nature **medicine**

Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses

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Vaccines and therapies are urgently needed to address public health needs stemming from emerging pathogens and biological threat agents such as the filoviruses Ebola virus (EBOV) and Marburg virus (MARV). Here, we developed replication-competent vaccines against EBOV and MARV based on attenuated recombinant vesicular stomatitis virus vectors expressing either the EBOV glycoprotein or MARV glycoprotein. A single intramuscular injection of the EBOV or MARV vaccine elicited completely protective immune responses in nonhuman primates against lethal EBOV or MARV challenges. Notably, vaccine vector shedding was not detectable in the monkeys and none of the animals developed fever or other symptoms of illness associated with vaccination. The EBOV vaccine induced humoral and apparent cellular immune responses in all vaccinated monkeys, whereas the MARV vaccine induced a stronger humoral than cellular immune response. No evidence of EBOV or MARV replication was detected in any of the protected animals after challenge. Our data suggest that these vaccine candidates are safe and highly efficacious in a relevant animal model.

Ebola virus (EBOV) and Marburg virus (MARV) of the virus family *Filoviridae* are emerging and reemerging pathogens that cause hemorrhagic fever with high mortality rates in humans and nonhuman primates[1–3]. The public health concern about filoviruses has increased in recent years as a result of increased awareness and frequency of cases in central Africa as evidenced by the current outbreak of MARV in Angola[4] and also because filoviruses are considered to be potential agents of bioterrorism[5]. Currently, there are no EBOV or MARV vaccines or therapies approved for human use. Recently, we generated live attenuated recombinant vesicular stomatitis viruses (rVSV) expressing the transmembrane glycoprotein of *Zaire ebolavirus* (ZEBOV; VSVΔG/ZEBOVGP) and MARV (VSVΔG/MARVGP)[6]. Our study evalu-

ated the utility of these rVSV vectors as candidate vaccines for EBOV and MARV using the cynomolgus macaque model.

Filovirus vaccine research has been extensively reviewed in the past and has primarily focused on EBOV[7,8]. The first EBOV vaccine to protect nonhuman primates was a DNA prime–adenovirus boost approach using both the glycoprotein and nucleoprotein as target antigens[9]. This approach required several months for immunity to develop, which limited the utility of this strategy. More recently, an accelerated vaccine was described. A single immunization of nonhuman primates with 2×10^{12} particles of an equal mixture of human adenovirus 5 vectors carrying either the gene encoding ZEBOV glycoprotein or the gene encoding ZEBOV nucleoprotein resulted in complete protection against ZEBOV[10]. Despite the intriguing success of the adenovirus vaccine, preexisting immunity rates of between 40 and 60% have been reported to adenovirus in the human population and this may eventually limit the utility of this approach[11–13].

A smaller number of efforts have focused on developing vaccines against MARV. Alphavirus replicons expressing MARV proteins protected cynomolgus monkeys from homologous MARV challenge[14]. Subsequent studies evaluating this platform as a vaccine for EBOV were less encouraging, as the EBOV counterpart of this alphavirus replicon platform was unable to protect any animal against lethal EBOV challenge under similar test conditions[7]. The ideal vaccine would protect humans from infection from all four EBOV species (ZEBOV, *Sudan ebolavirus* (SEBOV), *Reston ebolavirus*, *Ivory Coast ebolavirus*) and MARV. Although the adenovirus-based vaccine platform has completely protected nonhuman primates against ZEBOV[9,10], and the platform based on alphavirus replicons protected monkeys against MARV[14], no platform has demonstrably protected nonhuman primates against both of these viruses.

Vaccines based on live attenuated rVSV have been highly effective in animal models and are particularly attractive because they can be mucosally administered[15–18]. Furthermore, VSV infections in humans occur fairly rarely worldwide, mainly in the enzootic regions of the

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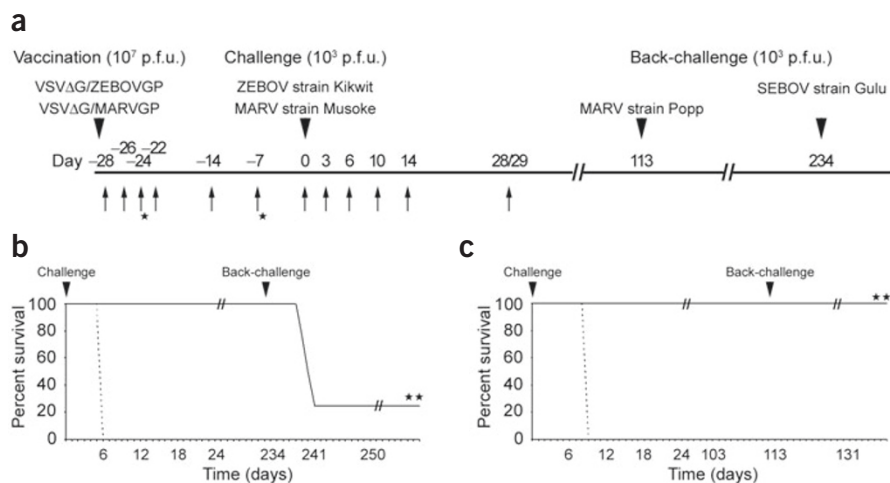
Figure 1 Immunization, challenge and rechallenge of nonhuman primates. (**a**) Flow chart of experimental design. Arrows indicate days of sampling (blood and swabs), asterisk indicates additional sampling days for the ZEBOV study. (**b**) Kaplan-Meier mortality chart of the EBOV vaccine study. Dotted line, animals immunized with VSVΔG/MARVGP and challenged with ZEBOV; solid line, animals immunized with VSVΔG/ZEBOVGP, challenged with ZEBOV and rechallenged with SEBOV. (**c**) Kaplan-Meier mortality chart of the MARV vaccine study. Dotted line, animals immunized with VSVΔG/ZEBOVGP and challenged with MARV (strain Musoke); solid line, animals immunized with VSVΔG/MARVGP, challenged with MARV (strain Musoke) and rechallenged with MARV (strain Popp).

Americas and consequently global preexisting immunity is negligible[19]. Preliminary immunization studies in mice[6] and guinea pigs (S.M.J., unpublished data) indicated the usefulness of VSVΔG/ZEBOVGP as a vaccine delivery system against ZEBOV. But rodent models are not generally predictive for efficacy of vaccines and antiviral drugs against filoviral infections in nonhuman primates[7]. Thus, in this study, we tested the protective efficacy of the replicating rVSV vector in nonhuman primates.

We used 12 cynomolgus macaques, of which 6 were immunized by intramuscular injection with a single dose of VSVΔG/ZEBOVGP (animal #105, #332, #480, #508, #725, #790) and the remaining 6 with a single dose of VSVΔG/MARVGP (animal #190, #338, #462, #652, #770, #831). The animals were monitored closely for clinical symptoms, shedding of rVSVs and viremia (**Figs. 1** and **2**). After vaccination, none of the nonhuman primates showed any signs of clinical symptoms, indicating that the rVSVs are apathogenic for these animals. All 12 animals were subsequently challenged on day 28 after immunization by intramuscular injection with a high dose (1×10^3 plaque-forming units (p.f.u.)) of either ZEBOV (animal #105, #332, #462, #508, #652, #725) or MARV (strain Musoke; animal #190, #338, #480, #770, #790, #831). The two VSVΔG/MARVGP-immunized animals (#462, #652), which served as controls in the ZEBOV challenge, started to show clinical signs of disease on day 3 after challenge and died on day 6. In contrast, none of the VSVΔG/ZEBOVGP-immunized macaques became sick and all four animals were fully protected against the ZEBOV challenge. The two VSVΔG/ZEBOVGP-immunized animals (#480, #790), which served as controls for the MARV challenge, showed first signs of disease on day 4 after challenge and both died on day 9. In contrast, none of the VSVΔG/MARVGP-immunized macaques became sick, and all four animals were fully protected against the MARV challenge. None of the protected animals in either challenge experiment showed any clinical signs or visual symptoms of EBOV or MARV disease. Results of blood chemistry and hematology did not differ substantially from values obtained before challenge and historical controls (data not shown).

To determine whether viremia or shedding of the rVSVs occurs after immunization, we analyzed whole blood and swab samples. We detected a mild viremia on day 2 after immunization by virus isolation (**Fig. 2a,c**) and RT-PCR (data not shown) in all six VSVΔG/ZEBOVGP-immunized monkeys and four of the six VSVΔG/MARVGP-immunized monkeys. Virus was undetectable in all remaining blood and swab samples, with the exception of virus detected using RT-PCR on a single blood and single nasal swab sample taken on day 6
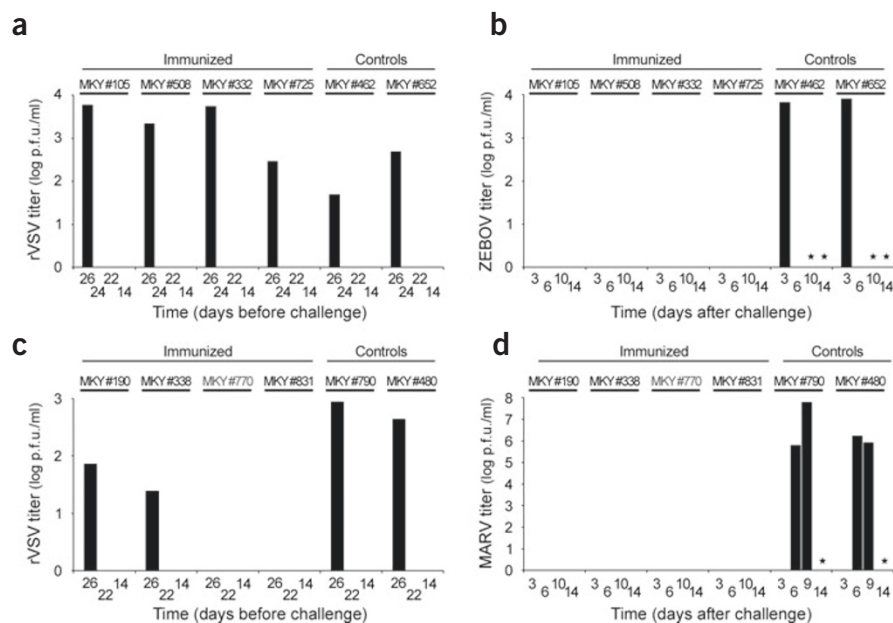
Figure 2 Viremia levels in nonhuman primates after immunization and challenge. VSV viremia levels were determined after immunization with VSVΔG/ZEBOVGP (**a**) or VSVΔG/MARVGP (**c**) and ZEBOV and MARV viremia levels after challenge with ZEBOV (**b**) or MARV (strain Musoke) (**d**) from plasma taken at the indicated time points (**Fig. 1a**). Animals MKY #105, MKY #508, MKY #332 and MKY #725 were vaccinated with VSVΔG/ZEBOVGP, whereas animals MKY #462 and MKY #652 served as the controls in this study and were vaccinated with VSVΔG/MARVGP; all were challenged with ZEBOV. Animals MKY #190, MKY #338, MKY #770, and MKY #831 were vaccinated with VSVΔG/MARVGP, whereas animals MKY #790 and MKY #480 served as the controls in this study and were vaccinated with VSVΔG/ZEBOVGP; all were challenged with MARV (strain Musoke). Asterisks indicate sample was not available because the animal had died.
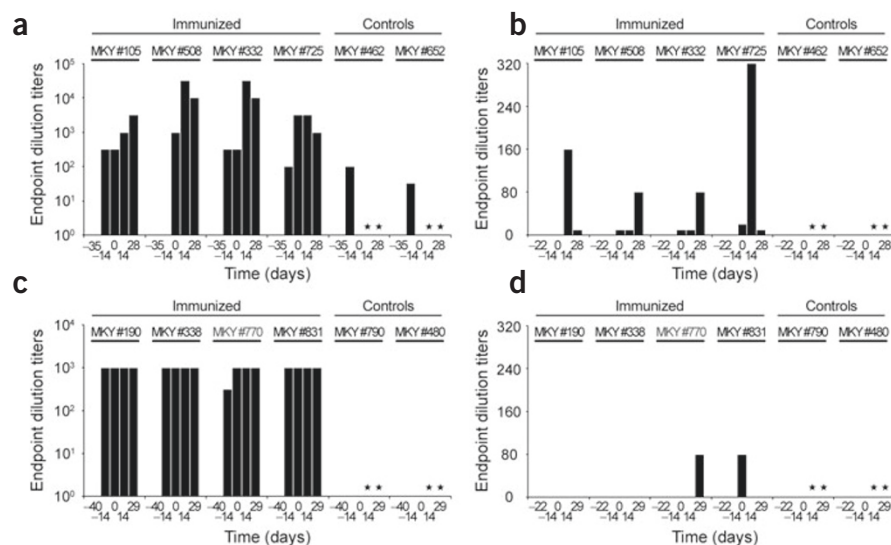
Figure 3 Humoral immune response in nonhuman primates to ZEBOV and MARV before and after challenge. (**a**) IgG response to ZEBOV. (**b**) ZEBOV neutralizing antibodies. (**c**) IgG response to MARV. (**d**) MARV neutralizing antibodies. IgG responses were measured using an established ELISA. Titers are presented as endpoint dilutions. Neutralizing antibodies were detected using a plaque reduction neutralization assay (PRNT$_{80}$). Titers are presented as endpoint dilutions. –, days before challenge; 0, day of challenge. Asterisks indicate sample was not available because the animal had died.

Fig. 4) showed a strong cellular response. In animals immunized with the glycoprotein- and nucleoprotein-expressing adenovirus vaccine, the highest cellular response detected was approximately 1.5% of CD8 cells producing IFN-γ using an identical assay[10]. This indicates that the VSVΔG/ZEBOVGP seems to be a potent stimulator of cellular immunity.

from animal #190, which had been vaccinated with VSVΔG/MARVGP; however, the same specimens were negative by virus isolation. Thus, inoculation led to transient viremia in most of the animals and probably resulted from localized virus replication at as yet undetermined sites. There is no compelling evidence to suggest that occasional virus shedding would lead to transmission. The inoculation dose was high (10^7 p.f.u.) and three logs greater than the doses successfully used to immunize mice[6] and guinea pigs (S.M.J., unpublished data) against ZEBOV. Thus, it seems feasible to reduce or even avoid transient viremia by using a lower immunization dose.

ZEBOV and MARV replication and shedding was analyzed from the blood and swab samples taken after the challenges (**Fig. 2b,d**). The two control animals of the ZEBOV challenge study developed high EBOV titers in blood (up to ~10^4 p.f.u./ml) by day 3 (**Fig. 2b**) and organs (10^4–10^8 p.f.u./g) after death (data not shown). Similarly, the controls of the MARV challenge experiment showed high viremia levels (10^6–10^8 p.f.u./ml by days 6 and 9; **Fig. 2d**) and organ titers (10^3–10^9 p.f.u./g; data not shown). In contrast, neither ZEBOV or MARV viremia (blood; **Fig. 2b,d**) nor ZEBOV or MARV shedding (data not shown) was detectable in the protected animals, which were immunized with VSVΔG/ZEBOVGP and VSVΔG/MARVGP, respectively.

By the day of ZEBOV challenge (day 0) all VSVΔG/ZEBOVGP-immunized animals had developed low- to moderate-level IgG antibody titers against ZEBOV glycoprotein (**Fig. 3a**). Notably, neutralizing antibody titers to ZEBOV were not detectable before challenge but became positive (1:80 to 1:320) 14 and 28 d after challenge (**Fig. 3b**). It remains unclear why the neutralizing antibody titers in two animals decreased after an initial rise. This has been seen in previous studies in nonhuman primates infected with ZEBOV (T. W. G., unpublished observation). By the day of MARV challenge (day 0) all animals vaccinated with VSVΔG/MARVGP had developed moderate IgG antibody titers against the MARV glycoprotein (**Fig. 3c**). We only detected neutralizing antibody titers to MARV (1:80) in two animals (**Fig. 3d**). The cellular responses in the VSVΔG/ZEBOVGP-immunized animals of this study mirrored the neutralizing antibody responses, as the specific production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α was not detectable before ZEBOV challenge (**Fig. 4**). After challenge, all VSVΔG/ZEBOVGP-immunized animals responded positively, ranging between 0.05 to 6% IFN-γ– or TNF-α–positive CD8 cells and 0.02 to 0.4% positive CD4 cells. It should be noted that some subjects (*e.g.*, animal #508;

Consistent with results in the ZEBOV portion of this study, the cellular responses in the MARV-immunized animals initially mirrored their neutralizing antibody responses, as the specific production of IFN-γ and TNF-α were not detectable before MARV challenge. In contrast to the ZEBOV results (**Fig. 4**), no evidence of a cellular immune response was detected after MARV challenge (data not shown). This indicates that protection of these animals against MARV might be caused by indices other than cellular immunity or neutralizing antibodies and may be partly associated with non-neutralizing antibodies.

Finally, we tested the protective efficacy against a rechallenge with a heterologous virus strain. All animals that were protected from the lethal ZEBOV (strain Kikwit) challenge were rechallenged with 1×10^3 p.f.u. of SEBOV (strain Gulu) 234 d after initial challenge (**Fig. 1a,b**). Three of the four animals died of the SEBOV infection on days 6 and 7 after rechallenge, with viremias ranging from 10^7–10^8 p.f.u./ml. Only one animal survived the rechallenge, showing transient viremia of ~10^3 p.f.u./ml on day 6 (data not shown). This single survivor cannot necessarily be attributed to vaccine protection because the SEBOV macaque model is not uniformly lethal (T. W. G., unpublished data). The lack of cross-protection was not unexpected, as the EBOV species differ from one another by 37–41% at the nucleotide and amino acid levels[20]. All VSVΔG/MARVGP-immunized macaques, which were protected against the lethal MARV (strain Musoke) challenge, were rechallenged with 1×10^3 p.f.u. of MARV (strain Popp) 113 d after initial challenge (**Fig. 1a,c**). In contrast to the SEBOV rechallenge, all four animals remained healthy and survived the rechallenge without showing clinical symptoms. The rechallenge results indicated that cross-protection can only be achieved against heterologous strains from the same virus species. Indeed, the MARV strains used in this study are genetically similar. Homology between nucleotide sequences of these two strains is 93.9%[21].

Although protection of monkeys by the rVSV EBOV vaccine seemed to be associated with humoral and cellular immune responses (**Fig. 3a,b** and **Fig. 4**), protection of monkeys by the rVSV MARV vaccine seemed to be primarily associated with the humoral immune response (**Figs. 3c,d**). Notably, neutralizing antibodies were poorly induced, suggesting that protection may result from rather higher levels of non-neutralizing antibodies in these animals. It is possible that the *in vitro* neutralization assay is not detecting the same neutralizing antibodies required to neutralize the ability of MARV or ZEBOV to infect their primary *in vivo* targets. But the MARV results in the current study are not without precedent.
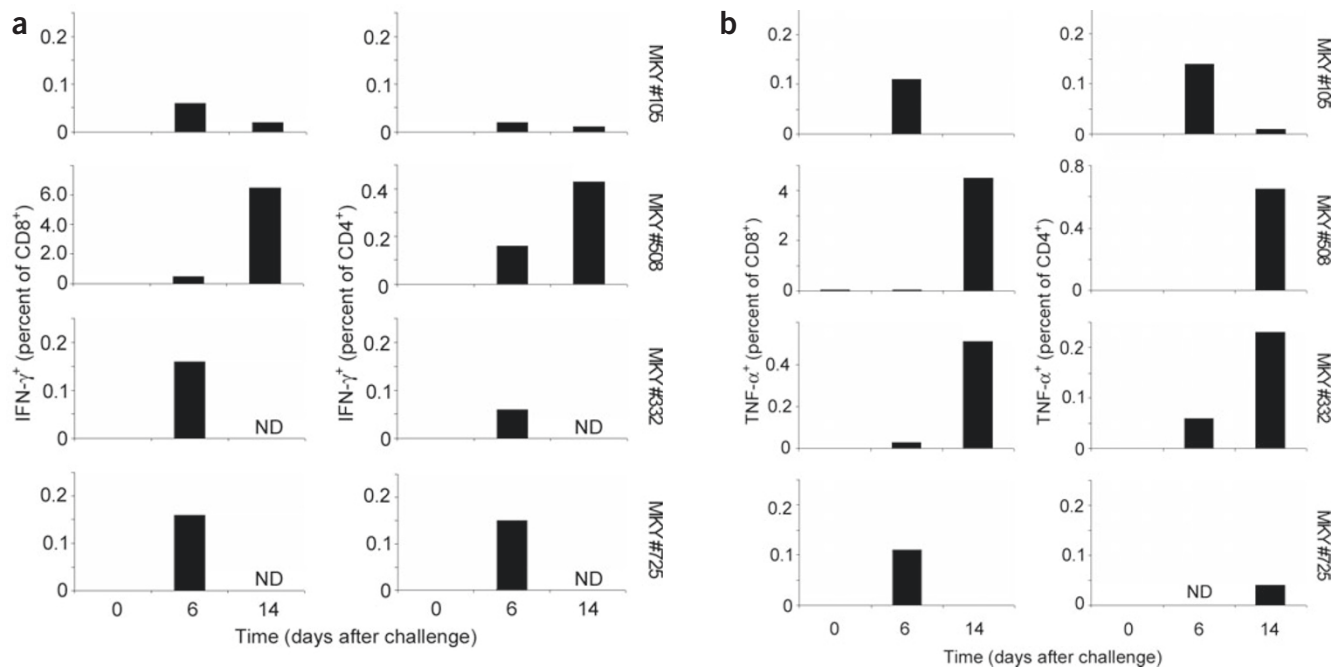
Figure 4 Cellular immune response in nonhuman primates before and after challenge with ZEBOV. (**a**) IFN-γ. (**b**) TNF-α. Intracellular levels of IFN-γ and TNF-α were determined in CD4+ and CD8+ T-cell populations. Cellular responses after restimulation with a glycoprotein peptide library were seen in all animals after challenge. One animal (MKY #508) had relatively high levels of circulating CD8+ T cells producing IFN-γ and TNF-α on day 14 after challenge. ND, no data (assay not performed).

In similar studies to evaluate alphavirus replicons expressing MARV genes including those encoding glycoprotein, cynomolgus monkeys were protected from homologous MARV challenge despite the absence of neutralizing antibody titers in prechallenge sera[14]. The potential importance of non-neutralizing antibodies to protection against MARV has also been noted in another study. Specifically, neutralizing antibodies were not detected in Rhesus monkeys immunized with an inactivated whole virion preparation. Although cellular responses were not detected in these animals, three of these six monkeys survived a lethal MARV challenge[22]. Notably, the investigators were unable to associate protection with the humoral or cellular immune response and concluded that protective immunity is determined by the indices of nonspecific immunity[22].

This current study is the first to show that nonhuman primates can be protected with a single-dose immunization using a vector expressing solely the ZEBOV glycoprotein. In addition, this is the first vaccine platform to show the ability to protect nonhuman primates against EBOV and MARV, which is an important first step toward developing a vaccine that will be effective against all filoviruses. Protection is dependent on immunization with an attenuated, replication-competent virus, which may raise questions regarding the safety of live attenuated vectors. There has been no evidence of pathogenicity in four species of animals (mouse, guinea pig, goat, nonhuman primate) tested so far (S.M.J. & H.F., unpublished data). Most notably, we showed here that despite a short-term viremia, rVSV replication and shedding were not detectable in nonhuman primates and that the animals did not develop fever or other symptoms, nor were there changes in blood chemistry or hematology.

The use of replicating rVSV-based vectors, shown here for EBOV and MARV, has proven to be a potent and promising concept for future vaccine development against these aggressive pathogens, and may be equally applicable to other lethal emerging and reemerging viruses.

METHODS

Vaccine vectors and viruses. The recombinant VSV expressing the glycoproteins of ZEBOV (strain Mayinga) and MARV (strain Musoke) were generated as described recently using the infectious clone for the VSV Indiana serotype (provided by J. Rose, Yale University School of Medicine)[6]. Briefly, the appropriate open reading frames for the genes encoding the glycoproteins were generated by PCR, cloned into the VSV genomic vectors lacking the VSV gene for glycoprotein, sequenced confirmed and originally rescued using the method described earlier[6,23]. ZEBOV (strain Kikwit) was isolated from a patient of the 1995 EBOV outbreak in Kikwit[24] whereas SEBOV (strain Gulu) was isolated from a patient of the 2000 EBOV outbreak in Gulu[25]. MARV strain Musoke was isolated from a human case in 1980 in Kenya[26] and strain Popp was isolated from a patient of the first MARV outbreak in 1967 (ref. 21).

Animal studies. We used 12 4–6 kg healthy adult cynomolgus macaques (*Macaca fascicularis*) for these studies. For the EBOV portion of this study, we intramuscularly immunized four animals with 10^7 p.f.u. of VSVΔG/ZEBOVGP (#105, #332, #508, #725) and two animals with $\sim5 \times 10^7$ p.f.u. of VSVΔG/MARVGP (#462, #652; controls). We intramuscularly challenged these six cynomolgus macaques 28 d after the single-dose immunization with 1×10^3 p.f.u. of ZEBOV. For the MARV portion of this study, we intramuscularly immunized four animals with $\sim5 \times 10^7$ p.f.u. (#190, #338, #770, #831) and two animals with 10^7 p.f.u. of VSVΔG/ZEBOVGP (#480, #790; controls). We intramuscularly challenged these six cynomolgus macaques 28 d after the single-dose immunization with 1×10^3 p.f.u. of MARV (strain Musoke). The rechallenge of the VSVΔG/ZEBOVGP-immunized animals, which were protected against the challenge with ZEBOV (#105, #332, #508, #725), was performed intramuscularly 234 d after initial challenge with 1×10^3 p.f.u. of SEBOV. We performed the intramuscular rechallenge of the VSVΔG/MARVGP-immunized animals (#190, #338, #770, #831), which were protected against the challenge with MARV (strain Musoke) 113 d after initial challenge with 1×10^3 p.f.u. of MARV (strain Popp). Swab samples (oral, nasal, rectal, vaginal) and blood were taken as indicated (**Fig. 1a**). Animal studies were performed in biosafety level 4 biocontainment at United States Army Medical Research Institute of Infectious Diseases (USAMRIID) and approved by the USAMRIID Laboratory Animal Care and Use Committee. Animal research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to

animals and experiments involving animals and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals* by the US National Research Council. The facility used is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Virus detection. RNA was isolated from blood and swabs using appropriate RNA isolation kits (QIAGEN). For the detection of VSV we used a RT-PCR assay targeting the matrix gene (nucleotides 2,355–2,661). ZEBOV and MARV RNA were detected using primer pairs targeting the L genes (ZEBOV: RT-PCR, nucleotides 13,344–13,622; nested PCR, nucleotides 13,397–13,590; MARV: RT-PCR, nucleotides 1,966–2,243; nested PCR, nucleotides 2,017–2,213). We performed virus titration by plaque assay on Vero E6 cells from all blood and selected organ (adrenal, ovary, lymph nodes, liver, spleen, pancreas, lung, heart, brain) and swab samples[24]. Briefly, $10g_{10}$ dilutions of the serum were adsorbed to Vero E6 monolayers in duplicate wells (0.2 ml per well); thus, the limit for detection was 25 p.f.u./ml.

Immune responses. IgG antibodies against ZEBOV and MARV were detected with ELISA using purified virus particles as an antigen source[10]. Neutralization assays were performed by measuring plaque reduction in a constant virus-serum dilution format as previously described[27]. Briefly, we incubated a standard amount of ZEBOV or MARV (~100 p.f.u.) with dilutions (1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640) of the serum sample for 60 min. We used the mixture to inoculate Vero E6 cells for 60 min. Cells were overlaid with an agar medium, incubated for 8 d, and plaques were counted 48 h after neutral red staining. We determined endpoint titers by the dilution of serum, which neutralized 80% of the plaque reduction neutralization test ($PRNT_{80}$).

Cellular immune responses. The method for assessment of T-cell responses to EBOV was previously published[10]. Briefly, peripheral blood mononuclear cells were isolated from samples of whole blood from cynomolgus macaques by separation over Ficoll. Approximately 1×10^6 cells were stimulated in 200 μl RPMI medium (GIBCO) for 6 h at 37 °C with antibodies specific for CD28 and CD49d and either dimethylsulfoxide or a pool of 15-nucleotide coding sequences for peptides spanning the open reading frames for the genes encoding ZEBOV glycoprotein (Mayinga strain) or the MARV glycoprotein (Musoke strain) in the presence of brefeldin A. The peptides were 15 amino acids in length, overlapping by 11, and were used at a final concentration of 2 μg/ml. We fixed cells and permeabilized them with FACS lyse (Becton Dickinson) supplemented with Tween-20, and stained them with a mixture of antibodies against lineage markers (CD3-phycoerythrin, CD4-peridinin chlorophyll protein, CD8-FITC) and either TNF-α–APC or IFN-γ–allophycocyanin. We ran samples on a FACSCalibur and analyzed them using the software FlowJo. Positive gating for lymphocytes using forward versus side scatter was followed by CD3+ CD8− and CD3+ CD4− gating, and specific populations were further defined by antibodies specific for CD4 and CD8 positivity, respectively. Cytokine-positive cells were defined as a percentage within these individual lymphocyte subsets, and at least 200,000 events were analyzed for each sample.

Accession numbers. The GenBank accession number for the vesicular stomatitis Indiana virus complete genome is NC_001560, for the *Zaire ebolavirus* (strain Mayinga) complete genome, AF272001 and for Marburg virus genomic RNA of L gene, X68494.

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