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# Virus-like particles exhibit potential as a pan-filovirus vaccine for both Ebola and Marburg viral infections<sup>☆</sup>

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## Abstract

A safe and effective pan-filovirus vaccine is highly desirable since the filoviruses Ebola virus (EBOV) and Marburg virus (MARV) cause highly lethal disease typified by unimpeded viral replication and severe hemorrhagic fever. Previously, we showed that expression of the homologous glycoprotein (GP) and matrix protein VP40 from a single filovirus, either EBOV or MARV, resulted in formation of wild-type virus-like particles (VLPs) in mammalian cells. When used as a vaccine, the wild-type VLPs protected from homologous filovirus challenge. The aim of this work was to generate a multi-agent vaccine that would simultaneously protect against multiple and diverse members of the Filoviridae family. Our initial approach was to construct hybrid VLPs containing heterologous viral proteins, of EBOV and MARV, and test the efficacy of the hybrid VLPs in a guinea pig model. Our data indicate that vaccination with GP was required and sufficient to protect against a homologous filovirus challenge, as heterologous wild-type VLPs or hybrid VLPs that did not contain the homologous GP failed to protect. Alternately, we vaccinated guinea pigs with a mixture of wild-type Ebola and Marburg VLPs. Vaccination with a single dose of the multivalent VLP vaccine elicited strong immune responses to both viruses and protected animals against EBOV and MARV challenge. This work provides a critical foundation towards the development of a pan-filovirus vaccine that is safe and effective for use in primates and humans.

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**Keywords:** Hybrid; Immunity; Antibody; Viremia; Adjuvant; Guinea pigs

## 1. Introduction

The filoviruses, Ebola (EBOV) and Marburg (MARV), are non-segmented negative-strand RNA viruses that cause

severe hemorrhagic fevers [1–3]. The first outbreak of MARV occurred in 1967 in Marburg, Germany, following exposure to monkeys imported from Uganda. This was followed almost 10 years later in 1976 by the first recognized outbreak of EBOV in the country Democratic Republic of the Congo, formerly known as Zaire [3–5]. The natural hosts of EBOV and MARV are unknown, although human cases occur most often due to contact with infected monkeys, humans, or their blood and tissues [4–6]. Natural outbreaks have been on the rise recently, most likely not only due in part to improved surveillance, but also due to more frequent contact between monkeys and humans because of the destruction of natural habitats in Africa (<http://www.who.int/disease-outbreak-news/disease/bydisease.htm>). In addition to their endemic

<sup>☆</sup> Disclaimer: opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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disease potential, filoviruses pose potential biological warfare threats. Both bioagents are highly stable under the proper conditions, easy to produce in large quantities, and can induce fatal disease via the aerosol route [3,7]. Currently, there are no licensed vaccines or therapeutics available to prevent or treat filovirus infections.

Classical-, subunit-, DNA-, and vector-based vaccine strategies have been tested for protective efficacy against filovirus challenge in rodents and non-human primates (reviewed in [8,9]). Several vaccine candidates, including DNA, liposome-encapsulated inactivated virus, and Venezuelan equine encephalitis virus replication-deficient particles (VRP) expressing filovirus proteins have been used with varying degree of success in the mouse and guinea pig models of filovirus infection [10–16]. For protection against MARV infection, a VRP vaccine encoding MARV GP was completely efficacious in both guinea pigs and non-human primates [9,11]. Additionally, vaccinating guinea pigs or non-human primates with a DNA vaccine encoding GP or purified GP is only partially protective against MARV challenge [9,10,17]. Administration of DNA vaccine encoding GP followed by  $>10^{10}$  plaque forming units (pfu) of a replication-defective, adenovirus-vectored vaccine expressing GP, or the adenovirus vaccine alone expressing GP and nucleoprotein protects non-human primates against EBOV challenge [18–20]. Collectively, these efforts indicate that protection against lethal filovirus infection is attainable. Unfortunately, questions remain about many of the vaccine strategies used thus far, including acceptable vaccine doses, safety considerations, the impact of prior immunity to the vaccine vector, and the ability of these vaccine strategies to cross-protect against multiple strains of EBOV and MARV [8,9,21]. Therefore, alternate approaches to filovirus vaccines are still needed.

We previously demonstrated that co-expression of filoviral glycoprotein (GP) and the matrix protein (VP40) in mammalian cells results in the spontaneous generation of enveloped filovirus-like particles (VLPs) [22–24]. VLPs potently stimulate functional maturation and activation of dendritic cells *in vitro*, as well as activate both humoral and cellular immune responses *in vivo* [23,25]. Vaccination with Ebola or Marburg VLPs (eVLPs and mVLPs, respectively) fully protected mice and guinea pigs from lethal challenge with the respective homologous virus [23,25]. VLPs are a highly desirable vaccine approach for these dangerous pathogens since they are a safe and efficacious non-replicating, subunit vaccine. Despite the proven efficacy of VLPs as vaccines, the individual role played by GP or VP40 in VLP-mediated protection remains unknown. Other vaccines against EBOV and MARV that used GP as an antigen have been the most successful in protecting against lethal homologous filovirus challenge (reviewed in [8,9]). However, vaccines based on VP40 alone have been only moderately successful. A VRP-based vaccine expressing VP40 protected ~50% of mice from homologous EBOV infection, with vaccinated BALB/c mice protected better than C57Bl/6 mice [15]. No significant protection was

observed with the same vaccination strategy for MARV, where only one out of six VRP-VP40-vaccinated guinea pigs survived challenge with lethal MARV infection [11].

In mice and guinea pigs, vaccine strategies that are protective against a homologous filovirus challenge are not efficacious against a heterologous challenge [23,25]. For instance, in guinea pigs, we found that eVLP vaccination protected against EBOV challenge, but was not sufficient to protect against MARV infection [25]. Additionally, mice vaccinated with inactivated MARV were not protected from challenge with EBOV [23]. Therefore, it is important to develop a pan-filovirus vaccine that can protect against multiple and diverse filovirus infections. The goal of our current study was to identify a vaccine candidate that could provide resistance against diverse members of the family Filoviridae, using EBOV-Zaire and MARV-Musoke as models.

## 2. Methods

### 2.1. Virus

The EBOV-Zaire 1995 or MARV-Musoke strains were used to generate expression plasmids and for all viral infections. Guinea pig-adapted strains of EBOV-Zaire 1995 or MARV-Musoke were used to challenge vaccinated guinea pigs [10,26,27]. All experiments with live MARV and EBOV were handled under maximum containment in a biosafety level (BSL)-4 laboratory at the United States Army Medical Research Institute of Infectious Diseases. EBOV or MARV infectious particles were enumerated by standard plaque assay on Vero E6 cells [28]. To produce purified viral preparations, EBOV- or MARV-infected cell supernatants were clarified at  $1500 \times g$  and then pelleted at  $9500 \times g$  for 4 h in a Sorvall GSA rotor. The partially purified viral preparations were then purified over a continuous 20–60% sucrose gradient and inactivated by irradiation with  $1 \times 10^7$  rads, as previously described [10].

### 2.2. Antibody titers

Levels of MARV- and EBOV-specific antibodies were determined, as previously described [10]. Briefly, 96-well plates were coated with sucrose-purified inactivated MARV or EBOV virions. Serial dilutions of each serum sample were tested and detected using a horseradish peroxidase (HRP)-labeled antibody. The endpoint titers were determined as the inverse of the last dilution where the optical density of the sample was  $\geq 0.2$  greater than control wells. Convalescent serum samples were removed from the BSL-4 laboratory after gamma-irradiation with  $2 \times 10^6$  rads from a  $^{60}\text{Co}$  source.

### 2.3. VLPs

VLPs were prepared as previously described, with minor modifications [22–24]. To generate VLPs, 293T cells were

co-transfected with combinations of pWRG7077 vectors encoding for MARV or EBOV VP40 and GP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To purify the VLPs, the cell supernatants were clarified at  $1500 \times g$ , and subsequently, the particles were pelleted at  $9500 \times g$  for 4 h in a Sorvall GSA rotor. The crude VLP preparations were then separated on a 20–60% continuous sucrose gradient centrifuged in a SW41 rotor at 38,000 rpm for 18 h (Beckman-Coulter Inc., Fullerton, CA, USA). The VLPs were concentrated by a second centrifugation and resuspended in endotoxin-free phosphate-buffered saline (PBS). The gradient fractions containing the VLPs were determined by Western blots and electron microscopy. Total protein concentrations of the VLP preparations were determined after lysis in NP40 detergent using a detergent-compatible protein assay (Bio-Rad, Hercules, CA, USA). The endotoxin levels in all VLP preparations used in this study were  $<0.03$  endotoxin units/vaccine dose, as assessed using the *Limulus* amoebocyte lysate test (Biowhittaker, Walkersville, MD, USA).

#### 2.4. Western blotting

Proteins of purified VLP preparations were separated on 4–12% Tris–glycine gels (Invitrogen, Carlsbad, CA, USA). Western blot analysis was performed using monoclonal anti-EBOV GP (6D8), anti-EBOV VP40 (AE11), anti-MARV GP (5E2/5D7/9G4), and anti-MARV VP40 (1H11) as primary antibodies followed by detection of the bound antibodies with a goat anti-mouse IgG HRP-conjugated antibody and enhanced chemiluminescence [22,24].

#### 2.5. Electron microscopy

Sucrose gradient-purified VLPs were processed as previously described [22–24]. Briefly, the particles were applied to formvar- and carbon-precoated 300-mesh grids. The grids were then treated with 1% glutaraldehyde in PBS, rinsed in distilled water, and negatively stained with 1% uranyl acetate. For immunoelectron microscopy, the samples were processed as previously described [22,24]. Briefly, after the VLPs were applied to grids, the grids were stained with a pool of EBOV- or MARV-specific antibodies against GP, washed, and incubated with goat-anti-mouse IgG labeled with 10 nm gold spheres. The grids were then rinsed in distilled water and stained with 1% uranyl acetate. Specimens were examined on a JEOL EX transmission electron microscope at 80 kV.

#### 2.6. VLP vaccinations and filovirus challenge

Inbred Strain 13 guinea pigs (USAMRIID, Frederick, MD, USA) were vaccinated once intramuscularly with 100  $\mu$ g of VLPs in 200  $\mu$ l of RIBI monophosphoryl lipid + synthetic trehalose dicorynomycolate + cell wall skeleton emulsion (Corixa Corporation, Hamilton, MT, USA) diluted in endotoxin-free PBS. Control guinea pigs were vaccinated with RIBI adjuvant in PBS alone. The guinea pigs were

challenged subcutaneously, 28 days after vaccination with  $\sim 1000$  pfu ( $\geq 2000$  50% lethal doses (LD<sub>50</sub>)) of guinea pig-adapted MARV or EBOV diluted in PBS [10,27]. After challenge, guinea pigs were observed at least twice daily for illness. Serum viremia was determined on day 7 by standard plaque assay, as previously described [28]. Vaccine experiments to test protective efficacy were performed twice.

Research 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 principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

#### 2.7. Plaque reduction–neutralization assay

To test for the presence of plaque-neutralizing antibodies, three-fold dilutions of guinea pig sera were incubated with  $\sim 100$  pfu of MARV or EBOV at 37 °C for 1 h in the presence of 5% guinea pig serum as a source of complement. The antibody–virus mixtures were then added to confluent Vero E6 cells and a standard plaque assay with Vero E6 cells was performed [11]. The percent of plaque reduction was calculated by comparing the number of pfu present in each sample to the pfu obtained with virus alone [11,29]. The data are displayed as the 80% plaque reduction–neutralization titer (PRNT<sub>80</sub>), which is defined as the inverse of the last dilution where  $>80\%$  inhibition of virus infection is observed.

#### 2.8. Statistical analysis

The proportion of treated and control animals surviving were compared by two-tailed Fisher exact tests within groups. The adjustments for multiple comparisons were made by stepdown Bonferroni correction. Analyses were conducted using SAS Version 8.2 (SAS Institute Inc., SAS OnlineDoc, Version 8, Cary, NC 2000). A  $p$ -value of  $\leq 0.05$  was considered significant.

### 3. Results

#### 3.1. Generation of hybrid filovirus-like particles

Previous observations determined that GP and VP40 are sufficient, in both EBOV and MARV, to produce VLPs with morphology similar to that of authentic virus [22–25]. As a first approach to generating a pan-filovirus vaccine, we sought to generate hybrid VLPs harboring proteins of different filoviruses. EBOV and MARV are members of the same family and cause similar diseases, but are genetically distinct, with only  $\sim 30\%$  homology at the amino acid level [2]. The structural requirements for filovirus assembly are poorly understood [22,30] and it was not known whether just these two

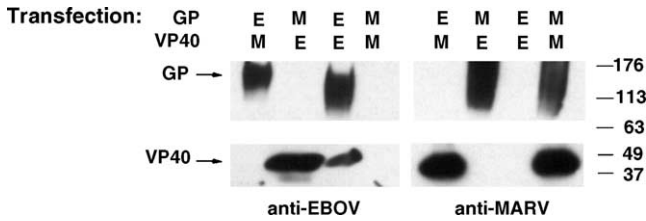


Fig. 1. Detection of Ebola and Marburg virus GP and VP40 by Western blot analysis. 293T cells were transfected with combinations of Ebola and Marburg virus (EBOV and MARV, respectively) GP and VP40, as indicated. The viral origin of the GP and VP40 proteins are specified by (E) for EBOV or (M) for MARV. The virus-like particles (VLPs) from supernatants of the transfected cells were purified on a 20–60% continuous sucrose gradient; successive gradient fractions were collected, and then analyzed by Western blotting. A representative fraction containing the indicated VLPs is shown here. The presence of wild-type or hybrid VLPs were determined using EBOV- or MARV-specific GP and VP40 monoclonal antibodies.

proteins from different filoviruses would cooperate to form VLPs. EBOV GP has been successfully incorporated into pseudotyped murine leukemia virus particles, indicating its promiscuity [31]. More recently, GP molecules from distinct filovirus subtypes and strains were incorporated into virus-like particles containing all seven EBOV structural proteins [32].

In order to assess the ability of GP and VP40 from EBOV and MARV to assemble and form hybrid VLPs, 293T cells were transfected with cDNAs encoding MARV GP and EBOV VP40, or alternatively, the cells were transfected with EBOV GP and MARV VP40. By Western blot, EBOV GP-specific anti-serum recognized the GP incorporated into the VLPs produced from cells transfected with EBOV GP and EBOV, or MARV VP40, while EBOV VP40 was found in preparations from cells transfected with either EBOV, or MARV GP and EBOV VP40 (Fig. 1). MARV GP-specific anti-serum detected GP in preparations containing MARV

GP and VP40 or MARV GP and EBOV VP40 (Fig. 1). MARV VP40 was detected in preparations from cells transfected with MARV GP and MARV VP40, or EBOV GP and MARV VP40 (Fig. 1).

To determine if the fractions isolated from the sucrose gradients contained filamentous particles, we used electron microscopy. As shown in Fig. 2, hybrid VLPs displayed morphology similar to the wild-type VLPs containing the homologous proteins or to the authentic filoviruses. The hybrid VLPs were designated e/m-VLPs (containing Ebola GP and Marburg VP40) and m/e-VLPs (containing Marburg GP and Ebola VP40). Using immunogold staining of the VLPs with EBOV GP antibodies, we confirmed the presence of EBOV GP spikes on the eVLP and e/m-VLPs (Fig. 3A and B), but not the mVLPs or m/e-VLPs (data not shown). Similarly, mVLP and m/e-VLPs displayed gold staining after incubation with MARV GP antibodies (Fig. 3C and D), but eVLPs and e/m-VLPs did not react with the MARV GP antibodies (data not shown). Taken together, these data show that heterologous EBOV and MARV proteins can cooperate to form hybrid VLPs.

### 3.2. Evaluation of hybrid VLPs as a potential pan-filovirus vaccine

Having the hybrid VLPs in hand, we sought to examine the ability of these structures, as vaccines, to generate protective immunity against both EBOV and MARV in guinea pigs. In addition, the hybrid VLPs gave us a powerful tool to examine the contribution of GP and VP40 in protective immunity against filoviruses. Guinea pigs were vaccinated once with wild-type eVLPs, mVLPs, hybrid e/m-VLPs, or m/e-VLPs in RIBI adjuvant, and their serum antibody levels against EBOV and MARV were measured by ELISA immediately prior to challenge (Table 1). Guinea pigs vaccinated with wild-type eVLP or e/m-VLPs generated high serum an-

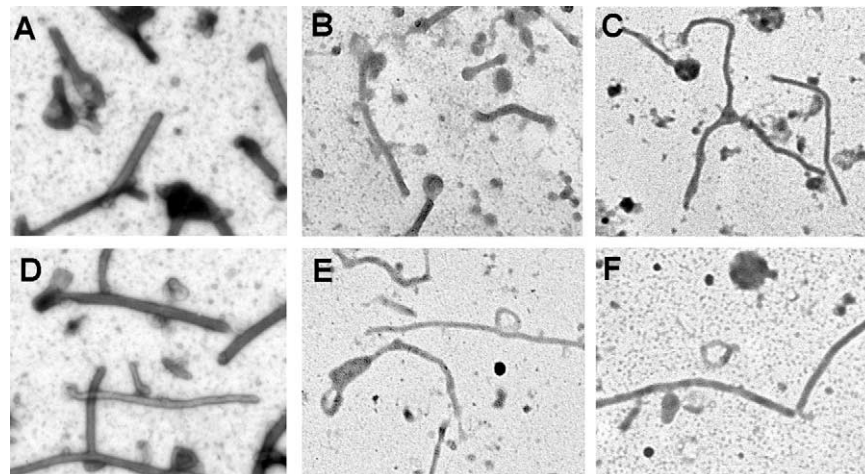


Fig. 2. Hybrid VLPs are morphologically similar to authentic filoviruses and wild-type VLPs. VLPs, purified from the supernatants of 293T cells transfected with combinations of EBOV and MARV GP and VP40, were negatively stained with uranyl acetate to reveal the ultrastructure. Electron micrographs of (A) authentic EBOV, (B) Ebola virus-like particles (eVLP), (C) VLPs containing EBOV GP and MARV VP40 (e/m-VLP), (D) authentic MARV, (E) Marburg virus-like particles (mVLP), or (F) VLPs containing MARV GP and EBOV VP40 (m/e-VLP) at 40,000 $\times$ .

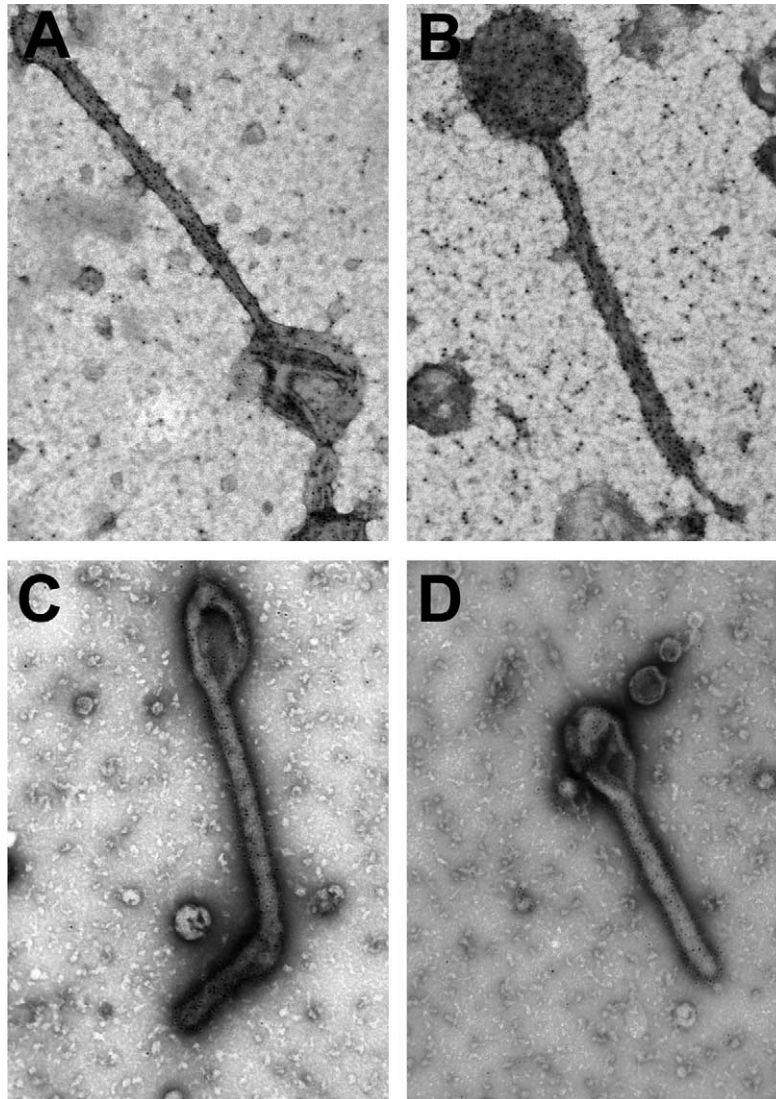


Fig. 3. Hybrid virus-like particles (VLPs) are antigenically similar to wild-type VLPs. Immunoelectron microscopy was performed to demonstrate the specificity of the GP on the (A) eVLPs, (B) e/m-VLPs, (C) mVLPs, or (D) m/e-VLPs at 40,000 $\times$ . To show that the VLPs contained the GP molecules of the correct specificity, the VLPs were labeled with EBOV- (A and B) or MARV-specific (C and D) monoclonal antibodies against GP followed by immunogold rabbit anti-mouse antibody and examined by electron microscopy.

tibody titers against EBOV (geometric mean titer (GMT): 8075 and 19,509, respectively), but not MARV (GMT: 53 and 30, respectively). Conversely, mVLP and m/e-VLP vaccination resulted in high titers against MARV (GMT: 19,595 and 13,856, respectively), but not EBOV (GMT: 47 and 54, respectively). Vaccination with EBOV GP in the form of eVLP or e/m-VLP resulted in induction of neutralizing antibodies against EBOV, but not MARV (Table 1). In contrast, guinea pigs vaccinated with mVLP or m/e-VLP did not generate significant neutralizing antibody titers against either MARV or EBOV after one dose of vaccine (Table 1). Control guinea pigs, vaccinated with RIBI adjuvant alone did not display EBOV- or MARV-specific antibodies (Table 1).

Because the VLP-vaccinated animals generated strong antibody responses after one vaccination, and in guinea pigs protective efficacy of filovirus vaccines correlate positively,

although imperfectly, with filovirus-specific antibody responses [9,11,33], the guinea pigs were challenged 28 days after a single VLP vaccination with  $\sim$ 1000 pfu of guinea pig-adapted EBOV or MARV. Guinea pigs vaccinated with VLPs containing the homologous GP were protected ( $\geq$ 90%) from lethal filovirus challenge (Table 1). A single vaccination with eVLP or e/m-VLP conferred significant protection against EBOV infection ( $\rho = 0.0002$  or 0.0014, respectively, when compared to RIBI-vaccinated animals) and mVLP or m/e-VLP completely protected MARV-challenged guinea pigs ( $\rho = 0.0026$  for both, when each was compared to RIBI-vaccinated animals). However, vaccines containing only heterologous proteins or homologous VP40 were not able to protect against lethal filovirus challenge. For instance, mVLP or m/e-VLP was entirely ineffective in preventing lethal EBOV infection (Table 1). Additionally,

Table 1

Homologous, but not heterologous GP, confers protection from Ebola virus (EBOV) and Marburg virus (MARV) in the context of virus-like particles (VLPs) containing EBOV or MARV glycoprotein (GP) or viral protein (VP40)

Vaccine <sup>a</sup>	Geometric mean titer <sup>b</sup>		PRNT <sub>80</sub> <sup>c</sup>		Viremia (log 10 pfu/ml) <sup>d</sup>		Survival <sup>e</sup>		Mean time to death (days)	
	EBOV	MARV	EBOV	MARV	EBOV	MARV	EBOV	MARV	EBOV	MARV
eVLP	8075	53	448	<10	<1.7	5.61	10/10	2/8	–	12.8
mVLP	47	19,595	<10	16	5.73	<1.7	0/10	10/10	10.3	–
e/mVLP	19,509	30	75.2	<10	<1.7	5.82	9/10	1/9	– <sup>f</sup>	12
m/eVLP	54	13,856	<10	14	5.98	<1.7	0/10	10/10	9	–
RIBI adjuvant	29	59	<10	<10	6.08	5.82	0/10	5/19	9.3	10.8
Naive	33	43	ND	ND	6.03	5.83	0/6	1/6	10	9.2

<sup>a</sup> Guinea pigs were injected with one dose of the indicated vaccine and 28 days later were challenged with 1000 pfu of guinea pig-adapted EBOV or MARV virus. The indicated vaccines contained 100 µg of VLPs comprised of EBOV or MARV GP and VP40 (eVLP and mVLP, respectively), or EBOV GP and MARV VP40 (e/mVLP), or MARV GP and EBOV VP40 (m/eVLP). The VLP vaccines were given in 200 µl of RIBI adjuvant. Control groups received 200 µl of RIBI adjuvant or were completely naive.

<sup>b</sup> Geometric mean titer ( $n = 6–10$ ) of EBOV- or MARV-specific antibodies, as measured by ELISA, from serum samples collected 28 days post-vaccination.

<sup>c</sup> Mean plaque reduction/neutralization titer (PRNT, >80% reduction) from serum samples collected 28 days post-vaccination where the dilutions began at 1:10.

<sup>d</sup> As measured by plaque assay from serum samples collected 7 days following challenge, with a limit of detection of ~50 pfu/ml.

<sup>e</sup> Indicates the number of survivors/total number of guinea pigs at 28 days post-challenge.

<sup>f</sup> A single vaccinee died on day 13 following challenge.

eVLP and e/m-VLP only provided 25 and 11% protection, respectively, against a MARV challenge (Table 1). The failure of the hybrid vaccines to protect against EBOV and MARV challenge was not due to challenge following administration of a single dose, as administering three doses of hybrid VLPs prior to virus challenge was not able to protect against both lethal infections (data not shown). Only 14 out of 19 RIBI adjuvant-vaccinated guinea pigs (77%) succumbed to challenge (Table 1). We were concerned that the guinea pig-adapted MARV-Musoke was not uniformly lethal, but previous studies caused death in only 60 out of 65 (92%) of Strain 13 guinea pigs [10,11,22,23]. The death rate in the guinea pigs vaccinated with RIBI adjuvant was slightly lower than we expected, despite the fact that our actual challenge doses (intended 1000 pfu) ranged between 452 and 2672 pfu. Naive guinea pigs were challenged to account for the effect of the RIBI adjuvant, which was given 28 days prior to challenge, and five out of six MARV-infected guinea pigs died (83%, Table 1). When taken with the previous data, this indicates that the MARV-Musoke adapted to guinea pigs is not uniformly lethal in the Strain 13 guinea pigs.

To determine if VLP vaccination induced sterile immunity, the levels of circulating virus were assessed 7 days after challenge. In correlation with the ability to confer protection against lethal filovirus infection, vaccination with VLPs containing homologous GP resulted in no detectable viremia on day 7 (Table 1). However, control guinea pigs or guinea pigs vaccinated with only heterologous proteins or homologous VP40 had high levels of circulating EBOV (range: 544,000–1,200,000 pfu/ml) or MARV (range: 409,000–681,000 pfu/ml) at 7 days post-challenge. These data indicated that GP is the critical protective antigen in the VLPs, and that VP40 may only be required to obtain the filamentous VLP structures, supporting previous observations about GP [9,11].

### 3.3. Pan-filovirus VLP vaccine protects against both MARV and EBOV lethal challenge

Because broad protection against both EBOV and MARV was not provided by the hybrid e/m- and m/e-VLPs, we sought to determine whether a mixture of eVLP and mVLP administered at the same time would protect guinea pigs against lethal challenge with both EBOV and MARV. To this end, animals were vaccinated once with a vaccine composed of an equal mixture of eVLPs and mVLPs and challenged with a lethal dose (~1000 pfu) of either EBOV or MARV. Before challenge, the guinea pigs vaccinated with eVLP and mVLP elicited high antibody titers against both EBOV and MARV (Fig. 4). The titers generated to the homologous antigen were similar to those developed by animals vaccinated with eVLP or mVLP alone, indicating that vaccinating with both antigens at the same time did not interfere with their ability to initiate humoral responses to the individual antigens (Fig. 4).

As shown in Fig. 5, vaccination with the pan-filovirus vaccine, comprising a mixture of eVLP and mVLP, conferred high levels of protection against a lethal challenge of EBOV (nine survivors out of 10 vaccinated guinea pigs) or MARV (10 survivors in 10 vaccinated guinea pigs), which was significant when compared to animals vaccinated with adjuvant alone ( $\rho = 0.0014$  or  $0.0026$ , respectively). The robust protection observed following vaccination with the mixture of eVLP and mVLP was similar to the protection observed in the groups of animals vaccinated with eVLP or mVLP alone and challenged with the homologous virus. Vaccination with adjuvant alone or the heterologous VLPs resulted in poor survival after lethal filovirus challenge (Fig. 5). All the VLP-vaccinated guinea pigs that survived lethal challenge did not have detectable circulating virus 7 days after challenge, unlike the guinea pigs that succumbed to disease (data not shown). Guinea pigs that survived challenge, including



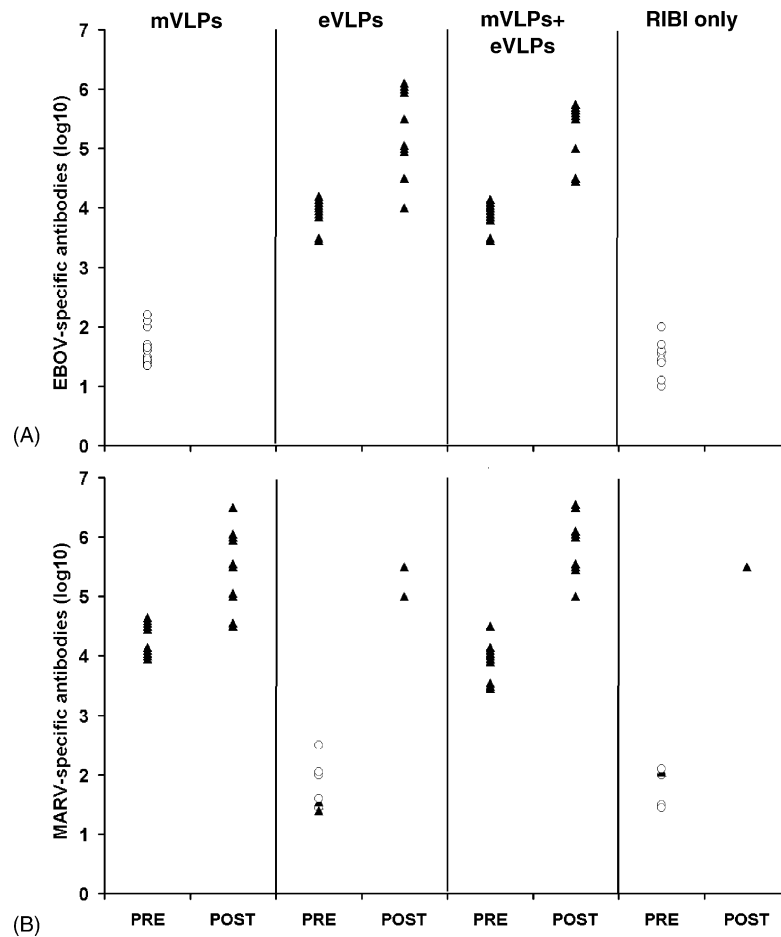


Fig. 4. Serum antibody responses to EBOV and MARV after VLP vaccination. Strain 13 guinea pigs were vaccinated once with eVLPs, mVLPs, or an equal mixture of eVLPs and mVLPs in RIBI adjuvant. Control guinea pigs were vaccinated with RIBI adjuvant alone. Serum samples from the guinea pigs were obtained immediately before (PRE) or 28 days post-challenge (POST). Total serum (A) anti-EBOV or (B) MARV antibodies were measured by ELISA. Antibody titers were measured in serum from individual guinea pigs and the results are graphed as the individual endpoint titers for each guinea pig in each group ( $n = 5-10$  per group). Guinea pigs that survived lethal challenge with (A) EBOV or (B) MARV are indicated by the closed triangles, and those that died are depicted by open circles.

naive animals, demonstrated an increase in their antibody titers indicating that they were exposed to the virus (Fig. 4).

#### 4. Discussion

We sought to develop a pan-filovirus vaccine using VLPs that could protect against multiple filovirus infections. As a first approach toward generation of pan-filovirus vaccines, we produced hybrid VLPs containing heterologous GP and VP40. These hybrid VLPs were useful in determining that the homologous GP, but not VP40, was required and sufficient for protection against lethal challenge with homologous virus in guinea pigs. However, the hybrid VLPs did not provide broad protection against both EBOV and MARV, so we developed a pan-filovirus vaccine comprising a mixture of eVLP and mVLP. This pan-filovirus vaccine induced strong humoral immune responses, similar to vaccination with eVLP or mVLP alone. Encouragingly, the multivalent VLP vaccine

provided almost complete protection ( $\geq 90\%$ ) against lethal challenge with either EBOV or MARV.

While MARV and EBOV are both members of the family Filoviridae, they have been classified in different genera and exhibit very little similarity at the amino acid level, with the GP and VP40 proteins having less than 30% identity between EBOV-Zaire and MARV-Musoke strains [2]. The incorporation of MARV GP has previously been shown onto 'wild-type' VLPs containing all seven structural EBOV proteins [32]. However, it was unknown whether GP and VP40 alone from the heterologous EBOV and MARV would associate within a cell, bud from the lipid rafts, and form functional VLPs without the presence of the other structural proteins. Here, we demonstrate that GP and VP40 from the genetically distinct viruses, EBOV-Zaire and MARV-Musoke, were able to co-associate and form VLPs. Furthermore, these hybrid VLPs exhibited morphological characteristics similar to live EBOV and MARV, as well as to Ebola and Marburg VLPs. The elements required for filovirus assembly are

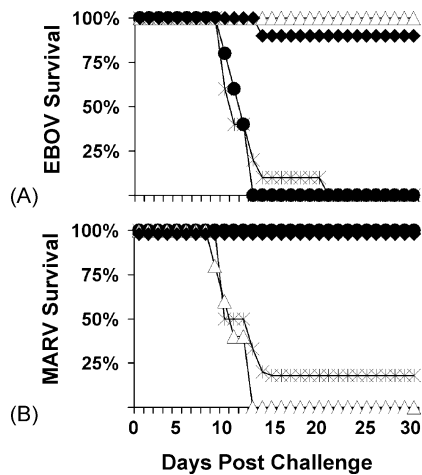


Fig. 5. Pan-filovirus VLP vaccine protects guinea pigs against both EBOV and MARV challenge. Strain 13 guinea pigs were vaccinated once with 100  $\mu$ g of eVLP (open triangle), mVLP (filled circle), or an equal mixture of both eVLP and mVLP (filled diamond), in RIBI adjuvant or RIBI adjuvant alone (star). The vaccinated guinea pigs were challenged with 1000 pfu of guinea pig-adapted EBOV-Zaire (A) or MARV-Musoke (B) virus 28 days post-vaccination. Results are plotted on Kaplan–Meier survival curves and presented as the percent survival for each vaccination group ( $n=5-10$  per group).

only beginning to be unraveled; however, we found that the generation of VLPs provides a useful tool to safely and easily dissect the cellular and viral requirements for assembly [22,30]. Because VP40 and GP naturally target the cellular lipid rafts [22,34], it is unknown at this time whether these molecules specifically interact to form VLPs, or whether it is a consequence of their localization to the same compartments within the cell. However, these data suggest that despite the limited homology, both viruses use similar mechanisms for assembly and release of filamentous structures.

Our finding that GP is sufficient and required for homologous protection is supported by previous studies showing that an immune response to GP is adequate for protection. Administration of MARV GP presented as a VRP or DNA vaccine successfully protected cynomolgus macaques from lethal MARV challenge (100 or 66%, respectively) [9,11,17]. Similarly, EBOV GP presented in a prime–boost strategy using DNA and adenovirus vaccines, protected monkeys from EBOV infection [20]. A VRP vaccine expressing GP protected mice and guinea pigs from lethal EBOV infection, but it was not sufficient to protect cynomolgus macaques from lethal EBOV infection [8,12,35]. Therefore, our findings further emphasize the essential role of GP in providing protective immunity against filoviruses and indicate the requirement for the relevant GP in a pan-filovirus vaccine. Further testing of our multivalent vaccine consisting of proteins derived from EBOV-Zaire and MARV-Musoke is required to determine if broad cross-protection against other strains of EBOV and MARV is provided by vaccination with eVLP and mVLP. In guinea pigs, mVLPs derived from MARV-Musoke, are able to broadly protect against MARV-Musoke, -Ravn, and -Ci67 infection (manuscript in preparation). We are also examining

the protective efficacy of multivalent VLPs containing GP from multiple filovirus strains generated in particles containing a single VP40 molecule as another candidate for broad protection against all known strains of EBOV and MARV.

The GP on the surface of the Ebola or Marburg virion is comprised of disulfide-linked GP1 and GP2 subunits, which are generated by proteolytic cleavage. For both EBOV and MARV, vaccination with either GP1 or GP2 expressed in a VLP backbone is sufficient for protection against homologous viral challenge (unpublished data). Further, monoclonal antibodies directed against either GP1 or GP2 confer protection from EBOV infection in mice [36]. Ongoing studies are focused on the requirements for GP1 and GP2 in VLP-mediated protection by generating and examining the protective efficacy of heterologous fusions of GP1 and GP2 from EBOV and MARV on a single VP40 backbone. A single component VLP-based multivalent vaccine would be preferable for broad protection against lethal infection with multiple filovirus strains.

Vaccination with a mixture of eVLP and mVLP induced high levels of filovirus-specific serum antibodies, similar to those induced by vaccination with eVLP or mVLP alone. Therefore, concurrent vaccination with eVLP and mVLP did not quench the immune response to the individual viruses. While a single vaccination with eVLP or mVLP induced strong humoral responses to the homologous antigen, there were only negligible levels of antibodies that recognized the heterologous antigen (Fig. 4). Boosting with the homologous VLP results in a slight increase (10- to 30-fold) in antibody responses towards the heterotypic virus [25]. However, the heterotypic responses induced by eVLP or mVLP vaccination alone are not sufficient to protect against lethal infection with heterologous virus [25]. Administration of repeated doses of a mixture of eVLP and mVLP, or alternating vaccinations with eVLP and mVLP may drive stronger heterotypic immune responses. A recent report showed that boosting papillomavirus-immune mice with chimeric papillomavirus VLPs could overcome inhibition of antigen presentation due to the presence of neutralizing antibodies [37]. Administration of the chimeric VLPs augmented both cellular and humoral homotypic and heterotypic responses, which could lead to protection against broader papillomavirus infections [37]. Therefore, altering the vaccine schedule or boosting with alternating VLP types or chimeric VLPs may broaden the heterotypic immune responses and increase protection against the multiple strains of EBOV and MARV.

Others and we have noted that in both rodents and non-human primates, ineffectual vaccination can cause an accelerated filovirus disease progression and “early-death” phenomenon [11,23,25,38]. In fact, we have observed that vaccination with eVLP appeared to decrease the time to death following MARV challenge, when compared to control guinea pigs [25]. A similar, potentiated “early-death” phenomenon was observed in MARV-immune mice challenged with EBOV, and inactivated MARV-vaccinated guinea pigs challenged with MARV [23,38]. Ineffectual

MARV vaccination of monkeys can also result in a decreased time to death compared to unvaccinated monkeys following MARV challenge [11,38,39]. However, in this set of experiments, we did not observe accelerated disease symptoms or lethality in VLP-vaccinated guinea pigs challenged with heterologous virus (Table 1). This difference in our current work may be due to administration of only a single dose of vaccine, compared to the use of multiple vaccine doses in our previous work. We feel it is likely that the induction of poor homotypic or heterotypic immune responses augments filovirus pathogenesis. A single VLP vaccination seems to be sufficient to induce protective immunity against homologous challenge, but does not induce more severe disease upon challenge with a heterologous virus.

In summary, our data demonstrated the ability of a Marburg and Ebola VLP-based vaccine to induce strong antibody responses that correlated with protection from EBOV and MARV challenge. Vaccination with this multivalent VLP vaccine protected guinea pigs from viremia and death caused by a lethal challenge with EBOV or MARV. Using hybrid VLPs consisting of heterologous GP and VP40 molecules from EBOV and MARV, we show that GP is required and sufficient to protect against a lethal filovirus challenge. The correlates and mechanisms of protective immunity generated by GP and other filovirus proteins are not fully understood at this time; however, elucidation of these markers are critical for eventual FDA licensing of filovirus vaccines, as efficacy trials of EBOV and MARV vaccines are unlikely. While VLPs are a promising candidate vaccine, the demonstration of protective efficacy in non-human primates is our current priority. In general, VLPs are unique when considering their advantages, including safety, ease of production and administration, lack of interference by an immunodominant vector backbone, concern of prior vector immunity, and the presentation of the relevant filovirus antigens in their native form.

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