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

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The family Filoviridae contains three genera, Ebola virus, Marburg virus, and Cuvaviruses1. Some members of the genus, including Zaire ebolavirus (ZEBOV), can cause lethal haemorrhagic fever in humans. During 2014 an unprecedented ZEBOV outbreak occurred in West Africa and is still ongoing, resulting in over 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 primates against ZEBOV2. 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 tenfold lower vaccine-associated viraemia compared to the first-generation vaccine and both provided complete, single-dose protection of macaques from lethal challenge with the Makona outbreak strain of ZEBOV.

Since discovery of the virus in 1976, outbreaks of ZEBOV have been detected sporadically in Africa. With increasing population growth the frequency of human contact with natural virus reservoirs is likely to 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.

There are currently no licensed filovirus vaccines or post-exposure 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 months4. 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 gene5. 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 virulence7–9. 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 virus8.

The first generation rVSV/ZEBOV vaccine that replaces the VSV glycoprotein G with the ZEBOV GP (rVSV/ZEBOVAG), 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 stock3. The rVSV/ZEBOVAG vector has also protected 50% of NHPs when administered shortly after ZEBOV challenge10, and has demonstrated safety in a NHP neurovirulence model11. However, there is a robust post-vaccination viraemia in macaques and a recent phase I trial of the rVSV/ZEBOVAG vaccine in Geneva was halted due to temporary joint pain in some patients. The level of vaccine-associated viraemia and frequency of adverse events will be more fully documented as data from ongoing phase 3 trials become available for this vector; but the early observation suggest that a further-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 (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 studies12,13, and replication is restricted to the IM inoculation site and draining lymph node following vaccination of mice14. 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 viraemia was detected in urine, saliva, and blood of vaccine recipients. The rVSVN4CT1gag1 vector described here (Fig. 1a, N4) is analogous in design to that of the rVSVN4CT1gag1 vaccine and expresses ZEBOV GP from genome position 1. The other attenuated rVSV/ZEBOV

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LETTER

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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 (orange 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 (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 ± s.d. from two biological replicates titrated by plaque assay in triplicate. Titre differences between ΔG and N1 vectors were statistically significant at 4 (P = 0.0001), 12 (P = 0.0055), and 24 h post infection (P = 0.0001). Likewise, ΔG and N4 vector titres were significantly different at 4 (P = 0.0001), 12 (P = 0.0005), 24 (P = 0.0001), and 48 h 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 h post infection. d. Flow chart showing the day of vaccination (triangles), days of sampling (arrows), day of challenge (*). Blue triangle, unvaccinated cohort; orange triangle, N1-vaccinated cohort; black triangle, N4-vaccinated cohort.

| Animal | Vaccine | Days post infection | PRNT<sub>50</sub> (%) | Clinical signs observed<sup>†</sup> | Final outcome |
|--------|---------|---------------------|-----------------------|--------------------------------|---------------|
| 129    | N/A     | 26<sup>‡</sup>       | 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     | 26<sup>‡</sup>       | 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                  | 0%                               | Survived      |
| 1001100| N1      | −                   | 0/160                 | CRP increase (6)                  | Survived      |
| 117    | N1      | −                   | 0/80                  | Lymphopenia (6), CRP increase (6, 10) | Survived      |
| 097095 | N1      | −                   | 0/160                 | Lymphopenia (6, 10), CRP increase (6, 10), ALT→(6), ALP→(6), AST→(6), GGT→(6) | Survived      |
| 0807174| N4      | +                   | 0/160                 | Lymphopenia (6), CRP increase (6, 10) | Survived      |
| 0901014| N4      | −                   | 0/80                  | 0%                               | Survived      |
| 119    | N4      | −                   | 0/80                  | 0%                               | Survived      |
| 0811013| N4      | +                   | 0/20                  | 0%                               | Survived      |

<sup>‡</sup> rVSV viraemia 2 days post vaccination. −, below limit of detection (25 PFU per ml); +, up to 3 × 10<sup>3</sup> PFU per ml.

<sup>†</sup> Days after ZEBOV challenge are in parentheses. Fever is defined as a temperature more than 38.9°C above baseline or at least 1.4°C above baseline in the last 6 h. 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: two- to threefold increase, ≥ 4- to fivefold increase, ≥ 5 fold increase, →, →→.

<sup>‡</sup> No symptoms observed.
Results from an in vitro growth kinetics study (Fig. 1b) indicate an approximate tenfold reduction in growth rate early in infection for rVSVN4CT1GP1 relative to rVSV/ZEBOVAG. Also noted during virus plaque assay were the larger more rapidly forming plaques generated by rVSV/ZEBOVAG 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 Guinea16. Groups of four cynomolgus macaques were inoculated intramuscularly with 2 × 10⁷ plaque-forming units (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 significant growth differences between rVSV/ZEBOVAG 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 per ml) were 10- to 50-fold lower than those detected for the more replication competent rVSV/ZEBOVAG9 (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 titres (Table 1, plaque 50% reduction neutralization test (PRNT₅₀)). Results showed neutralizing titres 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 interferon gamma (IFN-γ) ELISpot assay 10 days after vaccination (Extended Data 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 ZEBOV16 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), whereas 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) but 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. 3a–d) whereas ZEBOV antigen was not detected in tissues of the macaques vaccinated with rVSVN1CT1GP3 (1001100) or rVSVN4CT1GP1 (0807174) (Fig. 3e–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 viraemia 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

Figure 2 | N1 and N4 vaccination results in circulating anti-ZEBOV GP IgG and protection in cynomolgus macaques. a. Reciprocal endpoint dilution titres 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 s.e.m. b. Kaplan–Meier survival curve for each cohort post ZEBOV challenge. c. Circulating infectious virus load displayed as plaque forming units per ml. Data shown are from individual animals. Lower limit of detection is 25 PFU per ml.

Figure 3 | Comparison of ZEBOV antigen in tissues of cynomolgus macaques either vaccinated or unvaccinated. a. c, Liver, diffuse cytoplasmic immunolabelling (brown) of sinusoidal lining cells in both ZEBOV–infected control animals. b, d, Spleen, diffuse cytoplasmic immunolabelling of dendriform mononuclear cells in the red and white pulp of ZEBOV–infected control animals. e, f, Liver and spleen, respectively, with a lack of immunolabelling from N1 cohort animal 0910078. g, h, Liver and spleen, respectively, with a lack of immunolabelling from N4 cohort animal 0807174.
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. ZEBOV genome sequencing from cases later during the West Africa outbreak has revealed little drift in the GP gene, suggesting that this vaccine platform could 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.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**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.-S. and R.X. designed, conducted, and analysed 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. analysed 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.

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Methods

No statistical methods were used to predetermine sample size.

Generation of N4 and N1 ZEBOV vectors. As described previously, an rSVSNiN4CT1aggl vector (indian serotype) expressing HIV-1 gag was used as the backbone for the generation of artificial rSVSNiN4CT1 vectors expressing the Zaire ebolavirus (ZEOB) glycoprotein (GP). The corresponding rSVSNiN4CT1aggl genomic cDNA was modified by exchanging the gag gene expression cassette via Xhol/NotI restriction sites with an expression cassette encoding a full length ZEOB GP [1976, Mayinga strain], generating the rSVSNiN4CT1-1-ZEBOVGP1 cDNA (Fig. 1a, N4). The N1 vector was generated by first inserting ZEOB GP into a VSV-N1Ag backbone via Xhol/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/EcoRI sites, generating the rSVSNiN1(GP CT1)-1-ZEBOVGP3 cDNA (Fig. 1a, N1).

The rSVS-ZEBOV vectors were rescued from genomic cDNA as previously described. Recycled virus was plaque purified and amplified on Vero 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 30% sucrose cushion. Purified virus was resuspended in PBS, pH 7.0, mixed with a sucrose phosphate (SP) stabilizer (7 mM KH2PO4, 4 mM KH2PO4, 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 ΔG control virus to duplicate 3 ml Nunc-culture wells (ATCC, CCL-81) 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% CO2 for 30 min without agitation. The inoculum was aspirated, the cells washed 3× 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% CO2. 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 titres were determined in duplicate by plaque assay on Vero 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 rSVS titres 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 of a fatal case during the 2014 outbreak in Guekedou, Guinea (NCBI accession number KJ660347) and was passaged twice in Vero E6 cells (ATCC, CRL-1586). The virus stock was deep sequenced as 100% at the GP editing site (Fig. 1a).

Challenge and animal challenge. Ten, healthy, filovirus-naive, 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 × 107 PFU of the rSVSNiN4CT1GP1vaccine while animals in the other experimental group were vaccinated with approximately 2 × 106 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, 21 and 28 after ZEBOV challenge (Fig. 1d, arrows). Animals were monitored daily and scored for disease progression with an internal filovirus scoring protocol

Vaccination and animal challenge. A total of 20 macaques, 2 per group, were used for vaccination and animal challenge. The corresponding 3 pairs overlaps.

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Haematology and serum biochemistry. Total white blood cell counts, white blood cell differentials, red blood cell counts, platelet counts, haematocrit values, total haemoglobin concentrations, mean cell volumes, mean corpuscular volumes, and mean corpuscular haemoglobin concentrations were analysed from blood collected in tubes containing EDTA using a laser based haematologic analyser (Beckman Coulter). Serum samples were tested for concentrations of albumin, amylase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, glucose, cholesterol, total protein, total bilirubin, blood urea nitrogen, creatine, and C-reactive protein by using a Piccolo point-of-care analyser and Biochemistry Panel Plus analyser discs (Abaxis).

Histopathology and immunohistochemistry. Necropsy was performed on all subjects. Tissue samples of all major organs were collected for histopathological and immunohistochemical examination, immersion-fixed in 10% neutral buffered formalin, and processed for histopathology as previously described21. For immunohistochemistry, specific anti-ZEBOV immunoreactivity was detected using an anti-ZEBOV VP40 protein rabbit primary antibody (Integrated BioTherapeutics, Inc.) at a 1:4,000 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–horseradish peroxidase (Dako). Slides were developed with Dako DAB chromagen (Dako) and counterstained with haematoxylin. Non-immune rabbit IgG was used as a negative control.

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Extended Data Figure 1 | Relative immunogenicity of rVSV/ZEBOV vectors in cynomolgus macaques. At study day 28, cynomolgus macaques were immunized intramuscularly 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 quantified 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 s.e.m. indicated.