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TITLE:
Gene-Specific Countermeasures against Ebola Virus Based on Antisense Phosphorodiamidate Morpholino Oligomers

ABSTRACT:
The filoviruses Marburg virus and Ebola virus (EBOV) quickly outpace host immune responses and cause hemorrhagic fever, resulting in case fatality rates as high as 90% in humans and nearly 100% in nonhuman primates. The development of an effective therapeutic for EBOV is a daunting public health challenge and is hampered by a paucity of knowledge regarding filovirus pathogenesis. This report describes a successful strategy for interfering with EBOV infection using antisense phosphorodiamidate morpholino oligomers (PMOs). A combination of EBOV-specific PMOs targeting sequences of viral mRNAs for the viral proteins (VPs) VP24, VP35, and RNA polymerase L protected rodents in both pre- and post-exposure therapeutic regimens. In a prophylactic proof-of-principal trial, the PMOs also protected 75% of rhesus macaques from lethal EBOV infection. The work described here may contribute to development of designer, "druggable" countermeasures for filoviruses and other microbial pathogens.

Introduction:
The development of effective countermeasures to the filoviruses Ebola virus (EBOV) and Marburg virus (MARV) has been a long-sought and difficult endeavor, yielding little success [1,2]. Although the worst outbreaks have resulted in only several hundred deaths worldwide [3–6], the filoviruses are considered a significant global health threat, because as the reservoir remains unknown, the pathogen is extremely deadly and highly infectious by aerosol, and there is anecdotal evidence that the use of both MARV and EBOV were explored as potential biowarfare agents in the offensive program of the former Soviet Union [7–10]. The filoviruses are relatively simple viruses of 19-Kb genomes and consist of seven genes which encode nucleoprotein (NP), glycoprotein, four smaller viral proteins (VPs) (VP24, VP30, VP35, and VP40), and the RNA-dependent RNA polymerase (L protein), all in a single strand of negative-sensed RNA [11].
The development of an effective treatment for EBOV is hindered by lack of a clear understanding of filovirus pathogenesis, disparity between animal models, and both the difficulty and danger of working with EBOV under biosafety–level-4 conditions [1,2]. Although there has been significant progress toward vaccine development via demonstration of protection in nonhuman primates from EBOV illness and death [12,13], a vaccine will not fulfill all requirements for EBOV countermeasures. Administration of type I interferons, therapeutic vaccines, immune globulins, ribavirin, and other nucleoside analogues have been somewhat successful in rodent EBOV models, but all failed to benefit EBOV-infected nonhuman primates [1,14,15]. EBOV frequently causes severe disseminated intravascular coagulation, and administration of a recombinant clotting inhibitor was recently shown to protect 33% of rhesus monkeys [16]. It appears that host-directed consequence management of the disease alone may not be sufficient, and an additional well-orchestrated sequence-specific attack on viral replication may be more effective as a successful anti-filovirus treatment regimen.
The ability of virus-specific antisense oligonucleotides to inhibit viral growth by interfering with translation of viral RNAs was first demonstrated in 1978 [17,18]. Since then, remarkable progress has been made by modifying oligonucleotides to increase their stability, affinity, and delivery into cells [19]. Phosphorodiamidate morpholino oligomers (PMOs) are a subclass of antisense agents modified to include a phosphorodiamidate linkage and morpholine ring, and exhibit limited off-target effects, favorable base stacking, high duplex stability, high solubility, cell permeability, and no hybridization complexities [20,21]. Formation of a PMO:mRNA duplex can effectively block translation of viral RNA, thereby inhibiting viral replication [22,23]. Antisense PMO are effective as antivirals against vesiviruses [24], flaviviruses [23,25], and the SARS coronavirus [26]. The successful inhibition of viral replication by PMOs in these reports was demonstrated in vitro, but has not yet been shown in an animal model.

In Vitro Inhibition of EBOV Translation and Replication by Gene-Specific PMOs ::: Results:
We hypothesized that PMO inhibition of viral mRNA translation would provide protection against EBOV infections. In order to test our hypothesis, PMOs were designed to inhibit translation of the mRNAs for EBOV VP35, VP24, and L (Figure 1A). These EBOV-specific PMOs demonstrated sequence-specific inhibition when compared with a PMO molecule with a MARV-specific

sequence or PMOs containing EBOV-scrambled sequences in a reporter-based in vitro translation assay (Figure 1B–1D). No inhibition was observed for the scrambled or nonspecific PMO at up to 10 μM concentration for the EBOV VP24, VP35, or L RNA targets (Figure 1B–1D). To determine whether the EBOV-specific PMOs could reduce viral propagation in vitro, VeroE6 cells were pretreated with the PMOs at a concentration of 20 μM and then infected with a multiplicity of infection of 1. Treatment with the EBOV VP24-, VP35-, or L-specific PMO was able to consistently reduce the viral titers in the EBOV-infected VeroE6 cells (Figure 1E). A combination of the three PMOs also reduced the viral titers in the VeroE6 cells, but did not show an enhanced effect as compared with treatment with any one of the PMOs (Figure 1E). In the same assay, the viral titers in the scrambled PMO-treated cells were nearly identical to those observed in untreated VeroE6 cells, and the PMOs did not cause obvious cytotoxicity at concentrations used in this assay (unpublished data).

EBOV-Specific PMOs Protect Rodents against Lethal EBOV Infection ::: Results:
To evaluate the in vivo efficacy of the EBOV-specific PMOs, we first determined the survival of mice treated with individual PMOs at 24 h and 4 h before challenge with 1,000 plaque-forming units (pfu) of mouse-adapted EBOV. The three PMOs exhibited a wide range of efficacy against lethal EBOV infection. Both the VP24- and VP35-specific PMOs provided nearly complete protection when the mice were pretreated twice with 500 μg doses (Figure 2A–2B, $p < 0.001$ for both VP24 and VP35 PMO treatment versus PBS treatment). In contrast, and despite its strong activity in the cell-free translation assay, the L-specific PMO conferred survival to ~30% of treated mice at the 500-μg dose (Figure 2C, $p = 0.5$ for both doses of the L PMO compared with PBS-treated mice). As expected, treatment of the mice with a Marburg-specific VP24, VP35, or L PMO did not provide protection against EBOV (Figure 2A–2C). Next, we investigated whether a combination of all these PMOs could further enhance efficacy. We found that when the VP24-, VP35-, and L-specific PMOs were administered together at 24 h and 4 h before lethal EBOV challenge, this resulted in robust protection at all the doses tested (Figure 2D, $p = 0.00001$, 0.001, and 0.0136, respectively, for the 500-, 50-, and 5-μg EBOV-specific PMO-treated mice compared with the PBS-treated mice). To investigate the usefulness of the PMO in a post-exposure regimen, mice were treated with a single dose of the three PMOs administered 24 h after EBOV infection (Figure 2E). The PMO-treated EBOV-infected mice were fully protected at the 500-μg doses, and at lower doses the post-challenge treatment provided enhanced protection ($p = 0.00001$, 0.0136, and 0.2105, respectively, for the 500-, 50-, and 5-μg PMO-treated mice compared with the PBS-treated mice). Mice receiving a single PMO treatment 4 h before infection showed similar protection ($p = 0.00001$, 0.237, and 1.0, respectively, for the 500-, 50-, and 5-μg PMO-treated mice compared with the PBS-treated mice). Here, a single dose of PMO at 24 hpi was used and, at the highest concentration, the combination of PMOs completely protected EBOV-infected mice. In this single-dose therapeutic regimen, the lower PMO dosages did not provide complete protection. It may be possible that repeated injections of lower concentrations of EBOV-specific PMOs could provide equivalent protection to fewer administrations of higher amounts.
Importantly, multiple injections ($n > 5$) of nonspecific PMOs did not enhance survival of EBOV-infected rodents (unpublished data).
Examination of tissues 3 d after infection showed that treatment of mice with the combination of EBOV-specific PMOs slowed viral spread compared to mice treated with the scrambled PMO. Infected cells were easily observed in the spleens of the mice treated with the scrambled PMO (Figure 3A), whereas few EBOV-infected cells could be found in the spleens of the anti-EBOV PMO-treated mice (Figure 3B). By 6 d after viral inoculation, EBOV infection was fulminant in the spleens of both EBOV and scrambled PMO-treated animals (unpublished data) and had spread to the livers of both mice treated with scrambled and combination PMOs (Figure 3C–3D). However, the extent of the infection was limited in the EBOV-specific combination PMO-treated mice, and, unlike the scrambled PMO-treated mice, EBOV antigen was not detectable within hepatocytes (Figure 3C–3D). Viral antigen was not observed in the kidney on day 3; however, on day 6, viral antigen was more readily observed in the kidneys of the scrambled PMO-treated compared with the combination PMO-treated mice (unpublished data). Further, the viral titers in the spleen, liver, and kidney of the PMO-treated, infected animals corroborated the observed pattern of antigen staining within the tissues (Figure 3E).
To determine whether mice treated with the PMOs generated immune responses to EBOV, they were tested for EBOV-specific cell-mediated and humoral immune responses. 4 wk after infection, the mice demonstrated both CD4+ and CD8+ T cell responses to multiple EBOV-specific peptides, including NP and VP35 sequences (Figure 4A, unpublished data). They also generated

strong serum EBOV-specific antibody responses that were similar to the post-challenge antibody responses of mice protected by a therapeutic vaccine containing Ebola virus-like particles (Figure 4B) [15]. To find out if the generated immune responses were protective, PMO-treated mice were rechallenged with another dose of 1,000 pfu of EBOV 4 wk after surviving the initial challenge, and all the mice were completely protected from the second lethal EBOV infection (Figure 4C, $p < 0.000001$).

To verify the effectiveness of the PMO treatment in another EBOV animal model, we infected guinea pigs with EBOV and treated them with a single dose of the PMO combination either 24 h before or 24 h or 96 h after EBOV infection (Figure 5A). Survival was greatly increased in guinea pigs receiving the treatment 96 h after EBOV infection ($p = 0.029$, compared with untreated guinea pigs), in contrast to guinea pigs treated 24 h before or after infection ($p = 0.5$ and 0.227, respectively, compared with untreated guinea pigs). The mean viral titer for each group, measured on 7 dpi, closely mirrored the survival rates observed (Figure 5B). Results of a logistic regression indicated that reduction of the viral titer had a significant effect on the survival outcome of PMO-treated guinea pigs ($p = 0.0292$). With each log10 reduction in viral titer, there was a 56% decrease in the odds of surviving challenge (odds ratio = 0.434). It remains to be determined if administration of multiple or larger PMO doses might have increased the efficacy of the PMO treatments in the guinea pigs.

PMO Pretreatment Protects Rhesus Monkeys ::: Results:

Based on the encouraging results both in vitro and in rodents, we designed a small proof-of-concept trial in nonhuman primates. Rhesus monkeys were treated with the VP35 PMO only ($n = 4$) or a combination of the VP24, VP35, and L PMOs ($n = 4$) from 2 d prior to EBOV infection through day 9 of the infection (Figure 6A). An initial study tested the efficacy of the VP35 PMO, the compound that exhibited the highest activity in mice. Unfortunately, all of the VP35 PMO-treated monkeys, as well as the untreated control monkey, died within the time-to-death expected for EBOV-infected rhesus macaques (days 7, 7, 8, 8, and 7, respectively). In contrast, treatment of rhesus macaques with a combination of the VP24, VP35, and L PMOs showed substantial efficacy. Two PMO-treated monkeys survived the EBOV challenge with few clinical signs beyond mild depression from days 6–9 (Figure 6B). A third PMO-treated monkey cleared the EBOV infection and remained aviremic from days 9–14 (Figure 6C), but died of a severe bacterial infection, with no evidence of ongoing EBOV infection, on day 16 (Figure 6B, unpublished data). The fourth PMO-treated monkey succumbed to EBOV infection on day 10 (Figure 6B). Three naïve control monkeys used in this experiment received no treatment and succumbed to EBOV infection on days 7, 9, and 10. Since the VP35 PMO alone did not provide any protection to nonhuman primates, these data suggest that a combination of PMOs is more efficacious than a single-target PMO treatment and also suggests that protection in the combination PMO-treated monkeys is not due to nonspecific effects of PMO administration.

We were able to identify only a few early clinical signs or laboratory values that correlated with survival. The laboratory tests that most closely predicted survival were viral titers, platelet counts, and liver-associated enzymes in the blood. The monkeys that did not survive infection had detectable virus by day 5, in stark contrast to the PMO-treated monkeys that survived, which had little to no detectable viremia on days 3–14 (Figure 6C). Both the PMO-treated and naïve monkeys exhibited thrombocytopenia, although the PMO-treated monkeys that survived did not have platelet counts much below 100,000 at any time (Figure 6D). Similarly, all the monkeys experienced increases in their liver-associated enzyme levels, including alkaline phosphatase. However, the levels in the surviving monkeys did not climb as high as those that succumbed to the infection and returned to normal levels within the month after the EBOV infection (Figure 6E, unpublished data).

No correlation was found between survival and multiple other hematological values, body temperature, serum cytokines (measured by a Human 25-plex kit from Biosource, Camarillo, California, United States), or fibrin degradation products (unpublished data). Importantly, PMO administration did not induce early (0–2 dpi) detectable serum IFN, TNF, or other cytokines in the nonhuman primates (unpublished data), indicating that the protection was likely not due to nonspecific effects via induction of innate immunity. Since the surviving PMO-treated monkeys had low to undetectable viremias following infection, we assessed the immune responses of the surviving monkeys. By 28 d after EBOV challenge, the surviving rhesus monkeys had high levels of both anti-EBOV antibodies and T cell responses, similar to the PMO-protected mice (unpublished data).

Discussion:

EBOV infections represent a significant public health threat, with no reliable effective chemotherapeutics. Our current studies indicate the effectiveness of PMOs targeted to specific EBOV mRNAs against in vitro, rodent, and nonhuman primate EBOV infections. The data presented here are in line with our working hypothesis that a combination of PMOs that target multiple viral genes can slow EBOV replication, allowing enough time for development of antiviral immune responses and viral clearance.

Cell-free experiments demonstrated that the EBOV-specific PMOs were efficient at inhibiting EBOV mRNA translation and that MARV-specific PMOs had no effect on translation of corresponding EBOV genes. While the reporter-based in vitro assays appeared to be good indicators of sequence-specific activity of PMOs, it seems that in the case of EBOV, the in vitro translation assays were not fully predictive of the rodent efficacy data. Using VeroE6 cells we showed that EBOV replication was moderately suppressed in cells treated with EBOV-specific PMOs. However, no visible difference was observed when comparing the ability of a single PMO and the combination of three PMOs to reduce viral growth. The PMOs used in these studies were not conjugated with peptides that facilitate cellular uptake. We have observed lower cellular uptake of naked PMOs compared with peptide-conjugated PMOs by Vero E6 cells (unpublished data), and this may explain the moderate inhibition observed in the in vitro replication assay. Nonetheless, the data summarized in Figure 1 clearly indicate the feasibility of specific gene targeting by unconjugated PMO and the favorable antiviral activity of these compounds.

Here, we have presented data demonstrating a therapeutic intervention that interferes with viral replication. We hypothesize that the efficacy of this strategy relies on the ability of PMOs, by reducing viral replication, to create a window of opportunity for the immune system to clear the otherwise lethal infection. This approach does not result in sterile protection, as evidenced by the robust anti-EBOV immune responses in the surviving mice and nonhuman primates, and by the mild clinical signs (thrombocytopenia, liver enzyme elevations, depression, and lethargy) observed in the PMO-treated monkeys. It is likely that successful treatment of patients infected with EBOV will require a multifaceted approach consisting of an antiviral, and therapeutics for symptom and disease management [1,9].

Post-exposure treatment with PMOs was the most efficacious against EBOV in rodents, and, presumably, this is explained by the pharmacokinetic properties of PMO. Following parenteral injection, PMOs are cleared from the bloodstream within hours, but accumulate and remain within tissues such as spleen and liver for days (unpublished data). By administering PMO after challenge, it is likely that higher concentrations were available in the circulation and tissues during the peak of viral replication. Thus, the PMOs may have been able to reduce amplification of EBOV during this critical timeframe, allowing development of appropriate host immune responses to the otherwise lethal viral infection [27–29]. Since effective treatment seemed to require critical timing of PMO administration, and nonhuman primates are highly sensitive to EBOV infection, we decided to both pretreat and administer daily doses of PMOs after viral challenge in the proof-of-concept nonhuman primate studies. While short-term stability in vivo is an unfavorable property for a potential therapeutic, it should be noted that for a deadly acute infection that has only a short period of treatment in the clinic, repeated administration of a life-saving drug is entirely feasible. Along these lines, future studies may show that the pharmacokinetics of PMO can be enhanced by other modifications or alternative routes of administration or regimen to improve efficacy.

In order to corroborate the in vitro data and narrow down the number of antiviral lead candidates, the EBOV mouse model was an obvious choice for testing multiple candidate PMOs [30]. While the mouse model has several differences in pathogenesis compared with EBOV infection of nonhuman primates and humans [31–33], it is widely accepted as an appropriate model for rapid testing of potential antivirals before proceeding to NHP trials. While the differences in the pathology may severely hamper the predictive value of mouse studies using therapeutic strategies that rely on targeting mouse-specific virus–host interaction or the pathologic response by rodents, interventions that target viral replication can be evaluated with a high degree of confidence in this model. Antiviral efficacy in rodents may not always be predictive of efficacy in nonhuman primates, although an antiviral compound that is unsuccessful in rodents will most likely also fail in nonhuman primates, justifying initial screening in rodent models.

We have initiated a drug-discovery strategy to identify “druggable,” efficacious anti-filovirus PMOs. This proof-of-principle study using PMOs demonstrates favorable anti-EBOV activity both in vitro and in vivo; thus, these molecules represent a pool of potential lead compounds for further evaluation and optimization. Future studies will focus on determining the most favorable PMO

dose and regimen and also the therapeutic potential of these molecules. Additionally, antisense PMO designed to target other regions within the EBOV genome will be pursued. Genetically designed microbial therapeutics, such as antisense PMOs or other sequence-based approaches, allow quick hit-to-lead optimization and may accelerate time-to-drug development. Together, these strategies could result in a highly efficacious therapeutic treatment regimen for lethal viral infections. Nucleotide-based antivirals, such as PMOs, can be easily produced in large quantities, have already been tested in human clinical trials, and have appropriate safety profiles for use with humans [34]. The results presented here have far-reaching implications for the treatment of highly lethal hemorrhagic fever viruses, and for diseases caused by many other acute viral diseases including SARS, influenza, and dengue, or other emerging pathogens.

PMOs. ::: Materials and Methods:
PMOs were designed with sequence homology near or overlapping the AUG translational start site of EBOV VP35 (5′-CCTGCCCTTTGTTCTAGTTG-3′), EBOV VP24 (5′-GCCATGGTTTTTTCTCAGG-3′), and EBOV L (5′-TGGGTATGTTGTGTAGCCAT-3′). Similarly, PMOs were designed with sequence homology near or overlapping the AUG start site of MARV VP35 (5′-GTCCCACATTGTGAAAATTAT-3′), MARV VP24 (5′-CGTTGATAATTCTGCCATG-3′), and MARV L (5′-GATATTGAGTTGGATGCTGCAT-3′). Either the MARV-specific PMOs or an unrelated, "scrambled" PMO (5′-AGTCTCGACTTGCTACCTCA-3′) were used as controls in these experiments. The PMOs were synthesized by AVI BioPharma, (Corvallis, Oregon, United States), as previously described [35].

In vitro translation assay. ::: Materials and Methods:
The protein coding sequence for firefly luciferase, without the initiator-Met codon ATG, was subcloned into the multiple cloning site of plasmid pCiNeo (Promega, Madison, Wisconsin, United States). Subsequently, complementary oligonucleotides for EBOV VP35 (–98 to +39; bases 3020 to 3157), EBOV VP24 (–84 to +43; bases 10261 to 10390), EBOV L (–80 to +49; bases 11501 to 11632) were duplexed and subcloned into Nhe 1 and Sal 1 sites. The single AUG in each viral sequence leader is in frame with the coding sequence of luciferase. The plasmids were linearized with Not I, and in vitro transcribed RNA was produced using the T7 polymerase-based Megascript kit and protocol (Ambion, Austin, Texas, United States). In vitro translations were carried out by programming reactions with transcribed RNA at a final concentration of 1 nM, as previously described [36]. The average light units produced by the set of reactions for each treatment were normalized to the mean of all water-only control reactions and expressed as relative light units as compared to the luciferase signal of control reactions.

In vitro assessment of viral replication in the presence of PMOs. ::: Materials and Methods:
Vero E6 cells were grown to confluency in a 24-well plate. On day 0, the indicated PMOs were added in 100 μl of serum-free EMEM. After 2 h, 900 μl of EMEM containing 10% fetal calf serum was added for a final concentration of 20 μM of the VP24, VP35, L, or a combination of all three PMOs. On day 1, the medium was removed from the cells and one multiplicity of infection of EBOV-Zaire was added to each well in 100 μl of serum-free EMEM. After 1 h, the viral inoculum was removed and the cells washed twice with PBS. The PMOs were reintroduced for 2 hs in serum-free EMEM and then brought up to a total of 1 ml with EMEM containing 10% fetal calf serum. Supernatant was removed from the cells at 24, 48, or 72 hpi, and the viral titers were determined by standard plaque assay [37].

Animals. ::: Materials and Methods:
C57BL/6 mice, aged 8–10 wk, of both sexes, were obtained from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, Maryland, United States). Mice were housed in microisolator cages and provided autoclaved water and chow ad libitum. Mice were challenged by intraperitoneal injection with ~1,000 pfu of mouse-adapted EBOV diluted in phosphate buffered saline (PBS) [30]. Unless otherwise noted, mice were treated 2× at 24 h and 4 h prior to EBOV challenge with 0.5 mg, 0.05, or 0.005 mg of each of the EBOV or MARV VP24, VP35, and L PMOs, a combination of PMOs, or an unrelated PMO. In one presented study, the PMOs (0.5, 0.05, or 0.005 mg) were administered in a single dose 4 h before or 24 h after challenge. Previously, we showed Ebola virus-like particles given 24 h before viral challenge protects mice from lethal EBOV infection. Therefore, for comparison of post-challenge immune responses, a group of mice was injected with virus-like particles 24 h before EBOV challenge [15].

C57BL/6 mice were challenged intraperitoneally with 1,000 plaque-forming units of mouse-adapted EBOV [30].
Female Hartley guinea pigs, 400–500 g in weight, were obtained from Charles River Laboratories (Wilmington, Massachusetts, United States) and quarantined at the US Army Research Institute for Infectious Diseases for at least 72 h before manipulations. To determine efficacy of the PMOs in guinea pigs, the animals were treated intraperitoneally with a single 10-mg dose of each of the VP24, VP35, and L PMOs 24 h before or 24 h or 96 h after subcutaneous challenge with 1,000 pfu of guinea-pig adapted EBOV [38]. On day 7, the guinea pigs were bled from the retro-orbital sinus and the plasma samples were assessed for viral titers by plaque assay [37].
Female rhesus macaques of 3–4 kg in weight were challenged with ~1,000 pfu of EBOV-Zaire (1995 strain) [14] by intramuscular injection following PMO treatment. The monkeys were treated from days −2 through day 9 via a combination of parenteral routes (for treatment schedule, see Figure 6). The dose of the VP24 PMO was 12.5–25 mg at each injection, and the dose of the VP35 and L PMOs ranged from 12.5–100 mg per injection. All the monkeys in this study were found to be STLV-1, SIV-, and Herpes B-negative in testing prior to initiation of the study. Monkeys 1496 and 1510 both presented with redness in and bleeding from their vaginal area on days 3–5 post infection. The estrus status of the other monkeys in this study was unknown, although they did not present with vaginal bleeding throughout the 35 d that they were monitored during this study. The number of animals in each group was determined with the help of a statistician in order to use the least number of animals possible yet still to have the statistical power necessary to allow meaningful interpretation of the data produced. All EBOV-infected animals were handled under maximum containment in a biosafety–level-4 laboratory at the US Army Medical Research Institute for Infectious Diseases. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the National Research Council's Guide for the Care and Use of Laboratory Animals (1996). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Hematologic and pathologic sampling. ::: Materials and Methods:
Blood samples were obtained under anesthesia by cardiac puncture for mice and from the femoral vein of monkeys. Viremia was assayed by traditional plaque assay [37]. Hematological, cytokine, and D dimer levels, as well as liver-associated enzymes, were measured as previously described [16]. On day 3 and day 6 after EBOV infection, gross necropsies were performed on three mice from each group pretreated with either the combination of PMOs or the "scrambled" PMO. The tissues were collected in 10% neutral buffered formalin and held in the biosafety–level-4 laboratory for a minimum of 30 d. The tissues were then decontaminated, embedded in paraffin, and sectioned for histology. The sections were then stained with hematoxylin and eosin for routine light microscopy or exposed to EBOV-specific antisera to identify viral antigen within the tissue samples.

Immune responses following EBOV infection. ::: Materials and Methods:
Blood was collected from the surviving mice or monkeys, respectively, 28 d after EBOV challenge. Levels of EBOV-specific antibodies were determined from serum or plasma samples, as previously described [39]. Antibody titers were defined as the reciprocal of the highest dilution giving a net optical density value ≥0.2. Epitopes recognized by CD4+ and CD8+ T cells of surviving mice were determined as previously described [40]. Briefly, EBOV-specific responses were analyzed by culturing splenocytes with 1–5 μg overlapping 15-residue peptides representing the GP or VP40 of EBOV (Mimotopes, Clayton, Victoria, Australia) or 25 ng/ml phorbol 12-myristate 13-acetate and 1.25 μg/ml ionomycin in complete RPMI containing 10 μg/ml brefeldin A. After 5 h of culture, the cells were stained with anti-CD44, -CD8, or -CD4 (Pharmingen, San Diego, California, United States) in brefeldin A. After the cell surface staining, the cells were fixed in 1% formaldehyde, permeabilized with saponin, and stained with anti-IFN-γ PE (Pharmingen).

Statistical analysis. ::: Materials and Methods:
To compare the survival rates in the rodent experiments, we used a Fisher exact test with stepdown bootstrap adjustment. In the guinea pig experiments, the viral titers were analyzed by ANOVA with post hoc Dunnett's test, and the effect of viral titers on survival were analyzed by logistic regression. A p value of ≤0.05 was considered significant.