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FILOVIRUS SYMPOSIUM SUPPLEMENT

Preexisting immunity does not prevent efficacy of VSV-based filovirus vaccines in nonhuman primates

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Ebola virus (EBOV) and Marburg virus (MARV) made headlines in the past decade causing outbreaks of human disease in previously nonendemic yet overlapping areas. While EBOV outbreaks can be mitigated with licensed vaccines and treatments, there is not yet a licensed countermeasure for MARV. Here, we used nonhuman primates (NHPs) previously vaccinated with VSV-MARV and protected against lethal MARV challenge. After a resting period of 9 months, these NHPs were re-vaccinated with VSV-EBOV and challenged with EBOV resulting in 75% survival. Surviving NHPs developed EBOV GP-specific antibody titers and no viremia or clinical signs of disease. The single vaccinated NHP succumbing to challenge showed the lowest EBOV GP-specific antibody response after challenge supporting previous findings with VSV-EBOV that antigen-specific antibodies are critical in mediating protection. This study again demonstrates that VSVΔG-based filovirus vaccine can be successfully used in individuals with pre-existing VSV vector immunity highlighting the platform’s applicability for consecutive outbreak response.

Keywords: Vesicular stomatitis virus; NHP; Ebola virus; Marburg virus; cross-protection

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INTRODUCTION

Outbreak reports of filovirus disease caused by Ebola virus (EBOV) and Marburg virus (MARV) have been frequent in the last few years [1, 2]. While EBOV caused recent outbreaks mainly in the Democratic Republic of the Congo [1], human MARV infections were reported from areas where the virus had not been described previously including Guinea, Ghana and Equatorial Guinea [2]. For EBOV, vaccines and treatments have been licensed for human use [3, 4], however, there is not a licensed countermeasure for MARV yet, but several vaccine and therapeutic candidates are in clinical development [5]. With the expansion of MARV endemic areas to regions where EBOV is also endemic like Guinea it becomes increasingly more important to understand immune response dynamics after vaccination and treatment of one filovirus and how it may impact the efficacy of countermeasures against another filovirus. Previous studies showed that VSVΔG vectors can be blended and used for consecutive vaccinations against filo- and arenaviruses without compromising protective efficacy [6, 7]. In addition, we recently demonstrated that pre-existing immunity to the licensed vesicular stomatitis virus-based vaccine (VSV-EBOV, Ervebo) and EBOV did not negatively impact immunogenicity of a VSV-based Sudan virus (SUDV) vaccine when single-dose vaccinations were administered one year apart [8]. EBOV and SUDV are closely related ebolaviruses and the vaccinations elicited cross-reactive humoral responses.

Here, we expanded upon this data and investigated how pre-existing immunity to VSV-MARV and MARV could impact the immunogenicity of VSV-EBOV vaccination when the vaccines were administered approximately one year apart. We found that 75% of the nonhuman primates (NHPs) survived the EBOV challenge and the single vaccinated NHP succumbing to challenge had the lowest antigen-specific immune response.

METHODS

Ethics statement

All work involving MARV and EBOV was performed in the maximum containment laboratory (MCL) at the Rocky Mountain Laboratories (RML), Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. RML is an AAALACi-accredited institution. All procedures followed RML Institutional Biosafety Committee (IBC)-approved standard operating procedures (SOPs). Animal work was performed in strict accordance with the recommendations described in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, the Office of Animal Welfare and the Animal Welfare Act, United States Department of Agriculture. This study was approved by the RML Animal Care and Use Committee (ACUC), and all procedures were conducted on anesthetized animals by trained personnel under the supervision of board-certified clinical veterinarians. The NHPs were observed at least twice daily for clinical signs of disease according to a RML ACUC-approved scoring sheet and humanely euthanized when they reached endpoint criteria. NHPs were housed in adjoining
individual primate cages that enabled social interactions, under controlled conditions of humidity, temperature, and light (12 hours light - dark cycles). Food and water were available ad libitum. NHPs were monitored and fed commercial monkey chow, treats, and fruit at least twice a day by trained personnel. Environmental enrichment consisted of commercial toys, music, video, and social interaction. All efforts were made to ameliorate animal welfare and minimize animal suffering in accordance with the Weatherall report on the use of NHPs in research (https://royalsociety.org/policy/publications/2006/weatherall-report/).

**Cells and viruses**

Vero E6 cells (*Mycoplasma* negative, RRID: CVCL_0059) were grown at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Wisent Inc., St. Bruno, Canada), 2 mM L-glutamine, 50 U/mL penicillin, and 50 mg/mL streptomycin (all supplements from Thermo Fisher Scientific, Waltham, MA). Previously described VSV-based vaccines expressing either the EBOV-Mayinga glycoprotein (GP) (VSV-EBOV)[9] or the MARV-Angola GP (VSV-MARV)[10] were used in this study. MARV-Angola (GenBank KY047763) was used as challenge virus in the first study [11]. EBOV-Makona Guinea C07 (GenBank KP096421.1) was propagated once on Vero E6 cells, titered by median tissue culture infectious dose (TCID50) assay on Vero E6 cells and stored in liquid nitrogen. Deep sequencing revealed no contaminants and a target dose of 10,000 TCID50 (MARV back titration 14,000 TCID50; EBOV back titration 14,000 TCID50) was used for the IM MARV and EBOV challenges. Viral titers in blood and tissue samples were determined as previously described [12].

**NHP study design**

For this study we used cynomolgus macaques that were previously incompletely CD3 T cell depleted during VSV-MARV (n=4) or VSV-Lassa virus (LASV) (n=2) vaccination followed by a subsequent MARV or LASV challenge, respectively. The animals were rested for ~6 - 9 months prior to study start. Six male or female cynomolgus macaques, 7-9 years of age and 3.4-9.1 kg were used in this study. Four NHPs (2 male, 2 female) were intramuscularly (IM) vaccinated with 1x 10^7 PFU VSV-EBOV (Fig. S1A). The 2 control NHPs (2 male) were vaccinated with 1x 10^7 PFU VSV-MARV (control). All animals received a 1ml IM injection for vaccination into 2 sites in the caudal thighs containing the vaccine. All 6 NHPs were challenged IM on 0 DPC with 1,000 PFU EBOV-Makona C07 into 2 sites in the caudal thighs. Physical examinations and blood draws were performed as outlined in Fig. S1A. Following euthanasia, a necropsy was performed, and samples of key tissues were collected for analysis.

**Analysis of humoral immune responses**

Post-challenge NHP sera were inactivated by γ-irradiation (4 MRad)[13, 14], and removed from the MCL according to SOPs approved by the RML IBC. Titers for IgG specific to EBOV GP or MARV GP were determined in endpoint dilution ELISAs using recombinant EBOV GPΔTM

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levels of VSV-specific IgG were determined as previously described [8]. Neutralization of irradiated and heat-inactivated serum samples were assessed in Vero E6 cells as previously described [8]. VSV-EBOV-GFP or VSV-MARV-GFP were used as previously described [16], and the number of GFP-positive cells was counted on the FACSymphony A5 Cell Analyzer (BD Biosciences, Mississauga, ON, Canada). Data were analyzed using FlowJo V10.

**Statistical analysis**

Statistical analysis was performed in Prism 9 (GraphPad). Significant differences in the survival curves shown in Fig. 1A were determined performing Log-Rank analysis. Statistical significance is indicated in the figure panel.

**RESULTS**

**Pre-existing MARV immunity does not impact immunogenicity of VSV-EBOV**

Of the 6 NHPs used in this study 2 animals were previously vaccinated with VSV-LASV and survived the LASV challenge. Here, they were vaccinated with VSV-MARV ~ 6 months after the LASV challenge study was completed and served as the challenge control group. The 4 NHPs in the vaccine group were previously vaccinated with VSV-MARV, survived the lethal MARV challenge and, after a ~9 months resting period, were vaccinated with VSV-EBOV (Fig. S1A). All 6 NHPs were challenged with a lethal IM dose of EBOV 28 days after vaccination. Both control NHPs reached endpoint criteria 6 days post-infection (dpi) and were euthanized (Fig. 1A, B). None of the VSV-EBOV-vaccinated NHPs had detectable EBOV RNA levels in the blood at 3 dpi, however, at 6 dpi vaccinated NHP #1 presented with viremia (Fig. 1C) and thrombocytopenia (Fig. 1D). This NHP reached endpoint criteria on 8 dpi and was euthanized (Fig. 1A, B). Interestingly, this NHP had developed elevated white blood cell (WBC) and neutrophil levels at 3 dpi that returned to normal levels by 6 dpi (Fig. S2A, B). Serum chemistry analysis revealed that this NHP had developed other characteristic signs of Ebola virus disease (EVD) in addition to viremia and thrombocytopenia including decreased calcium and elevated liver enzyme levels (Fig. S2C, D) similar to the control NHPs. Further analysis for the 3 terminally ill NHPs resulted in elevated blood urea nitrogen (BUN) and creatinine levels (Fig. S2E, F) indicating impaired kidney function, another sign of EVD [17]. In tissue samples collected at the time of necropsy EBOV titers were comparable in lymph nodes between all 3 NHPs, however, the vaccinated NHP had lower EBOV titers in the liver, spleen and adrenal gland (Fig. S3A). Samples collected on day 42 from the surviving NHPs were negative for EBOV by titration.
The level of the EBOV GP-specific IgG response correlates with survival after EBOV challenge

Protection by VSV-based vaccines from filovirus disease is mainly mediated by the humoral immune response [9, 10, 18], therefore we focused our analysis on the antibody responses. Total IgG specific to MARV GP, EBOV GP and VSV were determined by ELISA. After the initial VSV-MARV vaccination, the 4 NHPs developed MARV GP-specific IgG that were boosted by the MARV challenge (Fig. S1B). During the resting period the MARV GP-specific IgG titers declined 16-fold and these NHPs had limited cross-reactive IgG specific to EBOV GP at the time of VSV-EBOV vaccination (Fig. S1B, day 322). The control NHPs only presented with background-levels of MARV or EBOV GP-specific IgG at the time of VSV-MARV vaccination (Fig. 2A, C) as they had previously been vaccinated with VSV-LASV and survived LASV challenge. As all 6 NHPs in this study had previously received a VSV-based vaccine, they developed a robust VSV-specific IgG response that was boosted by the vaccination on day -28 and mostly sustained throughout the entire study in the surviving NHPs (Fig. S3B).

There was only a limited and temporary boost-effect observed in the MARV GP-specific IgG and neutralizing response in the 4 NHPs surviving MARV challenge (Fig. 2A, B). In contrast, these 4 VSV-EBOV-vaccinated NHPs developed EBOV GP-specific IgG titers after vaccination ranging from 1:6,400-1:25,600 at the time of challenge (Fig. 2C). The EBOV challenge boosted the EBOV GP-specific IgG response to a level that was maintained for the duration of the study for surviving NHPs (Fig. 2C). One of the control NHPs had a temporary 1:400 for EBOV GP-specific IgG titer on day 4 but was again at background level at the time of euthanasia (day 6; Fig. 2C). The EBOV neutralizing response was assessed using VSV-EBOV-GFP, and results followed the observations made with the EBOV GP-specific binding IgG titers demonstrating a boost-effect of the EBOV challenge.

DISCUSSION

In this study we used previously VSV-MARV-vaccinated and MARV-challenged animals and showed that subsequent VSV-EBOV vaccination resulted in protective efficacy from lethal EBOV challenge. This confirms the consecutive use of VSVΔG-based vaccine vectors demonstrating that pre-existing VSV vector immunity does not affect the protective efficacy of a subsequent vaccination. This is important as nowadays overlapping endemic areas have to be considered for filoviruses and other emerging/reemerging pathogens.

Protection in the VSV-EBOV vaccine group was not uniform and one animal succumbed to EBOV challenge. The animals were CD3 depleted about 9 months prior to VSV-EBOV vaccination. CD3 depletion at the time was considered incomplete as evidenced by the development of MARV GP-specific antibodies following VSV-MARV vaccination. While the incomplete CD3 depletion did not impact the survival from the previous lethal MARV challenge, it may have had a long-term...
effect on the immune system of the NHPs, especially the animal that succumbed to EBOV challenge following VSV-EBOV vaccination approximately 9 months after CD3 depletion. Human clinical trial data show that CD3 depletion may affect the immune system for up to 2 years after anti-CD3 antibody is administered in some patients [19]. Interestingly, this NHP had the highest WBC and neutrophil levels at 3 days after EBOV challenge indicating a dysregulated immune response. Indeed, analysis of human samples from the West African EBOV epidemic suggested that neutrophils may dysregulate the adaptive immune response resulting in tissue damage contributing to fatal outcome [20]. Unfortunately, peripheral blood mononuclear cells (PBMCs) were not collected and analyzed during this study as previous studies had only identified a limited role for T cells in mediating protection against filoviruses using VSV-based vaccines [9, 10, 18]. This missed PBMC analysis could have shed light on WBC composition in this NHP compared to the control NHPs and vaccinated-surviving NHPs and strengthened the human data set. We may have also identified differences in stimulated cell populations during vaccination and possibly detected any impact of the previous CD3 depletion.

As stated above, EBOV GP-specific IgG has in the past been identified as the main mediator of protective immunity using VSV-based filovirus vaccines. Neutralizing titers 4 weeks after VSV-MARV and VSV-EBOV vaccination were in the range as previously observed [10, 12]. Interestingly, the titer of EBOV GP-specific IgG in the vaccinated NHP succumbing to disease was similar to the other 3 vaccinated NHPs at the time of challenge. However, the EBOV infection did not result in a boost of this IgG response in this NHP as it did in the 3 others in the group. Instead, the EBOV GP-specific IgG titer dropped on days 3 and 6 indicating antibody consumption by virus replication. The NHP was euthanized 2 days after the last control NHP demonstrating a limited benefit of the VSV-EBOV vaccination. This data highlights again the importance of the EBOV GP-specific IgG response mediating protection and survival from EBOV infection [9]. However, we cannot exclude that other immune factors are contributing to protection as the single non-surviving NHPs had similar antigen-specific IgG titers compared to the 3 surviving NHPs. Future studies will include extensive immune cell and antibody functionality analysis that was not conducted here.

This study also adds to the growing body of evidence demonstrating that VSVΔG-based vaccines can be used for several high-consequence emerging viral diseases including filoviruses for consecutive vaccination as VSVΔG vector immunity does not impact VSV replication, therefore, the immunogenicity of the viral antigen [6-8]. Indeed, previous studies showed that VSV vaccines for Lassa virus and EBOV are effective when used one after the other [7]. Additionally, these VSVΔG- vectored vaccines have been efficacious as a blended filovirus vaccine in the past [6] highlighting the platform’s versatility and applicability as a disease outbreak vaccine. However, we cannot exclude that the pre-existing vector immunity may have impacted vaccine efficacy in the single NHP that succumbed to disease. Future studies will investigate this possibility.

While the study supports previous findings with this vaccine platform, it also has its limitations. First, the NHPs were CD3 T cell-depleted and we did not confirm their CD3 depletion status at

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the time of VSV-EBOV vaccination because the depletion occurred almost 1 year prior and was considered incomplete as evidenced by the development of antigen-specific humoral responses. The study also was small with only 4 vaccinated and 2 control NHPs limiting analysis and data interpretation. Lastly, we used the EBOV-Makona as the challenge virus when the VSV-EBOV contained the EBOV-Mayinga GP as vaccine antigen. While there are only limited amino acid differences between both GPs, we cannot exclude, even though it seems unlikely, that this “heterologous” EBOV challenge may have contributed to the fatal outcome of the single vaccinated NHP.

Future studies will address different time intervals between vaccination with different VSV-based filovirus vaccines. Ideally, these studies should be expanded to investigate the efficacy of “mix-and-match” vaccines including adenovirus-vectored or subunit vaccines in order to inform the use of different vaccines for population-based and emergency outbreak vaccinations in the filovirus endemic areas.

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(1) H.F. claims intellectual property on VSV-based filovirus vaccines. All other authors do not have any conflict of interest.

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(3) Conferences where this work has been presented: N/A
Figure 1. Clinical disease after EBOV challenge. NHPs were challenged with a lethal dose of EBOV 28 days after VSV-EBOV vaccination and monitored daily for signs of disease. (A) Survival and (B) clinical disease scores are depicted. (C) Viremia and (D) platelet count in whole blood samples collected from NHPs during the acute phase of the disease. Individual values for each NHP are presented.
Figure 2. Humoral immune responses after VSV-EBOV vaccination and EBOV challenge. NHPs were vaccinated with VSV-MARV (control) or VSV-EBOV (vaccinated) and serum samples were collected throughout the study. (A) Levels of MARV GP-specific IgG or (C) EBOV GP-specific IgG were determined by ELISA. Serum titers neutralizing 50% of (B) VSV-MARV-GFP or (D) VSV-EBOV-GFP infected Vero E6 cells. Individual values for each NHP are presented. The dotted line indicates limit of detection.
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