Ebolaviruses and marburgviruses belong to the family Filoviridae and cause high lethality in infected patients. There are currently no licensed filovirus vaccines or antiviral therapies. The development of broad-spectrum therapies against members of the Marburgvirus genus, including Marburg virus (MARV) and Ravn virus (RAVV), is difficult because of substantial sequence variability. RNAi therapeutics offer a potential solution, as identification of conserved target nucleotide sequences may confer activity across marburgvirus variants.

Here, we assessed the therapeutic efficacy of lipid nanoparticle (LNP) delivery of a single nucleoprotein–targeting (NP-targeting) siRNA in nonhuman primates at advanced stages of MARV or RAVV disease to mimic cases in which patients begin treatment for fulminant disease. Sixteen rhesus monkeys were lethally infected with MARV or RAVV and treated with NP siRNA-LNP, with MARV-infected animals beginning treatment four or five days after infection and RAVV-infected animals starting treatment three or six days after infection. While all untreated animals succumbed to disease, NP siRNA-LNP treatment conferred 100% survival of RAVV-infected macaques, even when treatment began just 1 day prior to the death of the control animals. In MARV-infected animals, day-4 treatment initiation resulted in 100% survival, and day-5 treatment resulted in 50% survival. These results identify a single siRNA therapeutic that provides broad-spectrum protection against both MARV and RAVV.
siRNA rescues nonhuman primates from advanced Marburg and Ravn virus disease

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Introduction

From 1967 until late 2013, members of the 2 genera of filoviruses, Marburgvirus and Ebolavirus, have caused periodic occurrences of hemorrhagic fever (HF) in Central Africa, resulting in severe and often lethal disease in infected patients (1). Prior to 2013, these outbreaks were relatively small in size, ranging from a few cases to several hundred cases. However, the recent unprecedented epidemic of Ebola virus disease caused by the Ebola virus (EBOV, species Zaire ebolavirus) in West Africa that resulted in 28,616 cases and 11,310 deaths from December 2013 to January 2016 underscores the public health threat posed by filoviruses. This concern is further heightened by the lack of licensed vaccines and antiviral therapies. While substantial progress has been made in advancing promising candidate EBOV vaccines and antivirals as a result of the 2013–2016 epidemic, less work has been done on developing effective countermeasures against the marburgvirus genus.

Ebolavirus and Marburgvirus (MARV) virus and Ravn virus (RAVV) (2). In addition, the significant sequence variability (up to 21%) (2) between the 2 virus variants presents an obstacle to the development of therapies that are effective across all members of the Marburgvirus genus.

RNAi therapy is a promising drug modality that offers exquisite target specificity and a well-defined mechanism of action. A significant barrier to in vivo application of RNAi has been the development of efficient delivery vehicles that promote cellular uptake (3); however, use of a lipid nanoparticle (LNP) platform not only protects siRNAs from nuclease degradation in the bloodstream but also mediates effective delivery to hepatocytes, a major replication cell for MARV and RAVV. This technology has been used successfully to protect nonhuman primates (NHPs) against EBOV when given before or at the onset of illness (4, 5) and against Sudan virus (SUDV, species Sudan ebolavirus) when given at the late stage of disease (6). Although anti-EBOV siRNA-LNP was recently tested in a very small phase II clinical trial, in which it appeared safe but did not show clear benefit, the extremely high viral loads (>9 log_{10} copies/ml for all 12 cases) and existing organ injury in the enrolled end-stage patients probably obscured this trial’s ability to detect statistically significant treatment efficacy (7). In addition, LNP technology continues to advance beyond the formulations used in the preceding TKM-Ebola clinical trials, and novel LNP formulations with increased potency have been developed and shown to have utility in filovirus-infected NHPs (5).

In previous marburgvirus siRNA studies, we have (a) identified 2 siRNAs targeting the MARV NP (designated NP-718m and NP-143m) that, when encapsulated in a LNP, inhibited the replication of MARV and RAVV in vitro and provided complete protection to guinea pigs infected with several different MARV and RAVV isolates (8); and (b) showed that NP-718m–LNP conferred complete protection to MARV-infected rhesus macaques when administered at the onset of clinical illness, 3 days after MARV exposure.
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mals succumbed on day 8 p.i. (Figure 1A), which was consistent with a mean time to death of 7.86 days across 14 historical control animals (T.W. Geisbert, unpublished observations). To assess whether the 2 treated animals succumbed as a result of the emergence of viral escape mutants to the siRNA, virus was isolated from these animals and subjected to an in vitro infection assay in HepG2 cells. NP-718m–LNP treatment on infected HepG2 cells showed comparable antiviral activity between virus isolated from treated animals that had succumbed and the original virus inoculum (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI96185DS1).

NP-718m–LNP treatment results in the survival of RAVV-infected NHPs when treatment is initiated at early onset and late stages of the disease. A single nucleotide mismatch is present in the NP-718m target site in RAVV NP mRNA (Figure 1B). In vitro assessment of siRNA activity against MARV or RAVV NP transcript sequences in a dual-luciferase reporter assay predicted that this mismatch would have minimal impact on NP-718m anti-RAVV activity (Figure 1B). Previous results assessing the efficacy of NP-718m in lethally infected guinea pig models suggested a decreased efficacy against RAVV (treatment resulted in 60% survival), whereas NP-718m–LNP treatment of MARV-infected animals produced no survival benefit. Here, we assessed the utility of using NP-718m–LNP as a therapeutic intervention in uniformly lethal rhesus macaque models of MARV and RAVV infection when treatment was delayed until the animals were at advanced stages of illness, as evidenced by fevers and high viral loads. In doing so, importantly, we demonstrated the broad-spectrum activity of NP-718m–LNP as a therapeutic intervention against these sequence-divergent members of the Marburgvirus genus.

Results

NP-718m–LNP treatment results in the survival of MARV-infected NHPs when treatment is initiated at late stages of the disease. A total of 10 rhesus macaques were infected with a uniformly lethal dose of MARV. Initiation of NP-718m–LNP treatment occurred 4 days or 5 days post infection (p.i.) and consisted of 7 daily i.v. bolus doses. The control animals were not treated with NP-718m–LNP. All 4 animals given NP-718m–LNP beginning on day 4 p.i. survived MARV infection, while 2 of 4 animals given NP-718m–LNP beginning on day 5 p.i. survived infection. Both untreated control animals succumbed on day 8 p.i. (Figure 1A), which was consistent with a mean time to death of 7.86 days across 14 historical control animals (T.W. Geisbert, unpublished observations). To assess whether the 2 treated animals succumbed as a result of the emergence of viral escape mutants to the siRNA, virus was isolated from these animals and subjected to an in vitro infection assay in HepG2 cells. NP-718m–LNP treatment on infected HepG2 cells showed comparable antiviral activity between virus isolated from treated animals that had succumbed and the original virus inoculum (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI96185DS1).

NP-718m–LNP treatment results in the survival of RAVV-infected NHPs when treatment is initiated at early onset and late stages of the disease. A single nucleotide mismatch is present in the NP-718m target site in RAVV NP mRNA (Figure 1B). In vitro assessment of siRNA activity against MARV or RAVV NP transcript sequences in a dual-luciferase reporter assay predicted that this mismatch would have minimal impact on NP-718m anti-RAVV activity (Figure 1B). Previous results assessing the efficacy of NP-718m in lethally infected guinea pig models suggested a decreased efficacy against RAVV (treatment resulted in 60% survival), whereas NP-718m–LNP treatment of MARV-infected animals produced
Figure 2. NP-718m–LNP treatment effectively controls MARV or RAVV viral replication in blood and tissues. (A) NP-718m–LNP treatment mediates up to 4 log reductions in viremia in MARV-infected NHPs when compared with untreated controls. Protection was greatest when treatment was initiated at earlier p.i. time points. n = 4 treated animals and n = 1 untreated control each for 1 study 4 days p.i. and 1 study 5 days p.i. *P = 0.0159, by 1-tailed, unpaired t test (day 8). (B) Similar reductions in viremia were also observed in RAVV-infected animals treated with NP-718m alone or in a cocktail with NP-143m. n = 2 for NP-718m–LNP and NP-718m plus NP-143m–LNP treatment groups 3 days p.i.. n = 4 treated animals for the day-6 p.i. study. n = 1 untreated control for each study. NP-718m treatment resulted in reductions in viral RNA present in the blood for (C) MARV (*P = 0.0033, by 1-tailed, unpaired t test; day 8) and (D) RAVV, as assessed by viral RNA qRT-PCR. n = 4 treated animals and n = 1 untreated control each for the MARV day-4 p.i. study and the MARV day-5 p.i. study. n = 2 for NP-718m–LNP and NP-718m plus NP-143m–LNP treatment groups for the RAVV day-3 p.i. study and n = 4 treated animals for the RAVV day-6 p.i. study. n = 1 untreated control for each study. Treatment was also able to control RAVV (E) and MARV (F) viral replication in tissues, including those in immune-privileged sites such as the reproductive organs, brain, and eye. The lower limit of quantification (LLQ) for viral RNA qRT-PCR was $1 \times 10^5$ GEq/ml or GEq/g. LN, lymph node.
complete protection (8). Interestingly, in the previous study, treatment of RAVV-infected guinea pigs with a siRNA cocktail containing both NP-718m and a siRNA with perfect target site complementarity to RAVV (NP-143m) conferred complete protection. To determine whether these results translated into the more stringent NHP model of RAVV infection, we assessed NP-718m and the cocktail treatment consisting of NP-718m and NP-143m in 5 rhesus macaques lethally infected with RAVV, with treatment beginning 3 days p.i. In contrast to the previous results obtained in the guinea pig model, we found that treatment with NP-781m–LNP alone was sufficient to confer 100% survival to the lethally infected animals. Because no additional advantage was observed in survival or in the control of viral load in animals receiving the NP-718m plus NP-143m cocktail when compared with animals treated with NP-718m alone, we conducted a subsequent study in 5 RAVV-infected macaques to examine the efficacy of the single siRNA NP-718m when treatment was initiated 6 days p.i., only 1 day prior to when the untreated control animal in the study succumbed to disease (mean time to death across 3 historical control animals of 9 days; T.W. Geisbert, unpublished observations). Notably, even when treatment was initiated at this later stage of disease, all animals treated with NP-718m–LNP survived infection (Figure 1C), and treatment-associated survival was found to be statistically significant ($P = 0.0357$, Fisher’s exact test).

Viremia and viral RNA load are effectively reduced upon NP-718m–LNP treatment. NP-718m–LNP treatment on day 4 p.i. and day 5 p.i. with MARV was able to reduce peak viremia by up to 4 log_{10} PFU/mL, as measured by plaque assay, when compared with untreated control animals (Figure 2A). We observed similar reductions with NP-718m–LNP treatment of RAVV-infected animals, which was statistically significant for day 6 ($P < 0.0001$, 1-way ANOVA with Dunnett’s correction for multiple comparisons) (Figure 2B). Viral control was rapid, with reductions observed by the next sampling point, 3 days after treatment initiation. Treatment with NP-718m–LNP reduced the viral RNA load in the blood by a range of 2 to 4 log_{10} units by day 8 p.i. in MARV-infected macaques and by day 9 p.i. in RAVV-infected animals when compared with viral loads in the untreated animals (Figure 2, C and D). Animals administered NP-718m–LNP treatment also had reduced viral RNA loads in tissues at the study’s end when compared with viral loads in the untreated animals euthanized during peak viremia (Figure 2, E and F).

NP-718m–LNP treatment ameliorates clinical and pathological disease associated with MARV and RAVV HF. Animals treated with NP-718m–LNP exhibited less severe signs of clinical illness, such as depression, anorexia, petechial rash, and hemorrhage, which are associated with MARV or RAVV infection (Figure 3, A and B; see Tables 1 and 2 for individual animal data). We assessed serum samples from all infected animals for liver- and kidney-associated function markers such as alanine aminotransferase (ALT) and blood urea nitrogen (BUN), respectively, which dramatically increase during MARV or RAVV disease–mediated organ decompensation. We found that NP-718m–LNP treatment was able to protect against liver (Figure 3, C and D, and Figure 4, A–D) and renal damage (Figure 3, E and F; and Figure 4, E and F) induced by MARV or RAVV. All infected animals, independent of treatment, showed thrombocytopenia, lymphopenia, and/or granulocytosis during the peak viremia period, which is typical of MARV and RAVV HF (Figure 5, A–F). Examination of tissue sections showed various degrees of lesions and MARV (Figure 6, A–D) or RAVV (Figure 7, A–D) antigen in the untreated control animals, consistent with historical controls (10). Tissue lesions in NP-718m–LNP–treated animals that did not survive MARV infection were comparable to those in untreated control animals (Figure 6, I–L). None of the tissue sections from the NP-718m–LNP–treated animals that survived out to day 28 p.i. showed any lesions or clear immunoreactivity for MARV or RAVV antigen (Figure 6, E–H and Figure 7, E–H). This ability of NP-718m–LNP to protect against disease is also supported by the relative lack of change in the clinical scores of the treated animals from the baseline scores (Figure 3, A and B). These data collectively suggest that LNP delivery of NP-718m siRNA mediates effective and potent broad-spectrum therapeutic treatment of MARV or RAVV infection.

Discussion

Human fatality rates have ranged from 23% for some isolates of MARV and/or RAVV to as high as 90% for the Angola strain of MARV (1, 2). As evidenced by the recent Ebola virus disease epidemic of unprecedented size in West Africa (11, 12), there is a real and urgent need to develop effective countermeasures against these Tier 1 Select Agents (United States Federal Select Agent Program). Given the rapid disease onset associated with filoviral HF, the ability of a medical countermeasure to provide therapeutic treatment (upon or after initial clinical signs) and possess broad-spectrum activity against multiple species of virus would confer significant benefit in acute situations when the time to treatment initiation is at its most critical.

Historically, most studies in NHPs examined interventions against MARV infection at time points before the animals were viremic or showed any evidence of clinical illness. Furthermore, prior to this year, to our knowledge, there have been no studies of therapeutics against RAVV. Early studies using recombinant vesicular stomatitis virus–based vaccines (13, 14), polyclonal NHP antibodies (15), antisense phosphorodiamidate morpholino oligomers (16), and a broad-spectrum nuclease analog (17) all demonstrated potential in protecting NHPs against lethal MARV infection when treatment was initiated within 48 hours of exposure, before the onset of viremia or clinical illness. Subsequent work showed that antisense phosphorodiamidate morpholino oligomers could provide partial protection for NHPs when treatment was initiated as late as day 4 p.i. in a macaque model of MARV infection. However, an important consideration in these previous studies is the strain of MARV used. Specifically, 1 MARV postexposure treatment study in NHPs (15) evaluated efficacy against the Ci67 strain associated with the initial MARV outbreak in Europe in 1967 that resulted in 7 fatalities among 31 patients (18). All but 1 other MARV postexposure treatment study in NHPs evaluated efficacy against the Musoke strain. The Musoke strain was isolated from a nonfatal human case (19) and has been associated with a much more protracted disease course in NHPs, in which the animals typically succumb 10–13 days after exposure (13, 14, 16, 17). In contrast, the Angola strain is the causative agent of the largest outbreak of MARV HF thus far, with a mortality rate of 90% in over 200 confirmed cases (2). It would be highly desirable for advanced development of any MARV countermeasure to include the demonstration of efficacy against MARV-Angola, the most
Figure 3. NP-718m–LNP treatment ameliorates disease symptoms in MARV or RAVV HE. (A) Clinical scores of untreated or NP-718m–LNP–treated MARV-infected animals. $n = 4$ treated animals and $n = 1$ untreated control each for the MARV day-4 p.i. study and the MARV day-5 p.i. study. (B) Clinical scores of untreated or NP-718m–LNP–treated animals lethally infected with RAVV. $n = 2$ for NP-718m–LNP and NP-718m plus NP-143m–LNP treatment groups for the RAVV day-3 p.i. study and $n = 4$ treated animals for the RAVV day-6 p.i. study. $n = 1$ untreated control for each study. Scoring changes measured from baseline included posture and activity level, attitude and 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 the criteria for euthanasia. (C) Animals treated with NP-718m showed amelioration of liver dysfunction typical of MARV infection. (D) NP-718m–LNP treatment protected against liver dysfunction in macaques lethally infected with RAVV. (E) NP-718m–LNP treatment ameliorated kidney dysfunction resulting from MARV disease. (F) Both NP-718m alone or in a siRNA cocktail with NP-143m conferred similar protection against kidney dysfunction in RAVV-infected NHPs.
Table 1. Clinical description and outcome of MARV-infected NHPs

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Sex</th>
<th>siRNA Treatment regimen (d)</th>
<th>Clinical illness</th>
<th>Clinical and gross pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>F</td>
<td>None (ctrl for d 4–10)</td>
<td>Fever (d 7); depression (d 7, 8); lethargy (d 7, 8); loss of appetite (d 5–14); mild rash (d 7); ecchymotic rash (d 8); animal succumbed on d 8</td>
<td>Leukocytosis (d 8); granulocytosis (d 8); lymphopenia (d 7, 8); thrombocytopenia (d 7, 8); hypoalbuminemia (d 8); hypoamylasemia (d 8, 9); ALT &gt;6-fold ↑ (d 7); AST &gt;7-fold ↑ (d 8); AST &gt;10-fold ↑ (d 7, 8); ALP &gt;3-fold ↑ (d 7); GGT &gt;2-fold ↑ (d 7, 8); CRP &gt;6-fold ↑ (d 7); CRP &gt;5-fold ↑ (d 8)</td>
</tr>
<tr>
<td>Untreated</td>
<td>F</td>
<td>None (ctrl for d 5–11)</td>
<td>Fever (d 5); depression (d 7, 8); lethargy (d 7, 8); loss of appetite (d 6–8); moderate rash (d 7, 8); animal succumbed on d 8</td>
<td>Leukocytosis (d 8); granulocytosis (d 5); thrombocytopenia (d 5); ALT &gt;3-fold ↑ (d 5); ALT &gt;10-fold ↑ (d 5, 8); ALP &gt;5-fold ↑ (d 8); GGT &gt;10-fold ↑ (d 8); BUN &gt;2-fold ↑ (d 8); CRE &gt;6-fold ↑ (d 8); CRP &gt;5-fold ↑ (d 7); CRP &gt;2-fold ↑ (d 8)</td>
</tr>
<tr>
<td>NP-718m-1</td>
<td>F</td>
<td>NP-718m d 4–10</td>
<td>Loss of appetite (d 6–10)</td>
<td>Lymphopenia (d 6, 10); thrombocytopenia (d 14); AST &gt;3-fold ↑ (d 10); CRP &gt;5-fold ↑ (d 4, 10); CRP &gt;6-fold ↑ (d 6)</td>
</tr>
<tr>
<td>NP-718m-2</td>
<td>F</td>
<td>NP-718m d 4–10</td>
<td>Loss of appetite (d 8–14); depression (d 10–12)</td>
<td>Lymphopenia (d 14, 21); granulocytosis (d 10, 14, 21); thrombocytopenia (d 6, 10); hypoalbuminemia (d 10, 14, 21); hypoamylasemia (d 10); ALT &gt;2-fold ↑ (d 6); ALT &gt;7-fold ↑ (d 10); ALP &gt;2-fold ↑ (d 10, 14, 21); GGT &gt;4-fold ↑ (d 10); BUN &gt;2-fold ↑ (d 10); CRP &gt;4-fold ↑ (d 4); CRP &gt;2-fold ↑ (d 6); CRP &gt;8-fold ↑ (d 10); CRP &gt;5-fold ↑ (d 14)</td>
</tr>
<tr>
<td>NP-718m-3</td>
<td>M</td>
<td>NP-718m d 4–10</td>
<td>Loss of appetite (d 6–14)</td>
<td>Granulocytosis (d 10, 28); lymphopenia (d 6); thrombocytopenia (d 6); hypoalbuminemia (d 10, 14); ALT &gt;6-fold ↑ (d 10); AST &gt;2-fold ↑ (d 14); AST &gt;2-fold ↑ (d 6); AST &gt;8-fold ↑ (d 10); ALP &gt;3-fold ↑ (d 14); GGT &gt;2-fold ↑ (d 14); CRP &gt;5-fold ↑ (d 4, 10); CRP &gt;10-fold ↑ (d 6)</td>
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<tr>
<td>NP-718m-4</td>
<td>M</td>
<td>NP-718m d 4–10</td>
<td>Loss of appetite (d 5, 9–11)</td>
<td>Leukocytosis (d 4); granulocytosis (d 4, 10); AST &gt;3-fold ↑ (d 10); CRP &gt;10-fold ↑ (d 4, 6, 10)</td>
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<tr>
<td>NP-718m-5</td>
<td>M</td>
<td>NP-718m d 5–11</td>
<td>Loss of appetite (d 10–13)</td>
<td>Leukocytosis (d 15, 22); granulocytosis (d 11, 15, 22); lymphopenia (d 5, 8); thrombocytopenia (d 5); ALT &gt;3-fold ↑ (d 11); ALT &gt;3-fold ↑ (d 8); ALT &gt;10-fold ↑ (d 11); ALP &gt;3-fold ↑ (d 11); ALP &gt;6-fold ↑ (d 15); CRP &gt;5-fold ↑ (d 5, 11)</td>
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<tr>
<td>NP-718m-6</td>
<td>F</td>
<td>NP-718m d 5–11</td>
<td>Loss of appetite (d 6–15)</td>
<td>Leukocytosis (d 11, 15); lymphopenia (d 5, 8, 11); thrombocytopenia (d 8, 11); hypoalbuminemia (d 11, 15, 22); hypoamylasemia (d 8); ALT &gt;3-fold ↑ (d 8); ALT &gt;5-fold ↑ (d 11); AST &gt;3-fold ↑ (d 5); AST &gt;7-fold ↑ (d 8); AST &gt;9-fold ↑ (d 11); ALP &gt;2-fold ↑ (d 8); GGT &gt;6-fold ↑ (d 8, 9); BUN &gt;3-fold ↑ (d 9); CRE &gt;2-fold ↑ (d 9); CRP &gt;7-fold ↑ (d 5); CRP &gt;10-fold ↑ (d 8, 9)</td>
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<tr>
<td>NP-718m-7</td>
<td>M</td>
<td>NP-718m d 5–11</td>
<td>Loss of appetite (d 5–12); mild rash (d 6–15); animal succumbed on d 12</td>
<td>Leukocytosis (d 11, 15); lymphopenia (d 5, 11); thrombocytopenia (d 5, 8, 11); hypoalbuminemia (d 11, 15, 22); hypoamylasemia (d 8); ALT &gt;3-fold ↑ (d 8); ALT &gt;5-fold ↑ (d 11); AST &gt;3-fold ↑ (d 5); AST &gt;7-fold ↑ (d 8); AST &gt;9-fold ↑ (d 11); ALP &gt;2-fold ↑ (d 11, 15); CRP &gt;10-fold ↑ (d 5, 11)</td>
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<tr>
<td>NP-718m-8</td>
<td>F</td>
<td>NP-718m d 5–11</td>
<td>Loss of appetite (d 5, 7–9); depression (d 10–12); lethargy (d 12); loss of appetite (d 6–12); mild rash (d 10–12); animal succumbed on d 12</td>
<td>Leukocytosis (d 8, 11, 12); lymphopenia (d 8, 5, 8, 11, 12); thrombocytopenia (d 8, 11, 12); hypoalbuminemia (d 11, 12, 12); hypoamylasemia (d 11); ALT &gt;2-fold ↑ (d 5); ALT &gt;3-fold ↑ (d 8); ALT &gt;10-fold ↑ (d 11, 12); AST &gt;5-fold ↑ (d 5, 8, 11, 12); ALT &gt;10-fold ↑ (d 11, 12); ALP &gt;3-fold ↑ (d 8); ALT &gt;7-fold ↑ (d 11, 12); GGT &gt;2-fold ↑ (d 8); GGT &gt;10-fold ↑ (d 11); GGT &gt;8-fold ↑ (d 12); BUN &gt;10-fold ↑ (d 11, 12); CRE &gt;5-fold ↑ (d 12); CRP &gt;5-fold ↑ (d 8); CRP &gt;10-fold ↑ (d 11, 12)</td>
</tr>
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</table>

Days after MARV infection are in parentheses. Fever is defined as a temperature more than 2.5°F over baseline or at least 1.3°F over baseline and 103.5°F or higher or 1.1°F over baseline and 104.0°F or higher. Mild rash: focal areas of petechiae covering less than 10% of the skin. Moderate rash: focal areas of petechiae covering 10%–25% of the skin. Lymphopenia and thrombocytopenia are defined by a 35% or greater decrease in the number of lymphocytes and platelets, respectively. Leukocytosis and granulocytosis are defined by a 2-fold or greater increase in the number of white blood cells or granulocytes over baseline, respectively. Hypoalbuminemia, hypoproteinemia, and hypoamylasemia are defined by a 35% or greater decrease in the levels of albumin, total protein, and amylase, respectively. ctrl, control.

Pathogenic MARV strain, which also manifests as a more rapid disease course (7–9 days) in NHPs (10). Very recent studies are beginning to address the Angola strain of MARV as well as RAVV. Notably, it was reported that a single monoclonal antibody against the MARV glycoprotein provided complete protection against the Angola strain of MARV when treatment was initiated on day 4 and near-complete protection when treatment was initiated on day 5 (20). Importantly, the same antibody provided complete protection against RAVV when treatment of NHPs was delayed until day 5 after exposure. Prediction of broad-spectrum activity for monoclonal antibodies is difficult, and empirical testing is required to confirm activity against multiple virus species. In contrast, the sequence-dependent mechanism of action of RNAi therapeutics is amenable to design features that can incorporate broad-spectrum activity through the identification of conserved target nucleotide sequences present in multiple species.
### Table 2. Clinical description and outcome of RAVV-infected NHPs

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Sex</th>
<th>siRNA Treatment regimen (d)</th>
<th>Clinical Illness</th>
<th>Clinical and gross pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated 3 d p.i.</td>
<td>F</td>
<td>None None (ctrl for d 3–9)</td>
<td>Fever (d 6); depression (d 8–10); lethargy (d 9–10); loss of appetite (d 5–14); mild rash (d 8–10); animal succumbed on d 10</td>
<td>Lymphopenia (d 6); thrombocytopenia (d 6); AST &gt;2-fold ↑ (d 6); CRP &gt;6-fold ↑ (d 7); CRP &gt;4-fold ↑ (d 6)</td>
</tr>
<tr>
<td>Untreated 6 d p.i.</td>
<td>F</td>
<td>None None (ctrl for d 6–12)</td>
<td>Fever (d 6); depression (d 7); lethargy (d 7); loss of appetite (d 6, 7); mild rash (d 6); moderate rash (d 7); animal succumbed on d 7</td>
<td>Granulocytosis (d 7); lymphopenia (d 6, 7); thrombocytopenia (d 6, 7); hypoamylasemia (d 6, 7); ALT &gt;10-fold ↑ (d 6, 7); AST &gt;10-fold ↑ (d 6, 7); GGT &gt;3-fold ↑ (d 7); CRP &gt;2-fold ↑ (d 7); CRP &gt;10-fold ↑ (d 7)</td>
</tr>
<tr>
<td>NP cocktail-1</td>
<td>M</td>
<td>NP-718m + NP-143m d 3–9</td>
<td>Fever (d 4, 7); loss of appetite (d 5, 8–10)</td>
<td>Granulocytosis (d 6, 14); thrombocytopenia (d 6); AST &gt;2-fold ↑ (d 10); CRP &gt;10-fold ↑ (d 10); CRP &gt;3-fold ↑ (d 10)</td>
</tr>
<tr>
<td>NP cocktail-2</td>
<td>F</td>
<td>NP-718m + NP-143m d 3–9</td>
<td>None</td>
<td>Thrombocytopenia (d 10); AST &gt;2-fold ↑ (d 6); AST &gt;6-fold ↑ (d 10); CRP &gt;2-fold ↑ (d 6, 10)</td>
</tr>
<tr>
<td>NP-718m-9</td>
<td>M</td>
<td>NP-718m d 3–9</td>
<td>None</td>
<td>AST &gt;2-fold ↑ (d 10); CRP &gt;2-fold ↑ (d 10)</td>
</tr>
<tr>
<td>NP-718m-10</td>
<td>F</td>
<td>NP-718m d 3–9</td>
<td>None</td>
<td>AST &gt;4-fold ↑ (d 10); CRP &gt;2-fold ↑ (d 10)</td>
</tr>
<tr>
<td>NP-718m-11</td>
<td>F</td>
<td>NP-718m d 6–12</td>
<td>Fever (d 6–9); loss of appetite (d 8–16); diarrhea (d 13–15)</td>
<td>Leukocytosis (d 15, 22, 28); granulocytosis (d 12, 15, 22); lymphopenia (d 6, 9); thrombocytopenia (d 9, 12); hypoamylasemia (d 15); hypoamylasemia (d 9, 12, 15); ALT &gt;6-fold ↑ (d 9); ALT &gt;9-fold ↑ (d 12); ALT &gt;4-fold ↑ (d 15); AST &gt;5-fold ↑ (d 6); AST &gt;7-fold ↑ (d 9); AST &gt;10-fold ↑ (d 15); ALP &gt;2-fold ↑ (d 9, 22); ALP &gt;4-fold ↑ (d 12); ALP &gt;5-fold ↑ (d 15); CRE &gt;2-fold ↑ (d 12); CRP &gt;5-fold ↑ (d 15)</td>
</tr>
<tr>
<td>NP-718m-12</td>
<td>M</td>
<td>NP-718m d 6–12</td>
<td>Fever (d 6, 8); loss of appetite (d 8, 9)</td>
<td>Leukocytosis (d 12, 15, 22, 28); granulocytosis (d 9, 12, 22); lymphopenia (d 6, 9); thrombocytopenia (d 9, 12); hypoamylasemia (d 9, 12); ALT &gt;10-fold ↑ (d 9); ALT &gt;7-fold ↑ (d 12); ALT &gt;2-fold ↑ (d 15); AST &gt;2-fold ↑ (d 6, 15); AST &gt;10-fold ↑ (d 9); AST &gt;9-fold ↑ (d 12); ALP &gt;2-fold ↑ (d 12, 15); GGT &gt;2-fold ↑ (d 12, 15); CRP &gt;6-fold ↑ (d 6, 15); CRP &gt;2-fold ↑ (d 9); CRP &gt;7-fold ↑ (d 12)</td>
</tr>
<tr>
<td>NP-718m-13</td>
<td>M</td>
<td>NP-718m d 6–12</td>
<td>Fever (6–9); depression (d 9); lethargy (d 9); loss of appetite (d 6–15)</td>
<td>Granulocytosis (d 6, 9, 12, 15, 22, 28); lymphopenia (d 6, 9, 12); thrombocytopenia (d 9, 12); hypoamylasemia (d 6, 9, 12, 15); ALT &gt;10-fold ↑ (d 9); ALT &gt;7-fold ↑ (d 12); ALT &gt;2-fold ↑ (d 15); AST &gt;2-fold ↑ (d 6, 12); AST &gt;10-fold ↑ (d 9, 12); AST &gt;6-fold ↑ (d 15); ALP &gt;2-fold ↑ (d 12); ALP &gt;3-fold ↑ (d 15); GGT &gt;2-fold ↑ (d 12, 15, 22, 28); CRP &gt;3-fold ↑ (d 6, 12)</td>
</tr>
<tr>
<td>NP-718m-14</td>
<td>M</td>
<td>NP-718m d 6–12</td>
<td>Fever (d 6); depression (d 9–19); lethargy (d 9–13); loss of appetite (d 6–16); mild rash (d 6–8, 11–13); moderate rash (d 9, 10); diarrhea (d 18–21)</td>
<td>Leukocytosis (d 12, 15); granulocytosis (d 6, 9, 12, 15); lymphopenia (d 6, 9, 12); thrombocytopenia (d 9, 12, 15); hypoamylasemia (d 12, 15); hypoamylasemia (d 9, 12); ALT &gt;3-fold ↑ (d 6, 22); ALT &gt;10-fold ↑ (d 9, 12); ALT &gt;7-fold ↑ (d 15); ALT &gt;2-fold ↑ (d 28); AST &gt;10-fold ↑ (d 6, 9, 12); AST &gt;9-fold ↑ (d 12); AST &gt;5-fold ↑ (d 15); AST &gt;2-fold ↑ (d 22); ALP &gt;2-fold ↑ (d 6, 9, 12, 15, 28); ALP &gt;3-fold ↑ (d 22); CRP &gt;7-fold ↑ (d 6); CRP &gt;3-fold ↑ (d 9, 12); CRP &gt;2-fold ↑ (d 15)</td>
</tr>
</tbody>
</table>

Days after RAVV infection are in parentheses. Fever is defined as a temperature above 2.5°F over baseline or at least 1.3°F over baseline and 103.5°F or higher or 11°F over baseline and 104.0°F or higher. Mild rash: focal areas of petechiae covering less than 10% of the skin. Moderate rash: focal areas of petechiae covering 10%–25% of the skin. Lymphopenia and thrombocytopenia are defined by a 35% or greater drop in the number of lymphocytes and platelets, respectively. Leukocytosis and granulocytosis are defined by a 2-fold or greater increase in the number of white blood cells or granulocytes over baseline, respectively. Hypoamylasemia, hypoproteinemia, and hypoamylasemia are defined by a 35% or greater decrease in the levels of albumin, total protein, and amylase, respectively.

The goal of the current study was to determine whether a single siRNA, NP-718m, was able to confer broad-spectrum protection against the sequence-divergent MARV and RAVV variants in NHPs, when treatment was started at considerably advanced disease stages. We have now demonstrated that NP-718m-LNP treatment completely protects rhesus monkeys against lethal MARV and RAVV HF when treatment begins on days 4 and 6 after exposure, respectively. These studies are also the first to our knowledge to report 100% postexposure protection of NHPs from RAVV when treatment was delayed until day 6 after exposure, just 1 day prior to when the control animals in this study began to succumb to disease. This ability to confer protection against both MARV and RAVV is important, since all strains of MARV and RAVV are endemic to the same regions of Africa and have even been identified during the same outbreaks (21). NP-718m-LNP treatment resulted in 50% survival when administration began 5 days p.i. in animals infected with the MARV-Angola variant. We found no significant delay in the time to death of the treated animals that succumbed and no evidence of viral escape mutants. It is possible that at the start of...
Our results in the stringent NHP model of lethal infection with clinical virus corroborates earlier work demonstrating the ability of NP-718m to effectively protect against all major MARV strains and RAVV in guinea pigs (8). Interestingly, in the NHP model, which more closely approximates human disease, NP-718m–LNP treatment conferred better protection than what was observed previously in infected RAVV guinea pigs, despite the presence of a 1-nucleotide mismatch in the siRNA target sequence against RAVV. The location of this nucleotide change at position 9 in the antisense strand of NP-718m is within the previously predicted low-tolerance region (positions 5–11), in which mismatches have a higher probability of negatively impacting siRNA activity (23–26). However, mismatch tolerance is also dependent on the identity of the nucleotides involved, and the target:siRNA mismatch described here (a G:U wobble pair) is particularly well tolerated (26), a finding that is also consistent with our siRNA activity assessment (Figure 1B).

Figure 4. NP-718m–LNP treatment mitigates liver and kidney dysfunction in MARV or RAVV HF. (A and C) Reductions observed in AST and GGT elevations typical of MARV disease. (B and D) Similar reductions were observed in RAVV-infected animals treated with NP-718m–LNP alone or with the NP-718m plus NP-143m siRNA cocktail, with the greatest amelioration observed in animals in which treatment was initiated within an earlier p.i. time frame. NP-718m treatment was also able to maintain kidney function against (E) MARV or (F) RAVV infection. n = 4 treated animals and n = 1 untreated control each for the MARV day-4 p.i. study and the MARV day-5 p.i. study. n = 2 for NP-718m–LNP and NP-718m plus NP-143m–LNP treatment groups for the RAVV day-3 p.i. study, and n = 4 treated animals for the RAVV day-6 p.i. study. n = 1 untreated control for each study.
EST and most lethal outbreak in history represents a substantial advance in countermeasure development, particularly when coupled with the complete protection seen when treatment is administered late in the disease course. In addition, the ability of a single siRNA to protect against multiple sequence-divergent virus variants, as demonstrated here, represents a proof of principle of the utility of rational design in identifying siRNAs with broad-spectrum activity and a key milestone in progress toward RNAi therapeutics.

Methods
LNP encapsulation of siRNA. The design, chemical modification, and abrogated immunostimulation testing of NP-718m and NP143m siRNA have been previously described (8). NP-718m or NP143m siRNA (8) (synthesized by Integrated DNA Technologies) was encapsulated in LNP by the process of spontaneous vesicle formation as previously reported (30). The resulting LNPs were dialyzed against PBS and filter sterilized through a 0.2-μm filter before use. Particle sizes were highly consistent, ranging from 78 to 79 nm for all studies, with low polydispersity values (0.04–0.09). High encapsulation efficiencies (97%–98%) were obtained for material prepared for each study.

Figure 5. MARV- and RAVV-infected rhesus macaques experienced hematological changes after infection. Changes were observed independent of whether the animals were treated with NP-718m–LNP. (A and B) Thrombocytopenia after MARV and RAVV infection, respectively. (C and D) Lymphopenia and (E and F) granulocytosis. n = 4 treated animals and n = 1 untreated control each for the MARV day-4 p.i. study and the MARV day-5 p.i. study. n = 2 for NP-718m–LNP and NP-718m plus NP-143m–LNP treatment groups for the RAVV day-3 p.i. study, and n = 4 treated animals for the RAVV day-6 p.i. study. n = 1 untreated control for each study.

for NP-718m was not found to differ between the clinical and rodent-adapted MARV and RAVV virus used to infect NHPs and guinea pigs, respectively, the different survival outcomes with treatment in these 2 animal models may reflect differences in disease course (27), such as the more profound weight loss observed in guinea pigs during peak viremia or subtle changes potentially attributable to the genetic changes observed in the guinea pig-adapted virus strains. These observations further emphasize the need to evaluate medical countermeasures in NHP models that recapitulate multiple aspects of human filovirus HF.

In this study, siRNA encapsulated in a LNP was administered through daily i.v. injection, a route of administration that may present challenges in resource-scarce settings such as Central Africa. The development of alternate routes such as intradermal (28) or subcutaneous (29) routes for the delivery of siRNA may enable simpler and easier methods of administration for antifiloviral siRNA therapeutics in the future.

Together, the results presented here strongly support the further development of NP-718m–LNP as a broad-spectrum therapeutic treatment for MARV and RAVV infection in humans. Its success against the strain of MARV responsible for the largest and most lethal outbreak in history represents a substantial advance in countermeasure development, particularly when coupled with the complete protection seen when treatment is administered late in the disease course. In addition, the ability of a single siRNA to protect against multiple sequence-divergent virus variants, as demonstrated here, represents a proof of principle of the utility of rational design in identifying siRNAs with broad-spectrum activity and a key milestone in progress toward RNAi therapeutics.
Dual-luciferase reporter assay. siRNA activity against MARV and RAVV NP mRNAs was confirmed in vitro using a dual-luciferase reporter assay assessing reductions in the expression of MARV or RAVV NP transgenes cloned into the psiCHECK2 vector (Promega) in HepG2 cells (ATCC; HB-8065) grown in MEM-supplemented media (Gibco, Thermo Fisher Scientific). Gene synthesis and subcloning into psiCHECK2 plasmid and LNP (20–0.8 nM) were used to conduct four separate studies (4 treated animals and 1 control per study; control animals were untreated, as previous studies treating filovirus-infected NHPs with nonspecific siRNAs encapsulated in LNP showed no evidence of nonspecific protection or delay in death). Animals were randomized using Microsoft Excel into treatment or control groups. Ten animals were inoculated i.m. with a target dose of 1,000 PFU MARV-Angola H.sapiens-tc/ANG/2005/Angola-200501379 (Vero E6, passage 2 [ATCC; CRL-1586]) (the actual dose of MARV was determined to be 1,063 PFU for the 96-hour delay to treatment study and 1,138 PFU for the 120-hour delay to treatment study). In addition, 10 animals were inoculated i.m. with a target dose of 1,000 PFU RAVV H.sapiens-tc/KEN/1987/Kitum Cave-810040 (Vero E6, passage 3) (the actual dose of RAVV was determined to be 1,125 PFU for the 72-hour delay to treatment study and 1,163 PFU for the 120-hour delay to treatment study). In the first study, NP-718m–LNP (0.5 mg/kg total siRNA dose) was administered to 4 macaques by i.v. bolus infusion 96 hours after MARV infection, while 1 control animal received no treatment. All treated animals were given a total of 7 daily doses of NP-718m–LNP after MARV infection. In the second study using 5 macaques, NP-718m–LNP (0.5 mg/kg) was administered to 4 macaques by i.v. bolus infusion 120 hours after MARV infection, while the control animal received no treatment. The 4 animals received additional treatments of NP-718m–LNP on days 6, 7, 8, 9, 10, and 11 after MARV infection. In the third study involving 5 macaques, NP-718m–LNP (0.5 mg/kg) was administered to 2 macaques, and an equal mixture of NP-718m–LNP and NP-143m–LNP (0.5 mg/kg total dose) was administered to 2 animals by i.v. bolus infusion 72 hours after RAVV infection, while the control animal received no treatment. The 4 animals received additional treatments of NP-718m–LNP after RAVV infection. In the fourth study using 5 macaques, NP-718m–LNP (0.5 mg/kg) was administered to 4 macaques by i.v. bolus infusion 144 hours after RAVV infection, while the control animal was not treated. These animals received additional treatments of NP-718m–LNP on days 7, 8, 9, 10, 11, and 12 after RAVV challenge. All 20 animals underwent physical examination, and blood was collected at the time of infection and at various times after MARV or RAVV infection. In addition, all animals were monitored daily and scored for disease progression with an internal filovirus scoring protocol approved by the IACUC of the University of Texas Medical Branch. The scoring changes measured from baseline included posture and activity level, attitude and behavior, food and water intake, weight, respiration, and disease manifestations such as visible rash, hemorrhage, ecchymosis, or flushed skin. A score of 9 or higher indicated that an animal met the criteria for euthanasia. These studies were not conducted in a blinded fashion.

Detection of viremia. RNA was isolated from whole blood using the Viral RNA Mini Kit (QIAGEN), with 100 μl of blood added to 600 μl of buffer AVL. The primers and probe targeting the NP gene...
of MARV were used for quantitative real-time PCR (qRT-PCR). The probe used here was 6-carboxyfluorescein (6FAM)-5′5′CCCATA-AGGTCAACCTCTT-3′-6-carboxytetramethylrhodamine (TAMRA) (Life Technologies, Thermo Fisher Scientific) (8). MARV RNA was detected using the CFX96 Detection System (Bio-Rad Laboratories) and One-Step Probe qRT-PCR kits (QIAGEN) with the following cycle conditions: 50°C for 10 minutes, 95°C for 10 seconds, and 40 cycles of 95°C for 10 seconds and 59°C for 30 seconds. The Ct values representing MARV genomes were analyzed with CFX Manager Software, and data are shown as genome equivalents (GEq). To create the GEq standard, RNA from MARV stocks was extracted, and the number of MARV genomes was calculated using Avogadro’s number and the molecular weight of the MARV genome.

Virus titration was performed by plaque assay with Vero E6 cells (ATCC; CRL-586) from all serum samples as previously described (10, 13, 14, 31). Briefly, 10-fold serial dilutions of the samples were adsorbed to Vero E6 monolayers in duplicate wells (200 μl); the limit of detection was 15 PFU/ml.

**Hematology and serum biochemistry.** Total white blood cell counts, white blood cell differentials, red blood cell counts, platelet counts, 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, ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), glucose, cholesterol, total protein, total bilirubin (TBIL), 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 IHC.** Necropsy was performed on all subjects. Tissue samples from all major organs were collected for histopathological and immunohistochemical examination, immersion-fixed in 10% neutral-buffered formalin, and processed for histopathology as previously described (9). For IHC, anti-Marburgvirus genus immunoreactivity was detected using an anti-MARV VP40 protein rabbit primary antibody (Integrated BioTherapeutics; 0303-001) at a 1:4,000 dilution. In brief, tissue sections were processed for IHC using the Dako Autostainer. The secondary antibody used was biotinylated goat anti-rabbit IgG (VECTOR Laboratories) at 1:200, followed by Dako LSAB2 streptavidin-HRP. Slides were developed with Dako DAB chromogen and counterstained with hematoxylin. Nonimmune rabbit IgG was used as a negative control.

**Statistics.** Statistical analyses were conducted using GraphPad Prism 6.0 (GraphPad Software). A 1-tailed, unpaired Student’s t test was used to determine whether differences in the means were present. A Fisher’s exact test was used to compare differences in survival between the treated and control groups. A P value of less than 0.05 was considered significant.

**Study approval.** The animal studies were performed at the Galveston National Laboratory of the University of Texas Medical Branch at Galveston and approved by the IACUC of the University of Texas Medical Branch.

**Author contributions**

RUB and MR designed the siRNA, and RUB conducted the dual luciferase reporter studies. EPT, CEM, ACHL, IM and TWG conceived and designed the NHP studies. CEM, JBG, DJD, RWC, and TWG performed the NHP infection and treatment experiments and conducted clinical observations of the animals. JBG, KNA, and DJD performed the clinical pathology assays. JBG performed the MARV and RAVV infectivity assays. CEM and KNA performed the PCR assays. EPT, CEM, JBG, KNA, DJD, RWC, KAF, ASK, ACHL, and TWG analyzed the data. KAF performed histological and immunohistochemical analyses. EPT, CEM, ACHL, and TWG wrote the manuscript. All authors had access to all of the data and approved the final version of the manuscript.

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BSL-4 operations support of the Galveston National Laboratory. The 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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