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# Single dose attenuated Vesiculovax vaccines protect primates against Ebola Makona virus

**Chad E. Mire**[#1,2], **Demetrius Matassov**[#3], **Joan B. Geisbert**[1,2], **Theresa E. Latham**[3], **Krystle N. Agans**[1,2], **Rong Xu**[4], **Ayuko Ota-Setlik**[4], **Michael A. Egan**[4], **Karla A. Fenton**[1,2], **David K. Clarke**[3], **John H. Eldridge**[3,4], and **Thomas W. Geisbert**[1,2,†]

[1]Galveston National Laboratory, University of Texas Medical Branch, Galveston, TX, USA.

[2]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA.

[3]Department of Virology and Vaccine Vectors, Profectus BioSciences, Inc., Tarrytown, NY, USA

[4]Department of Immunology, Profectus BioSciences, Inc., Tarrytown, NY, USA

[#] These authors contributed equally to this work.

## Abstract

The family *Filoviridae* contains three genera, Ebolavirus (EBOV), Marburg virus, and Cuevavirus[1]. Some members of the EBOV genus, including *Zaire ebolavirus* (ZEBOV), can cause lethal hemorrhagic fever in humans. During 2014 an unprecedented ZEBOV outbreak occurred in West Africa and is still ongoing, resulting in nearly 10,000 deaths, and causing global concern of uncontrolled disease. To meet this challenge a rapid acting vaccine is needed. Many vaccine approaches have shown promise in being able to protect nonhuman primate (NHPs) against ZEBOV[2]. In response to the current ZEBOV outbreak several of these vaccines have been fast tracked for human use. However, it is not known whether any of these vaccines can provide protection against the new outbreak Makona strain of ZEBOV. One of these approaches is a first generation recombinant vesicular stomatitis virus (rVSV)-based vaccine expressing the ZEBOV glycoprotein (GP) (rVSV/ZEBOV). To address safety concerns associated with this vector, we developed two candidate, further attenuated rVSV/ZEBOV vaccines. Both attenuated vaccines produced an approximately ten-fold lower vaccine-associated viremia compared to the first

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[†]To whom correspondence should be addressed. twgeisbe@utmb.edu.

**Author contributions:** D.K.C., D.M., and T.E.L. designed the vaccine vectors and did preparative work. J.H.E., M.A.E., A.O., and R.X. designed, conducted, and analyzed the *in vitro* vaccine characterization studies. C.E.M., J.H.E., and T.W.G. conceived and designed the NHP study. C.E.M., J.B.G., and T.W.G. performed the NHP vaccination and challenge experiments, and conducted clinical observations of the animals. J.B.G. and K.N.A. performed the clinical pathology assays. J.B.G. performed the ZEBOV infectivity assays. C.E.M., D.M., J.B.G., K.N.A., M.A.E., K.A.F., D.K.C, J.H.E, and T.W.G. analyzed the data. K.A.F. performed histologic and immunohistochemical analysis of the data. C.E.M., D.M., D.K.C., and T.W.G. wrote the paper. All authors had access to all of the data and approved the final version of the manuscript.

**Competing interests:** The N1 and N4 rVSV vectors described in this manuscript are the subject of patents licensed to Profectus BioSciences, Inc. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the University of Texas Medical Branch.

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generation vaccine and both provided complete, single dose protection of macaques from lethal challenge with the Makona outbreak strain of ZEBOV.

Outbreaks of ZEBOV have been sporadic in Africa since discovery of the virus in 1976. With increasing population growth the frequency of human contact with natural virus reservoirs[3] will likely rise, potentially leading to more catastrophic outbreaks such as the current epidemic in West Africa, thus increasing the need for effective antiviral strategies. A highly effective countermeasure would be a preventive vaccine that can be simply and widely administered to people in regions of virus zoonosis and provide a "blanket immunity" curtailing any future outbreaks. Also important will be the ability to rapidly combat deliberate misuse of these deadly viruses. Therefore, a preventive vaccine should ideally confer rapid, single dose protection.

Currently, there are no licensed filovirus vaccines or postexposure treatments available for human use. However, there are at least ten different vaccine approaches that have shown the potential to protect nonhuman primates (NHPs) from lethal ZEBOV infection including platforms based on recombinant adenovirus serotype 5 (rAd5) vectors, combined DNA/rAd5 vectors, combined rAd serotype 26 and 35 vectors, recombinant chimpanzee adenovirus serotype 3 (rChAd3) vectors, combined rChAd3 and modified vaccinia Ankara (MVA) vectors, virus-like particles (VLPs), alphavirus replicons, recombinant human parainfluenza virus 3 (rHPIV3), rabies virus, and recombinant vesicular stomatitis virus (rVSV)[2]. Of the vaccines advancing to Phase I trials, the rChAd3 and rVSV vectored vaccines have shown success in single dose protection of NHPs against ZEBOV challenge; with the caveat that the rChAd3/ZEBOV vaccine requires a boost with an MVA/ZEBOV vector for protection past 6 months[4]. Also, NHPs inoculated with the rChAd3/ZEBOV vaccine were challenged with a ZEBOV seed stock containing a large virus population encoding 8 uridines (U) at a critical transcription editing site in the GP gene[4]. This specific genetic feature typically arises following prolonged passage of ZEBOV in Vero E6 cells and results in higher levels of expression of full length GP. In contrast, low passage ZEBOV isolates retain 7U at the GP editing site, resulting in higher levels of secreted GP (sGP) expression, which is associated with greater viral virulence[5-7]. Importantly, studies have shown that rAd-based ZEBOV vaccines that completely protect NHPs against ZEBOV stocks containing high populations of 8U virus are not able to completely protect vaccinated macaques challenged with ZEBOV stocks containing high populations of 7U virus[8].

The first generation rVSV/ZEBOV vaccine that replaces the VSV glycoprotein G with the ZEBOV GP (rVSV/ZEBOVΔG), originally developed by Drs Feldmann and Geisbert and currently licensed by Merck, has demonstrated solid single dose NHP protection against a low passage 7U ZEBOV stock[8]. The rVSV/ZEBOVΔG vector has also protected 50% of NHPs when administered shortly after ZEBOV challenge[9], and has demonstrated safety in a NHP neurovirulence model[10]. However, there is a robust post vaccination viremia in macaques and a recent Phase I trial of the rVSV/ZEBOVΔG vaccine in Geneva was halted due to temporary joint pain in some patients. The level of vaccine associated viremia and frequency of adverse events will be more fully documented as data from ongoing Phase 3 trials becomes available for this vector; but the early observation suggest that a further

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attenuated rVSV vector may be more desirable for widespread administration in endemic regions of Africa.

To address this possible safety concern we have developed and tested two further attenuated rVSV/ZEBOV vaccine candidates for efficacy. One of these vaccines is based on an rVSV vector that has advanced through clinical evaluation. It was attenuated by translocating the VSV nucleoprotein (N) gene from position 1 to position 4 in the genome (N4) and truncating the cytoplasmic tail (CT) of the VSV G protein from 29 amino acids (aa) to 1 aa (CT1)[11]. This rVSVN4CT1 vector was modified to maximally express HIV-1 gag from position 1 in the genome (rVSVN4CT1gag1) by positioning the gag gene immediately adjacent to the single strong 3' VSV transcription promoter. The rVSVN4CT1gag1 vector has demonstrated safety in mouse and NHP neurovirulence studies[11,12], and replication is restricted to the IM inoculation site and draining lymph node following vaccination of mice[13]. The rVSVN4CT1gag1 vector has demonstrated safety and immunogenicity in two Phase I clinical trials (HVTN 090 and HVTN 087: http://clinicaltrials.gov/) and no post vaccination viremia was detected in urine, saliva, and blood of vaccine recipients. The rVSVN4CT1GP1 vector described here **(Fig. 1a, N4)** is analogous in design to that rVSVN4CT1gag1 vaccine and expresses ZEBOV GP from genome position 1. The other attenuated rVSV/ZEBOV vaccine described here (rVSVN1CT1GP3) expressing a truncated form of VSV G was designed to be of intermediate attenuation between rVSVN4CT1GP1 and the first generation rVSV/ZEBOVΔG vaccine (**Fig. 1a, N1**). Both attenuated rVSV/ZEBOV vectors express GP from the ZEBOV Mayinga strain, as do most other candidate ZEBOV vaccines currently under evaluation. Sequence homology between GPs from the new West African Makona strains analyzed to date and the 1976 Mayinga strain is approximately 97%. While this difference is not likely to affect the protective efficacy of the current ZEBOV vaccines against the heterologous West African strains, it is possible that small changes in sequence could lead to reduced efficacy of a vaccine[14]. It is well established that small variations in sequence and even single amino acid changes in sequence for other viruses including influenza, respiratory syncytial virus, polio, equine infectious anemia virus, and SIV can reduce vaccine efficacy. Here, we assessed the ability of our newly developed next generation rVSV-based vaccines expressing ZEBOV Mayinga GP to protect against heterologous challenge with the new outbreak Makona strain of ZEBOV in cynomolgus monkeys.

Results from an *in vitro* growth kinetics study **(Fig. 1b)** indicate an approximate 10-fold reduction in growth rate early in infection for rVSVN4CT1GP1 relative to rVSV/ZEBOVΔG. Also noted during virus plaque assay were the larger more rapidly forming plaques generated by rVSV/ZEBOVΔG compared to rVSVN4CT1GP1, with rVSVN1CT1GP3 showing intermediate growth and plaque size (**Fig. 1c**).

We next tested if the further attenuated rVSV/ZEBOV vaccines could provide NHPs with single dose protection against challenge with ZEBOV isolated from the current outbreak in Guinea[15]. Groups of four cynomolgus macaques were inoculated intramuscularly with $2 \times 10^7$ PFU of either rVSVN4CT1GP1 or rVSVN1CT1GP3; a group of two control macaques were unvaccinated (**Fig. 1d, arrow heads**). None of the macaques showed any sign of illness or distress following vaccine administration. Consistent with the statistically

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significant growth differences between rVSV/ZEBOVΔG and the more attenuated vectors seen during *in vitro* growth kinetics studies, levels of both attenuated vaccine viruses detected in the blood of vaccinated macaques (500 PFU $ml^{-1}$) were 10 to 50-fold less than those detected for the more replication competent rVSV/ZEBOVΔG[16] **(Table 1, Day −26)**. The ZEBOV GP-specific humoral immune response was assessed for all animals before vaccination (**Fig 2A, −28**) and after vaccination (**Fig. 2a, −18 and 0**) by IgG capture ELISA and neutralizing antibody titers (**Table 1, $PRNT_{50}$)**. Results showed neutralizing titers at terminal days for vaccinated cohorts and detectable circulating levels of anti-ZEBOV GP IgG for both vaccine cohorts after vaccination and before challenge with no detectable levels for the unvaccinated control animals (**Fig. 2a**). A cell mediated immune response was also detected in all vaccinated animals by ZEBOV GP specific IFN-γ ELISpot assay 10 days post vaccination (**Extended Fig. 1a and b**).

The eight vaccinated and two unvaccinated control macaques were challenged by intramuscular injection with 1,000 PFU of a low passage 100% 7U Makona strain stock of ZEBOV[15] 28 days after the single injection vaccination **(Fig. 1d, asterisk)**. None of the animals vaccinated with either of the two further attenuated rVSV/ZEBOV vectors showed any severe signs of illness following challenge with ZEBOV (**Table 1**), while the two unvaccinated control macaques succumbed to disease on days 7 and 8 (**Fig. 2b**). Circulating infectious ZEBOV was isolated from both of the unvaccinated control macaques on days 3 and 6 post challenge (**Fig. 2c, blue**) while no circulating infectious ZEBOV could be detected in any of the vaccinated animals. Examination of tissues by immunohistochemistry showed abundant ZEBOV antigen in tissues of the unvaccinated control animals (129 and 276) (**Fig. 3, a-d**) while ZEBOV antigen was not detected in tissues of the rVSVN1CT1GP3- (1001100) or rVSVN4CT1GP1- (0807174) vaccinated macaques (**Fig. 3, e-h**).

Here we show protection against a new West African Makona strain of ZEBOV using a novel filovirus vaccine platform. The large reduction in vaccine-associated viremia indicates a significant increase of *in vivo* attenuation for these next generation rVSV/ZEBOV vaccine vectors, which should translate into greater safety and reduced adverse events in humans. Importantly, single dose vaccination of NHPs with highly attenuated forms of rVSV expressing ZEBOV Mayinga GP provides complete protection from heterologous challenge with a highly virulent 7U ZEBOV isolated early during the current West African outbreak[15]. ZEBOV genome sequencing from cases later during the West Africa outbreak has revealed little drift in the GP gene[17,18] suggesting that this vaccine platform would also be efficacious against currently circulating ZEBOV. These findings pave the way for the identification and manufacture of safer, single dose, high efficacy vaccine(s) to combat current and future filovirus outbreaks in Africa and their potential use as biological weapons.

## METHODS

### Generation of N4 and N1 ZEBOV vectors

As described previously[11,19] an $rVSV_{IN}$N4CT1gag1 vector (Indian serotype) expressing HIV-1 gag was used as the backbone for generating the attenuated rVSVN4CT1 vector

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expressing the *Zaire ebolavirus* (ZEBOV) glycoprotein (GP). The corresponding $rVSV_{IN}$N4CT1gag1 genomic cDNA was modified by exchanging the gag gene expression cassette via XhoI/ NotI restriction sites with an expression cassette encoding a full length ZEBOV GP [1976, Mayinga strain], generating the $rVSV_{IN}$N4CT1-ZEBOVGP1 cDNA (**Fig. 1a, N4**). The N1 vector was generated by first inserting ZEBOV GP into a VSV-N1ΔG backbone via XhoI/ NotI restriction sites within a transcriptional cassette located at position 3 in the genome; followed by the insertion of a PCR fragment containing a portion of VSV L, a modified VSV G CT1 gene and trailer into the N1 genome at position 6 via the HindIII/ RsrII sites, generating the $rVSV_{IN}$N1(G CT1)6-ZEBOVGP3 cDNA (**Fig. 1a, N1**).

The rVSV-ZEBOV vectors were rescued from genomic cDNA as previously described[20]. Rescued virus was plaque purified and amplified on Vero E6 cell monolayers (ATCC CCL-81). For animal studies, virus vectors were purified from infected BHK-21(ATCC CCL-10) cell supernatants by centrifugation through a 10% sucrose cushion. Purified virus was resuspended in PBS, pH 7.0, mixed with a sucrose phosphate (SP) stabilizer (7 mM $K_2HPO_4$, 4mM $KH_2PO_4$, 218 mM Sucrose), snap frozen in ethanol/dry ice and stored at −80°C until ready for use.

## Growth kinetics study of ΔG, N4 and N1 ZEBOV vectors

Single-step growth curves were performed by adsorbing the N4, N1 and a ΔG control virus to duplicate monolayers of Vero-E6 cells (ATCC, CRL-1586) in 6-well plates at a multiplicity of infection (MOI) of 10 for 15 min at room temperature with continued rocking followed by incubation at 37°C with 5% $CO_2$ for 30 min without agitation. The inoculum was aspirated, the cells washed 3X with serum-free Dulbecco's Minimal Eagle's Medium (DMEM) and then DMEM containing 5% fetal bovine serum (FBS) was added to the plates, which were placed at 32°C with 5% $CO_2$. Samples for titration were taken at 4, 8, 12, 16, 24 and 48 h post infection and replaced with the same volume of fresh media. Virus titers were determined in duplicate by plaque assay on Vero-E6 cells. Growth curves were performed in triplicate for each virus. Plaque images for each vector were taken at 48 h post infection, after staining with a 1% crystal violet solution. Statistical analysis of rVSV titers were performed using unpaired *t*-test with a 95% confidence level ($p < 0.05$) with the GraphPad Prism program.

## Challenge virus

The ZEBOV Makona strain seed stock originated from serum from a fatal case early during the 2014 outbreak in Guékédou, Guinea[15] (NCBI Accession # KJ660347) and was passaged twice in Vero E6 cells (ATCC, CRL-1586). The virus stock was deep sequenced as 100% 7U at the GP editing site in the viral genome (see below).

## Deep sequencing

Approximately 1 ml of the ZEBOV Makona strain seed stock was removed from the seed stock vial and placed in 5 ml of Trizol LS and vortexed 3 times and allowed to sit for 10 minutes. The 6 ml were then placed into 2 separate 3ml Nunc cryo-vials for removal from the BSL-4. RNA was isolated from the Trizol LS/sample mixture using Zymo Research Direct-zol RNA mini-prep per manufacturer's instructions. Approximately 150 ng of

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purified RNA were used to make cDNA using the NuGen Ovation RNA-seq 2.0 kit ultimately for the preparation of the double stranded DNA library using Encore Ion Torrent library prep kit. Sequencing was performed by the UTMB Molecular Core on the Ion Torrent using 318-v2 deep sequencing chips. Sequence analysis was performed using DNA Star Seqman NGen software based on paired-end analysis of 100 bp overlaps.

### Vaccination and animal challenge

Ten, healthy, filovirus-naïve, adult (~ 3 to 9.5 kg, 7 female and 3 male), Chinese origin cynomolgus macaques (*Macaca fascicularis*) were randomized with Microsoft Excel into two experiment groups of four animals each and a control group of two animals. Animals in one experimental group were vaccinated by intramuscular injection of approximately $2 \times 10^7$ PFU of the rVSVN4CT1GP1vaccine while animals in the other experimental group were vaccinated with approximately $2 \times 10^7$ PFU of the VSV-N1CT1 ZEBOVGP vaccine. The two control animals were not vaccinated. Four weeks after the single injection vaccination all ten animals were challenged by intramuscular injection with 1,000 PFU of the ZEBOV Makona strain virus. All animals were given physical exams and blood was collected before vaccination, at day 10 after vaccination, at the time of ZEBOV challenge and on days 3, 6, 10, 14, 22, and 28 after ZEBOV challenge (Fig. 1B, arrows). Animals were monitored daily and scored for disease progression with an internal filovirus scoring protocol approved by the UTMB Institutional Animal Care and Use Committee. The scoring changes measured from baseline included posture/activity level, attitude/behavior, food and water intake, weight, respiration, and disease manifestations such as visible rash, hemorrhage, ecchymosis, or flushed skin. A score of ≥ 9 indicated that an animal met criteria for euthanasia. This study was not blinded.

### Anti-ZEBOV GP IgG ELISA

Serum collected at indicated time points was tested for immunoglobulin G (IgG) antibodies against ZEBOV. Enzyme-linked immunosorbent assay (ELISA) using recombinant ZEBOV GPdTM purified protein (Integrated BioTherapeutics, Inc.) was used to detect cross-reactive IgG. ZEBOV GPdTM was diluted to an optimal working concentration of 100 ng/well in 0.1 ml carbonate/bicarbonate buffer (Carbonate/bicarbonate buffer with azide tablets from Sigma Cat#08058-50TAB-F) and used to coat Immulon 2HB flat bottom ELISA plates (Thermo Labsystem Cat#3455) for 18 h at 4°C. Coated plates were blocked (10% FBS + 1 x PBS) for at least 2 h. The serum samples were assayed at 2-fold dilutions starting at a 1:100 dilution in ELISA diluent (1% heat inactivated fetal bovine serum (HI-FBS), 1X PBS, and 0.2% Tween-20). Samples were incubated for 1 h at room temperature, removed, and plates were washed. Wells were then incubated at room temperature for 1 h with anti-monkey IgG conjugated to horseradish peroxidase (Fitzgerald Industries International) at a 1:2500 dilution. These wells were washed and then incubated with 2,2'-azine-di(3ethylbenzthiazoline-6-sulfonate) peroxidase substrate system (KPL) at room temperature for approximately 10 min. Reaction was stopped with 1% SDS and read for dilution endpoints at 405 nm on a microplate reader (Molecular Devices Emax system). O.D. values were normalized by subtraction of background O.D.405 from uncoated wells for each serum dilution. Antigen-specific serum IgG end-point titers were defined as the reciprocal of the last normalized serum dilution giving an O.D.405 greater than 0.1.

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### ZEBOV neutralization assay

Neutralization assays were performed by measuring plaque reduction in a constant virus:serum dilution format as previously described[16]. Briefly, a standard amount of ZEBOV (~ 100 PFU) was incubated with serial two-fold dilutions of the serum sample for 60 min. The mixture was used to inoculate Vero E6 cells (ATCC, CRL-1586) for 60 min. Cells were overlayed with an agar medium, incubated for 7 days, and plaques were counted 48 h after neutral red staining. Endpoint titers were determined by the dilution of serum, which neutralized 50% of the plaques ($PRNT_{50}$).

### IFN-γ ELISpot assay

Ninety-six-well flat-bottomed ELISpot plates (Millipore) were coated overnight with a mouse anti-human IFN-γ monoclonal antibody (clone 27; BD-Pharmingen) at a concentration of 10 μ/mL, after which the plates were washed three times with 1x PBS and then blocked for 2 h with PBS containing 5% heat-inactivated fetal bovine serum (FBS). Heparinized whole blood was collected 10 days after immunization of macaques, and peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation, and resuspended in complete R05 culture medium. The isolated macaque PBMCs were washed once with complete R05 culture medium and resuspended in complete R05 culture medium containing either 5 μ/mL phytohemagglutinin mucoprotein (Sigma), peptide pools (15-mers overlapping by 11 amino acids; final peptide concentration, 1 μM [each]) spanning the ZEBOV Mayinga strain GP, or medium alone. The input cell number was $2 \times 10^5$ PBMCs per well ($2 \times 10^6$ PBMCs/mL), and cells were assayed in duplicate wells. Cells were incubated for 18 to 24 h at 37°C and then removed from the ELISpot plate by first being washed with deionized water and then being washed six times with 1x PBS containing 0.25% Tween 20. Thereafter, plates were treated with a rabbit polyclonal anti-human IFN-γ biotinylated detection antibody (0.65 μ/well; Life Technologies) diluted with 1x PBS containing 1% bovine serum albumin (BSA) and were incubated at 37°C for 2 h. ELISpot plates were then washed 6 times with 1x PBS containing 0.25% Tween 20, treated with 100 uL per well of streptavidin-HRP conjugate (BD Biosciences) diluted 1:250 with 1xPBS containing 10% FBS and 0.005% Tween 20, and incubated for an additional 1 h at room temperature. Unbound conjugate was removed by rinsing the plate six times with 1x PBS containing 0.25% Tween 20 and three times with 1x PBS. A chromogenic substrate (100 μL/well) (one-step nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate [NBT/BCIP]; Pierce) was then added for 3 to 5 min before being rinsed away with water, after which the plates were air dried and the resulting spots counted using an immunospot reader (CTL Inc.). Peptide-specific IFN-γ ELISpot responses were considered positive if the responses (minus the medium background) were 3-fold above the medium response and 50 spot-forming cells (SFC)/$10^6$ PBMCs. Unpaired t-test analysis of IFN-γ ELISpot data was performed on GraphPad Prism version 5.02 software. Two-tailed p-values less than 0.05 indicated that the tests were statistically significant.

### Detection of viremia

Virus titration of the rVSV vaccine vectors and ZEBOV was performed by plaque assay with Vero E6 cells (ATCC, CRL-1586) from cell culture or serum samples as previously

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described[16]. Briefly, increasing 10-fold dilutions of the samples were adsorbed to Vero E6 monolayers in duplicate wells (200 μL); the limit of detection was 25 PFU/ml.

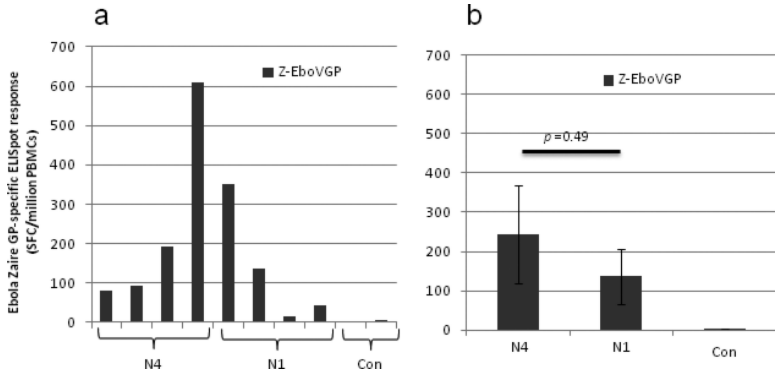
### Hematology and serum biochemistry

Total white blood cell counts, white blood cell differentials, red blood cell counts, platelet counts, hematocrit values, total hemoglobin concentrations, mean cell volumes, mean corpuscular volumes, and mean corpuscular hemoglobin concentrations were analyzed from blood collected in tubes containing EDTA using a laser based hematologic analyzer (Beckman Coulter). Serum samples were tested for concentrations of albumin, amylase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), glucose, cholesterol, total protein, total bilirubin (TBIL), blood urea nitrogen (BUN), creatine (CRE), and C-reactive protein (CRP) by using a Piccolo point-of-care analyzer and Biochemistry Panel Plus analyzer discs (Abaxis).

### Histopathology and immunohistochemistry

Necropsy was performed on all subjects. Tissue samples of all major organs were collected for histopathologic and immunohistochemical examination, immersion-fixed in 10% neutral buffered formalin, and processed for histopathology as previously described[21]. For immunohistochemistry, specific anti-ZEBOV immunoreactivity was detected using an anti-ZEBOV VP40 protein rabbit primary antibody (Integrated BioTherapeutics, Inc.) at a 1:4000 dilution. In brief, tissue sections were processed for immunohistochemistry using the Dako Autostainer (Dako). Secondary antibody used was biotinylated goat anti-rabbit IgG (Vector Laboratories) at 1:200 followed by Dako LSAB2 streptavidin-HRP (Dako). Slides were developed with Dako DAB chromagen (Dako) and counterstained with hematoxylin. Nonimmune rabbit IgG was used as a negative control.

## Extended Data




**Extended Data Figure 1. Relative immunogenicity of rVSV/ZEBOV vectors in cynomolgus macaques**

At study day −28, cynomolgus macaques were immunized IM with $2 \times 10^7$ PFU of either N4 or N1 vectors. Ten days after a single immunization, PBMCs were prepared and ZEBOV GP specific T cell responses were quantitated by IFN-γ ELISpot assay. **a.** ZEBOV GP-specific IFN-γ ELISpot responses in individual macaques. **b.** Average ZEBOV GP-specific IFN-γ ELISpot responses with standard error of the means indicated.

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## Supplementary Material

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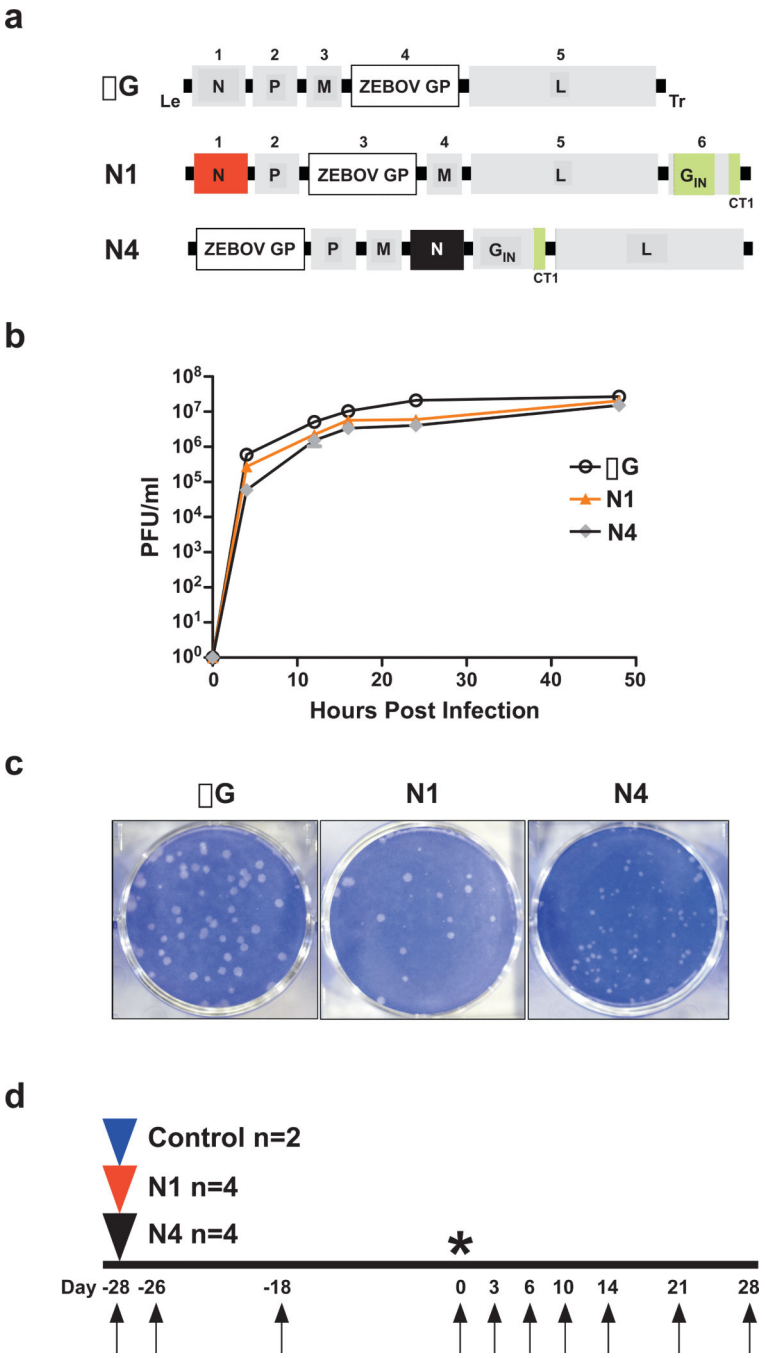


**Figure 1. rVSV/ZEBOV vector design, growth kinetics and vaccine study strategy**

**a.** Genome organization comparing ZEBOV GP (Mayinga strain) expressing rVSV vectors as described in methods. The rVSV/ZEBOVΔG (ΔG) vector had the natural VSV G gene replaced with the ZEBOV GP at position 4 within the genome. rVSVN1CT1GP3 (N1) vector retained the position of VSV N in position 1 (red box), insertion of ZEBOV GP at position 3 and a truncated form of VSV G containing the CT1 truncation was inserted at position 6. The rVSVN4CT1GP1 (N4) vector had the insertion of ZEBOV GP in position 1, attenuating N gene translocation (N4) (black box) and truncated G protein cytoplasmic tail

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(CT1). Numbers above vector constructs designate genome positions. Virus leader (Le), trailer (Tr), and intergenic regions are shown in black. Shaded regions represent deleted amino acid regions. **b.** Single-cycle growth kinetics comparing the ΔG, N1, and N4 vectors depicted in (**a**). Data shown are mean ± SD from two biological replicates titrated by plaque assay in triplicate. Titer differences between ΔG and N1 vectors were statistically significant at 4 ($p = 0.0001$, 12 ($p = 0.0055$), and 24 hours post infection ($p = 0.0001$). Likewise, ΔG and N4 vector titers were significantly different at 4 ($p = 0.0001$), 12 ($p = 0.0005$), 24 ($p = 0.0001$), and 48 hours post infection ($p = 0.0068$). Unpaired *t*-test, $p = 0.05$. **c.** Crystal violet stained Vero cell monolayers showing plaques generated by the ΔG, N1, and N4 vectors at 48 hours post infection. **d**. Flow chart showing the days of vaccination (triangles), days of sampling (arrows), day of challenge (*). Blue triangle, unvaccinated cohort; orange triangle, N1 vaccinated cohort; black triangle, N4 vaccinated cohort.

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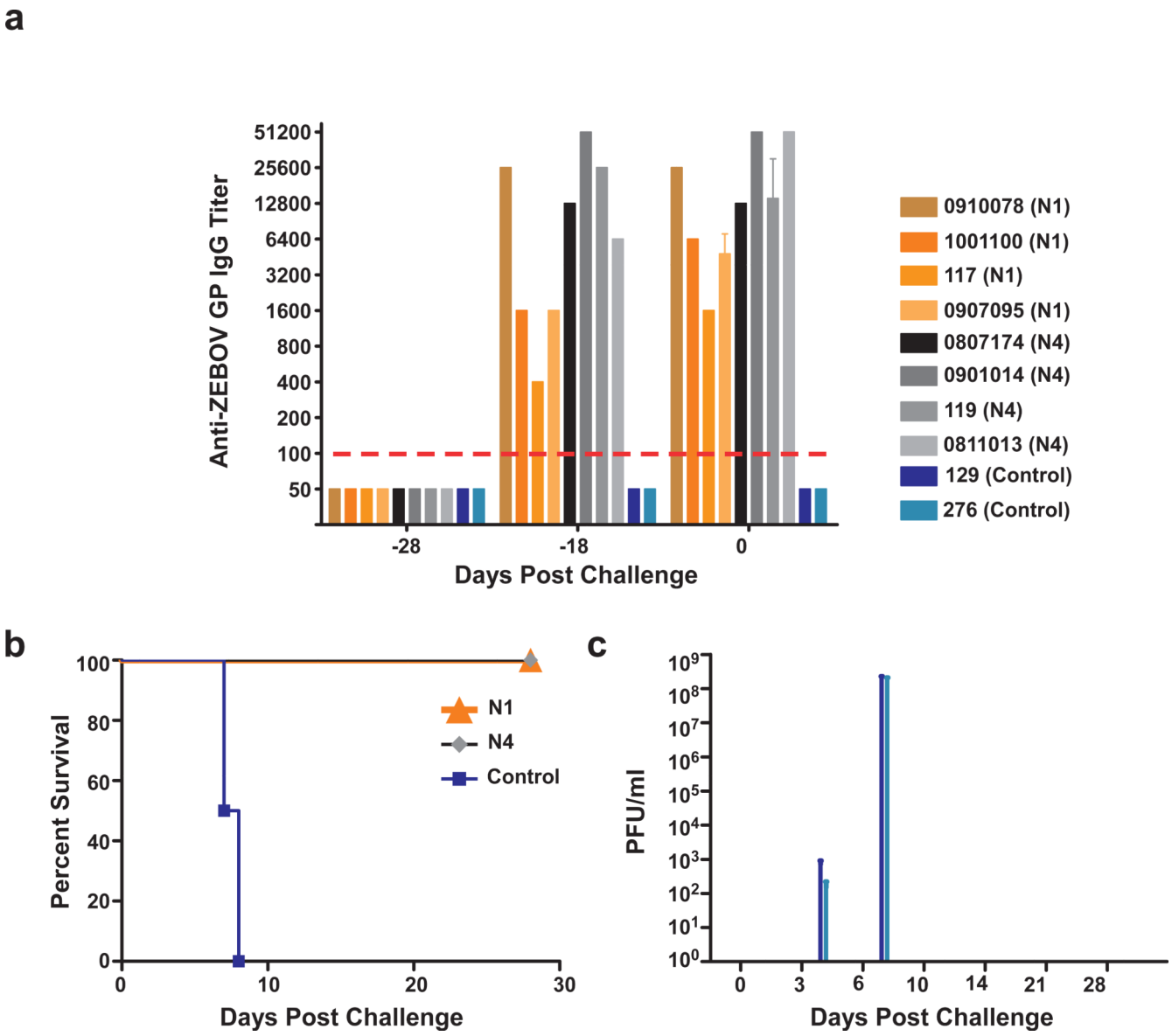


**Figure 2. N1 and N4 vaccination results in circulating anti-ZEBOV GP IgG and protection in cynomolgus macaques**

**a.** Reciprocal endpoint dilution titers for circulating IgG against ZEBOV GP for control (blue), N1 (orange), and N4 cohorts (black-grey) on day of vaccination (−28), 10 days post vaccination (−18), and on day of challenge (0). Red dashed line depicts limit of detection for ELISA assay. Error bars represent the standard error of the means. **b.** Kaplan-Meier survival curve for each cohort post ZEBOV challenge. **c.** Circulating infectious virus load displayed as plaque forming units per ml (PFU/ml). Data shown are from individual animals. Lower limit of detection is 25 PFU/ml.

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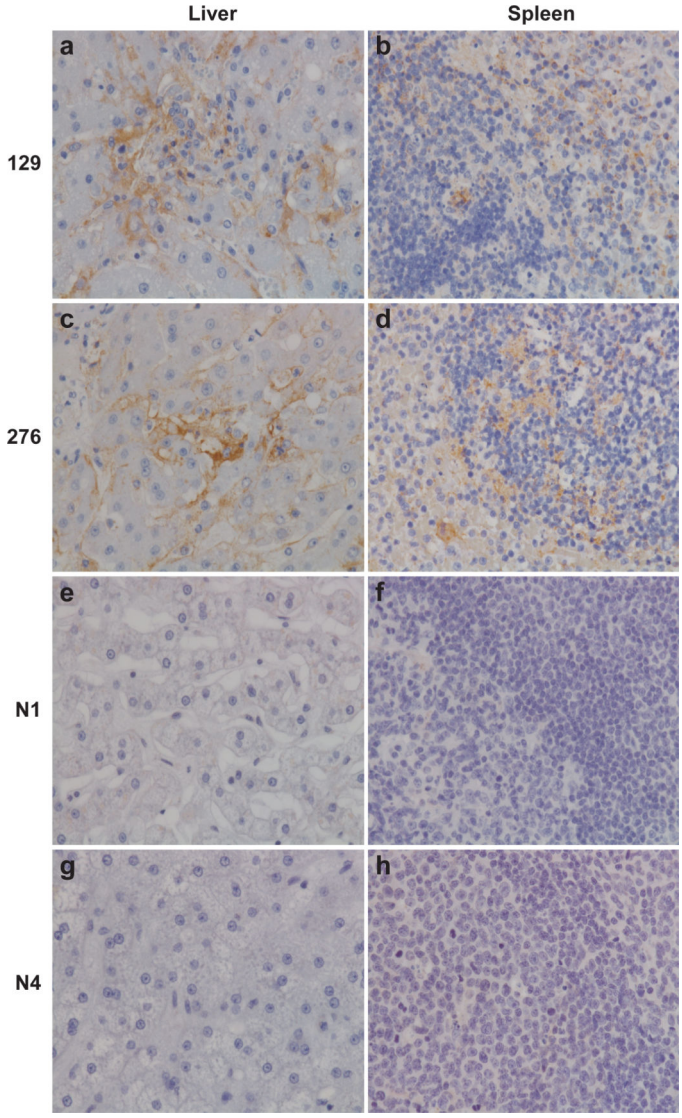


**Figure 3. Comparison of ZEBOV antigen in tissues of cynomolgus macaques either vaccinated or unvaccinated**

**a. and c.** Liver, diffuse cytoplasmic immmunolabeling (brown) of sinusoidal lining cells in both ZEBOV-infected control animals. **b. and d.** Spleen, diffuse cytoplasmic immunolabeling of dendriform mononuclear cells in the red and white pulp of ZEBOV-infected control animals. **e. and f.** Liver and spleen respectively with a lack of immunolabeling from N1 cohort animal 0910078. **g. and h.** Liver and spleen respectively with a lack of immunolabeling from N4 cohort animal 0807174.

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## Table 1

Clinical findings for NHPs challenged with ZEBOV-Guinea

| Animal | Vaccine | Day -26[a] | $PRNT_{50}$[b] | Clinical Signs Observed[c] | Final Outcome |
|---|---|---|---|---|---|
| **129** | N/A | neg | 0/0 | Fever (6), Anorexia (5-8), Depression (6-8), Mild rash (6-8), Lymphopenia (3, 6), Thrombocytopenia (6), ALT→(6), ALP→→ → (6), AST→→→ (6), GGT→→→ (6), CRP increase (6) | Expired day 8 |
| **276** | N/A | neg | 0/0 | Fever (6), Anorexia (6-7), Depression (6-7), Mild rash (6-7), Thrombocytopenia (6, 10), ALT→(6), ALP→→→ (6), AST→→→ (6), GGT→ (6), CRP increase (6) | Expired day 7 |
| **0910078** | N1 | + | 0/40 | Ø[d] | Survived |
| **1001100** | N1 | neg | 0/160 | CRP increase (6) | Survived |
| **117** | N1 | neg | 0/80 | Lymphopenia (6), CRP increase (6, 10) | Survived |
| **0907095** | N1 | neg | 0/160 | Lymphopenia (6, 10), CRP increase (6, 1o), ALT→→(6), ALP→(6), AST→→(6) | Survived |
| **0807174** | N4 | + | 0/160 | Lymphopenia (6), CRP increase (6, 10) | Survived |
| **0901014** | N4 | neg | 0/80 | Ø | Survived |
| **119** | N4 | + | 0/80 | Ø | Survived |
| **0811013** | N4 | + | 0/20 | Ø | Survived |

[a] rVSV viremia 2 days post vaccination: (neg) below limit of detection (25 PFU/ml), + up to $3 \times 10^2$ PFU/ml

[b] 50% Plaque reduction neutralization titer at day of challenge and terminal day presented as day of challenge/terminal day

[c] Days after ZEBOV challenge are in parentheses. Fever is defined as a temperature more than 2.5°F over baseline or at least 1.5°F over baseline and ≥103.5°F. Lymphopenia and thrombocytopenia are defined by a ≥35% drop in numbers of lymphocytes and platelets, respectively. (ALT) Alanine aminotransferase, (ALP) alkaline phosphatase, (AST) aspartate aminotransferase, (GGT) gamma glutamyltransferase, (CRP) C-Reactive Protein: 2- to 3-fold increase,→; 4- to 5-fold increase, →→; >5 fold increase, →→→

[d] No symptoms observed.