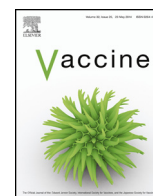




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Immunization with vesicular stomatitis virus vaccine expressing the Ebola glycoprotein provides sustained long-term protection in rodents



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ABSTRACT

Ebola virus (EBOV) infections cause lethal hemorrhagic fever in humans, resulting in up to 90% mortality. EBOV outbreaks are sporadic and unpredictable in nature; therefore, a vaccine that is able to provide durable immunity is needed to protect those who are at risk of exposure to the virus. This study assesses the long-term efficacy of the vesicular stomatitis virus (VSV)-based vaccine (VSVΔG/EBOVGp) in two rodent models of EBOV infection. Mice and guinea pigs were first immunized with 2×10^4 or 2×10^5 plaque forming units (PFU) of VSVΔG/EBOVGp, respectively. Challenge of mice with a lethal dose of mouse-adapted EBOV (MA-EBOV) at 6.5 and 9 months after vaccination provided complete protection, and 80% (12 of 15 survivors) protection at 12 months after vaccination. Challenge of guinea pigs with a lethal dose of guinea pig-adapted EBOV (GA-EBOV) at 7, 12 and 18 months after vaccination resulted in 83% (5 of 6 survivors) at 7 months after vaccination, and 100% survival at 12 and 18 months after vaccination. No weight loss or clinical signs were observed in the surviving animals. Antibody responses were analyzed using sera from individual rodents. Levels of EBOV glycoprotein-specific IgG antibody measured immediately before challenge appeared to correlate with protection. These studies confirm that vaccination with VSVΔG/EBOVGp is able to confer long-term protection against Ebola infection in mice and guinea pigs, and support follow-up studies in non-human primates.

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1. Introduction

Ebola virus (EBOV) infections cause severe disease characterized by hemorrhage, multi-organ failure, and death in up to 90% of untreated human cases. Past EBOV outbreaks have been sporadic in nature and confined to central Africa, in which the aggregate number of infections up to early 2014 have caused 1093 deaths from 1393 total cases, for a 78.5% case fatality rate [1]. The EBOV outbreak in the West African countries of Guinea and Liberia in 2014, as well as confirmed imported cases in Sierra Leone and several suspected cases in the surrounding countries, indicates that EBOV is geographically more widespread than previously thought. The identity of the EBOV reservoir remains elusive despite efforts, although it has been suggested that fruit bats are one of the potential reservoir species [2]. This complicates the strategy of eradicating EBOV and

preventing its transmission to humans by targeting the reservoir for vaccination. As a result, there is a need for a preventive vaccination program tailored for humans at high risk of exposure to EBOV, such as residents of areas considered endemic for EBOV, primary health care providers in these endemic areas, as well as biosafety level (BSL)-4 laboratory researchers.

There are several factors to consider before the potential implementation of an EBOV vaccination program, which include cross-protection against other Ebola species, pre-existing immunity of the recipient population to the vaccine vector, ease of implementing the vaccine regimen and sustained vaccine-induced protection. Several experimental vaccine platforms, most of which express the EBOV glycoprotein (GP) as the viral antigen of choice for immunizations, have demonstrated protection in nonhuman primates (NHPs) against lethal EBOV challenge. These vaccine platforms include a DNA prime with a human adenovirus serotype 5 (Ad5) boost [3], Ad5 alone [4], virus-like particles [5], human parainfluenza virus type 3 [6], rabies virus [7] as well as vesicular stomatitis virus (VSV) [8].

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From the candidates listed above, the live-attenuated VSV-based vaccine (VSVΔG/EBOVGP) is unique in that it has demonstrated both prophylactic and post-exposure efficacy [8,9], suggesting that the vaccine is able to rapidly establish protective immunity in the recipient after immunization. Mucosal vaccination with VSVΔG/EBOVGP via the intranasal (IN) or oral (OR) routes still protects NHPs [10], easing the difficulty of administration by eliminating the use of needles. VSV-based vaccines expressing GP from either Marburg virus (MARV) or Sudan virus (SUDV) (known as VSVΔG/MARVGP or VSVΔG/SUDVGP, respectively) are able to protect against a homologous virus challenge [11,12]. A blended vaccine consisting of equal parts VSVΔG/EBOVGP, VSVΔG/MARVGP and VSVΔG/SUDVGP conferred complete protection against EBOV, MARV or SUDV challenge [13]. Complete protection was also achieved in these NHPs after challenge with Tai Forest virus (TAFV), suggesting evidence of cross-protection [13]. VSVΔG/EBOVGP has been previously administered as an emergency treatment to a researcher on compassionate grounds after a suspected EBOV exposure in the laboratory. Aside from a fever detected 12 h after vaccination, the recipient remained healthy [14]. While clinical trials will be needed, this is a positive sign for the safety of VSV-based vaccines. Pre-existing immunity to VSV is not prevalent in humans [15–17], and therefore it is not expected to be a significant factor in a potential vaccination campaign with VSV-based vaccines.

There has been a lack of studies investigating sustained protection conferred by VSV-based vaccines. In studies involving other pathogens, protection was observed after 4 months in mice immunized with a VSV encoding the Severe Acute Respiratory Syndrome coronavirus spike protein [18]. Additionally, mice vaccinated with recombinant VSV encoding the hemagglutinin antigen of H5N1 influenza virus were still completely protected 1 year after vaccination [19], and VSV expressing the Andes virus glycoproteins was still efficacious after 6 months in Syrian hamsters [20]. Protection from Andes virus challenge was still observed in Syrian hamsters 1 year after VSV immunization; however, it was not possible to distinguish vaccine efficacy from an increased resistance to infection with age in the control animals [20]. Recently, the VSVΔG/MARVGP vaccine was shown to provide full protection in cynomolgus macaques challenged 14 months after immunization [21], demonstrating that the VSV platform has the potential to provide durable immunity.

Long-term protection against EBOV infection has not yet been published with any of the experimental vaccines listed above. The aim of this study is to investigate whether VSVΔG/EBOVGP administration can provide long-term protection against EBOV challenge in both mice and guinea pigs. For the purpose of this study, we define long-term as extending beyond 6 months after vaccination. Two past studies have demonstrated the importance of the antibody response in VSVΔG/EBOVGP-mediated protection of NHPs [22], and that elevated IgG levels statistically correlated with survival in VSVΔG/EBOVGP-vaccinated, EBOV-challenged NHPs [23]. This study will also test whether humoral responses, in the form of neutralizing antibody (nAb) and IgG antibody, are indicative of sustained protection.

2. Results

2.1. Survival and weight change after challenge in mice

To determine whether long-term protection could be achieved after a single injection of VSVΔG/EBOVGP, mice were vaccinated via the intraperitoneal (IP) route with either 2×10^4 plaque forming units (PFU) of VSVΔG/EBOVGP ($n=25$), or DMEM ($n=20$) as a mock treatment. After the initial immunization, groups of five

animals for each treatment were challenged IP with $1000 \times LD_{50}$ of mouse-adapted EBOV (MA-EBOV) at 6.5 or 9 months after vaccination. Weight change and clinical symptoms were monitored daily for 15 days, and survival was monitored until 28 days post-infection (dpi). Of note, a larger number of animals (10 for DMEM and 15 for VSVΔG/EBOVGP) were challenged at 12 months post-vaccination, because some previously vaccinated and unchallenged mice housed in BSL-2 containment unexpectedly died due to old age [24]. A decision was made to challenge the remaining mice, as longer intervals between immunization and challenge could not be reliably assessed beyond 12 months in this animal model. Mock-treated animals rapidly lost weight after MA-EBOV challenge and succumbed to disease by 7 dpi. The mean time to death (MTD) of all control mice was 5.4 ± 1.0 days. In contrast, mice immunized with VSVΔG/EBOVGP were completely protected against MA-EBOV when challenged at 6.5 and 9 months after vaccination, and did not display observable weight loss (Fig. 1A–D). When challenge was performed 12 months after vaccination, 12 of 15 (80%) animals were protected from MA-EBOV. The survivors did not display weight loss, whereas the three non-surviving mice lost weight and succumbed to disease with a MTD of 8.0 ± 1.0 days (Fig. 1E and F).

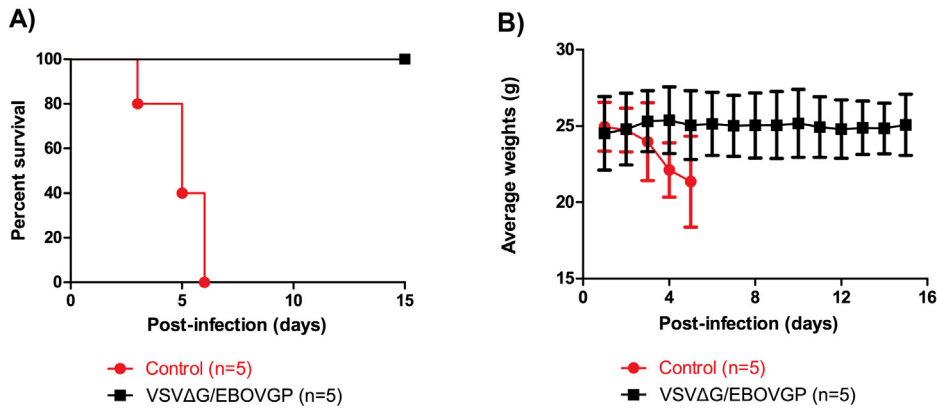
2.2. Antibody response in vaccinated mice over time

Since past studies have described the importance of humoral immunity in survival from EBOV infection, antibody responses from the mock and VSVΔG/EBOVGP vaccinated mice were measured. Serum samples were obtained from animals that were bled at 3, 6.5 and 12 months after vaccination. The humoral immune response was evaluated for levels of EBOV-specific neutralizing antibody (nAb) and GP-specific IgG antibody. EBOV-specific nAbs were only detected in 7 of 25 VSVΔG/EBOVGP vaccinated mice when measured 3 months after vaccination. The titers ranged from 13 to 53 (Fig. 2A, supplementary data). In contrast, high titers of GP-specific IgG were detected in all immunized mice at all times post-vaccination. The IgG titer reached between $\sim 10^4$ and $\sim 10^5$ at 3 months post-vaccination in all but two mice. In these two animals, the IgG titer only reached $\sim 10^3$, which was 4- to 32-fold less than the other mice. Twelve months after vaccination, GP-specific IgG titers decreased in some mice. One previously strong responder had its IgG titer decline to the level of the 3 month low responders (Fig. 2B, supplementary data). The three mice with lower IgG titers as measured at 12 months after vaccination ultimately did not survive MA-EBOV challenge.

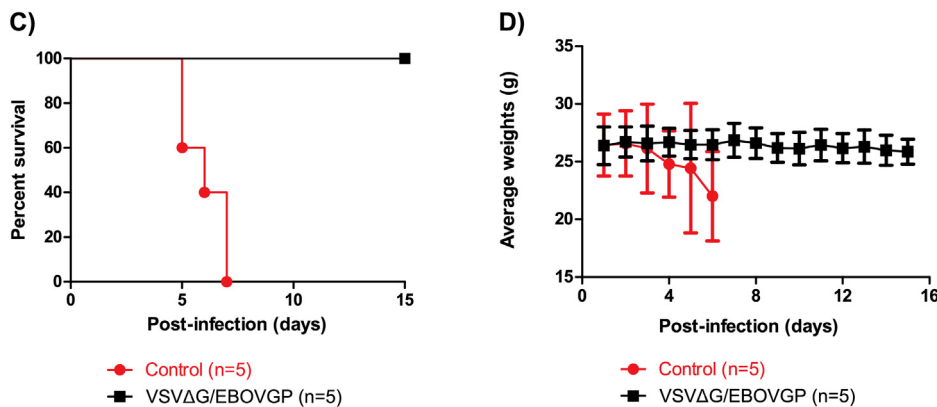
2.3. Survival and weight change after challenge in guinea pigs

To determine whether VSVΔG/EBOVGP is efficacious beyond 12 months, guinea pigs were vaccinated IP with either 2×10^5 PFU of VSVΔG/EBOVGP ($n=18$), or DMEM as a mock treatment ($n=12$). Groups of 6 VSVΔG/EBOVGP immunized and 4 DMEM treated animals were challenged IP with $1000 \times LD_{50}$ of guinea pig-adapted EBOV (GA-EBOV) at 7, 12 or 18 months after vaccination. Weight change and clinical symptoms were monitored daily for 15 days, and survival was monitored until 28 dpi. After GA-EBOV challenge, DMEM-treated animals rapidly lost weight and succumbed to disease by 8 dpi (MTD = 7.1 ± 0.5 days). In contrast, protection was observed in guinea pigs given VSVΔG/EBOVGP. Five of six (83%) animals were protected when challenged 7 months after vaccination (Fig. 3A and B), with the non-surviving guinea pig succumbing to disease at 7 dpi. Surprisingly, complete protection was observed in the animals that were challenged in the 12 and 18 month groups (Fig. 3C–F) without observable weight loss.

Challenged at 6.5 months



Challenged at 9 months



Challenged at 12 months

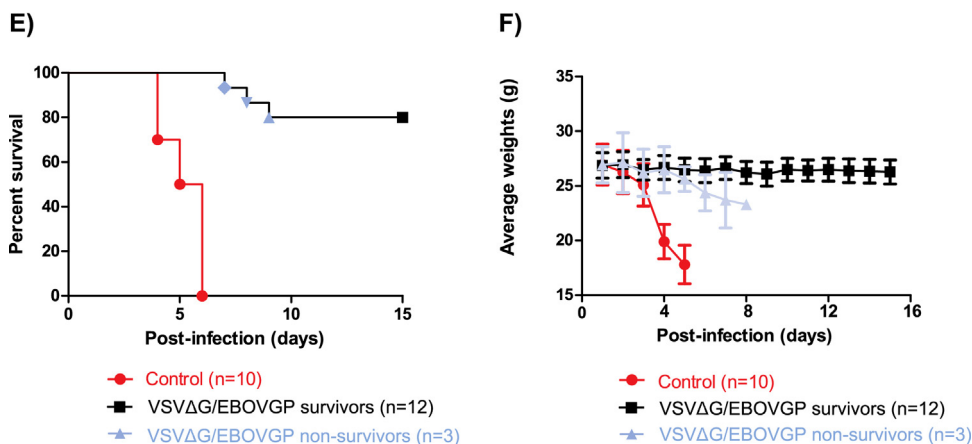


Fig. 1. Survival and weight loss of control and vaccinated BALB/c mice. Animals were vaccinated with VSVΔG/EBOVGP via the intraperitoneal (IP) route, and then challenged with $1000 \times LD_{50}$ of MA-EBOV IP at (A and B) 6.5 months, (C and D) 9 months, and (E and F) 12 months, respectively, after vaccination. Mice were monitored daily for survival as well as percentage change in weight. Error bars indicate 95% confidence intervals.

2.4. Antibody response in vaccinated guinea pigs over time

To determine the level of antibody responses of VSVΔG/EBOVGP vaccinated guinea pigs, serum samples were obtained from individual animals that were bled at 3, 7, 12 and 18 months after vaccination. Humoral responses were then evaluated for titers of both nAb and total GP-specific IgG antibody. EBOV-specific nAbs were detected in all 18 of the VSVΔG/EBOVGP vaccinated guinea

pigs, with titers ranging between 33 and 373 when measured at 3 months after vaccination. The sole non-surviving guinea pig had lower nAb levels, measured to be 40 at the time of challenge. Neutralizing antibody levels do not appear to be a reliable measure of protection, as there were other animals challenged at 12 and 18 months after vaccination that exhibited a similar level of nAbs shortly before challenge (Fig. 4A, supplementary data). GP-specific IgG was detected in all immunized guinea pigs in all time points

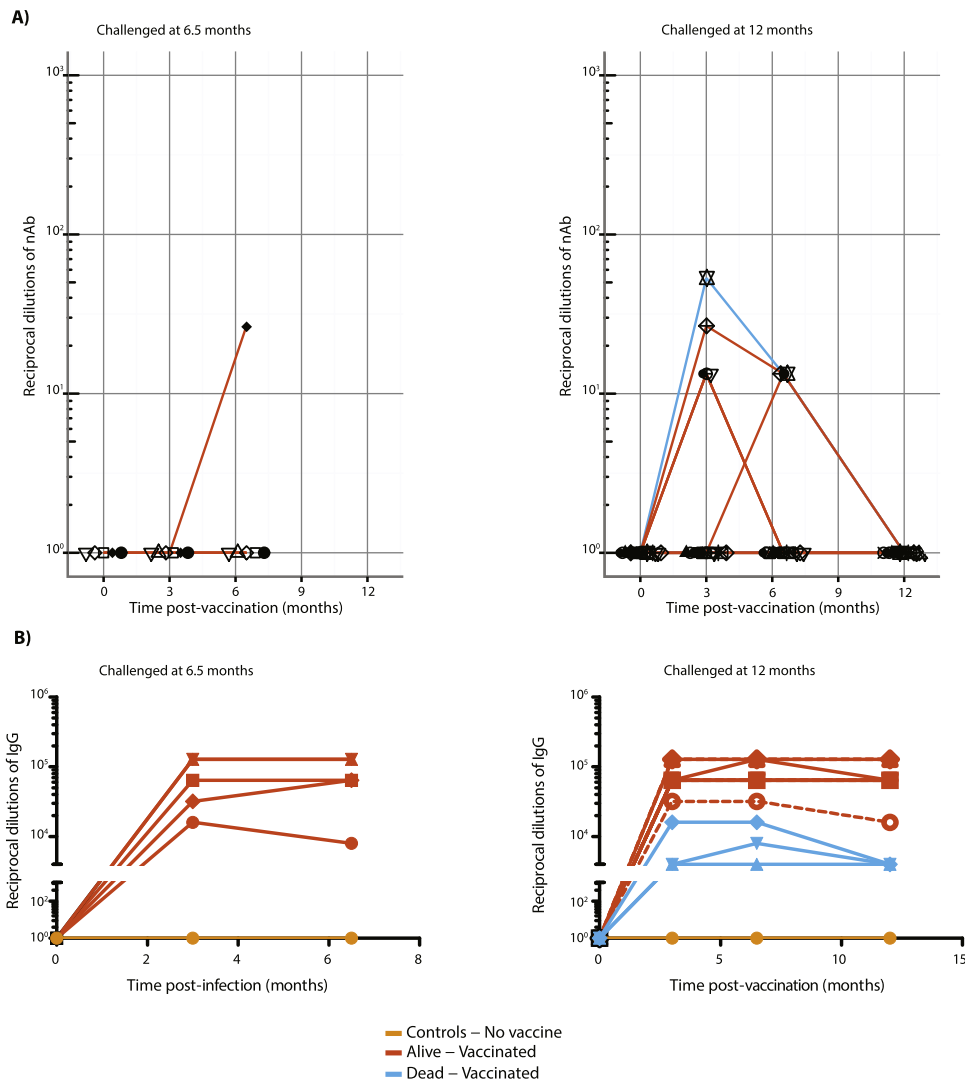


Fig. 2. Antibody responses in mice. Levels of (A) EBOV-specific neutralizing antibodies and (B) GP-specific IgG were measured from mouse serum harvested at 3, 6.5 and 12 months after immunization with VSVΔG/EBOVGP. Data points in panel A are dodged for better visualization of individual animals. VSV-vaccinated survivors are in red, VSV-vaccinated non-survivors are in blue, and DMEM-treated animals are in orange. The animals are separated by the time from immunization to challenge. Neutralizing and IgG antibody titers are expressed as reciprocal dilutions of nAbs and IgG, respectively.

post-vaccination. The titers at 3 months post-vaccination reached between $\sim 10^5$ and $\sim 10^6$ in all but one guinea pig. In this animal, the IgG titer only reached $\sim 10^3$, which was 25- to 4000-fold less than the other guinea pigs. A decrease in circulating IgG titers was not observed in the surviving guinea pigs, even 18 months after vaccination (Fig. 4B, supplementary data). The animal with the lowest levels of circulating GP-specific IgG did not survive GA-EBOV when challenged 7 months after vaccination.

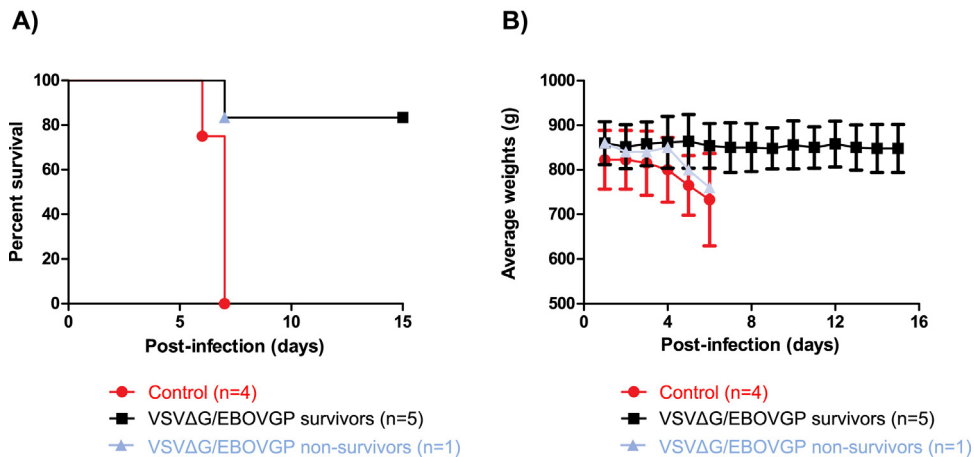
3. Discussion

Past efforts against EBOV have focused on the design of an efficacious vaccine or treatment to achieve short-term protection, and to control the immediate negative impact stemming from outbreaks. Space limitations in high bio-containment laboratories and the prohibitive costs of maintaining NHPs over a longer period of time are also reasons behind why there are very few studies investigating the long-term protection of EBOV vaccines. The data presented in this study show that a single injection of VSVΔG/EBOVGP provides durable protection in rodents. Similar to a previous study [25], VSVΔG/EBOVGP viremia was not detected in the immunized animals when RT-qPCR was performed on sera at 3 months after

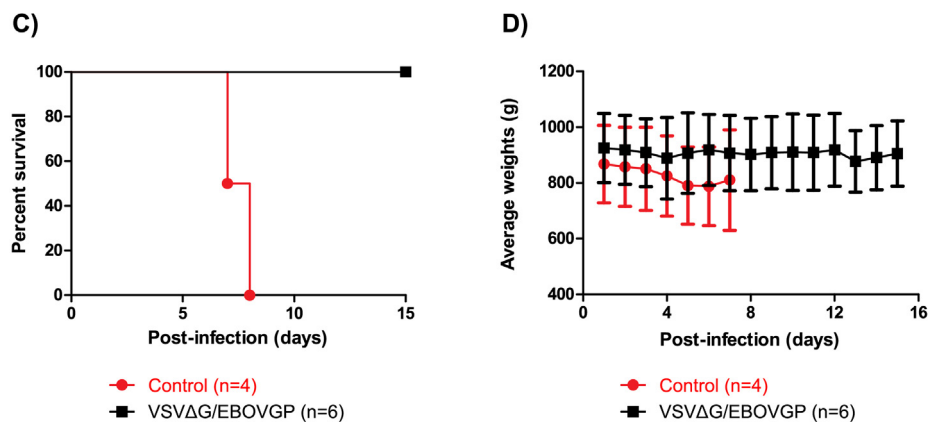
vaccination (data not shown), and there were no clinical signs after immunization. As highlighted by the 100% survival in guinea pigs challenged 18 months after immunization, the VSV-based vaccine is a promising long-term vaccine candidate. However, it was unknown whether sterile immunity was observed in these animals, as EBOV viremia was not measured after challenge. Specific IgG responses decreased at 12 months after vaccination in mice. This could be due to the old age of the animals, and may indicate the need for booster vaccinations later in life. Additionally, a prime-boost regimen may also be necessary to elicit a protective immune response in older animals.

Consistent with previous studies which demonstrate the importance of antibodies to VSV-mediated immunity [22,23], this study showed that levels of circulating EBOV GP-specific IgG may also be a reliable measure for predicting long-term VSVΔG/EBOVGP-induced protection. A standard ELISA and several microliters of sera were, in hindsight, sufficient to predict survival outcome in each rodent. Decreased levels of circulating specific IgG may be indicative of declining immunity, as observed in the non-surviving mice, or a poor vaccine response, as observed in the non-surviving guinea pig. Follow-up studies will be necessary to confirm these trends. It should be noted that since IgG antibody levels did not

Challenged at 7 months



Challenged at 12 months



Challenged at 18 months

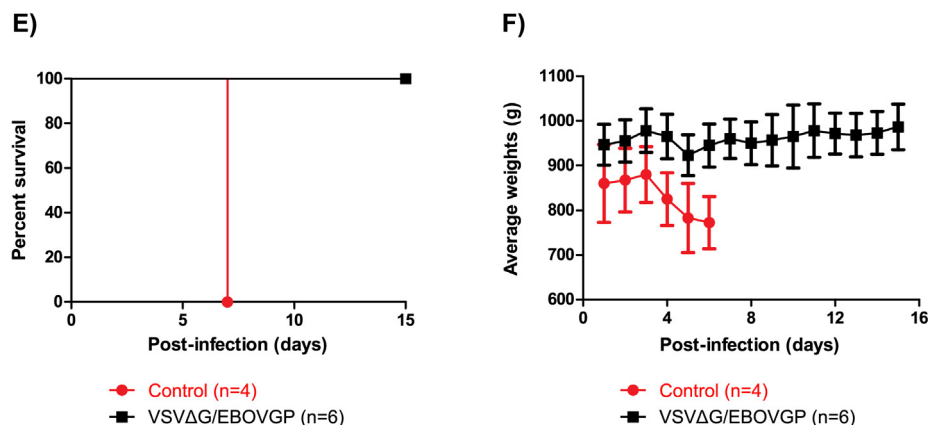


Fig. 3. Survival and weight loss of control and vaccinated Hartley guinea pigs. Animals were vaccinated with VSVΔG/EBOVGP via the intraperitoneal (IP) route, and then challenged with $1000 \times LD_{50}$ of GA-EBOV IP at (A and B) 7 months, (C and D) 12 months, and (E and F) 18 months, respectively, after vaccination. Guinea pigs were monitored daily for survival as well as percentage change in weight. Error bars indicate 95% confidence intervals.

decrease in guinea pigs at the time of latest challenge, the antibody data suggest that protection may be possible beyond 18 months after immunization. Furthermore, these studies warrant further investigation in NHPs, which allows for full characterization of the immune response. This was not possible in the current experiments either due to small blood volumes from individual mice or the lack of available reagents to more thoroughly study the immune responses in guinea pigs.

At present, very little is known about the nature of the immune responses required for long-term protection against EBOV. A study was conducted to characterize the sustained immune responses in 6 NHPs that had survived an initial EBOV infection with the help of a monoclonal antibody treatment (ZMAb) [26]. Four of six animals were protected against a subsequent re-challenge that took place 13 weeks after the original exposure. Comparison of the immune responses between surviving and non-surviving animals showed

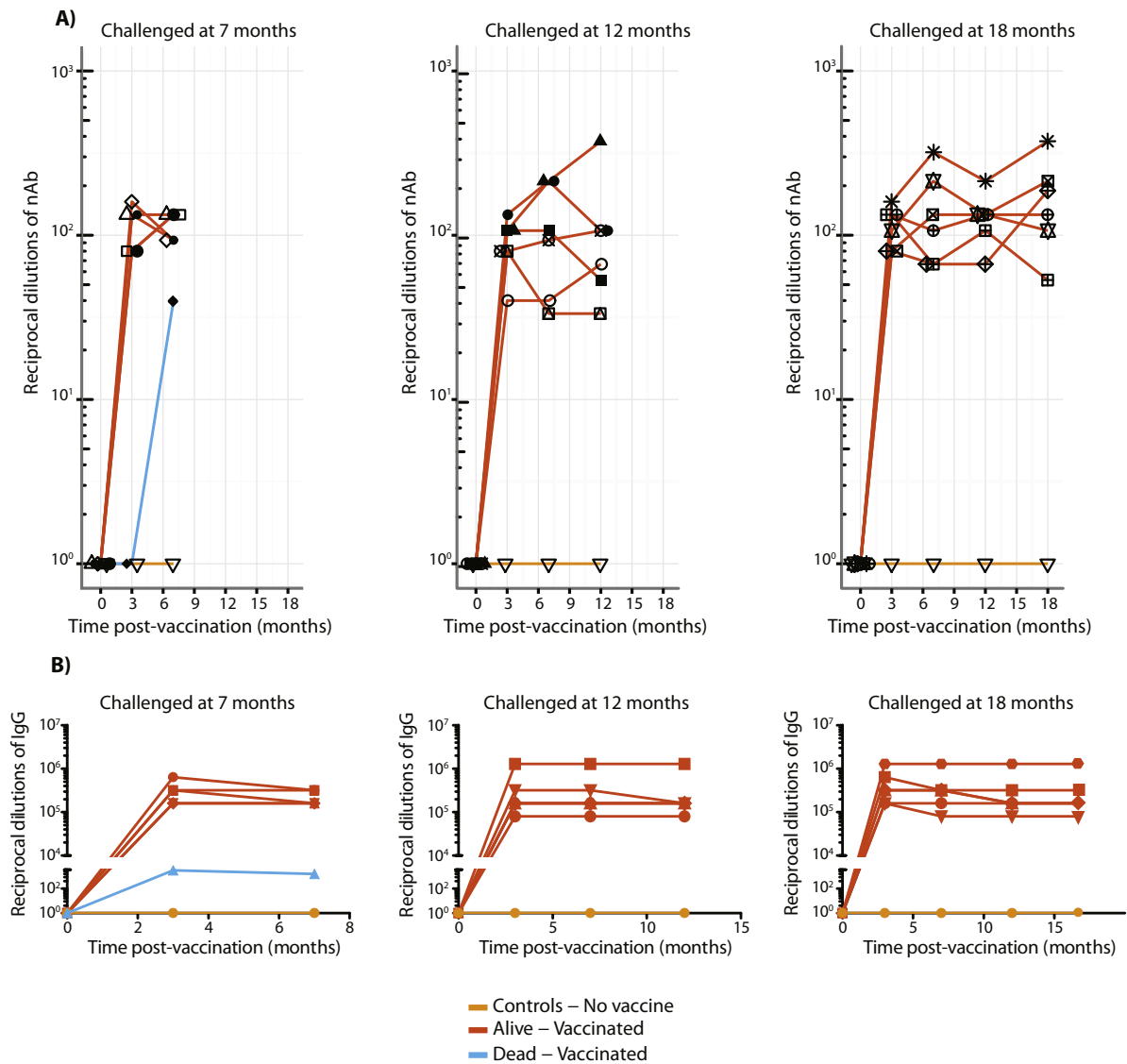


Fig. 4. Antibody responses in guinea pigs. Levels of (A) EBOV-specific neutralizing antibodies and (B) GP-specific IgG were measured from guinea pig serum harvested at 3, 7, 12, and 18 months after immunization with VSVΔG/EBOVGP. Data points in panel A are dodged for better visualization of individual animals. VSV-vaccinated survivors are in red, VSV-vaccinated non-survivors are in blue, and DMEM-treated animals are in orange. The animals are separated by the time from immunization to challenge. Neutralizing and IgG antibody titers are expressed as reciprocal dilutions of nAbs and IgG, respectively.

that a non-protective immune response was generated after the first challenge, as indicated by the absence of memory IFN- γ secreting EBOV GP-specific CD4⁺ T-cells, coupled with 10-fold lower IgG levels [26].

Immune responses were also characterized in human survivors after several outbreaks. For EBOV infections, analysis of 14 survivors from the Kikwit outbreak in 1995 showed that EBOV-specific IgG antibodies were still detectable up to 2 years after the onset of symptoms [27], whereas two survivors from the Yambuku outbreak in 1976 had persistent IgG responses when sampled 10 years after infection [28]. When survivors of the 1996 and 2001 Gabon outbreaks were sampled, a decrease in the levels of EBOV-specific IgG was observed in 5 of 9 surviving patients, however circulating IgG was still readily detectable [29].

For SUDV infections, a robust SUDV- and GP-specific IgG response was detectable from 6 survivors of the Gulu outbreak in 2000, when sampled 12 years after infection. Neutralizing antibodies were detected in only 4 of the 6 survivors [30]. The prevalence of SUDV-specific IgG antibodies amongst the survivors was also found to decline over time, as 61% of survivors (33 individuals/54

total) initially tested positive for SUDV-specific antibodies during the original outbreak, but this changed to 73% (29/40), 66% (32/48), and 41% (13/32) when re-tested after 6 months, 2 and 10 years [31]. In summary, a specific IgG response can be detected in human survivors of Ebola infections. However, conclusions and trends cannot be drawn based on these results, as samples from non-survivors would not be available for direct comparison. A laboratory-based approach using animals will be required to determine whether these sustained immune responses are sufficient for protection from disease in case of potential re-exposure. The results presented in this study are consistent with the findings in humans, and give weight to the hypothesis that levels of total GP-specific IgG antibodies can be used to predict protection from EBOV.

As several EBOV vaccine candidates progress through clinical trials [32–35], the day when a licensed medical countermeasure becomes available is drawing closer. However, in order to mount an effective immunization campaign, the vaccine must also demonstrate sustained and durable protection over a reasonable period of time. Therefore, these results will be of particular relevance for those who are at a constant risk of EBOV infection. The

development of an efficacious vaccine capable of sustained protection, in addition to establishing a simple but dependable measure to test for the strength of long-term vaccine-induced immunity, will provide more options in the control of future EBOV outbreaks.

4. Materials and methods

4.1. Ethics statement

All animal work was performed according to Animal Use Document (AUD) #H-11-007, which has been approved by the Animal Care Committee (ACC) based at the Canadian Science Center for Human and Animal Health (CSCHAH), in accordance with the guidelines outlined by the Canadian Council on Animal Care (CCAC). All infectious work was performed in the BSL-4 facility at the National Microbiology Laboratory in Winnipeg, Canada.

4.2. Production of vesicular stomatitis virus-vectored vaccine

The recombinant VSV expressing EBOV GP was generated as described previously, using the infectious clone for VSV Indiana serotype [36]. Briefly, the open reading frame for EBOV GP was generated using polymerase chain reaction and cloned into the VSV plasmid lacking its wild-type glycoprotein. The sequence was confirmed and transfected into BSR-T7 cells using Lipofectamine 2000 (Invitrogen) according to manufacturer instructions, and then rescued. Stock virus was grown on VeroE6 cells and purified using a standard sucrose cushion gradient before use in animals.

4.3. Animals, vaccination and challenge

Female BALB/c mice, 4–6 weeks old, were used for these experiments (Charles River). Mice were immunized via the IP route with 2×10^4 PFU of VSV Δ G/EBOVGP in 200 μ l DMEM at two sites, with each injection containing 1×10^4 PFU. Control animals were given DMEM as a mock treatment. Female Hartley guinea pigs, 6–8 weeks old, were also purchased for these experiments (Charles River). Guinea pigs were immunized IP with 2×10^5 PFU of VSV Δ G/EBOVGP in 1 ml DMEM at two sites, with each injection containing 1×10^5 PFU. Control animals were given DMEM. Mice were challenged IP with $1000 \times LD_{50}$ of Ebola virus USAMRIID/BALB/c-lab/COD/1976/Mayinga-MA-p3 (MA-EBOV) [37] at 6.5, 9 and 12 months after vaccination, whereas guinea pigs were challenged IP with $1000 \times LD_{50}$ of Ebola virus VECTOR/C.porcillus-lab/COD/1976/Mayinga-GPA-p7 (GA-EBOV) [38] at 7, 12 and 18 months after vaccination.

4.4. Serology

Serum was harvested from individual mice at 3, 6.5 and 12 months after immunization, as well as from individual guinea pigs at 3, 7, 12 and 18 months after vaccination. EBOV GP-specific IgG antibodies were quantified by ELISA, using a His-tagged recombinant glycoprotein [39] as the capture antigen. High binding, half-well, flat bottom polystyrene microtiter plates (Thermo Scientific) were coated with 30 ng of capture antigen diluted in PBS and incubated overnight at 4 °C. The plates were then washed with PBS 0.1% Tween-20, blocked with PBS 5% skim milk and incubated for 1 h, 37 °C. Sera diluted in PBS 2% skim milk (30 μ l) was added, in triplicate and incubated for 1 h, 37 °C. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (KPL) diluted 1:2000 in PBS 2% skim milk was added for 1 h at 37 °C. After washing, the substrate (ABTS + H₂O₂, KPL) was added for 30 min at 37 °C, before reading on a Versamax Microplate reader (Molecular Devices) at 405 nm. The data were analyzed with the SoftMaxPro

6.1 software. The resulting data are expressed as endpoint dilutions and a sample was deemed positive when the absorbance was greater than five standard deviations from the pre-vaccination control sample for each animal. The titers were plotted using GraphPad Prism 5 and the limit of detection for this assay was 100 reciprocal dilutions. Samples were assayed in triplicate and the average titer was reported.

Harvested serum samples were also assessed for their ability to neutralize EBOV-eGFP in VeroE6 cells. After serum inactivation at 56 °C for 45 min, samples were initially diluted 1:10 in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum (FBS) (DMEM 2% FBS) and then serially diluted 2-fold in DMEM 2% FBS, mixed with 100 transducing units of EBOV encoding the enhanced green fluorescent protein (eGFP) reporter gene Ebola virus NML/H.sapiens-lab/COD/1976/Mayinga-eGFP-p3 (EBOV-eGFP), and incubated at 37 °C, 5% CO₂ for 60 min. The mixture was then transferred onto subconfluent VeroE6 cells and incubated for 90 min at 37 °C, 5% CO₂. DMEM/2% FBS was added, and plates were incubated at 37 °C, 5% CO₂ for 48 h. The highest serum dilution with cells scoring for greater than 50% reduction in quantity of eGFP expression, when observed under the iSpot FluoroSpot Reader System (AID), was considered positive for neutralizing antibodies. Neutralizing titers were reported as the reciprocal of this dilution, and the limit of detection for this assay was 10 reciprocal dilutions. Samples were assayed in triplicate and the average titer was reported.

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Conflict of interest statement: The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2014.08.028>.

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