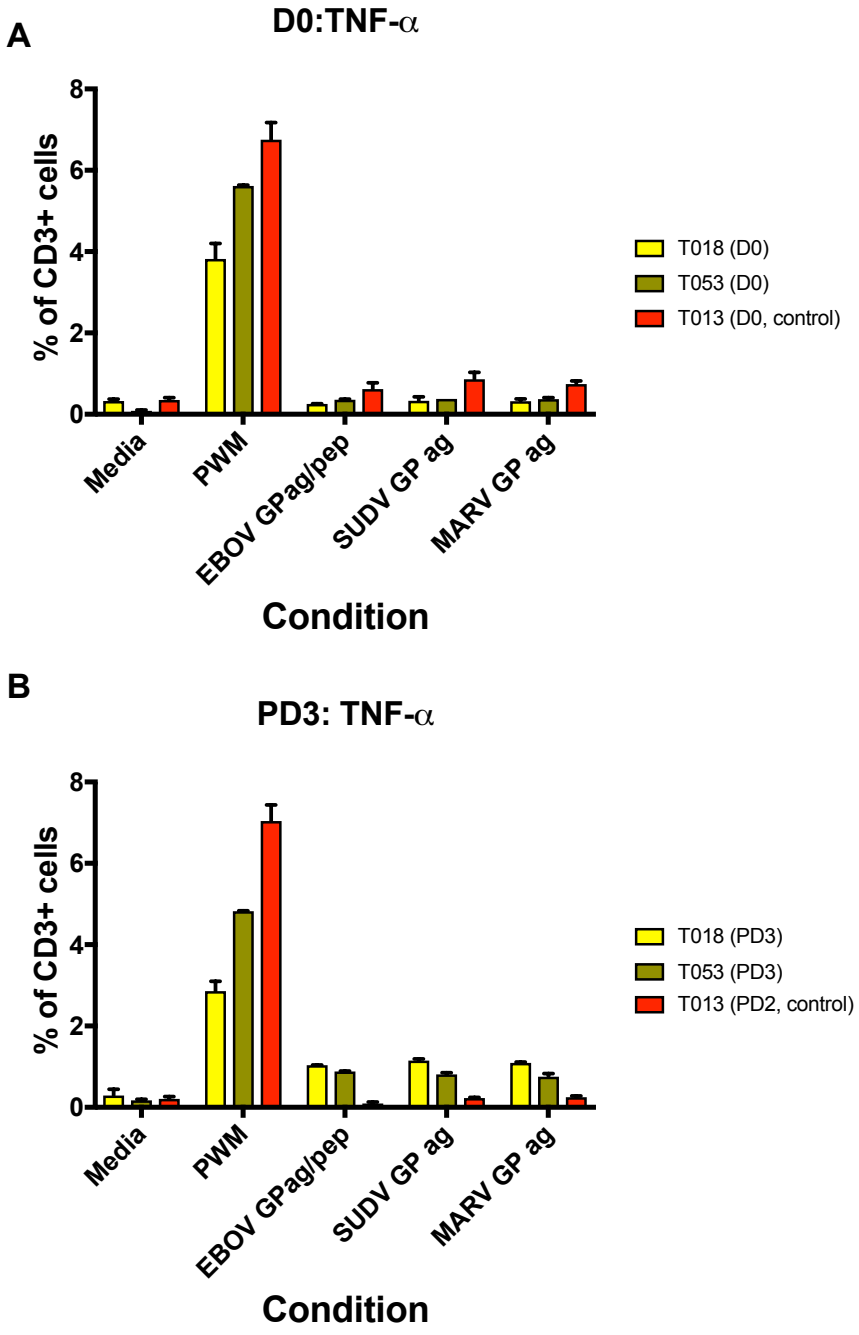


Figure 18: TNF-α production of NHP CD3+ cells after GP stimulation with a combination of GP antigen and peptide pool (EBOV GP), or antigen alone (SUDV and MARV GP). A) Very little TNF-α production is seen after GP stimulation in any of the animals prior to immunization. However, after 3 doses B) immunized animals show more TNF-α production compared to cells from the unvaccinated animal

At day 0, very little TNF-α is seen from any of the animals when stimulated with any filovirus GP. At the post dose 3 time point, CD3+ cells from both vaccinated animals secrete TNF-α in response to not only EBOV GP antigen/peptide stimulation, but also in response to stimulation with SUDV and MARV GP antigen. This demonstrates that there is a specific response to not only the antigen used in the vaccine (EBOV GP), but also cross-reactive responses to GPs from closely related species. These responses are not different between cells stimulated with SUDV or MARV GP, and are also very similar to the response seen in cells stimulated with EBOV GP antigen/peptide. This potential for cross-reactive cellular responses is confirmed by the IFN-γ ELISpot data from these same animals (**figure 19**). These data show no IFN-γ secretion from cells in any of the animals at day 0, but at post-dose 3 both of the vaccinated animals show secretion in response to stimulation with filovirus GP. Cytokine production is greater in the cells stimulated with SUDV or MARV GP antigen than it is in cells stimulated with EBOV GP antigen/peptide, despite the fact that neither of the vaccinated animals received those antigens suggesting potential additional innate immune stimulation was triggered by these whole antigens.

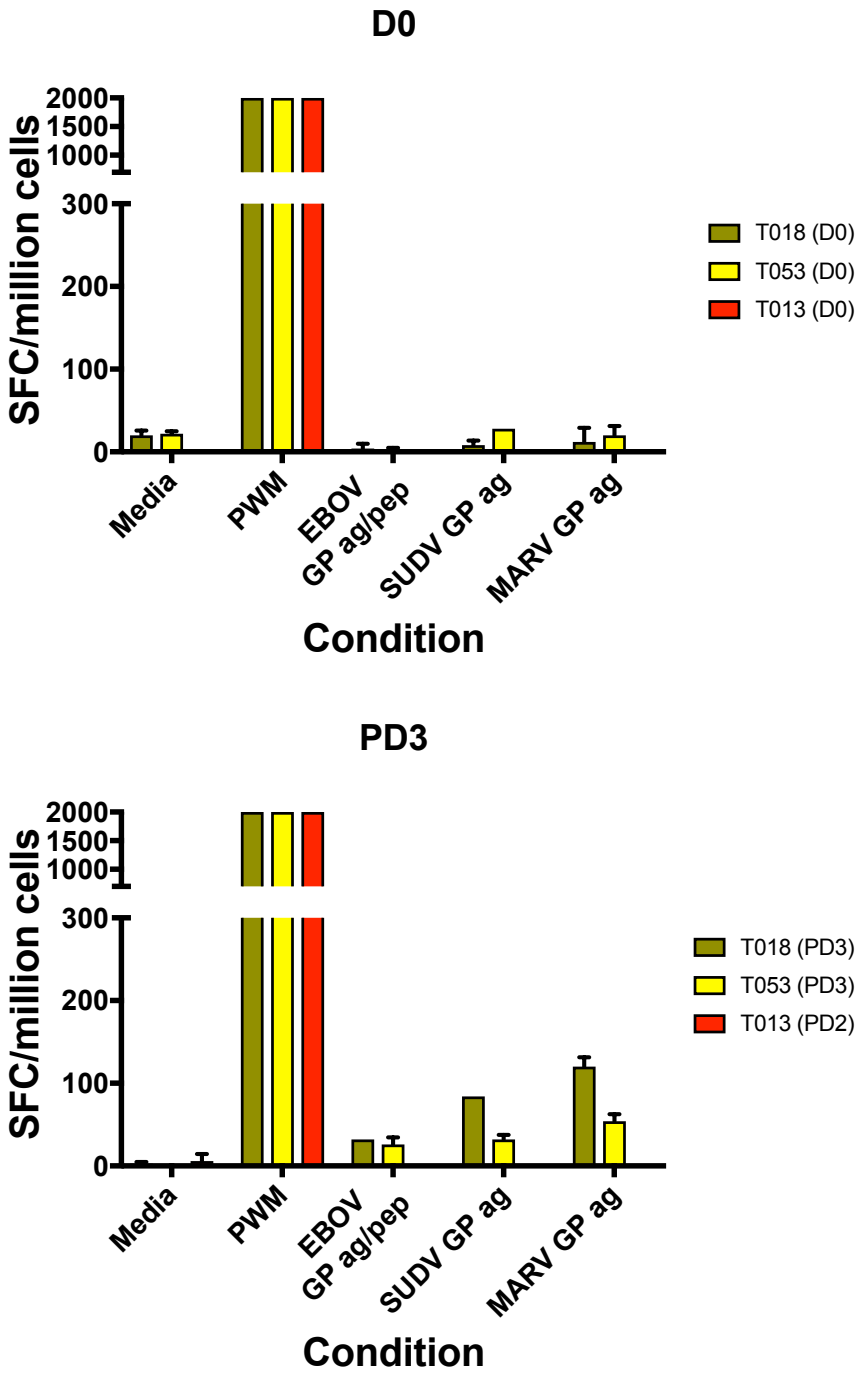


Figure 19: ELISpot data showing IFN-γ secreted from PBMCs from NHPs vaccinated with three doses of EBOV GP with CoVaccine HT (T018 and T053) versus a control animal (T013) on day 0 and post-dose 2 or 3

Discussion

The search for a panfilovirus or multivalent vaccine has been ongoing for at least 15 years, with many different platforms being employed including VLPs, chimpanzee and human adenovirus vectored vaccines, with or without DNA vector priming, and recombinant VSV vaccines [31-33, 103-109]. Most of the strategies and studies, whether they are done in rodents or nonhuman primates initially focused on the development of binding or neutralizing antibodies in response to vaccination with filovirus vaccines [31-33, 103-109]; however, as the potential role for the cell mediated response in protection grew, experiments to determine the cell mediated response to vaccination were implemented [32, 33, 106, 109]. Some studies focused only on the development of homologous immune responses to individual vaccine components [33, 104, 105, 107, 109] while some tried to determine the extent of cross-reactive and potentially cross-protective responses [31, 32, 103, 106, 108]. Some even try to determine the cross-reactive cell mediated responses [32], although very few have done this in nonhuman primates [106]. Although many multivalent vaccine strategies are under development, none are currently approved for human use and only one vaccine with multiple filovirus components is currently being employed in human clinical trials, MVA-BN-Filo (NCT04028349, NCT03583606).

Early work in guinea pigs demonstrated that the use of VLPs containing either MARV GP or EBOV GP, when combined were able to confer high levels of protection after challenge with either virus using only a single dose [103]. Following this, a single rVSV vector was used to express the GPs of three filoviruses (EBOV, SUDV, and MARV), which was able to protect four out of four guinea pigs from challenge with EBOV,

MARV, or SUDV [107]. It is of note however, that the SUDV guinea pig model used in these studies was not uniformly lethal, and circulating SUDV GP IgG titers were the lowest of the three filovirus GP IgG titers analyzed [107]. Other work in guinea pigs sought to develop a cross-protective vaccine using a single rVSV type, rather than a blended rVSV vaccine [31]. This was done using a SUDV GP and VP40 encoding rVSV, which was given in two doses. This vaccine was shown to protect five out of six animals after challenge with a lethal dose of EBOV, demonstrating that cross-protection against heterologous challenge was possible with a monovalent vaccine [31]. This vaccine was able to elicit a humoral immune response against SUDV GP and VP40, however the response against EBOV GP and VP40 was not analyzed to determine the cross-reactive immunogenicity of the vaccine [31]. In contrast our vaccine using a recombinant subunit platform showed reduced efficacy (**figure 12**) with the addition of SUDV or MARV antigens, demonstrating that developing an efficacious multivalent vaccine using this platform will require careful formulation.

Monovalent and trivalent rVSV based vaccines were also developed and tested in both mice and cynomolgus macaques [32]. These studies were some of the few that examined the cross-reactive cellular response in vaccinated mice and nonhuman primates. In mice it was seen that after vaccination with 2 doses of monovalent or trivalent vaccine formulations using an rVSV vector that expressed EBOV, MARV, and/or SUDV GP, IFN-γ secreting splenocytes were lower in response to EBOV GP stimulation in the trivalent vaccine recipients than in the monovalent vaccine recipients, and mice that received a monovalent EBOV vaccine cross-reacted to SUDV GP

stimulation but animals that received a monovalent SUDV GP vaccine did not respond to EBOV GP stimulation [32]. The humoral response in mice showed that EBOV and SUDV GP specific IgG responses were similar in the monovalent and trivalent recipients, however the MARV GP specific IgG response was lower in animals receiving the trivalent formulation than in those that received the monovalent formulation [32].

Several different approaches have also been used in NHPs. One used a chimpanzee adenovirus vector (CAdVax-Panfilo), resulting in a nonreplicating virally vectored vaccine that was comprised of four vectors, expressing the GPs of five filoviruses and NPs from two. This vaccine demonstrated full protection of NHPs following an initial challenge with either MARV or EBOV [105]. In an attempt to develop a single injection multivalent filovirus vaccine, a multivalent vaccine with recombinant VSV expressing the GPs of TAFV, SUDV, MARV or EBOV was tested in either cynomolgus or rhesus macaques [104]. When challenged with TAVF or EBOV none of the animals succumbed to challenge, however one animal out of three in the group challenged with SUDV did develop clinical signs of disease and did succumb to infection [104]. In cynomolgus macaques receiving a trivalent vaccine formulation using an rVSV vector that expressed EBOV, MARV, or SUDV GP, six of fifteen developed a cell mediated immune response as measured by IFN-γ secreting, filovirus GP stimulated, PBMCs, and four of those six developed a balanced cell mediated immune response to all three filovirus components, with PBMCs taken from these animals secreting equal amounts of IFN-γ when stimulated with GP peptide pools of each filovirus [32]. While we did not use equal stimulants for all three filovirus GP's, our preliminary work using whole antigen as

stimulants for SUDV and MARV GP's did not strongly activate PBMCs from NHPs that received the trivalent subunit filovirus vaccine (**figure 16**), since PBMCs collected from the animal receiving the trivalent vaccine secreted more IFN-γ when stimulated with SUDV or MARV GP antigens than with EBOV antigen stimulation. All four animals receiving our trivalent formulation succumbed to lethal EBOV challenge while similar data for the other two viruses was not obtained. These results in NHPs confirm what was seen in our earlier guinea pig experiments, suggesting that continued antigenic balancing of our trivalent vaccine candidate is required, as data suggests that the SUDV and MARV components interfere with the development of a protective response to EBOV.

Many obstacles exist in the way of the development of an effective multivalent filovirus vaccine, particularly in regard to immune competition or interference, where the immune response to one or more components of a multivalent vaccine is reduced [110]. This phenomenon has primarily been studied in Influenza [110] where the optimal vaccination strategy involves using multiple viral strains in one vaccine, but has also been reported with vaccines for different Human Papilloma Virus (HPV) strains [60], DENV [62], as well as Hepatitis A and B [61]. Immune interference could be the reason why our vaccine formulations containing the GP of multiple filovirus species offered reduced protective efficacy in the NHP model against challenge with EBOV compared to a vaccine containing only the GP of EBOV. Although the humoral response to all three filovirus components (EBOV, SUDV, and MARV GP) looks balanced in terms of

IgG titers, with most animals showing MFI values against each GP within a log of each other, the cell mediated response does not show this same balance. PBMCs from vaccinated animals show that a disproportionate number of cells collected from animals receiving the trivalent formulation secrete IFN-γ in response to stimulation with SUDV, and MARV GP in comparison to the response seen after stimulation with EBOV GP (**figure 16**). The secretion of IL-4 in trivalent formulation recipients is higher in cells stimulated with SUDV GP than in those stimulated with EBOV or MARV GP. In fact, the number of cells secreting IL-4 in response to EBOV GP stimulation in the trivalent vaccine recipient is very low in comparison to the number of IL-4 secreting cells in the animal that received the monovalent formulation (**figure 17**). Even in animals that did not receive vaccines containing MARV or SUDV GP (**figure 18**, **figure 19**), stimulation with these antigens was able to elicit IFN-γ and IL-4 responses as measured by ELISpot, as well as TNF-α secretion from CD3+ cells as measured by flow cytometry. This indicates either a very strong cross-reactive cellular immune response, or, due to the fact that cytokine secretion was occasionally seen at day 0 or in unvaccinated controls (**figure 16**, **figure 18**), could mean that these antigens are innate immune stimulators (or that the purity levels of the whole antigen preparations is different causing this effect due to impurities). Knowing that certain vaccine components may elicit stronger immune responses than others is helpful in moving forward with formulating an effective multivalent vaccine. The goal is to develop a vaccine that elicits a balanced immune response to all components in the vaccine and is therefore more likely to provide full protection against multiple filoviruses.

Chapter 5: Summary and Future Directions

There are many different approaches to developing viral vaccines. Historically, attenuation and inactivation were used [1], but modern vaccines may use other methods such as mRNA, DNA, or antigenic subunits [3, 111, 112]. Advances have also allowed us to better characterize and understand complex immune responses and in turn to correlate them with vaccine protection. This facilitates rational vaccine design that is tailored to elicit a specific and protective response. The first step in steering the design and refining vaccines using this method is to explore and understand the immune response elicited by vaccine formulations. For some infectious agents, antibody responses remain key to protecting from a natural infection, as is the case with flaviviruses [46, 66, 69, 83]. The protection against ZIKV infection using a recombinant subunit vaccine platform is no different. Our vaccine candidate elicited high levels of both binding and neutralizing antibody titers in most vaccinated animals, and we were able to show, through comparison of total IgG and virus neutralizing titers as well as a passive transfer experiment, that neutralizing antibodies were correlated with protection.

For other pathogens, such as filoviruses, establishing correlates of protection has not been as successful. In the case of natural infections, differences between survivors and non-survivors tend to be related to an early but transient release of pro-inflammatory cytokines in humans and animals that survive EBOV infections, although this may not be the case for SUDV infections [113]. Studies to determine vaccine correlates of protection have yielded variable results, some of which may have to do with the vaccine platform used as well as the route of infection [44, 113]. Vaccines using rVSV vectors elicit high titers of binding antibodies that have been correlated with protection in this

platform, while in contrast rAd based vaccines elicit lower levels of antibody titers yet are also protective [104, 113]. This suggests that both the humoral and cell mediated response can be important in protection, and that their importance will vary depending on the vaccine platform used.

We have demonstrated that by using a recombinant subunit vaccine platform, we are able to elicit high titers of EBOV GP binding IgG antibodies. We have not, however, been able to directly correlate this response to protection, as there were no differences in the antigen binding titers found between survivors and non-survivors. This clearly does not mean that antibody responses are not important as is clearly seen in comparison with non-protective formulations (data not shown), and it seems possible that the quality and functional capability of the antibodies and not the quantity of antibodies elicited are important for protection. Further work to characterize the antibody response needs to be done to determine if neutralizing antibodies, antibody affinity, avidity, or antibodies against specific epitopes are necessary to measure a successful vaccine response [43, 96]. The role of cell mediated immunity is likewise unclear. We have successfully developed assays that have allowed us to detect vaccine specific cell mediated responses, using both flow cytometry and ELISpot assays. Based on the results of the flow cytometry assays we have shown that the cell-mediated response elicited by our vaccine candidate stems primarily from CD4+ T cells, a result that is not surprising considering the difficulty in inducing strong CD8+ T cell responses using subunit vaccines [3]. The only cytokine we were able to detect using this assay was TNF-α, a pro-inflammatory cytokine. This cytokine was secreted by CD4+ T cells

originating from vaccinated monkeys, and only after being stimulated with EBOV GP peptide and antigen. In ELISpot assays we saw secretion of both IFN-γ and IL-4 from PBMCs of vaccinated animals following stimulation with homologous antigen and peptide, but due to the nature of the assay we are unable to determine from which cell subset this response originates. We were unable to find any difference in cytokine secretion between animals that survived challenge and those that did not, based on the work conducted to date. This could be due to the small number of animals that were analyzed, and it is possible that having a larger data set could yield differences between survivors and non-survivors. As non-human primates are from a genetically diverse outbred population, the large heterogeneity in the level of cytokine production between animals makes direct comparisons difficult. This is particularly notable in the level of IL-4 production seen in different animals and can be seen prior to vaccination. It is possible that analysis of cellular immune activation after viral challenge may give better insights into the differences between successful and unsuccessful vaccination.

Analysis of cell mediated immune responses to the multivalent vaccine formulation as well as the cross-reactive responses elicited by the monovalent EBOV vaccine candidate was attempted as well. Preliminary results show that the trivalent vaccine was able to elicit binding IgG to all three filovirus GP, and that the monovalent EBOV GP formulation resulted in GP specific IgG titers that cross-reacted strongly to SUDV GP and weakly to MARV GP. We also saw that MARV GP and to a lesser extent SUDV GP are able to stimulate the production of IL-4 and IFN-γ from immune cells, even from animals vaccinated only with EBOV GP, suggesting cross-reactive cell-mediated

responses may be expected (or some unspecific reactivity caused by whole antigen preparations). In the animal vaccinated with the trivalent formulation, frequency of cytokine-secreting cells to SUDV and MARV GP antigen stimulation was higher than that in response to EBOV GP antigen and peptide stimulation. While it is difficult to draw conclusions from very few animals included in the analysis, it is tempting to speculate that the magnitude of the response to MARV and SUDV could potentially have reduced the response to EBOV GP. This is supported by the fact that the animal that received the monovalent vaccine had a much stronger cytokine response to EBOV GP than the trivalent vaccine recipient. Further characterization of the immune response elicited not only by SUDV and MARV GP vaccination, but also of the cross-reactivity to these other filoviruses elicited by vaccination with EBOV GP alone remains to be done. Of particular interest will be determining the presence of antibodies capable of cross-neutralizing other filoviruses in animals vaccinated only with EBOV GP, as well as the neutralizing antibodies elicited against all three filoviruses in animals receiving multivalent formulations.

Comparing the results of the analysis of the immune responses to a recombinant subunit vaccine against a flavivirus (ZIKV) and a filovirus (EBOV), it is clear that correlates of protection are not universal and must be determined for each pathogen. We were able to determine that protection against ZIKV using a subunit vaccine platform is contingent on the development of neutralizing antibodies. In the case of a recombinant subunit vaccine against EBOV, while we have been able to protect both guinea pigs and NHPs from lethal challenge, we have not yet been able to identify a correlate of protection. We have, however, found both cell mediated and humoral

components in the immune response to the vaccine. Exploring and understanding these responses further is essential to achieve the ultimate goal of developing a multivalent filovirus vaccine. Current formulations include EBOV, SUDV, and MARV components, but lack protection against EBOV challenge. While bivalent combinations of EBOV GP with SUDV or MARV GP protect against SUDV or MARV challenge, respectively (data not shown), demonstrating that EBOV GP will not impact the efficacy of a protective SUDV or MARV vaccine but SUDV or MARV GP may impact the efficacy of an EBOV vaccine. If specific types of cell mediated or humoral responses are important for survival against EBOV, understanding these characteristics can be applied to guide the development of a trivalent vaccine, as currently the data suggest that MARV and SUDV GP specific responses may dominate over EBOV GP specific responses while protection against EBOV infection seems to be the most difficult to achieve.

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