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SUPPLEMENT ARTICLE

Role of Antibodies in Protection Against Ebola Virus in Nonhuman Primates Immunized With Three Vaccine Platforms

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Background. Several vaccine platforms have been successfully evaluated for prevention of Ebola virus (EBOV) disease (EVD) in nonhuman primates and humans. Despite remarkable efficacy by multiple vaccines, the immunological correlates of protection against EVD are incompletely understood.

Methods. We systematically evaluated the antibody response to various EBOV proteins in 79 nonhuman primates vaccinated with various EBOV vaccine platforms. We evaluated the serum immunoglobulin (Ig)G titers against EBOV glycoprotein (GP), the ability of the vaccine-induced antibodies to bind GP at acidic pH or to displace ZMapp, and virus neutralization titers. The correlation of these outcomes with survival from EVD was evaluated by appropriate statistical methods.

Results. Irrespective of the vaccine platform, protection from EVD strongly correlated with anti-GP IgG titers. The GP-directed antibody levels required for protection in animals vaccinated with virus-like particles (VLPs) lacking nucleoprotein (NP) was significantly higher than animals immunized with NP-containing VLPs or adenovirus-expressed GP, platforms that induce strong T-cell responses. Furthermore, protective immune responses correlated with anti-GP antibody binding strength at acidic pH, neutralization of GP-expressing pseudovirions, and the ability to displace ZMapp components from GP.

Conclusions. These findings suggest key quantitative and qualitative attributes of antibody response to EVD vaccines as potential correlates of protection.

Keywords. adenovirus; Ebola virus; immune correlate; vaccine; virus-like particles.

Over the past 50 years, filoviruses, primarily Ebola virus (EBOV), have caused multiple human outbreaks with high fatality rates. The 2014–2016 EBOV disease (EVD) epidemic in West Africa, caused by 2 new isolates of Zaire EBOV (Mak-1 [GenBank accession no. KP178538] and Mak-2 [GenBank accession no. KP240932]), led to 28 616 infections and 11 310 deaths (http://www.who.int/csr/disease/ebola/en/). During the 2014–2016 EVD outbreak, a vaccine based on replication-competent vesicular stomatitis virus (VSV) lacking G protein and expressing EBOV glycoprotein (rVSVΔG-ZEBOV-GP) was tested in a ring vaccination phase III efficacy trial. This trial was reported to be 100% efficacious in preventing transmission of EVD among vaccinated adults, indicating the prospect of an effective prophylactic EBOV vaccine [1]. Other virus vector-based vaccines, including chimpanzee adenovirus vector [2, 3], and a prime boost regimen of adenovirus-vectored vaccine with recombinant modified vaccinia Ankara (MVA) [4] expressing full-length EBOV GP have also been tested for safety and immunogenicity in healthy individuals. Furthermore, other vaccine platforms such as virus-like particles (VLPs) expressing EBOV GP, matrix protein VP40, and the nucleoprotein (NP) [5, 6], a rabies-based inactivated vaccine expressing EBOV GP [7], a GP-expressing Venezuelan equine encephalitis virus-based replicon [8], and replication-competent VSV-based vaccines [9, 10] have shown remarkable efficacy in preclinical challenge studies in nonhuman primates (NHPs).

Despite these advances, the mechanisms of vaccine-mediated protection and correlates of protective immunity against EVD remain poorly understood. Vector-based vaccines such as adenovirus [3] and VSV [11] induce both strong GP-specific CD4 and CD8 T-cell and antibody responses. Virus-like particle vaccination induces dominant NP-specific T-cell and GP-targeted antibody responses [6, 12]. Recent advances in immunotherapy of EVD with polyclonal convalescent macaque immunoglobulin (Ig)G [13] or monoclonal antibody (mAb) cocktails such as ZMapp [14], ZMab [15], MB-003 [16] indicate that antibodies can protect against filoviruses, supporting a vaccine approach focused on generation of antibody responses. However, it is not

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known which attributes of vaccine-elicited antibody response are a reliable predictor of survival in EVD.

Studies in knockout mice indicate that CD8 T cells are absolutely required, whereas both CD4 and B cells partially contribute to protective efficacy of EBOV VLPs expressing GP, NP, and matrix protein (VP40) (referred to hereafter as triple VLP) [17]. Several studies in rodents [18, 19] and NHPs demonstrated the protective efficacy of triple VLPs (reviewed in [12]). Virus-like particle-immunized NHPs exhibit strong T-cell responses to NP and antibody responses to GP, whereas the T-cell response to GP is less pronounced [6]. However, the requirement for NP-directed responses in the efficacy of EBOV VLPs remains unknown.

In this study, we generated VLPs expressing only GP and VP40 (referred to as double VLP hereafter) to simplify the VLP vaccine and evaluated its efficacy in comparison to triple VLPs. We found that vaccination with the double VLPs, despite their induction of higher antibody titers, provided less protection than vaccination with triple VLPs. Protection in double VLP-vaccinated NHPs was strictly dependent on anti-GP antibody titer, and a clear cutoff for protective IgG enzyme-linked immunosorbent assay (ELISA) titer could be defined for this vaccine platform. Further examination of a larger number of NHPs vaccinated with either VLPs or adenovirus-vectored GP showed highly significant correlation between survival and anti-GP antibody titers, neutralization, and the ability of the serum antibodies to bind to GP at acidic pH. In contrast, antibody titers against NP or VP40 exhibited no correlation with protection similar to previous observations in mice [20] and guinea pigs [21]. These findings strongly suggest that anti-GP antibodies are reliable predictors of protection in NHPs; however, the pattern of antibody response and its correlation with protection varies depending on the vaccine platform.

MATERIALS AND METHODS

Production of Ebola and Marburg Virus-Like Particles

Two types of VLPs were produced using baculovirus expression system in insect cells: (1) VLPs expressing the glycoprotein, the matrix protein, and (2) the NP (triple VLPs) and VLPs expressing only GP and VP40 (double VLPs), as we have described previously in detail [18, 22, 23]. Double and triple VLPs were characterized using a battery of assays including total protein (BCA), identity (Western blotting using mouse monoclonal or epitope-specific rabbit antibodies recognizing EBOV GP, VP40, and NP), electron microscopy, and endotoxin content, as previously described [18, 22, 23].

Vaccination of Nonhuman Primates

Vaccination of cynomolgus macaques (4 to <9 kg; Worldwide Primates, Miami, FL) was performed at US Army Medical Research Institute of Infectious Diseases ([USAMRIID] Frederick, MD) or Covance (Denver, PA). The animals were found to be antibody-negative for filovirus, simian T-lymphotropic virus, simian immunodeficiency virus, and herpes B virus before study initiation. For the NHP study described here, cohorts of 2–5 NHPs (Table 1) were vaccinated via intramuscular (i.m.) injection with EBOV "double" or "triple" VLPs, supplemented with Alhydrogel, RIBI (Corixa, Hamilton, MT) or QS-21 adjuvant (Antigenics, Lexington, MA), or no VLP (QS-21 only) on study days 0 and 42. All injection sites were observed daily for 7 days after each dose and weekly thereafter. Each site was scored for redness and swelling according to the method of Draize et al [24]. Blood samples were obtained under anesthesia from the femoral veins of monkeys on study days −1, 14, 42, 56, and 63 and processed within 4–6 hours. Plasma or serum samples were aliquoted and frozen until analysis.

The vaccination portions of the studies were conducted in compliance with the current standard operating procedures (SOPs) of Covance Research Products, Inc. (Denver, PA and Alice, TX) and with any applicable amendments. All planned changes or revisions of the study protocols at Covance were written in the form of a protocol amendment, signed by the Study Director and the Sponsor, dated and maintained with the protocol. All procedures were in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. In the opinion of the Sponsor and Study Director, the studies conducted at Covance did not unnecessarily duplicate any previous work (US Department of Agriculture [USDA] Regulation: Animal Welfare Regulations 9 Code of Federal Regulations [CFR], Subchapter A). Both Covance facilities are Association for the Assessment and Accreditation of Laboratory and Care International (AAALAC) accredited.

Certain portions of this animal research were conducted according to research protocols approved by the USAMRIID Institutional Animal Care and Use Committee (IACUC). Work at USAMRIID was performed 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, National Research Council, 2011. The USAMRIID is fully accredited by AAALAC. All challenge studies and necropsies were conducted under maximum containment in an animal biosafety level (BSL)-4 facility at USAMRIID.

Studies performed at University of Texas Medical Branch (UTMB) were conducted in compliance with the approved study protocol approved by the institutional IACUC, as well as applicable UTMB SOPs and any applicable amendments. All planned changes or revisions of the study protocol were documented. All procedures in this study were conducted in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory. The UTMB Animal Resource Center is AAALAC accredited, and UTMB operates as follows: to comply with the USDA Animal

Table 1. Summary of the Study Designs and Antibody Titers of NHPs Vaccinated With Triple VLPs (GP/VP40/NP) or Double VLPs (GP/VP40) (Study ID: IBT_01220)[a]

Group No., Vaccine, Dose	NHP ID No.	Time of Death (dpi)	Peak Viremia (dpi; pfu/mL)	Peak Viremia (dpi; GE/mL)	Antibody Titers (AU/mL)		
					GPΔTM	GPΔMuc	VP40
1 Triple VLP, 3 mg	AR791	Survived	<LOD	Day 7; 1.0E+04	1553	1057	2842
	AP229	Survived	<LOD	<LOD	412	770	3461
	AR920	Survived	<LOD	<LOD	1324	1267	2258
2 Double VLP, 3 mg	AT233	7	Day 7; 4.25E+07	Day7; 2.6E+10	645	732	2802
	AR280	8	Day 7; 2.95E+06	Day 7; 6.6E+09	573	1011	757
	SZ77	Survived	<LOD	Day 7; 4.2E+05	970	1218	1382
	AR960	10	Day 10; 6.80E+06	Day 10; 1.2E+07	938	1551	3545
	BM669	Survived	<LOD	Day 7; 9.8E+06	2067	2149	6606
3 Double VLP, 200 µg	AP601	7	Day 7; 3.50E+07	Day 7; 7.9E+08	634	672	5150
	AP360	Survived	<LOD	<LOD	3069	2428	1412
	AR546	10	Day 10; 5.50E+01	Day 10; 1.2E+07	1392	1815	5113
	AT164	Survived	<LOD	<LOD	2218	2764	4332
4 Double VLP, 75 µg	YS87	8	Day 8; 4.80E+04	Day 7; 3.2E+06	575	613	350
	AP633	9	Day 7; 7.50E+06	Day 7; 2.3E+10	612	569	2470
	AR965	10	Day 7; 3.60E+01	Day 7; 7.5E+05	1170	1045	3593
5 Double VLP, 25 µg	AT105	Survived	<LOD	<LOD	1476	2543	2525
	AT3	Survived	<LOD	<LOD	1882	2024	4950
	AT237	9	Day 7; 5.45E+06	Day 7; 1.1E+09	835	635	8039
6 Control	AP355	6	Day 6; 1.45E+07	Day 6; 1.5E+10	1.2	5.8	44.1
	AR919	6	Day 6; 4.50E+07	Day 6; 6.3E+10	1.2	5.8	92.6

Abbreviations: dpi, day postinfection; GE, genome equivalent; LOD, limit of detection; NHP, nonhuman primates; pfu, plaque-forming units; qRTPCR; quantitative reverse-transcription polymerase chain reaction; VLP, virus-like particles.

[a]All vaccinations were performed with QS-21 as adjuvant on days 0 and 42. Antibody titers are from the last bleed before challenge (3–4 weeks after the last vaccine). Peak viremia levels from plaque assay and qRTPCR analysis are shown.

Welfare Act (Public Law 89–544) as amended by PL91-579 (1970), PL94-279 (1976), and 45 CFR37618 (6-30-80); to comply with Health Research Extension Act of 1985 (Public Law 99–158); follows the Public Health Service Policy on Humane Care and Use of Laboratory Animals (revised September 1986); and follows the Guide for the Care and Use of Laboratory Animals, Department of Health, Education, and Welfare (National Institutes of Health) 85-23. The UTMB is a registered Research Facility under the Animal Welfare Act.

Protein Production and Determination of Imunnoglobulin G Antibody Titers Against Glycoprotein and Matrix Protein VP40

Ebola virus GP with the transmembrane domain deleted (GPΔTM) or the mucin-like domain (MLD) and transmembrane domain-deleted ectodomain (GPΔMuc) were produced in insect cells and purified by chromatography as previously described [5]. Production of VP40 in *Escherichia coli* was previously described [25]. Details of production of NP in *E coli* is provided in Supplemental Methods. A serology ELISA method was developed to determine the serum IgG titers against GPΔTM, GP-ΔMuc, VP40, and NP, as detailed in the Supplemental Methods. To determine the ability of serum antibodies to bind GP at acidic pH and competition with ZMapp, the ELISAs were performed in acidic buffer or in presence of ZMApp components as detailed the Supplemental Methods.

Ebola Virus Seed and Nonhuman Primate Challenge Study

The strain of EBOV used in this study was isolated from an infected patient in the 1995 outbreak in Kikwit, Zaire at the Centers for Disease Control and Prevention (Atlanta, GA). The virus stock used at USAMRIID was propagated 2 passages in Vero cells and 4 passages in VeroE6 cells and had a titer of 1.4×10^8 plaque-forming units (pfu)/mL. The virus stock used at UTMB was propagated 2 passages on VeroE6 cells and had a titer of 5.25×10^5 pfu/mL. For studies performed at USAMRIID, animals were transferred from BSL-2 to BSL-4 ≥48 hours before challenge. In the remaining studies, animals were transferred from Covance to the UTMB BSL-4 facility 7 days before challenge. Animals were challenged via i.m. injection with EBOV on study day 70 (referred to as 0 days postinfection [dpi]) with 1000 pfu in 0.5 mL phosphate-buffered saline i.m. in the thigh. The leg in which the virus was injected was recorded for observation purposes. A scoring sheet was used for assisting in determining the time of euthanasia depending on clinical signs (eg, respiratory distress, weakness, inability to move when prodded, hemorrhage, macular rash, etc). Animals were euthanized after deep anesthesia (~9 mg/kg i.m. injection of Telazol) by intracardiac administration of Euthasol (~1 mL/4.5 kg) in accordance with the 2007 American Veterinary Medical Association Guidelines on Euthanasia. Euthanasia was performed by qualified personnel. Death was verified by the absence of a palpable

heartbeat at no less than 5 minutes post-exsanguination. All procedures were approved by USAMRIID, Covance, or UTMB IACUC. Blood samples were collected from animals on 0, 3, 5, 7, 21, and 28 dpi and used for blood chemistry, hematology, and viral load analysis as described in Supplemental Methods.

Historical Serum Samples

Serum samples from previous vaccination studies performed at USAMRIID and Public Health Agency of Canada were obtained. All of these animal studies were performed under approval of the local IACUC committees. Historical samples were stored at −80°C until use in the serology assays.

Vesicular Stomatitis Virus-Pseutotype Neutralization Assay

Neutralizing potency of the sera was tested in a VSV-pseudotype system expressing EBOV GP as described previously [26] and detailed in the Supplemental Methods.

Statistical Methods

The data analyses were from NHP studies in which animals received different vaccine types. The sera from the last time point before challenge with the virus were used for analysis, as well as survival outcome and day of death. For survivors, the day of death is censored at 28 dpi (study termination). The parameters for analysis were as follows: (1) serum antibody titer against Ebola GPΔTM; (2) serum antibody titer against Ebola GPΔMuc; (3) serum antibody titer against Ebola VP40 (double and triple VLP groups only); (4) serum antibody titer against Ebola NP (triple VLP group only); (5) ratio of the binding of each serum diluted at 1:100 to GP at pH 4.5 divided by the binding of the same serum sample to GP at pH 7.4; (6) ratio of the binding of each serum to GP at pH 5.5 divided by the binding of the same serum sample to GP at pH 7.4; (7) percentage of displacement of ZMapp by each sample measured at a single dilution (NOTE: negative values indicate that the serum increased the binding of ZMapp to GP instead of competing with it, positive values show competition); (8) percentage of neutralization of a pseudotype virus carrying Ebola GP at a 1:25 dilution of the immune serum.

The number and percentage of NHPs were tabulated by survival outcome at 28 dpi. Each of the above-listed parameters was summarized descriptively by survival outcome (dead vs alive) and compared statistically using 2-sided hypothesis tests without adjustment for multiplicity. Nominal *P* values are presented.

Before the above analyses, the data were assessed for normality on both the original and log-transformed scale. If deviations from the normality assumption were detected, the comparison of study parameters by survival outcome was performed using Wilcoxon rank-sum tests, with the 2-sample 2-sided *t* test performed on log-transformed data as a sensitivity analysis. It was noted, based on visual inspection of the normal quantile plots, that the titer endpoints deviate from normality inconsistently on both the original and log scales. For percentage of displacement of ZMapp and percentage of neutralization, the original scale includes negative values; therefore, the log-transformation was not appropriate. However, the normality assumption appears to be appropriate on the original scale for these 2 endpoints (Supplementary Table S2). As a result, comparisons for each of the endpoints by survival outcome were performed using Wilcoxon rank-sum text as well as by 2-sample, 2-sided *t* test on the log scale as a sensitivity analysis, with the exception of the percentage of displacement of ZMapp components and percentage of neutralization endpoints, for which the *t* test was performed on the original scale.

The time to death (in days) was analyzed to assess the relationship of each parameter listed above with survival outcomes. Estimates of median survival time along with corresponding 95% confidence intervals were summarized by vaccine type where calculable (ie, at least 50% of NHPs with outcome of death required for calculation of median), and Kaplan-Meier survival curves were plotted. Day of death was censored at the time of last assessment for NHPs who survived the challenge (day 28 postinfection). Separate Cox proportional hazards models were fitted to the time to death data, with each parameter listed above included as a continuous covariate. Hazard ratios and 95% confidence intervals were presented.

RESULTS

Comparative Efficacy of Ebola Virus Virus-Like Particles Vaccine in Presence or Absence of the Nucleoprotein

We have previously demonstrated that administration of 2 or 3 doses of triple VLP vaccine along with RIBI adjuvant in cynomolgus macaques provides full protection against lethal challenge with EBOV [5, 6, 12]. Nucleoprotein is not required for the formation of EBOV VLPs [22], but it can increase the yield of VLP [27, 28] and provide additional CD8 T-cell epitopes for enhanced cell-mediated immunity (CMI) [6, 29–31]. To examine whether double VLPs are as immunogenic and induce similar levels of protection as triple VLPs, NHPs were randomized into 6 groups and vaccinated twice at days 0 and 42 (Table 1). Group 1 received triple VLPs at 3-mg dose and groups 2–5 received a range of double VLP doses from 25 μg to 3 mg, along with 100 μg of QS-21 as adjuvant on days 0 and 42. Immunizations did not affect the body weight, leukocyte counts, hematological parameters, or serum chemistry, except for mild and transient erythema and swelling at the injection site, presumably related to the adjuvant (data not shown).

Animals were bled on days 0, 14, 42, 56, and 63 for serological analysis before being challenged on day 70, and the antibody response was measured by ELISA against GPΔTM, GPΔMuc, and VP40 (Table 1). As shown in Figure 1, immunization with 1 dose of triple and double VLPs induced a moderate antibody response to GP, and this response was boosted by approximately 10-fold upon second vaccination. Unexpectedly, no correlation was observed between the double VLP vaccine

dose administered and the resulting magnitude of the antibody response.

Animals were challenged on study day 70 with 1000 pfu of EBOV (913 pfu based on back-titering). Consistent with our previous reports [5, 6], all 3 triple VLP-vaccinated monkeys survived the challenge with no abnormal clinical symptoms; in contrast, 9 of the 15 NHPs vaccinated with double VLP succumbed to infection.

Viremia was measured by quantitative reverse-transcription polymerase chain reaction (qRTPCR) and standard plaque assays. Control animals became viremic on day 3 (based on qRTPCR) or day 4 (based on plaque assay) with peak levels of 1.45E+07 and 4.50E+07 pfu/mL or 1.5E10 and 6.3E10 GE/mL before death at 6 dpi (Table 1). None of the surviving animals showed any viremia detectable by plaque assay, and all fatal cases showed onset of viremia at 5 or 6 dpi and peaking on the day of death (Table 1). However, virus was transiently detectable by qRTPCR in 2 surviving animals in double VLP groups and 1 animal in the triple VLP group (Table 1).

Blood chemistry data are shown in Supplementary Figure S1. Glucose levels were generally maintained in surviving animals but decreased in fatal cases, likely due to anorexia. Nonsurvivors displayed unchanged or moderately increased blood urea nitrogen and creatinine in blood after infection. Only a single animal (AT233) showed increased uric acid. Levels of calcium, albumin, and total protein remained unchanged in survivors, whereas most nonsurvivors showed slightly reduced levels. Alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and gamma glutamyltransferase levels remained stable in survivors except for SZ77 that showed slight elevation and was sick through day 12, whereas the levels for these enzymes were elevated in most nonsurvivors. Amylase remained stable among survivors, but it varied in nonsurvivors, with 5 animals showing 2- to 7-fold increase and 3 animals showing 30%–50% decreased levels. All surviving animals had normal levels of C-reactive proteins (CRP) except for SZ77, whereas all nonsurvivors had greatly increased levels of CRP. Most animals exhibited a spike in white blood cell count after challenge, most notably among fatal cases, whereas hematocrit and hemoglobin levels remained largely stable. Percentage of lymphocytes was significantly decreased among nonsurvivors. Platelet counts decreased in most animals and was more pronounced among nonsurvivors.

Although survival in animals vaccinated with double VLPs did not correlate with the vaccine dose (Figure 2A), it did correlate directly with antibody titers against GP. All double VLP-vaccinated, surviving animals had an anti-GPΔTM antibody titer of higher than 1400 AU/mL, except for 1 animal (SZ77) with an antibody titer of 970 AU/mL, and this animal was sick through day 12 before recovering (Figure 2B). All animals that succumbed to EVD had an anti-GPΔTM titer of less than 1400 AU/mL, and the antibody titer appeared to correlate with the day of death (Figure 2B). This correlation was more pronounced for antibodies to GPΔMuc with a cutoff of ~1900 AU/mL separating survivors from nonsurvivors except SZ77, which was sick through day 12 (Figure 2C).

The GPΔTM antibody titers of all 3 animals vaccinated with triple VLP were similar to fatal cases of double VLP-vaccinated animals or close to the cutoff level, although all 3 animals survived with no clinical symptoms (Figure 2B). The GPΔMuc titers of the triple VLP-vaccinated animals were far below the apparent cutoff for survival of double VLP-vaccinated animals (Figure 2C). A similar pattern was observed with respect to virus neutralization and fatality using the rVSV-GP pseudotype assay at a 1:200 dilution of the sera. (Figure 2D).

There was no correlation between survival and antibody titer to VP40 (P = .8186, unpaired t test) (Table 1). The requirement for higher anti-GP titer for survival in double VLP-vaccinated animals suggested that, in the triple VLP-vaccinated animals, antibody or T-cell response to NP may be contributing to the protection. Due to unavailability of peripheral blood mononuclear cells from these animals, we were unable to evaluate T-cell

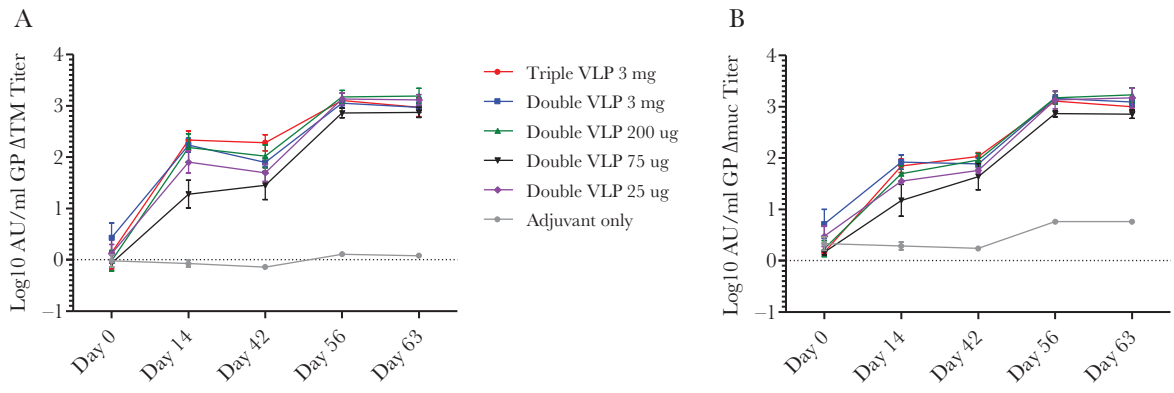

Figure 1. Antibody response to double and triple virus-like particle (VLP) vaccination. Total immunoglobulin G titers was determined against GPΔTM (A) and GPΔMuc (B) in cynomolgus macaques vaccinated with the indicated doses of Ebola virus VLPs along with QS-21 as adjuvant.

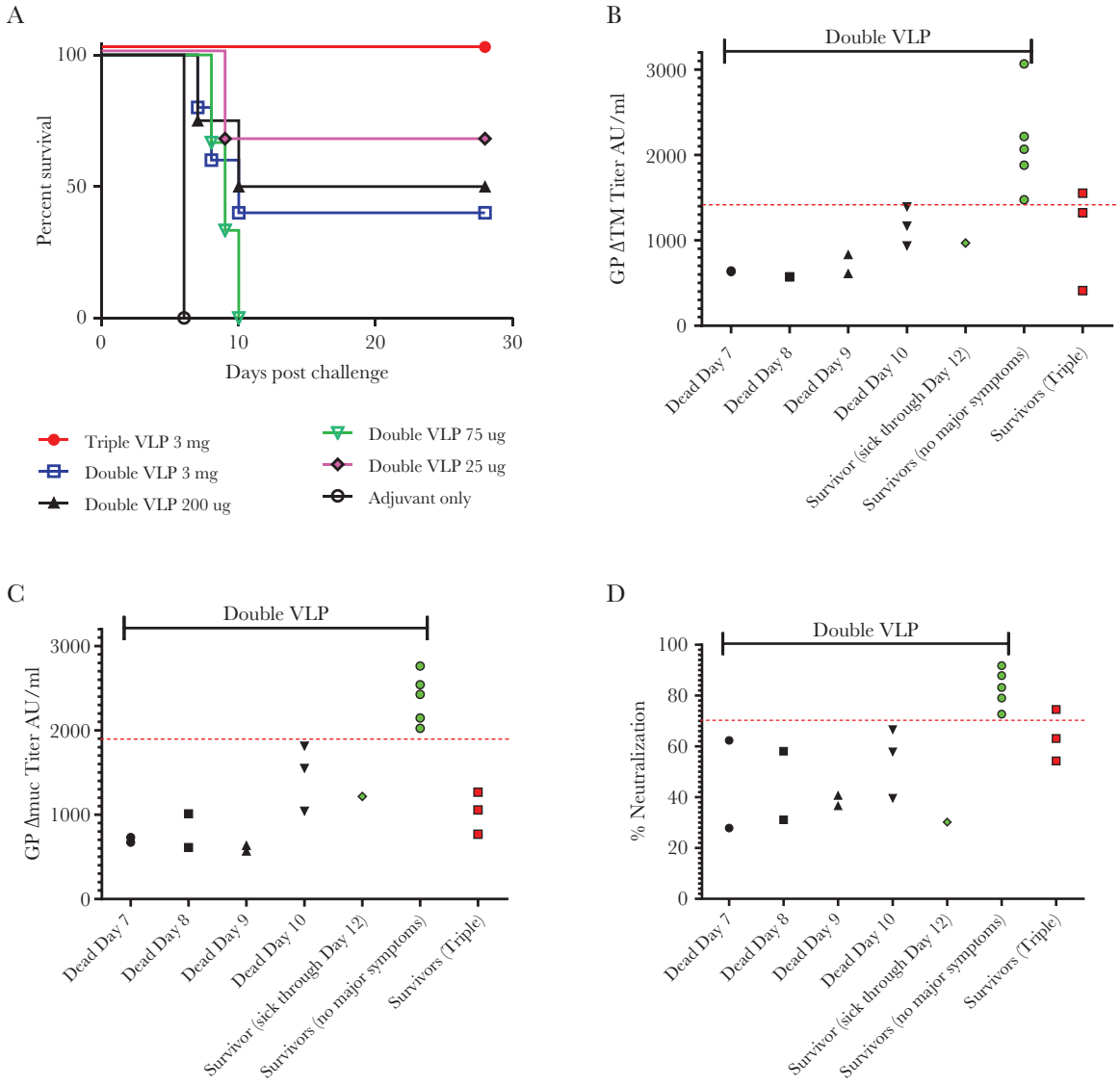

Figure 2. Efficacy of double and triple virus-like particles (VLPs) against Ebola virus (EBOV) challenge in cynomolgus macaques. Survival of macaques vaccinated with VLPs after EBOV challenge was monitored for 28 days (A). Antibody titers against were GPΔTM (B) and GPΔMuc (C) as well as percentage of neutralization (D) are shown for individual animals with the day of death or survivorship indicated on the x-axis. Black symbols signify dead animals and colored symbols indicate the survivors. The red line separates the double VLP-vaccinated survivors from lethal cases with a single exception.

responses. However, we tested the antibody responses to NP in the sera from the 3 triple VLP-vaccinated NHPs using an NP ELISA assay. For this purpose, we used 3 variations of NP proteins fused to maltose binding protein as coating antigen: NP25-457, NP25-739, and full-length NP1-739. Deletion of the first 24 residues in Ebola NP decreases the ability of NP to oligomerize [32], whereas constructs ending at residue 457 represent the shared domain organization of negative sense ribonucleic acid virus-specific region, and the NP458-739 is the filoviral-specific region [33]. As shown in Figure 3A, the animals showed high antibody titers to the full-length and NP25-739 but very low titers against NP25-457, suggesting that the response is primarily directed against the filoviral specific C-terminal domain of NP.

Serological Analysis of Historical Nonhuman Primate Sera

To further evaluate the protective role of GP-specific antibody responses against EVD, we sought to determine the antibody titers in a larger number of sera from vaccinated NHPs in previous studies. Sera from several studies using triple VLP vaccines (Table 2) and sera from a vaccine study using GP-expressing adenovirus along with adenovirus-expressed interferon alpha, a vaccine known to induce strong T-cell responses [34] (Supplementary Table S.3), were collected for this analysis. We determined the antibody titers to GPΔTM, GPΔMuc, VP40, and NP (for triple VLP samples) in these sera (Table 2 and Supplementary Table S.3). Furthermore, we tested these sera for neutralization of rVSV-GP pseudotype assay. Due to limited

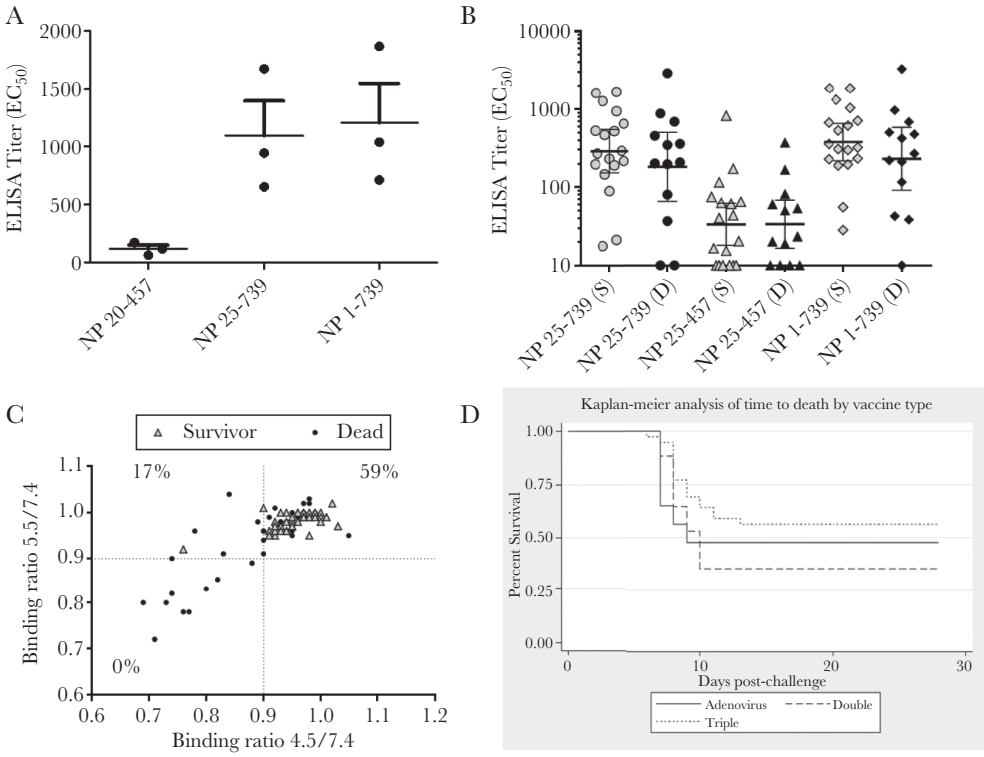

Figure 3. (A) The immunoglobulin G response to 3 nucleoprotein (NP) constructs from the 3 animals in study IBT_01220 vaccinated with triple virus-like particles (VLPs). (B) Antibody response of all triple VLP-vaccinated animals listed in Table 2, stratified by survivors (S) and dead (D). (C) Binding of the sera was determined at pH 4.5 or 5.5 relative to binding at pH 7.4 by enzyme-linked immunosorbent assay (ELISA) and plotted against each other. Sera are stratified based on survival from challenge. The numbers in quadrants represent percentage of survival for each quadrant. (D) Kaplan–Meier survival curve of the animals in the 3 vaccine groups.

amount of sera available, we were unable to perform neutralization on samples from the adenovirus vaccine study, as well as 3 NHP samples from VLP studies (A0023, AP690, and 201033).

Because the GP-receptor interactions occur in the endosomes [35–37], the ability of antibodies to effectively neutralize the virus may relate to the stability of the GP-antibody interaction at acidic pH. To address this question, we also examined the ability of the sera to bind to GP at pH 5.5 and 4.5.

A cocktail of 3 mAbs known as ZMapp has shown remarkable efficacy in NHPs [14]. Induction of antibodies that target the epitopes recognized by ZMapp [38] may be critical for vaccine efficacy. To examine this hypothesis, we also performed a competition ELISA to determine the relative presence of the murine versions of ZMapp component antibodies in the vaccinated NHP sera. These data were subjected to statistical analysis to examine potential correlation with protection from lethal challenge as described below.

Analysis of Survival Outcome

The summary of the survival outcomes by vaccine type is shown in Supplementary Table S.4. As shown in Table 3, overall, titers for GPΔTM and GPΔMuc were significantly higher ($P < .001$) in surviving NHPs compared with NHPs that died; no significant difference in VP40 titers was observed ($P > .05$). Analysis of sera from triple VLP-vaccinated animals showed no significant difference in NP titers between survivors and dead animals (Figure 3B). Results from the *t* test were consistent with those from the non-parametric Wilcoxon rank-sum test for titer endpoints. Binding ratio at pH 4.5/7.4 was significantly higher ($P < .001$) in surviving NHPs compared with NHPs who died; however, the binding ratio at pH 5.5/7.4 was only marginally higher in the surviving NHPs ($P = .05$, Wilcoxon rank sum) (Table 3). As shown in Figure 3C, sera from all but 1 surviving animal (38 of 39) maintained more than 90% of binding to GP at pH of 5.5 or less, whereas 26 of 40 fatal cases showed the same property. Overall, 59% of sera that maintained over 90% binding at both pH 4.5 and 5.5 were from survivors. In contrast, only 17% of the animals with >10% loss of binding at pH 4.5 despite >90% binding at pH 5.5 survived the challenge. None of the 10 animals whose sera lost more than 10% binding at both acidic pH values survived the challenge. These data suggest that the ability of the antibodies to bind GP at low pH is important for protection. Percentage of displacement of ZMapp and percentage of neutralization was significantly higher in surviving NHPs compared with NHPs who died ($P < .05$ and $P < .001$, respectively) (Table 3). Of note, some serum samples increased ZMapp binding resulting in negative displacement values.

Table 2. Summary of the Study Designs and Antibody Titers of NHP Vaccinated With Tripe VLP (GP/VP40/NP) Used for Statistical Analysis[a]

					Antibody Titers (AU/mL)										
Study ID[b]	NHP ID	Day of Death	eVLP	mVLP	GPΔTM	GPΔMuc	VP40	NP 1–739	NP 25–457	NP 25–739	VLP Source	Adjuvant	No. of Vaccinations	Vaccination Schedule (Week)	Reference
VLP No. 2	C250B	Survived	x		920	2147	355	670	10	525	Mam.	RIBI	Two	Week 0 and 6	Unpublished
	305B	9	x		427	720	102	686	10	692					
	E358A	7	x		219	452	531	10	10	10					
VLP No. 3	C573	Survived	x		573	858	433	NT	NT	NT	Mam.	RIBI	Three	Week 0, 6, 12	Unpublished
	91–495	Survived	x		702	706	884	NT	NT	NT					
	120–334	11	x		281	382	173	NT	NT	NT					
VLP No. 5	C0167	Survived	x	x	815	1322	1897	234	44	147	Mam.	RIBI	Three	Week 0, 6, 12	[19]
	C0219	Survived	x	x	1245	2052	1476	196	20	191					
	C0379	Survived	x	x	896	1004	2345	308	17	217					
	C0548	Survived	x	x	370	451	626	323	10	270					
	C1019	Survived	x	x	738	1262	1081	228	62	197					
VLP No. 7	304021	Survived	x	x	229	1803	3663	357	40	231	BV/IC	RIBI	Three	Week 0, 6, 12	Unpublished
	204005	Survived	x	x	249	1530	4515	NT	NT	NT					
	201033	13	x	x	424	2105	4644	NT	NT	NT					
	104003	Survived	x	x	593	2653	4201	NT	NT	NT	BV/IC	QS-21			
	201007	10	x	x	351	1506	3202	969	373	876					
	201013	8	x	x	424	465	4402	271	10	202					
VLP No. 9	502090	Survived	x	x	93	441	1379	28	10	21	BV/IC	QS-21	One		[5]
	502084	6	x	x	19	108	3244	43	19	37					
	504060	Survived	x	x	117	332	3082	1321	10	1267					
	J16	Survived	x	x	387	1761	7373	616	75	531	BV/IC	QS-21	Two	Week 0, 6	
	112713	Survived	x	x	320	1279	9077	299	10	293					
	A0023	Survived	x	x	358	2515	7337	1872	824	1616					
	A0024	Survived	x	x	798	1991	9760	56	10	18	BV/IC	QS-21	Three	Week 0, 6, 12	
	302557	Survived	x	x	356	1483	13 684	532	65	470					
	206345	11	x	x	847	2207	5276	3293	168	2906					
	208411	9	x	x	256	1130	17 577	39	82	10	BV/IC	Alum	Two	Week 0, 6	Unpublished
	204225	Survived	x	x	281	983	18 895	190	15	89					
	107309	9	x	x	124	387	18 250	117	54	80					
	206517	8	x	x	102	437	14 305	NT	NT	NT	BV/IC	Alum	Three	Week 0, 6, 12	
	202419	8	x	x	501	987	4734	NT	NT	NT					
	204535	8	x	x	405	1320	15 036	424	23	349					
IBT_00534	GA759	8	x	x	55	91	1376	503	20	455	BV/IC	QS-21	Two	Week 0, 3	Unpublished
	AR601	8	x	x	43	99	2537	221	10	198					
	GA689	10	x	x	21	35	1418	212	61	208					
	AP690	8	x	x	4	10	2710	478	50	360					
IBT_01220	AR791	Survived	x		1553	1057	2842	1038	172	944	BV/IC	QS-21	Two	Week 0, 6	This report
	AP229	Survived	x		412	770	3461	1869	63	1675					
	AR920	Survived	x		1324	1267	2258	711	116	652					

Abbreviations: BV, baculovirus; eVLP, Ebola virus-like particles; IC, insect cell expression system; Mam., mammalian expression system; mVLP, Marburg virus-like particles; NT, not tested; USAMRIID, US Army Medical Research Institute of Infectious Diseases; UTMB, University of Texas Medical Branch; VLP, virus-like particles.

[a]Antibody titers are from the last bleed before challenge.

[b]Studies with VLP designation were entirely performed at USAMRIID. Studies with IBT designation were performed at Covance (vaccination) and UTMB (challenge).

Table 3. Summary Statistics by Survival Outcome

Variable	Survival Outcome	N	Mean (SD)	Median	Range	*P* Value
Ebola GP-ΔTM titer	Alive	39	1062.34 (881.00)	798.15	93.21–3879.08	<.001[a]
	Dead	39	337.49 (343.62)	202.51	4.45–1391.59	<.001[b]
	Overall	78	699.91 (757.87)	425.47	4.45–3879.08	
Ebola GP-ΔMuc titer	Alive	39	1253.00 (790.20)	1218.49	28.56–2764.40	<.001[a]
	Dead	39	582.80 (622.40)	437.11	2.29–2207.26	<.001[b]
	Overall	78	917.90 (782.99)	751.01	2.29–2764.40	
Ebola VP40 titer	Alive	28	4351.16 (4299.68)	2961.99	354.90–18 895.37	.904[a]
	Dead	28	5202.59 (5168.83)	3568.98	101.79–18 250.06	.544[b]
	Overall	56	4776.87 (4730.29)	3352.22	101.79–18 895.37	
Binding ratio pH 4.5/7.4	Alive	39	0.95 (0.044)	0.95	0.76–1.03	<.001[a]
	Dead	40	0.89 (0.091)	0.92	0.69–1.05	<.001[b]
	Overall	79	0.92 (0.078)	0.94	0.69–1.05	
Binding ratio pH 5.5/7.4	Alive	39	0.98 (0.020)	0.98	0.92–1.02	.003[a]
	Dead	40	0.94 (0.080)	0.96	0.72–1.04	.050[b]
	Overall	79	0.96 (0.062)	0.98	0.72–1.04	
%Displacement of ZMapp	Alive	39	8.17 (16.70)	7.49	−42.95 to 42.74	.014[c]
	Dead	40	−1.44 (17.13)	−3.50	−47.27 to 30.41	.015[b]
	Overall	79	3.30 (17.50)	5.75	−47.27 to 42.74	
%Neutralization	Alive	27	64.95 (20.06)	71.3	27.3–95.2	<.001[c]
	Dead	26	38.29 (25.86)	37.35	−27.4 to 85.7	<.001[b]
	Overall	53	51.87 (26.53)	56.4	−27.4 to 95.2	

Abbreviation: SD, standard deviation.

[a]Two-sample, 2-sided *t* test for log-transformed values.

[b]Wilcoxon rank-sum test.

[c]The *t* test is performed on untransformed values.

For each vaccine type, titers for GPΔTM and GPΔMuc were significantly higher in surviving NHPs compared with NHPs who succumbed to infection ($P < .01$) (Table 4). Overall, the antibody response to GP tends to be higher in double VLP-vaccinated animals. Among surviving animals vaccinated with adenovirus-based vaccine, the anti-GPΔTM titers were significantly higher than the response to GPΔMuc ($P < .05$) (Supplementary Table S.5). In surviving animals vaccinated with double VLP, the anti-GPΔTM response was slightly but not significantly lower ($P > .05$); in triple VLP-vaccinated survivors, response was significantly lower ($P < .001$) than GPΔMuc. No significant difference in VP40 or NP titers was observed ($P > .05$) (Figure 3B and Table 4).

Overall, the time to death was shorter for NHPs immunized with adenovirus or double VLP vaccine, with longer times to death observed in NHPs treated with triple VLP vaccine, as illustrated in Figure 3D. Median times to death and corresponding 95% confidence intervals are shown in Supplementary Table S.6; however, due to the low number of NHPs with observed deaths in the follow-up period, the median is not calculable for triple VLP vaccine, and upper 95% confidence intervals are not calculable overall and for all vaccine types.

Cox-proportional hazards models were fitted to the time to death data for each vaccine type and titer endpoint separately, as well as by titer endpoint overall (Table 5). In general, the risk of death is significantly reduced for each 1-unit increase in log GPΔTM or log GPΔMuc titer ($P < .01$). The risk of death does not appear to be impacted by changes in VP40 or NP titer ($P > .05$).

DISCUSSION

Several lines of evidence suggest that antibodies against EBOV GP play a critical role in protection against EVD. Multiple mAbs and antibody cocktails have been reported to protect against EBOV as well as other filoviruses [14, 16, 39–42]. A previous analysis of 54 macaques vaccinated with various adenovirus constructs expressing GP or GP plus NP showed a strong correlation between total anti-GP IgG titer and survival [30]. However, this study does not define a clear cutoff separating survivors from fatal cases, and, despite statistically significant correlations, the spectrum of IgG response among survivors and fatal cases are largely overlapping, suggesting that other factors, such as CMI, play a role in protection. It is also likely that qualitative attributes of the antibody response that cannot be captured in a total antibody ELISA are important for protection.

Here, we report a comparative study using a VLP-based vaccine known to induce high NP-directed T-cell responses (triple VLP) [6] with double VLPs lacking NP. In the absence of NP, the vaccine induced higher levels of anti-GP antibody, and higher IgG levels were needed for protection compared with the triple VLP. It remains to be determined why VLPs lacking NP induced higher anti-GP titers. A previous studies indicated

Table 4. Summary Statistics by Vaccine Type and Survival Outcome

Vaccine Type	Variable	Survival Outcome	N	Mean (SD)	Median	Range	*P* Value
Adenovirus	Ebola GP-ΔTM	Alive	11	1492.76 (1109.60)	1251.5	275.64–3879.08	<.001[a]
		Dead	11	101.62 (64.35)	96.01	16.66–202.51	<.001[b]
		Overall	22	797.19 (1046.48)	239.08	16.66–3879.08	
	Ebola GP-ΔMuc	Alive	11	551.95 (484.64)	408.44	28.56–1551.47	<.001[a]
		Dead	11	41.50 (28.09)	35.84	2.29–94.1	<.001[b]
		Overall	22	296.73 (424.81)	80.48	2.29–1551.47	
Double	Ebola GP-ΔTM	Alive	6	1947.14 (711.02)	1974.40	970.10–3069.28	.003[a]
		Dead	11	685.53 (397.83)	634.29	34.27–1391.59	.002[b]
		Overall	17	1130.80 (801.94)	938.10	34.27–3069.28	
	Ebola GP-ΔMuc	Alive	6	2187.98 (544.87)	2288.68	1218.49–2764.40	.002[a]
		Dead	11	893.67 (461.94)	732.49	199.23–1814.64	.002[b]
		Overall	17	1350.48 (795.38)	1044.53	199.23–2764.40	
	Ebola VP40	Alive	6	3534.51 (2108.72)	3428.44	1381.94–6606.422	.958[a]
							.763[b]
		Dead	11	4196.16 (2878.17)	3592.58	350.36–10 024.99	
		Overall	17	3962.64 (2583.26)	3592.58	350.36–10 024.99	
	%Neutralization	Alive	6	0.74 (0.225)	0.81	0.30–0.92	.011[c]
		Dead	11	0.39 (0.223)	0.40	0.01–0.67	.012[b]
		Overall	17	0.51 (0.279)	0.58	0.01–0.92	
Triple	Ebola GP-ΔTM	Alive	22	605.82 (398.47)	492.5	93.21–1552.93	.004[a]
		Dead	17	264.90 (227.58)	256.22	4.45–847.34	.006[b]
		Overall	39	457.22 (372.67)	370.28	4.45–1552.93	
	Ebola GP-ΔMuc	Alive	22	1348.521 (656.19)	1273.30	331.79–2652.72	.002[a]
		Dead	17	731.90 (703.32)	452.36	10.28–2207.26	.008[b]
		Overall	39	1079.74 (736.34)	1003.85	10.28–2652.72	
	Ebola VP40	Alive	22	4573.88 (4740.05)	2961.99	354.90–18 895.37	.956[a]
		Dead	17	5853.80 (6225.77)	3243.74	101.79–18 250.06	.630[b]
		Overall	39	5131.79 (5399.08)	3201.5	101.79–18 895.37	
	NP	Alive	18	602.66 (568.80)	340.3	28.4–1872	.335[a]
		Dead	13	558.70 (867.89)	270.5	10–3293	.337[b]
		Overall	31	584.23 (696.50)	323.2	10–3293	
	%Neutralization	Alive	21	0.62 (0.191)	0.64	0.27–0.95	.010[c]
		Dead	15	0.38 (0.289)	0.36	−0.27 to 0.86	.010[b]
		Overall	36	0.52 (0.263)	0.55	−0.27 to 0.95	

Abbreviations: NP, nucleoprotein; SD, standard deviation.

[a]Two-sample, 2-sided *t* test for log-transformed values.

[b]Wilcoxon rank-sum test.

[c]The *t* test is performed on untransformed values.

Table 5. Survival Analysis Results by Vaccine Type: Cox Proportional Hazards Model

Vaccine Type	Variable	Hazard Ratio[a]	95% Confidence Interval	*P* Value[b]
Adenovirus	Ebola GP-ΔTM	0.39	0.22–0.68	.001
	Ebola GP-ΔMuc	0.35	0.17–0.74	.006
Double	Ebola GP-ΔTM	0.51	0.31–0.84	.008
	Ebola GP-ΔMuc	0.05	0.01–0.34	.003
	Ebola VP40	0.89	0.14–5.47	.897
Triple	Ebola GP-ΔTM	0.57	0.42–0.78	<.001
	Ebola GP-ΔMuc	0.30	0.15–0.61	.001
	Ebola VP40	1.04	0.42–2.56	.928
	Ebola NP	0.71	0.46–1.11	.132
Overall	Ebola GP-ΔTM	0.57	0.47–0.70	<.001
	Ebola GP-ΔMuc	0.43	0.30–0.62	<.001
	Ebola VP40	1.05	0.49–2.27	.894

[a]Hazard ratio per 1 unit increase in the log-transformed variable.

[b]Log-rank test.

that although expression of NP increases the rate of VLP release [27], the overall morphology of VLPs is not affected by coexpression of NP [43]; however, to our knowledge, it is not known whether NP affects the density of GP spikes on the surface of particles. Because the antibody titers are lower in triple VLP-vaccinated NHPs (Table 4 and Supplementary Table S5), the increased efficacy is most likely related to NP-mediated T-cell responses. In a larger cohort of macaques vaccinated with VLPs or adenovirus-expressed GP, we confirmed significant correlation between anti-GP titer and survival irrespective of the platform, whereas survival did not correlate with IgG titers for VP40 or NP. Although a cutoff for protection based on anti-GP titers could not be defined for triple VLP and adenovirus vaccines, all of the double VLP-vaccinated survivors had an antibody titer of >1900 AU/mL against GPΔMuc except for a single animal, which was very sick through day 12 but eventually survived.

The current studies expand beyond any other published studies to date by examining qualitative attributes of the antibody response that correlate with protection. Our data show that neutralization of rVSV-GP, as well as the ability of the antibodies to bind to GP at acidic pH, correlate with survival. Filoviruses use macropinocytosis to enter the acidic environment of the endosomes, where GP is proteolytically cleaved by cathepsins, exposing the receptor-binding site of GP to interact with its endosomal receptor Nieman Pick C 1 (NPC-1). Triggering the productive fusion of viral and endosomal membranes is dependent on this cleavage and additional low pH-dependent events [44, 45]. Therefore, the ability to bind at acidic pH is an important attribute of neutralizing antibodies, specifically those targeting the viral fusion mechanism, as we and others have recently reported [42, 46]. Our current data show that vaccine induced antibodies from the surviving macaques bind GP much more strongly at acidic pH compared with animals that succumb to infection.

ZMapp, a cocktail of 3 mAbs that target the glycan cap and the base of EBOV GP, protects NHPs from lethal EBOV challenge. To evaluate whether vaccine-mediated protection correlates with the ability of the vaccine to induce ZMapp-like antibodies, we evaluated the ability of the immune sera to displace components of ZMapp. These data showed that survivors had a higher titer of antibodies that compete with ZMapp. Some of the sera increased binding of ZMapp components to GP. Among survivors, only sera from 1 of 39 animals showed >20% increase in ZMapp binding, whereas this was observed in 4 of 40 sera from lethal cases. It is possible that binding of certain classes of antibodies may have an allosteric effect on binding to these epitopes. This would be consistent with our recent report showing cooperative binding between 2 classes of anti-EBOV mAbs [41].

Our findings suggest that the quality of antibody response is critically important. In a recent study, Khurana et al [47] performed a comprehensive analysis of the antibody responses to the rVSVΔG-ZEBOV-GP vaccine tested in a phase I clinical trial. These data showed that the response was dominated by antibodies against MLD, a highly glycosylated domain believed to mask key neutralizing epitopes [48]. In contrast, the study shows poor to moderate response among vaccinees to the regions of GP2 encompassing the internal fusion loop and the N terminus of GP1 that forms the base of GP trimer along with GP2. Nonetheless, the success of VSV-based EBOV vaccines suggests that sufficient protective antibodies are elicited and/or a major involvement of CMI responses. These protective antibodies could include antibodies binding to MLD that can activate antiviral effector functions as previously proposed [49]. In the current study, we evaluated antibody responses to both full-length GPΔTM and MLD-deleted GP (GPΔMuc). Antibody titers to GPΔTM was significantly higher than GPΔMuc in adenovirus-vaccinated animals, suggesting that a large portion of these antibodies may be MLD-specific. In contrast, VLP-vaccinated survivors showed higher titers against GPΔMuc than GPΔTM, suggesting that the antibody response was not skewed towards MLD.

CONCLUSIONS

In summary, although lack of data on T-cell responses of these animals remains a limitation of our study, our findings provide strong evidence that antibodies to GP can be used as a reliable correlate of protective immune response in NHPs. This study further emphasizes the importance of evaluating the qualitative attributes of the antibody response. As more protective epitopes are being identified, it is important that future studies focus on the analysis of the epitope-specific antibody profiles. Obtaining a comprehensive understanding of the correlates of protective antibody response in NHPs is critically important for development of EBOV vaccines under US Food and Drug Administration Animal Rule and will also support efforts to define immune correlates to predict efficacy in humans.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. M. J. A. and F. W. H. have stocks or stock options in Integrated Biotherapeutics. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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