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SUPPLEMENT ARTICLE

An Inactivated Rabies Virus–Based Ebola Vaccine, FILORAB1, Adjuvanted With Glucopyranosyl Lipid A in Stable Emulsion Confers Complete Protection in Nonhuman Primate Challenge Models

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The 2013–2016 West African Ebola virus (EBOV) disease outbreak was the largest filovirus outbreak to date. Over 28 000 suspected, probable, or confirmed cases have been reported, with a 53% case-fatality rate. The magnitude and international impact of this EBOV outbreak has highlighted the urgent need for a safe and efficient EBOV vaccine. To this end, we demonstrate the immunogenicity and protective efficacy of FILORAB1, a recombinant, bivalent, inactivated rabies virus–based EBOV vaccine, in rhesus and cynomolgus monkeys. Our results demonstrate that the use of the synthetic Toll-like receptor 4 agonist glucopyranosyl lipid A in stable emulsion (GLA-SE) as an adjuvant increased the efficacy of FILORAB1 to 100% protection against lethal EBOV challenge, with no to mild clinical signs of disease. Furthermore, all vaccinated subjects developed protective anti–rabies virus antibody titers. Taken together, these results support further development of FILORAB1/GLA-SE as an effective preexposure EBOV vaccine.

Keywords. Ebola; vaccine; rabies; nonhuman primate; GLA-SE; protection; challenge model.

Ebola virus (EBOV) was first identified as the causative agent of a severe hemorrhagic fever in 1976 [1]. Since then, at least 20 human outbreaks have been reported in Central Africa [2]. The largest outbreak occurred in late 2013, in West Africa, with the peak number of cases occurring from late December 2014 through December 2015 [3]. The total number of suspected, probable, and laboratory-confirmed cases topped 28 000, with an overall fatality rate of 53% as of 3 March 2016. The fatality rate of laboratory-confirmed cases (15 250 cases) was substantially higher, at 74%. The best preventive measure for stemming the outbreak was raising public awareness about the transmission routes and implementing infection control procedures at hospitals and Ebola treatment units. The obvious outcome of such an outbreak was reinforcement of the need for development of suitable countermeasures, including vaccines and small-molecule inhibitors.

EBOV infection is characterized by malaise, high fever followed by coagulopathy, and gastrointestinal symptoms, which can ultimately progress to multiorgan system failure [4]. Case-fatality rates vary between outbreaks, but case-fatality rates can reach 90% [5]. In addition, outbreaks of EBOV infection among gorillas, chimpanzees, and other nonhuman primates (NHPs) have been reported in the EBOV-endemic region, with death rates in the thousands [6–10]. The definitive reservoir for EBOV has yet to be identified, but bats are suspected [11].

Several strategies have been used to develop EBOV vaccines. Considering the rapid spread and high lethality of EBOV infections, vaccines should be easy to administer, safe for both the general public and special populations (ie, elderly individuals, young individuals, pregnant individuals, and immunosuppressed individuals), and provide long-term protection. Two vaccine strategies exist to combat EBOV infections: widespread vaccination campaigns in the EBOV-endemic area, and ring vaccination during outbreaks. Widespread vaccination was effective against smallpox and is proving effective against poliovirus. A ring vaccination response requires early detection of an outbreak, contact tracing, maintenance of a stockpile, and the ability to deploy the stockpile to the effected region. In either scenario, the vaccine must be easily administered and have suitable stability for long-term storage, ideally in a lyophilized form. While 2 vaccines have been recently used in phase 2 and 3 clinical trials and 1 has demonstrated efficacy in humans, it is unclear whether they will fulfill the above criteria [12, 13].

Previous work has demonstrated that immunization with EBOV glycoprotein (GP), which mediates viral attachment and entry, will confer protection against homologous virus challenge in small-animal models and NHPs [14]. Multiple

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platforms have been developed, including rabies virus (RABV), adenovirus, vesicular stomatitis virus (VSV), and paramyxoviruses [15–17]. Humoral and cellular immunity to EBOV has also been demonstrated by these platforms, but correlates of protection have not been established [17–27]. An ideal vaccine would provide protection against the multiple species, but cross-protection studies with EBOV GP–based vaccines were not successful against heterologous challenge. For example, in guinea pigs, recombinant VSV with its own G protein deleted and expressing only EBOV GP failed to protect against heterologous challenge with other ebolaviruses, such as Sudan virus (SUDV). Furthermore, a blended SUDV and EBOV vaccine demonstrated 33% efficacy when challenged with Bundibugyo virus (BUDV) [28, 29]. In contrast, DNA/adenovirus prime boost vaccination with SUDV and EBOV or a single immunization with a blended VSV vaccine both demonstrated 100% cross-protection against BUDV, indicating the potential for heterologous protection [21, 28].

Previously, we established the efficacy and immunogenicity of a bivalent RABV-EBOV vaccine in mice and rhesus monkeys [15, 30–33]. We demonstrated that the live replication-competent vaccine provided 100% protection following EBOV challenge, while the inactivated candidates provided 50% protection [15]. Further development by codon optimization of GP increased efficacy of the inactivated RABV-EBOV vaccine [30]. In these studies, we established that protection is partially dependent on the quality of the antibodies, rather than the quantity [15]. Further development and characterization of the RABV-EBOV platform is warranted. Here we report that the potent synthetic Toll-like receptor 4 (TLR4) agonist glucopyranosyl lipid A in a stable oil-in-water emulsion (GLA-SE) improves the immunogenicity and efficacy of FILORAB1 in a 2-dose or 3-dose prophylactic regimen in cynomolgus and rhesus monkeys.

MATERIALS AND METHODS

Generation of FILORAB1

The vaccine vector BNSP333-coZGP (FILORAB1) was constructed, recovered, purified with sucrose, inactivated with β-propiolactone, and characterized as previously described [30].

Formulation of GLA-SE

GLA (Immune Design Corp) is a synthetic TLR4 agonist that is formulated in SE, consisting of squalene, glycerol, phosphatidylcholine, poloxamer surfactant, and ammonium phosphate buffer, at a final concentration of 15 μg GLA/2% SE per vaccine dose.

Animal Ethics Statement

This study was carried out in strict adherence to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, the Office of Animal Welfare, and the US Department of Agriculture. All work was approved by the National Institute of Allergy and Infectious Diseases (NIAID) Division of Clinical Research Animal Care and Use Committee and performed at the NIAID Research Facilities. Procedures were carried out after animals had been anesthetized by trained personnel under the supervision of veterinary staff. Food and water were available ad libitum.

Vaccination and Challenge

The experiment was performed in 2 phases, using rhesus and cynomolgus monkeys. For simplicity and consistency with our previous experiments, the experiment with Rhesus monkeys is referred to as NHP 3 (phase 1), and the experiment using cynomolgus monkeys is referred to as NHP 4 (phase 2). For both NHP 3 and NHP 4, study investigators were blinded to the groups until study end. NHP 3 involved a mixture of 20 male and female rhesus monkeys with ages ranging from 3.4 to 9.3 years and weights ranging from 4.2 to 11.2 kg, referred to as NHP 3. Groups were as follows: the RabAvert group (n = 6) received half of the human dose, the FILORAB1 plus GLA-SE group (n = 4) received 100 μg of FILORAB1 plus 15 μg of GLA-SE, the 100-μg FILORAB1 group (n = 4) received 100 μg of FILORAB1, and the 200-μg FILORAB1 group received 200 μg of FILORAB1 and were vaccinated on study days 0 and 42 by intramuscular injections in the caudal thigh. For NHP 4, a mixture of male and female cynomolgus monkeys with ages ranging from 2.9 to 3.8 years and weights ranging from 2.5 to 3.4 kg were vaccinated as follows: the RabAvert group (n = 6) received half of the human dose, the FILORAB1 plus GLA-SE group (n = 6) received 100 μg of FILORAB1 plus 15 μg of GLA-SE, and the 200-μg FILORAB1 group (n = 6) received 200 μg of FILORAB1 on study days 0, 7, and 28. For both phases, subjects received periodic, complete physical examinations, including blood specimen collection for complete blood count with differential and serum chemistry analysis to monitor health. For efficacy testing, NHPs were intramuscularly injected with 1000 plaque-forming units (PFU) of EBOV Makona C05 [34] on day 85, followed by periodic physical examinations, which included blood specimen collection for complete blood count with differential and serum chemistry analysis. Subjects were also evaluated daily to monitor health for clinical assessment.

Analysis of Humoral Response to Immunization by ELISA

Individual NHP serum and control sera were tested for the presence of EBOV GP or RABV G–specific IgG by enzyme-linked immunosorbent assay (ELISA), as previously described [15].

RABV Neutralizing Antibodies (NAbs)

Sera of NHPs and control sera were heat inactivated at 56°C for 30 minutes. Neutralizing activity was determined using the rapid fluorescent focus inhibition test assay as described previously [35].

Fluorescence Reduction Neutralizing–50% Assay ($FRNA_{50}$)

$FRNA_{50}$ were performed to determine the neutralizing activity of anti-EBOV antibody samples. Vero E6 cells (4×10^4 cells/well)

were plated in 96-well Operetta-compatible plates (PerkinElmer). Serum samples were first inactivated by γ irradiation before being heat inactivated. A total of 5.0 μL of each sample was serially diluted (1:40 to 1:10 240) in Dulbecco's modified Eagle's Medium (Lonza) in a dilution plate/block. Fifty microliters of EBOV virus stock (multiplicity of infection, 1) was then added to each well and inoculated for 1 hour at 37°C for 1 hour with shaking every 15 minutes. A total of 100 μL of the diluted sera and virus stock was added to each well of the 96-well Operetta plate and was incubated for 48 hours at 37°C and 5% CO_2. Negative control samples (no virus and no serum) and positive control samples (virus plus virus-like particles; IBT Bio Sciences, lot 1 302 001) were used. Cells were fixed by adding 20% neutral-buffered formalin and were processed by labeling with a primary anti-EBOV VP40 antibody. A secondary antibody goat anti-mouse IgG (heavy and light chain) conjugated to Alexa Fluor 488 (A-11028, Thermo Fisher Scientific) detected the primary antibody and was visualized on an Operetta High Content Imager (Perkin Elmer). The dilution at which 50% inhibition of relative fluorescence intensity was observed was reported as the $FRNA_{50}$.

RNA Isolation From Whole Blood and Quantification of Viral RNA by Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Whole blood (50 μL) was resuspended in 150 μL of TRIzol LS Reagent (Life Technologies). The RNA extraction protocol for biological fluids using TRIzol LS Reagent was followed until the phase separation step. The remaining RNA extraction was done using the PureLink RNA Mini Kit from Ambion (Ambion). The quantity and quality (260/280 ratios) of RNA extracted was measured using NanoDrop (Fisher). Two microliters of RNA/sample was run in triplicate in 2 independent qRT-PCR experiments, using the Ebola Zaire (2014) Assay and Control set (Applied Biosystems). The kit is standardized and contains a synthetic RNA as a control along with macaque PPIA gene as a sample preparation control. A set of EBOV-specific primers and probe (FAM/MGB) were designed and used based on the Kulesh EBOV MGB-based assay [36].

RESULTS

To further evaluate the efficacy of FILORAB1, we continued to use NHPs as the animal model for EBOV disease. In this study, the TLR4 agonist GLA-SE, which has an excellent safety record in clinical studies [37], was evaluated in 2 independent experiments.

NHP 3 was composed of 4 groups of rhesus monkeys that were immunized as follows: 6 received the approved RABV vaccine RabAvert (half the human dose); 6 received 200 μg of FILORAB1, 4 received 100 μg FILORAB1, and 4 received 100 μg of FILORAB1 plus GLA-SE at days 0 and 42. All inoculations were performed intramuscularly in the right caudal thigh. Immune responses were analyzed on study days −21, −14, 0, 7, 14, 28, 42, and 56. Day 0 was the first vaccination day. As show in Figure 1, some subjects developed EBOV GP–specific immune responses as early as day 7, and all responded by day 14. Immune responses continued to increase over time until day 28 and slightly decreased by the booster vaccination (day 42). As expected, none of the RabAvert group control animals developed EBOV-specific immune response above the background signal (Figure 1). Moreover, the groups of NHPs immunized with 200 μg of FILORAB1 or 100 μg of FILORAB1 plus GLA-SE developed higher EBOV GP–specific ELISA titers than the subjects immunized with 100 μg of FILORAB1. The FILORAB1 boost increased the GP-specific ELISA titers further, with 2 of the animals of the FILORAB1 plus GLA-SE group reaching titers higher than the control serum, which consists of pooled sera of 8 NHPs from our first NHP study that survived EBOV challenge [15]. Additionally, we observed a high RABV G–specific IgG response in all of the groups, with the highest titers observed for the FILORAB1 plus GLA-SE group, followed by the 200-μg FILORAB1 group, and then the 100-μg FILORAB1 group. The lowest observed RABV G response was observed in the RABV-only group (data not shown).

We also analyzed RABV NAbs. As expected, the prime and boost yielded an increase in RABV NAbs, with increases observed in the 200-μg FILORAB1 and 100-μg FILORAB1 plus GLA-SE groups (Figure 2). Two subjects in the 100-μg FILORAB1 group did not develop RABV NAbs prior to the day 42 boost. However, based on the NAb results 2 weeks after boost immunization, the NAb against RABV in all subjects from all 4 groups reached titers that are considered protective against RABV by the WHO standard (eg, >0.5 IU).

Since correlates of protection against EBOV are not established and may vary between vaccines, the NHPs were challenged at study day 85 with 1000 PFU of the 2013–2016 outbreak strain EBOV Makona C05 intramuscularly [34] in the lateral head of the right triceps muscle. The results are shown in Figure 3*A*. All 4 subjects in the 100-μg FILORAB1 plus GLA-SE group were protected against challenge, whereas only 1 of 4 subjects in the 100-μg FILORAB1 group survived EBOV challenge. Five of six animals in the 200-μg FILORAB1 group survived. All RabAvert control subjects met moribund end point criteria by day 9 after challenge and were humanely euthanized. The improved protection due to the different vaccination strategies was reflected in the development of NAbs and circulating viral loads (Figure 3*B* and 3*C*). The lowest average clinical disease scores were detected in the group immunized with 100 μg of FILORAB1 plus GLA-SE, and viral RNA was only observed in 2 animals and only at low levels in the other 2 NHPs. The subjects in the 100-μg FILORAB1 group had high average clinical disease scores, and high viral loads were detected by qRT-PCR in 3 of 4 subjects. Increasing the antigen dose in the vaccine to 200 μg clearly increased the efficacy of FILORAB1 but did not add as much benefit as the addition of GLA-SE.

Last, to evaluate the immune response after EBOV challenge, EBOV GP–specific ELISAs were performed. As shown in

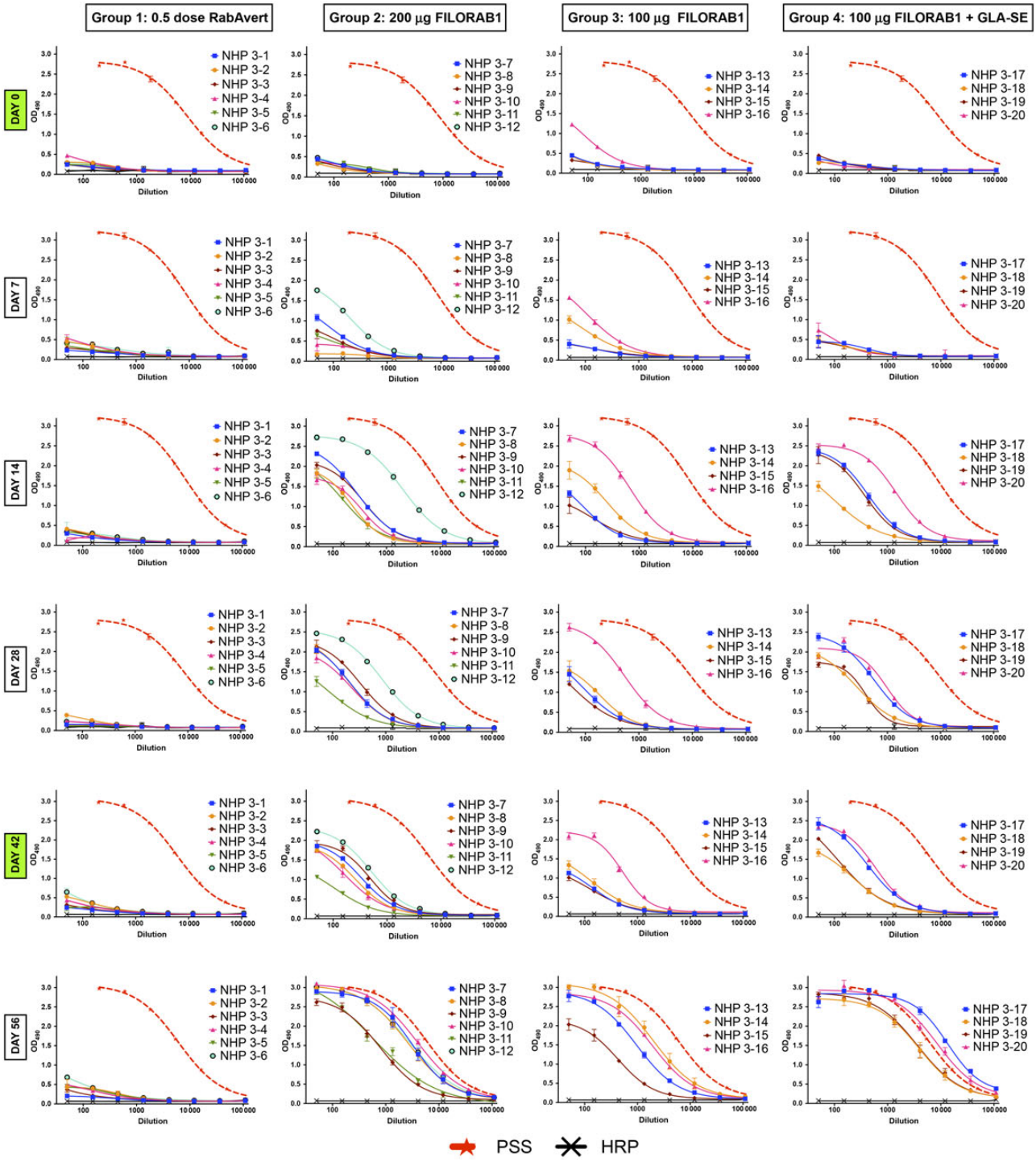

Figure 1. Humoral immune response to Ebola virus (EBOV) glycoprotein (GP) in nonhuman primate (NHP) study 3 (NHP 3). Subjects were vaccinated on day 0 and boosted on day 42 (highlighted in green). Sera from NHPs were analyzed for total immunoglobulin G with an EBOV GP (Zaire)–specific enzyme-linked immunosorbent assay (ELISA). OD_{490} readings were compared to those for pooled sera from the 8 surviving NHPs from a previous study (NHP 1) as a positive control (PSS). Sera obtained prior to immunization on day 0 were used as a negative control. All sera were diluted 1:50 and serially diluted 3-fold before ELISA. Abbreviations: GLA-SE, glucopyranosyl lipid A in stable emulsion; HRP, horseradish peroxidase. This figure is available in black and white in print and in color online.

Figure 4, there was no boost effect seen in the GP response 6 day after challenge when compared to the day of the EBOV challenge. However, EBOV GP–specific ELISA findings on the day of necropsy indicated that all surviving subjects developed higher titers against EBOV GP than were observed before virus challenge. All subjects that met end point criteria and were

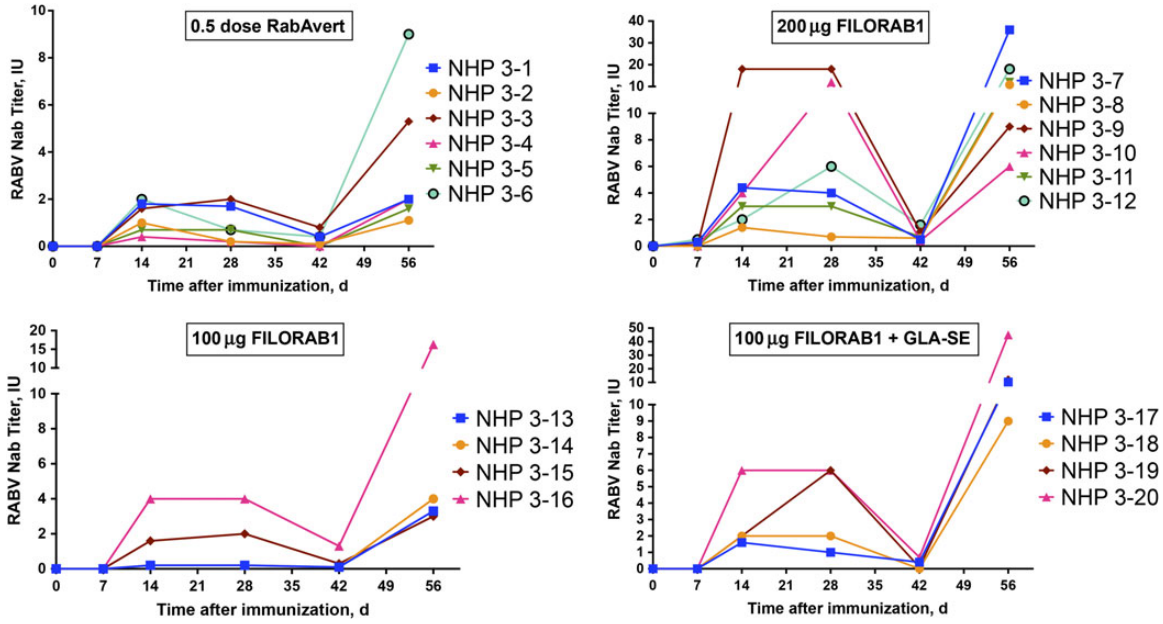

Figure 2. Development of rabies virus (RABV) neutralizing antibodies (NAbs) over time in immunized rhesus monkeys (nonhuman primate study 3 [NHP 3]). Animals were immunized at day 0 and 42 with the indicated vaccine and RABV NAbs were analyzed over time and determined as international units (IUs), using the World Health Organization standard. Abbreviation: GLA-SE, glucopyranosyl lipid A in stable emulsion.

euthanized did not mount increased antibody responses or demonstrated reduced anti-GP titers. Interestingly, all NHPs of the group immunized with 100 µg of FILORAB1 plus GLA-SE showed increased GP antibody titers, but this response was less dramatic than the titers observed in surviving animals from the other vaccine groups. Viral replication of EBOV after challenge most likely was better controlled in this group of animals, as indicated by the low viral RNA levels detected (Figure 3*C*). Less viral replication possibly results in reduced EBOV immune responses.

Before infection, no subjects developed clinical signs of disease or adverse reactions at the vaccine injection site, supporting the safety of FILORAB1 in NHPs. Clinical disease scores were assigned on the basis of the subjects' physical activity, rash, appetite, signs of respiratory distress, and motor function. Clinical disease scores corresponded with antibody titers and viral loads. The animals that received 100 µg of FILORAB1 plus GLA-SE demonstrated few or short-lived clinical signs and high antibody titers and low viral loads. In contrast, the RabAvert group demonstrated clinical signs consistent with EBOV disease, low to no antibody titers, and high viral loads.

In nonsurviving subjects, gross necropsy observations were consistent with EBOV disease. However, at the EBOV injection site, a mild-to-moderate and occasionally severe injection site reaction was observed in 6 of 6 animals in the 200-µg FILORAB1 group, in 3 of 4 in the 100-µg FILORAB1 group, and in 4 of 4 in the 100-µg FILORAB1 plus GLA-SE group. The mechanism of this reaction is currently unknown. Grossly, severe myofiber degeneration and necrosis was observed in 1 of 6 RabAvert subjects, and a moderate myofiber degeneration and necrosis was observed in the subject that died of disease in the 200-µg FILORAB1 group. Although injection site swelling was observed in the 100-µg FILORAB1 plus GLA-SE group, it was not as severe as that observed in the other groups, and myofiber degeneration and necrosis were not grossly observed.

The lack of 100% protection for the 200-µg FILORAB1 group was a concern. One method to consider increasing protection against EBOV was to switch to a prime-boost-boost vaccine strategy, which is in keeping with the human RABV preexposure prophylaxis vaccination schedule (days 0, 7, and 28). Generally, multiple inoculations are commonly used for inactivated vaccines because inactivated vaccines are less immunogenic but provide improved safety when compared to live-replication competent vaccines.

A follow up study was composed of 3 groups of 6 cynomolgus monkeys, which were immunized with RabAvert, 200 µg of FILORAB1, or 100 µg of FILORAB1 plus 15 µg GLA-SE on study days 0, 7, and 28. Similar to the previous experiments, the challenge was performed on day 85 after initial vaccination, and the challenge was performed with 1000 PFU of the 2013–2016 outbreak strain EBOV Makona C05 [36] via the intramuscular route. Similar to NHP 3, EBOV-specific immunity was detected as early as 7 days after immunization (Figure 5) for the 200-µg FILORAB1 and 100-µg FILORAB1 plus GLA-SE groups but not for the RabAvert group. The responses were boosted to almost the level detected for pooled sera from NHP 1 survivors used as a

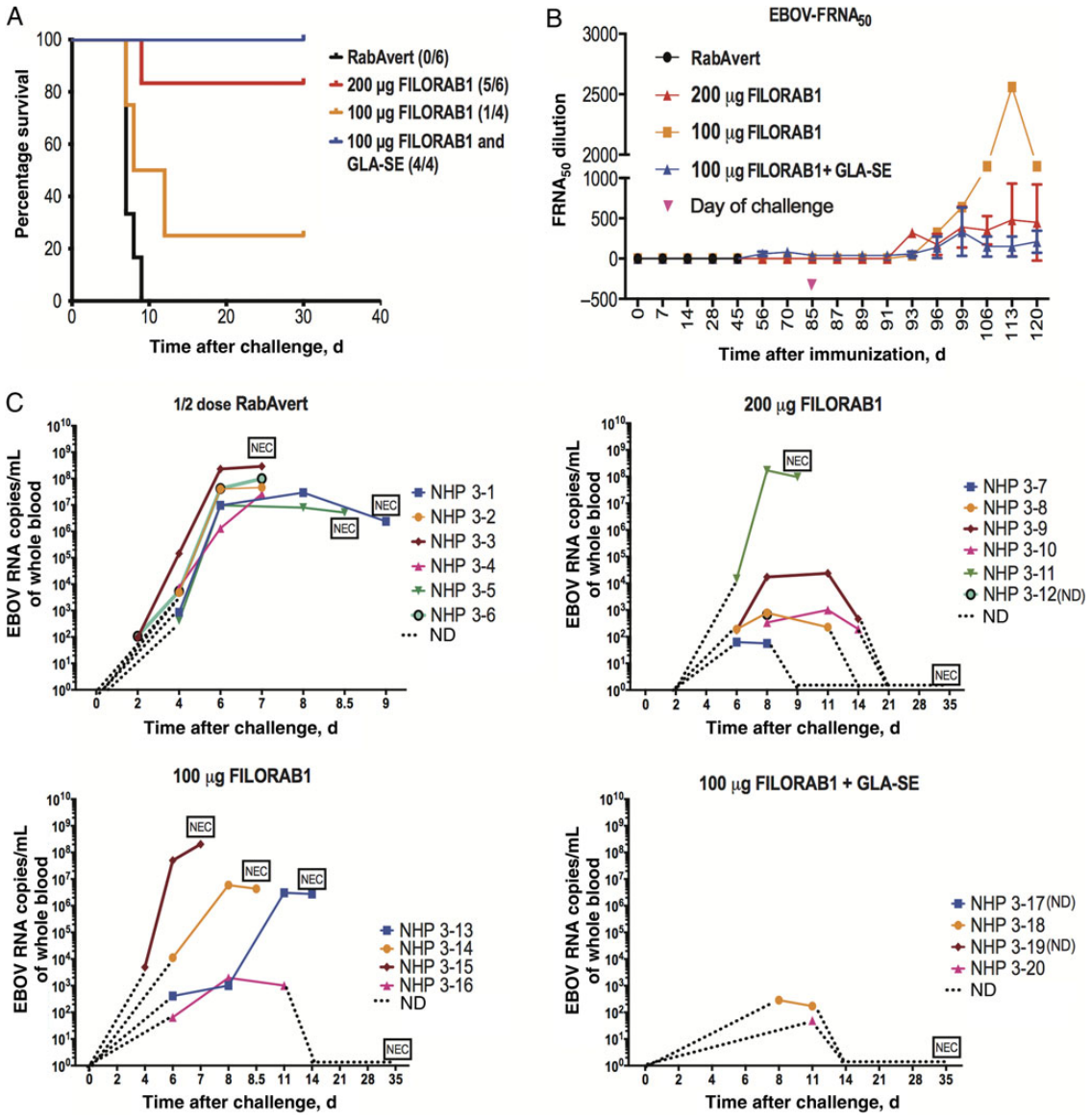

Figure 3. Survival (*A*), neutralizing antibody (NAb) titer against Ebola virus (EBOV; *B*), and RNA loads after challenge of immunized nonhuman primates (NHPs) in NHP study 3 (NHP 3; *C*). Immunized NHPs were challenged with 1000 plaque-forming units (PFU) of EBOV Makona C05 intramuscularly on day 85 after the first immunization. *A*, FILORAB1 adjuvanted with glucopyranosyl lipid A in stable emulsion (GLA-SE) provided 100% protection. *B*, NAb titer as measured by a fluorescence reduction neutralizing–50% assay ($FRNA_{50}$) indicates increased response in surviving NHPs. *C*, Total viral RNA (genomic RNA and messenger RNA) levels at different time points after challenge indicate protection associated with FILORAB1. Abbreviation: ND, not detectable.

positive control. There was a small reduction in the titer for EBOV GP antibody detected by ELISA for both groups from days 14 to 28, but the second boost increased EBOV GP–specific titers for NHPs in the 200-µg FILORAB1 and 100-µg FILORAB1 plus GLA-SE groups. The anti-EBOV antibody responses for the 100-µg FILORAB1 plus GLA-SE group was more stable over time and did not decrease, whereas the detected anti-GP response for NHPs immunized with 200 µg of FILORAB1 decreased over time. Evaluating the immune response against RABV by NAbs indicated that all 3 groups mounted sufficient NAbs to RABV, which is consistent with findings from NHP 3 (Figure 6).

Unblinding of the study revealed that 6 of 6 subjects in the RabAvert group met end point criteria on day 7, on average; that 4 of 6 subjects from the 200-µg FILORAB1 group met end point criteria on day 9 after challenge, on average; and that 0 of 6 subjects from the 100-µg FILORAB1 plus GLA-SE group met end point criteria (Figure 7*A*) or developed clinical signs of disease. The lack of clinical signs in the 100-µg FILORAB1 plus GLA-SE group was paralleled by low levels of viral RNA (Figure 7*C*). Low levels of viral RNA were also detected in the 2 surviving NHPs from the 200-µg FILORAB1 group (Figure 7*C*). NAb against EBOV was detected as early as day

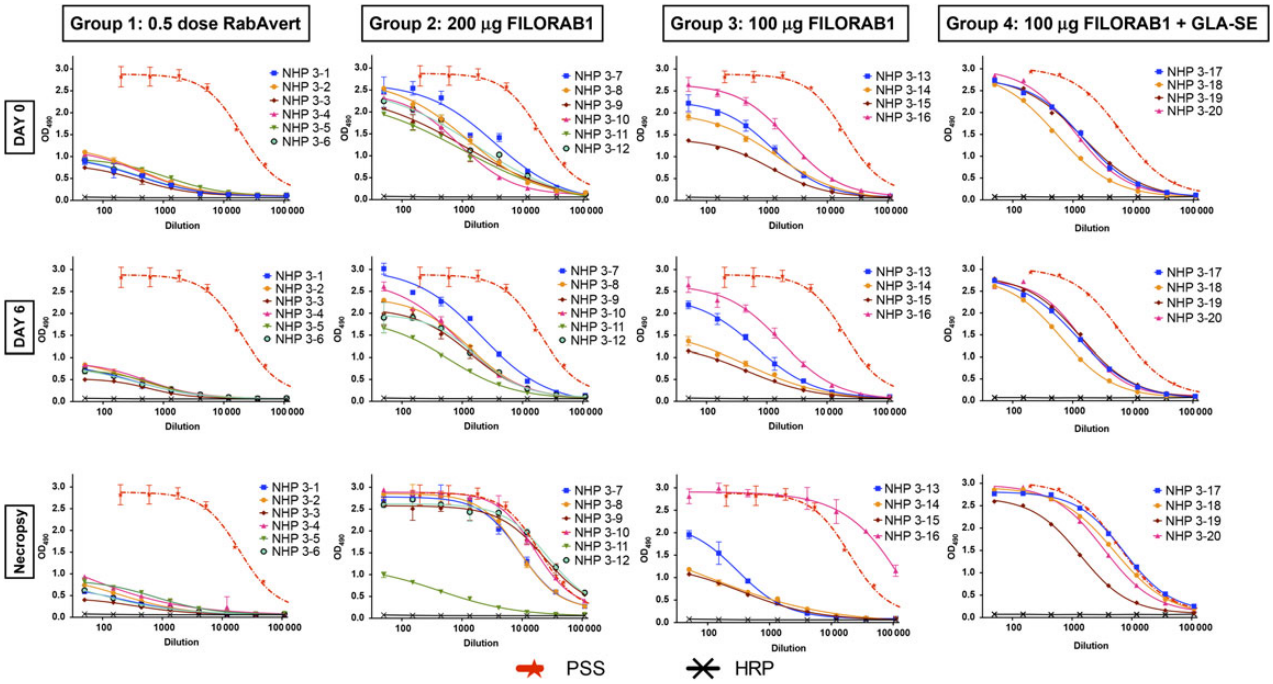

Figure 4. Humoral immune response to Ebola virus (EBOV) glycoprotein (GP) after challenge in nonhuman primate (NHP) study 3 (NHP 3). Sera from NHPs were analyzed for total immunoglobulin G (IgG) with an EBOV GP (Zaire)–specific enzyme-linked immunosorbent assay (ELISA). OD_{490} readings were compared to those for pooled sera from the 8 surviving monkeys from NHP 1 study as a positive control (PSS). All sera were diluted 1:50, followed by 3-fold serial dilutions, and were evaluated by ELISA. Abbreviations: GLA-SE, glucopyranosyl lipid A in stable emulsion; HRP, horseradish peroxidase.

9 after challenge (Figure 7*B*). The 100-µg FILORAB1 plus GLA-SE group developed higher NAb titers against EBOV than the 200-µg FILORAB1 group. The RabAvert group did not develop NAbs against EBOV. Taken together, these results indicate that vaccination with GLA-SE-adjuvanted FILORAB1 resulted in the best protection against EBOV challenge, resulting in 100% survival with no clinical signs. As discussed by Geisbert et al [38], the cynomolgus or rhesus macaque models are both well suited for EBOV vaccine studies, but some differences in the sensitivity to the challenge exist, which could explain the lower survival rates among the cynomolgus macaques immunized with 200 µg of FILORAB1, compared with the previous study in rhesus macaques.

To evaluate the immune response after EBOV challenge, EBOV-GP–specific ELISAs were performed. As shown in Figure 8, there was no boost effect seen in the GP response 6 days after challenge when compared to the day of the EBOV challenge. However, the EBOV GP ELISA at the necropsy time point indicated that all surviving subjects developed higher titers against EBOV GP than what was observed before challenge.

Comparison with NHP 3 indicated that subjects in NHP 4 developed clinical signs of disease 1 day earlier than what was observed in NHP 3 (day 5 vs day 6). Clinical signs for the RabAvert group were consistent with EBOV disease. The surviving subjects receiving 200 µg of FILORAB1 developed mild-to-moderate clinical signs, whereas the nonsurvivors developed severe clinical signs that were consistent with EBOV disease. Subjects in the 100-µg FILORAB1 plus GLA-SE group did not develop clinical signs of disease. However, 5 of 6 in the 200-µg FILORAB1 group and 4 of 6 in the 100-µg FILORAB1 plus GLA-SE group developed mild-to-severe reactions at the challenge virus injection site. Grossly, severe myofiber degeneration was observed in 3 of 6 subjects that received 200 µg of FILORAB1. Mild-to-moderate myofiber degeneration and necrosis was observed in 1 of 6 subjects that received 100 µg of FILORAB1 plus GLA-SE. Mild myofiber degeneration was observed in 1 RabAvert recipient. One of the 2 subjects from the 200-µg FILORAB1 group that developed severe myofiber degeneration also developed intussusception with ischemic necrosis of the bowel. The other subject from the 200-µg FILORAB1 group developed locally extensive, severe, subacute ischemic necrosis with mesenteric vascular infarction in the distal jejunum.

DISCUSSION

The RABV-based EBOV vaccine has been studied in mice and NHPs previously [15, 31–33, 39]. The first-generation of the RABV-based EBOV vaccine (BNSP333-ZGP) induced protection against EBOV challenge in NHPs when used as a replication-competent vaccine and partial protection (50%) when used as an inactivated vaccine [15]. The second generation of this

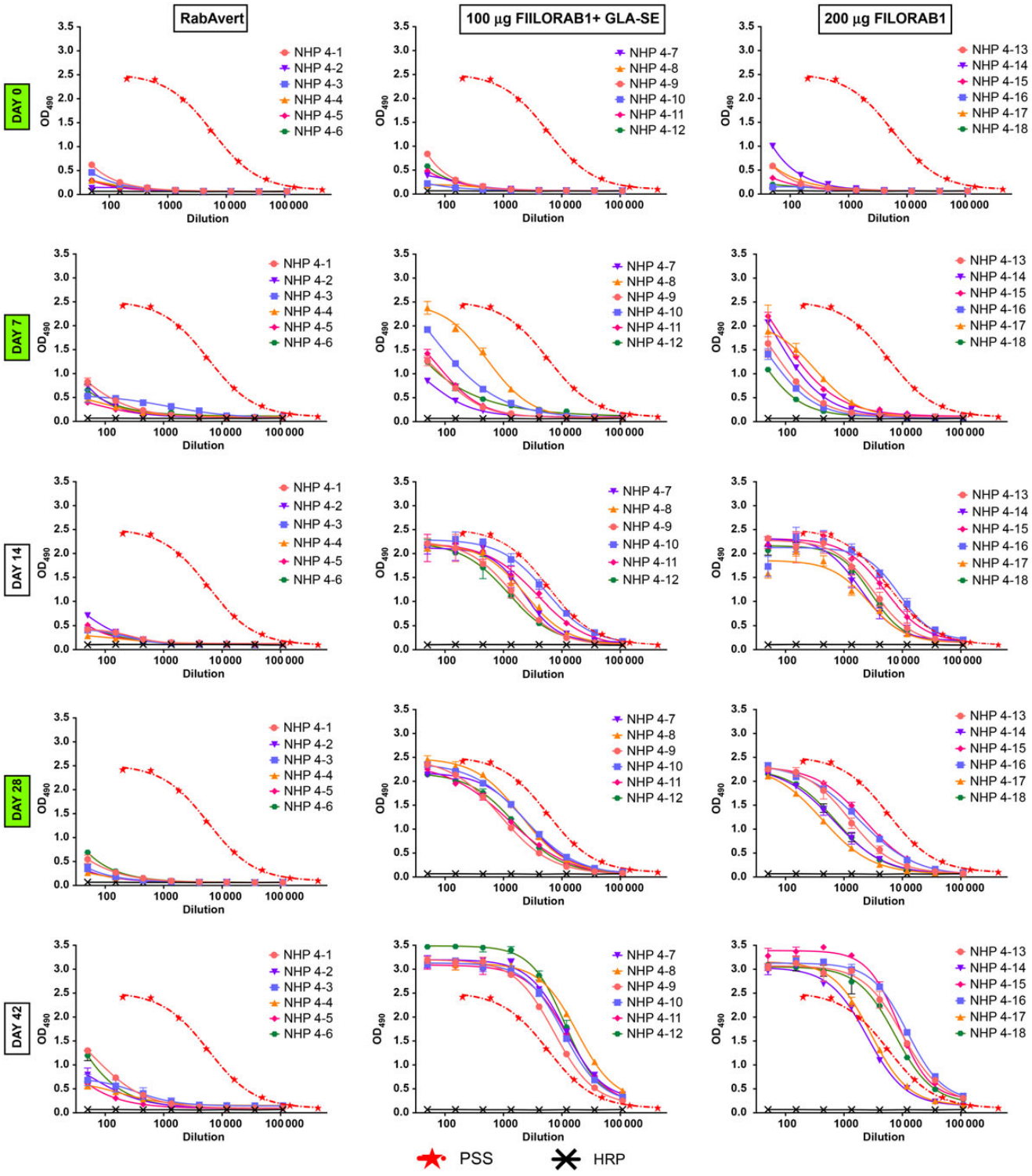

Figure 5. Humoral immune response to Ebola virus (EBOV) glycoprotein (GP) in nonhuman primate (NHP) study 4 (NHP 4). NHPs were vaccinated on days 0, 7, and 28 (green). Sera from NHPs were analyzed for total immunoglobulin G (IgG) with an EBOV GP (Zaire)–specific enzyme-linked immunosorbent assay. OD_{490} readings were compared to those for pooled sera from the 8 surviving NHPs from a NHP 1 study as a positive control (PSS). Sera obtained prior to immunization on day 0 were used as a negative control. All sera were diluted 1:50 and analyzed in a 3-fold serial dilution. Abbreviations: GLA-SE, glucopyranosyl lipid A in stable emulsion; HRP, horseradish peroxidase. This figure is available in black and white in print and in color online.

vaccine was modified to express codon-optimized EBOV GP (BNSP333-coZGP or FILORAB1). The codon-optimized EBOV GP was expressed at higher levels in Vero cells as compared to wild-type GP, and approximately 2.5-fold more EBOV

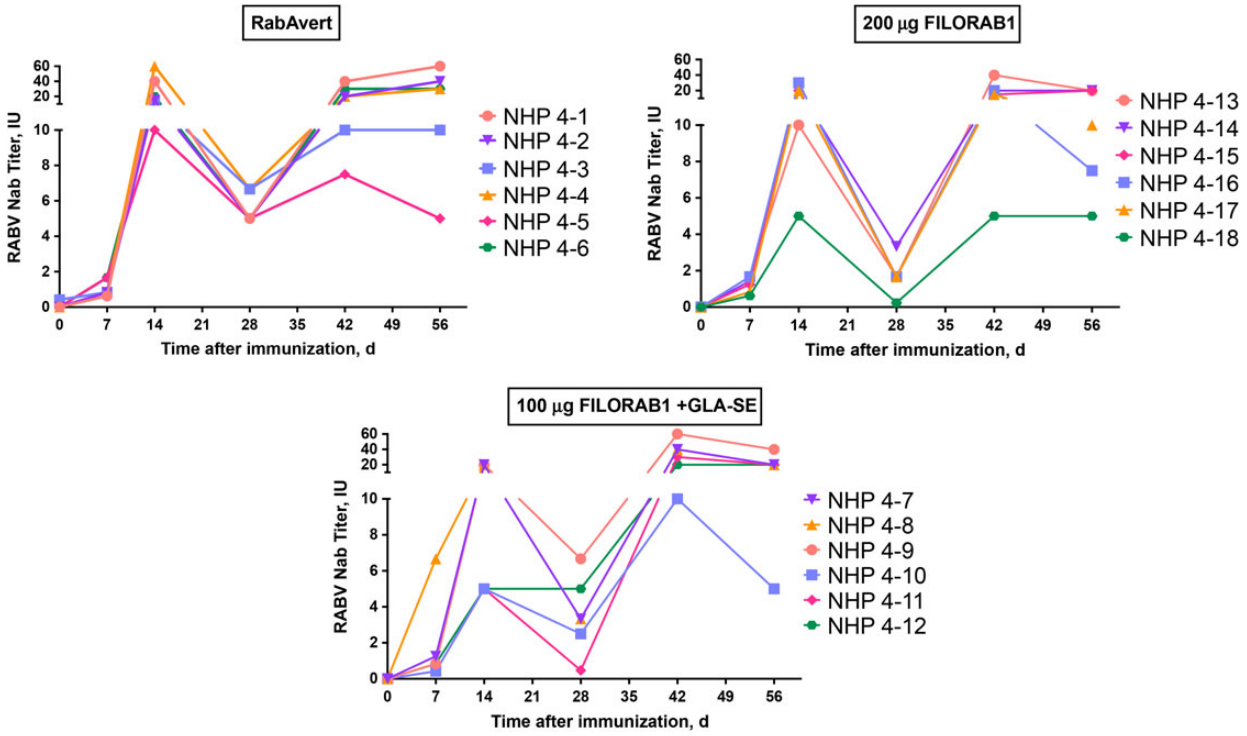

Figure 6. Development of rabies virus (RABV) neutralizing antibodies (NAbs) over time in immunized rhesus monkeys (from nonhuman primate [NHP] study 4). Animals were immunized on days 0, 7, and 28 (green) with the indicated vaccine, and RABV NAbs were analyzed over time and determined as international units (IUs), using a World Health Organization standard. Abbreviation: GLA-SE, glucopyranosyl lipid A in stable emulsion. This figure is available in black and white in print and in color online.

GP was incorporated into RABV virions. In general, codon-optimization of transgenes expressed by RABV-based vectors increased the expression levels of GP but also enhanced the incorporation of the foreign proteins into RABV particles [30, 40]. In a small follow-up study in NHPs, the inactivated second-generation RABV/EBOV vaccine FILORAB1 proved to be safe, immunogenic, and efficacious in NHPs against a 100-PFU EBOV challenge when combined with 2 different experimental adjuvants [41].

Here we present results of larger experiments evaluating the efficacy and 2 vaccine regimens of inactivated FILORAB1 in NHPs. Two antigenic doses of unadjuvanted FILORAB1 and a GLA-SE–adjuvanted low dose of FILORAB1 on 2 different vaccination schedules in 2 NHP species were evaluated. The interval from final immunization to challenge was kept as similar as logistically possible to the interval used in the initial experiments. A total of 100 µg of FILORAB1 plus GLA-SE provided 100% protection with no or minimal clinical signs observed after challenge. GLA-SE has been shown to stimulate production of several inflammatory cytokines, type I interferons, elicit maturation of dendritic cells, prime T-helper type 1 (Th1) cell responses, and induce both antibody and T-cell–mediated protective immunity in multiple animal models of infectious diseases [42–45]. Importantly, GLA-SE has been safely administered to >1000 human subjects in clinical trials as a vaccine adjuvant and shown to induce potent neutralizing antibody and $CD4^+$ T-cell responses [37].

NHPs immunized with the unadjuvanted vaccine mounted higher EBOV antibody responses after 3 immunizations than after 2 immunizations. However, 2 of 6 animals in the 200-µg group, and 1 of 4 in the 100-µg group were protected. Since EBOV antibody titers in the unadjuvanted 200 µg FILORAB1 group were comparable or only slightly inferior to the GLA-SE–adjuvanted 100-µg FILORAB1 group, these data suggest that the additional protection provided by GLA-SE is due to qualitative and quantitative changes in the antibody response against EBOV. In addition, T-cell responses are known to contribute to protection against EBOV, and GLA-SE has been shown to prime strong Th1-type $CD4^+$ T-cell responses and to a lesser extent also $CD8^+$ T-cell responses [46]. To this end, the contribution of the T-cell response to the protection that GLA-SE–adjuvanted FILORAB1 provided against EBOV challenge is currently under investigation.

Interestingly, we observed reactions at the site of the injection of the challenge virus. The cause of these reactions is unknown at this time. One possible explanation is response to Vero cell protein. In these experiments, FILORAB1 and the EBOV stock were both propagated on Vero cells, thus it is possible that the edema is caused by a localized reaction of varying severity to Vero proteins. To explore this possibility and others,

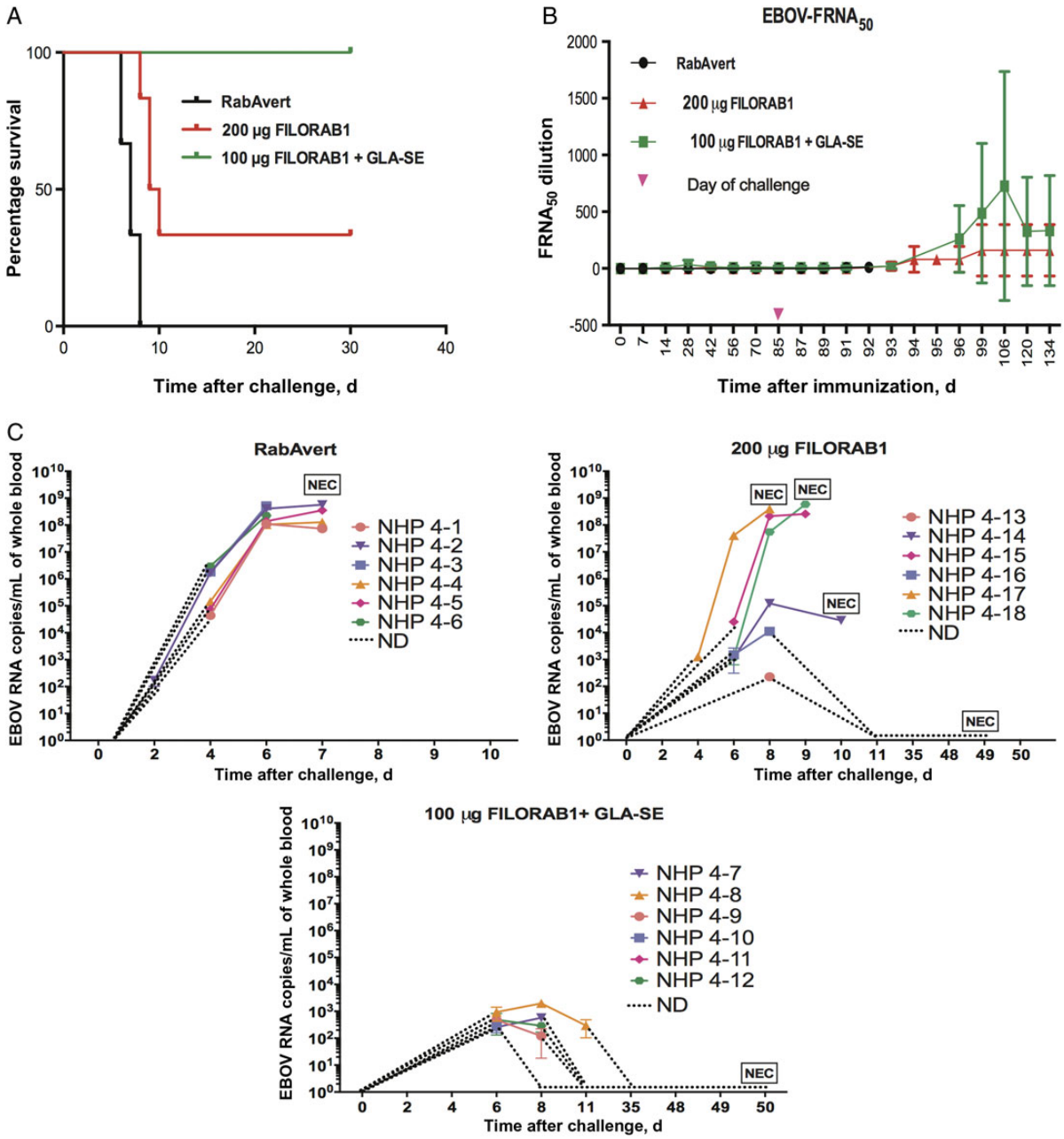

Figure 7. Survival (*A*), neutralizing antibody (NAb) titer against Ebola virus (EBOV; (*B*), and RNA loads after challenge of immunized nonhuman primates (NHPs) in NHP study 4 (NHP 4; *C*). Immunized NHPs were challenge with 1000 plaque-forming units of EBOV Makona C05 intramuscularly on day 85 after the first immunization. *A*, Survival curve demonstrating 100% efficacy of FILORAB1 plus glucopyranosyl lipid A in stable emulsion (GLA-SE). *B*, NAb titer as measured by a fluorescence reduction neutralizing–50% assay ($FRNA_{50}$). *C*, Total viral RNA (genomic and messenger RNA) at different time points after challenge. Abbreviation: ND, not detectable.

a follow-up experiment will be performed in which groups of vaccinated NHPs will be challenged in parallel with EBOV and γ-irradiated EBOV.

In this experiment, we used well-characterized stocks that were sequenced to ensure fidelity with the published sequences for Makona isolates of EBOV [36]. Historically, the most commonly used EBOV for countermeasure efficacy studies is the prototype EBOV strain Mayinga. Marzi et al reported that there is a slight delay in onset of the disease with Makona when compared to Mayinga in cynomolgus monkeys [47]. However, the mean day to end point and final outcome of EBOV-induced disease was similar with both viral strains, indicating that the use of either virus in challenge studies is justified. In our 2 studies, the observed differences in disease and survival for the RabAvert only group was 0.8 days and not statistically significantly different by Kaplan–Meier analysis (GraphPad Prism 6.0).

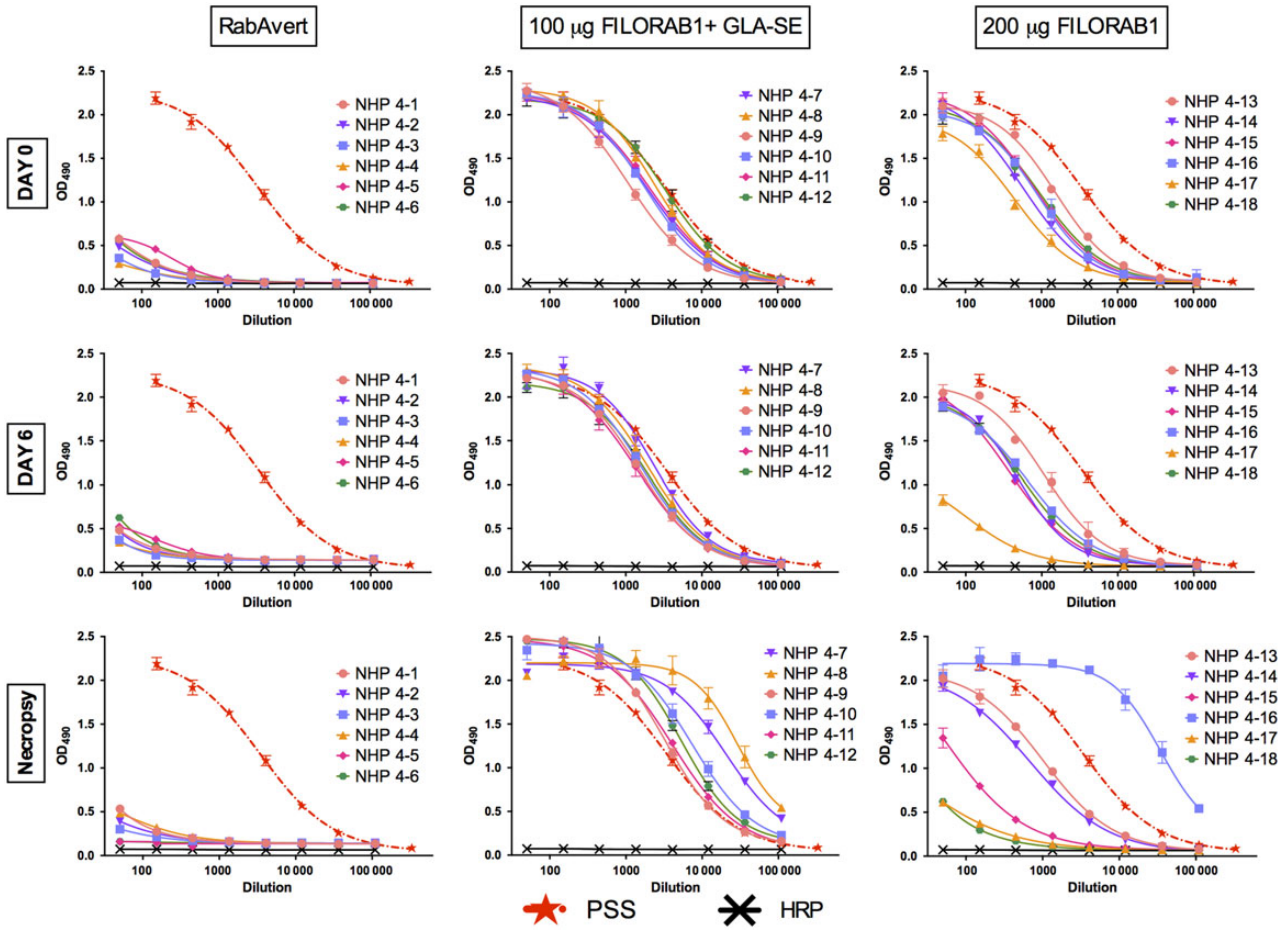

Figure 8. Humoral immune response to Ebola virus (EBOV) glycoprotein (GP) after challenge in nonhuman primate (NHP) study 4 (NHP 4). Sera from NHPs were analyzed for total immunoglobulin G (IgG) with an EBOV GP (Zaire)–specific enzyme-linked immunosorbent assay. OD_{490} readings were compared to those for pooled sera from the 8 surviving NHPs from a previous study (NHP 1) as a positive control (PSS). All sera were diluted 1:50, followed by 3-fold serial dilutions for analysis. Abbreviations: GLA-SE, glucopyranosyl lipid A in stable emulsion; HRP, horseradish peroxidase.

Currently, the lead EBOV vaccine candidates are a chimpanzee adenovirus-based and a VSV (rVSV-GP)–based vaccine. The latter was recently reported to have 100% efficacy in the preliminary analysis of a phase 2/3 trial using a ring-vaccination cluster-randomized design during the EBOV epidemic in Guinea [48]. While virally vectored vaccines can protect against EBOV infection with a single immunization and are thus well suited for ring vaccination, recent modeling has raised the question whether this would have been sufficient to stop the EBOV epidemic [49]. The model predicted that if more than a few cases were missed, large outbreaks could occur under ring vaccination. The authors conclude that mass vaccination or hybrid strategies involving mass and ring vaccinations might need to be considered alongside ring vaccination when planning for future outbreaks [49]. In addition, replication-competent vaccines have drawbacks with regard to safety, especially in immunocompromised persons, and will likely not be suitable for mass immunization of the general population. Thus far, the candidate vaccines require low-temperature cold chains for long-term storage and have limited stability at ambient temperature, in the case of EBOV-VSV, 24 hours at 25°C [50]. This makes them less well suited for stockpiling and mass immunization in tropical conditions. In contrast, inactivated RABV vaccine can be lyophilized without loss of antigenicity [51], indicating that lyophilized FILORAB1 should be explored. One concern with FILORAB1 is that 2–3 immunizations are required, but several currently approved and used vaccines need to be administered multiple times, and this is performed successfully. Of note, FILORAB1 builds on the excellent safety record of the current inactivated RABV vaccine approved for human use, which has been administered to tens of millions of people, including pregnant women and children, and addresses several shortcomings of the virally vectored EBOV vaccines currently in development by offering an improved safety profile, bivalency for a more common pathogen, and possibly improved stability for long-term storage. Further development

would include production and testing of a GMP-produced lot, monitoring and understanding of the injection site reaction, dose ranging of adjuvanted vaccine, and development and testing of lyophilized FILORAB1.

Notes

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Potential conflicts of interest. P. B. J., R. F. J., and M. J. S. have a pending patent application entitled "US Prov. Appl. Multivalent Vaccines for Rabies Virus and Filovirus." J. t. M. is a full-time employee of Immune Design. J. T. W. is a full-time employee of Genetic Sciences, Thermo Fisher Scientific. All other authors report no potential conflicts. 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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