Cell Reports

An mRNA Vaccine Protects Mice against Multiple Tick-Transmitted Flavivirus Infections

Graphical Abstract

Highlights

- A Powassan virus LNP-mRNA vaccine induces potently neutralizing antibodies in mice

- One dose of the mRNA vaccine protects against lethal Powassan virus challenge

- The antibody response to the vaccine neutralizes other tick-borne flaviviruses

- The vaccine cross-protects against disease following challenge with Langat virus
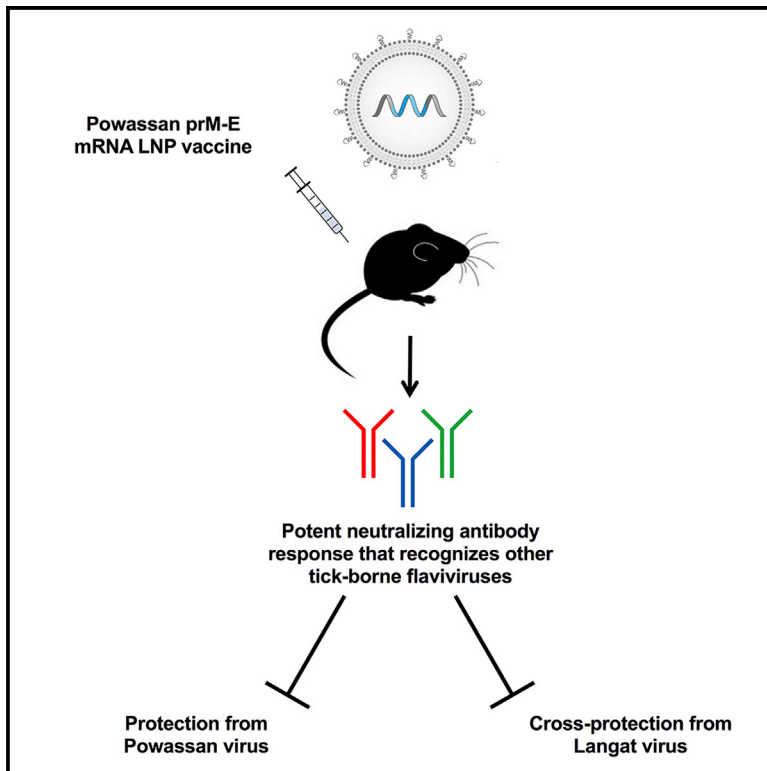
Authors

Laura A. VanBlargan, Sunny Himansu, Bryant M. Foreman, Gregory D. Ebel, Theodore C. Pierson, Michael S. Diamond

Correspondence

diamond@wusm.wustl.edu

In Brief

VanBlargan et al. demonstrate a lipid nanoparticle-encapsulated mRNA vaccine against Powassan virus, an emerging tick-borne flavivirus, is highly immunogenic in mice and protects against lethal Powassan virus infection. Furthermore, the vaccine induces a cross-reactive antibody response against other tick-borne flavivirus that is protective against disease caused by Langat virus infection in mice.

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An mRNA Vaccine Protects Mice against Multiple Tick-Transmitted Flavivirus Infections

Laura A. VanBlargan,[1] Sunny Himansu,[2] Bryant M. Foreman,[3] Gregory D. Ebel,[4] Theodore C. Pierson,[3] and Michael S. Diamond[1,5,6,7,8,*]

[1]Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA
[2]Moderna, Inc., 500 Technology Square, Cambridge, MA 02139, USA
[3]Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892, USA
[4]Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, USA
[5]Department of Pathology & Immunology, Washington University School of Medicine, St. Louis, MO 63110, USA
[6]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110, USA
[7]The Andrew M. and Jane M. Bursky Center for Human Immunology and Immunotherapy Programs, Washington University School of Medicine, St. Louis, MO 63110, USA
[8]Lead Contact
*Correspondence: diamond@wusm.wustl.edu
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SUMMARY

Powassan virus (POWV) is an emerging tick-transmitted flavivirus that circulates in North America and Russia. Up to 5% of deer ticks now test positive for POWV in certain regions of the northern United States. Although POWV infections cause life-threatening encephalitis, there is no vaccine or countermeasure available for prevention or treatment. Here, we developed a lipid nanoparticle (LNP)-encapsulated modified mRNA vaccine encoding the POWV prM and E genes and demonstrated its immunogenicity and efficacy in mice following immunization with one or two doses. The POWV mRNA vaccine induced high titers of neutralizing antibody and sterilizing immunity against lethal challenge with different POWV strains. The mRNA vaccine also induced cross-neutralizing antibodies against multiple other tick-borne flaviviruses and protected mice against the distantly related Langat virus. These data demonstrate the utility of the LNP-mRNA vaccine platform for the development of vaccines with protective activity against multiple flaviviruses.

INTRODUCTION

Powassan virus (POWV) is a tick-borne flavivirus (TBFV) that was first described following its isolation from the brain of a child who died of encephalitis in Powassan, Ontario, in 1958 (McLean and Donohue, 1959). Human cases of POWV have been reported in the United States, Canada, and Russia (reviewed in Ebel, 2010; Hermance and Thangamani, 2017). Though POWV infections are relatively rare, they can cause severe or even fatal neuroinvasive disease, including encephalitis, meningoencephalitis, and meningitis. Approximately 10% of neuroinvasive POWV cases are fatal, and 50% of survivors suffer long-term neurological sequelae (Ebel, 2010; Hermance

and Thangamani, 2017). Unfortunately, POWV is emerging; increasing numbers of cases have been diagnosed in the United States over the past decade (Hermance and Thangamani, 2017; Krow-Lucal et al., 2018), and up to 5% of Ixodes scapularis ticks isolated in parts of New York, Connecticut, and Wisconsin now test positive for POWV (Aliota et al., 2014; Anderson and Armstrong, 2012; Knox et al., 2017).

Two genetic lineages of POWV circulate in North America, lineage I and lineage II (also called deer-tick virus [DTV]), although they are serologically and clinically indistinguishable and share at least 96% amino acid identity in their envelope (E) proteins (Ebel et al., 2001). POWV lineage I strains are predominantly maintained in Ixodes cookei ticks and include isolates from New York and Canada, whereas lineage II strains are found in Ixodes scapularis deer ticks and include strains from regions infested by these ticks (Ebel et al., 2001). Because deer ticks are more aggressive at biting humans, lineage II viruses may have greater epidemic potential. Although POWV has been found predominantly in north-central and northeastern parts of the United States in Ixodes species ticks, POWV also has been isolated from Dermacentor andersoni ticks in Colorado (Thomas et al., 1960), indicating the vector and geographical range may be larger than previously estimated.

The TBFVs are divided into three groups: the mammalian group, the seabird group, and the Kadam virus group (Grard et al., 2007). The mammalian TBFV group includes POWV and several other human pathogens, including tick-borne encephalitis virus (TBEV), Omsk hemorrhagic fever virus (OHFV), Kyasanur forest disease virus (KFDV), and Alkhurma hemorrhagic fever virus (AHFV). Within their E proteins, the mammalian TBFV group shares ≥70% amino acid identity but only about 54%–60% identity with seabird TBFVs. One exception is Gadgets Gully virus (GGYV), which is more closely related to the mammalian TBFVs even though it causes infection of seabirds (Grard et al., 2007). Vaccines have been developed against several mammalian TBFVs, although only TBEV vaccines have proven efficacy (Ishikawa et al., 2014). TBEV vaccines induce antibodies capable of neutralizing closely related TBFVs, including OHFV, KFDV, and AHFV (McAuley et al., 2017). However, TBEV immune sera
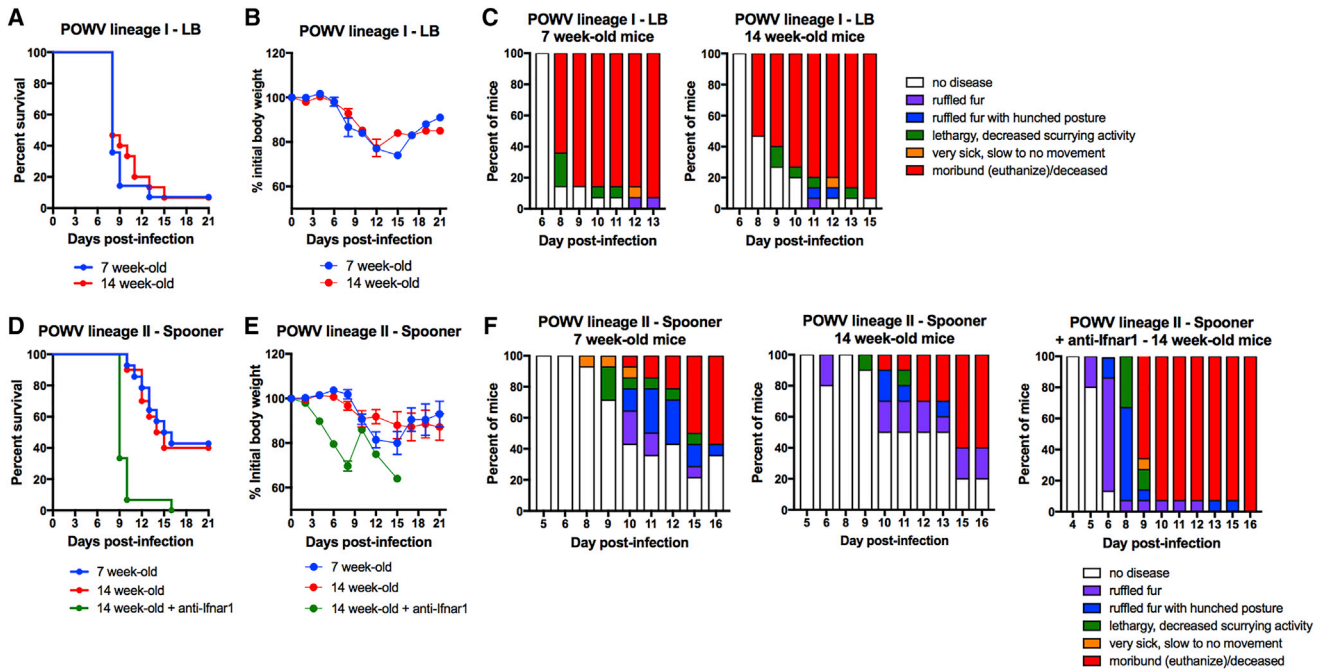
Figure 1. Lineage I and II POWV Strains Cause Lethal Disease in Adult Mice

(A–C) C57BL/6 mice were inoculated via subcutaneous inoculation in the footpad with 10^2 FFU of POWV lineage I LB at 7 weeks (n = 14) or 14 weeks (n = 15) of age. Mice were monitored for (A) mortality, (B) weight loss, and (C) clinical disease for 21 days.

(D–F) C57BL/6 mice were inoculated subcutaneously at 7 weeks (n = 14) or 14 weeks (n = 10) of age with 10^2 FFU of POWV Spooner or at 14 weeks of age after treatment with 0.5 mg of anti-Ifnar1 mAb administered via intraperitoneal one day before inoculation with POWV lineage II Spooner (n = 15). Mice were monitored for (D) mortality, (E) weight change, and (F) clinical disease for 21 days.

(B and E) Error bars represent SEM. Data were collected from two (Spooner infection of 7-week-old mice) or three (all other data) independent experiments.

had limited cross-neutralizing activity against the more distantly related POWV. No approved vaccines for POWV exist.

The lack of a POWV-specific vaccine, limited induction of cross-neutralizing antibodies by other TBFV vaccines, and epidemic potential for POWV prompted us to design a vaccine and test its immunogenicity and efficacy. We selected a vaccine platform that we developed for a distantly related flavivirus, Zika virus (ZIKV): a lipid nanoparticle (LNP)-encapsulated modified mRNA encoding the structural premembrane (prM) and E protein genes (Richner et al., 2017a). Co-expression of the flavivirus structural proteins prM and E results in the secretion of subviral particles (SVPs) that share many functional and antigenic features with infectious virions and elicit neutralizing antibodies. SVPs are heterogeneous in size and likely display E proteins in distinct chemical environments, with the potential to affect epitope display (Allison et al., 1995; Heinz et al., 1995; Kiermayr et al., 2009; Konishi and Fujii, 2002; Konishi et al., 1992). In addition to selecting the POWV prM-E structural gene components, we used an mRNA platform, because some modified mRNA have been reported to enhance follicular helper T cell and germinal center B cell responses that are essential for inducing memory B cells and durable levels of neutralizing antibody (Pardi et al., 2018).

Here, we describe an LNP-encapsulated modified mRNA vaccine encoding the prM-E of POWV that induces a potent neutralizing antibody response in mice and protects against lethal viral challenge by strains of both POWV lineages. Furthermore, the POWV mRNA vaccine induced cross-neutralization antibody titers against other TBFVs and conferred protection against disease following challenge of mice with the distantly related Langat virus (LGTV).

RESULTS

A Lethal Model of POWV Infection in Adult Mice

In planning for vaccine efficacy studies, we developed challenge models of POWV infection in adult mice. Although others have described murine models of POWV infection, these have been in juvenile mice (4–5 weeks old), which are not ideal for experiments requiring iterative immunization and resting periods (Hermance and Thangamani, 2015; Holbrook et al., 2005; Mlera et al., 2017; Santos et al., 2016; Wang et al., 2013). Accordingly, we tested the ability of two POWV strains, LB (a lineage I strain) and Spooner (a lineage II DTV strain) to cause morbidity and mortality in wild-type (WT) C57BL/6 mice at 7 or 14 weeks of age. Following subcutaneous inoculation of 10^2 focus-forming units (FFU) of POWV LB, lethal infection ensued in 93% of 7- and 14-week-old mice, with a mean time to death of 8.6 and 9.5 days post-infection (dpi), respectively (Figure 1A). All mice inoculated with POWV LB lost approximately 15%–25% of their body weight (Figure 1B). POWV LB-infected mice exhibited ruffled fur, hunched posture, lethargy, and partial paralysis,
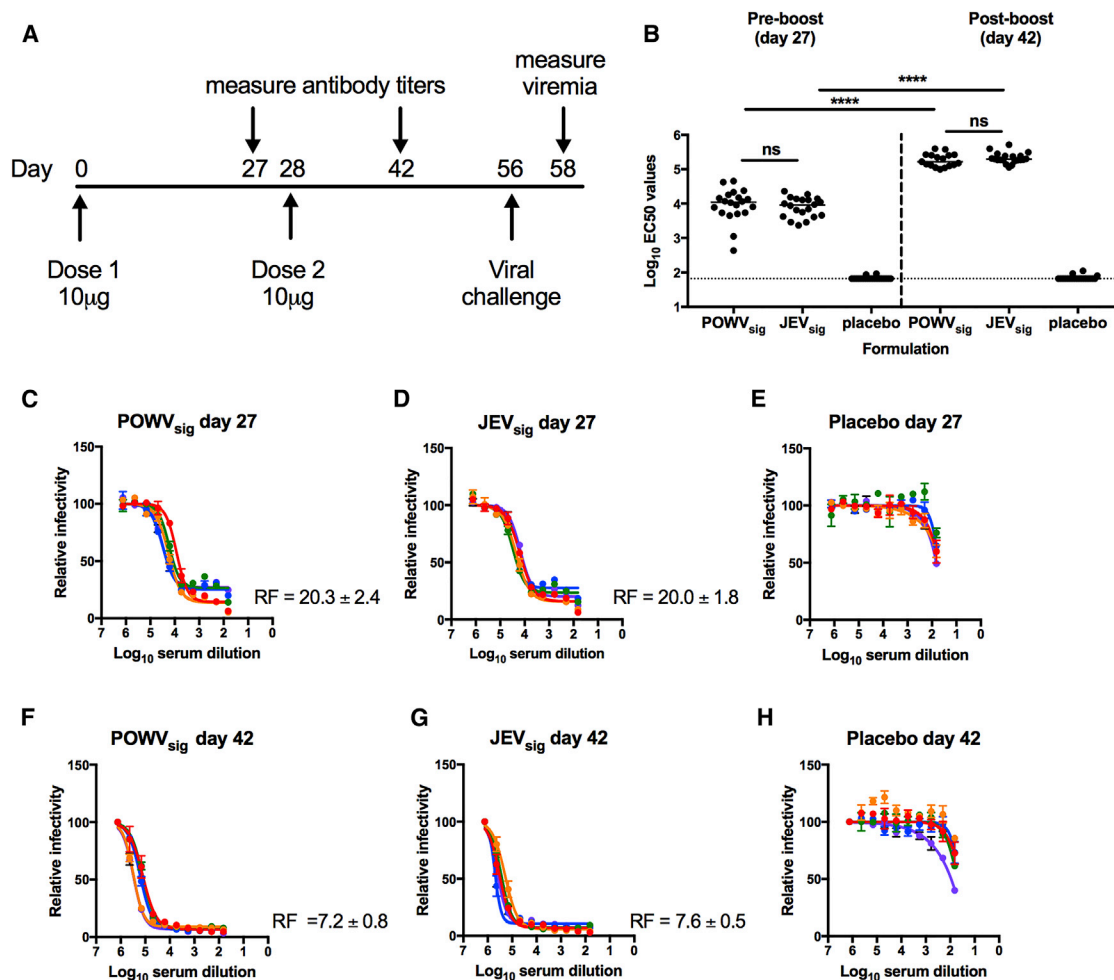
Figure 2. POWV mRNA LNP Vaccine Induces High Levels of Neutralizing Antibody in Mice

(A) Scheme of the two-dose POWV Spooner mRNA LNP vaccination schedule.

(B–H) Serum samples collected pre- and post-boost were assayed for neutralization potency by an RVP-based assay using a lineage II POWV strain (P0375) and Raji-SIGNR cells (n = 20 for JEV$_{sig}$ and placebo; n = 19 for POWV$_{sig}$).

(B) The reciprocal neutralization titers (EC$_{50}$ values) are shown. Bars represent median values. Statistical significance was determined using an ANOVA followed by Tukey's multiple comparisons test (ns, not significant; *p < 0.05; ****p < 0.0001).

(C–H) Representative dose response curves are shown for each vaccine and time point. The mean resistant fraction (RF) ± SEM is indicated on each panel. Error bars represent ± SEM from two technical replicates. Serum samples were collected over four independent experiments and assayed once each for neutralization potency. See also Figure S1.

although ~50% of mice did not exhibit these signs before death (Figure 1C). In comparison, POWV Spooner resulted in lower mortality rates; 57% and 60% of 7- and 14-week-old mice succumbed to infection with mean times to death of 13.0 and 12.7 days, respectively (Figure 1D). Because of the partial lethality caused by POWV Spooner in WT mice, we created a more susceptible host by transiently blocking type I interferon (IFN) signaling with an anti-IFN receptor (Ifnar1) monoclonal antibody (mAb), MAR1-5A3 (Sheehan et al., 2006). When 0.5 mg of anti-Ifnar1 mAb was administered one day before infection, POWV Spooner resulted in 100% lethality, with a mean time to death of 9.7 days (Figure 1D). Whereas POWV Spooner-infected WT mice typically displayed ~15%–20% body weight loss, infected mice treated with anti-Ifnar1 mAb sustained greater

weight loss of ~30% (Figure 1E). Most POWV Spooner-infected WT mice (70%–85%) displayed ruffled fur, hunched posture, and lethargy, with a minority developing limb paralysis (Figure 1F). In comparison, anti-Ifnar1 mAb-treated, POWV Spooner-infected mice uniformly displayed signs of morbidity before death.

An mRNA Vaccine against POWV Induces High Levels of Neutralizing Antibody in Mice

Having developed lethal challenge models in older mice, we engineered a LNP-encapsulated modified mRNA vaccine against POWV that was based on a prM-E construct developed for ZIKV (Richner et al., 2017a, 2017b). We designed a base-modified mRNA encoding the prM and E genes of POWV Spooner that was preceded by the prM signal sequence of POWV (POWV$_{sig}$) or
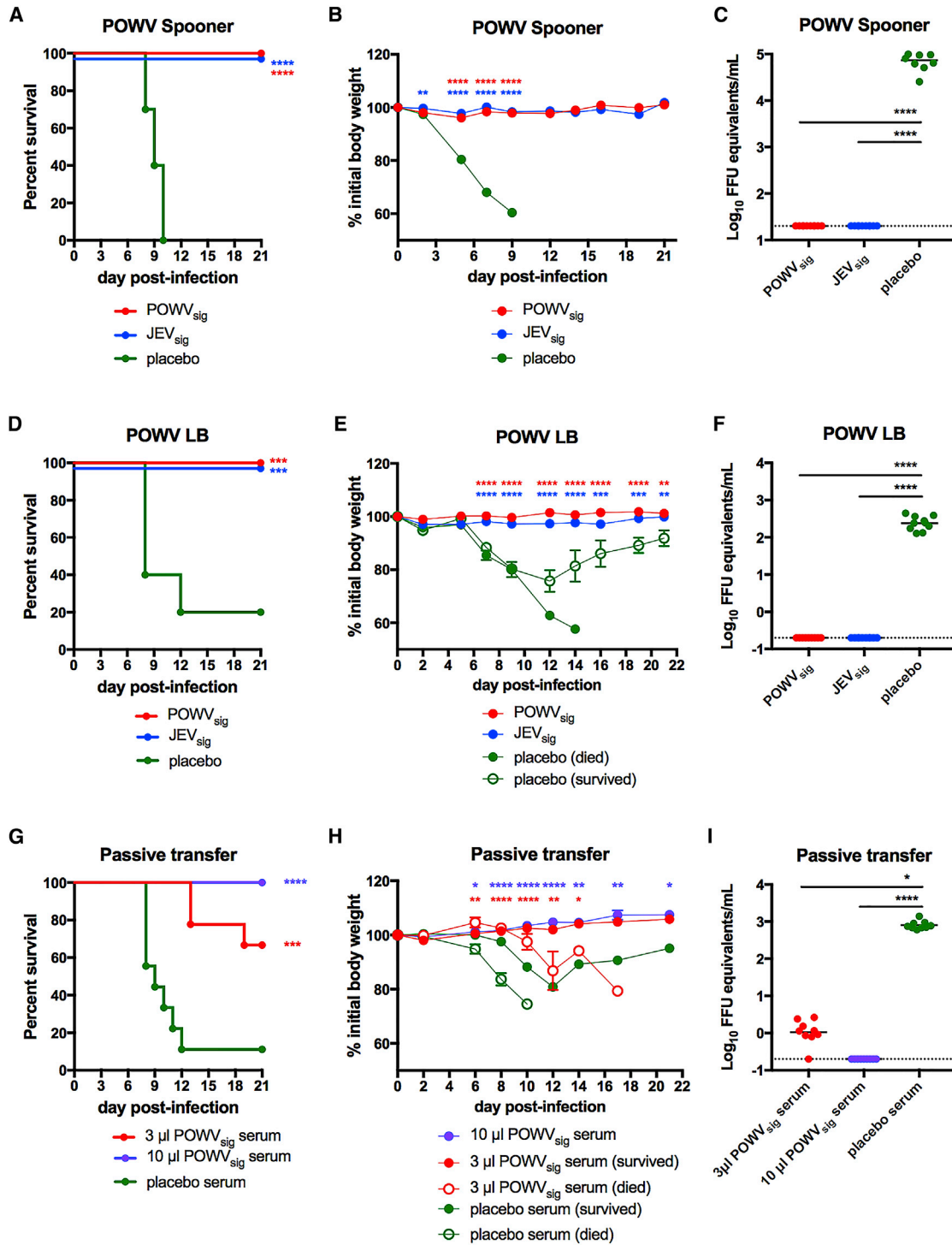
Figure 3. POWV mRNA LNP Vaccines Induce Protective Immunity against Lineage I and II POWV Strains

(A–F) C57BL/6 mice receiving two doses of POWV$_{sig}$ or JEV$_{sig}$ vaccines or a placebo were challenged 4 weeks after the second dose with 10^2 FFU of (A–C) POWV lineage II strain Spooner (following anti-Ifnar1 mAb treatment) (n = 10 per group) or (D–F) POWV lineage I strain LB (n = 10 for JEV$_{sig}$ and placebo; n = 9 for POWV$_{sig}$).

(G–I) Serum samples were collected from mice that received two doses of POWV$_{sig}$ or a placebo and passively transferred to naive C57BL/6 mice one day before challenge with 10^2 FFU POWV LB (n = 9 per group). Mice received a dose of 3 or 10 μL of vaccine immune serum or 10 μL of placebo serum.

Mice were monitored for mortality (A, D, and G) for 21 days following viral challenge. Statistical significance was determined by the log rank test with a Bonferroni correction (***p < 0.001; ****p < 0.0001). Mean weight change (B, E, and H) post-viral challenge is shown. Error bars represent SEM. Statistical significance was
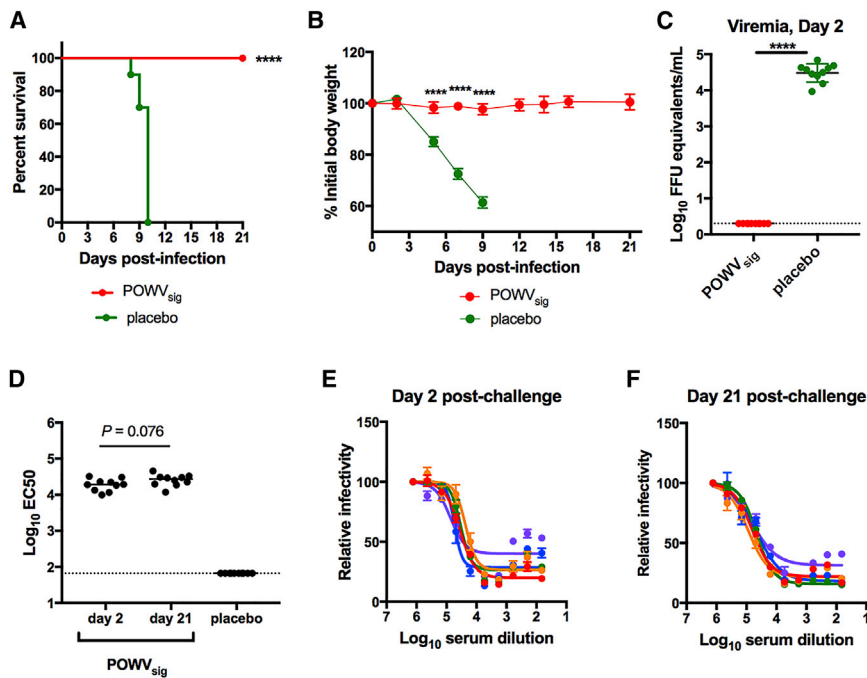
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Figure 4. Single Dose of an mRNA LNP Vaccine Induces Protective Immunity against POWV Challenge

C57BL/6 mice receiving one dose of the POWV$_{sig}$ mRNA vaccine or a placebo vaccine were challenged 29 days later with 10^2 FFU of POWV Spooner (following anti-Ifnar1 mAb treatment) (n = 10 per group).

(A) Mice were monitored for mortality for 21 days after viral challenge. Statistical significance was determined by log rank test (****p < 0.0001).

(B) Mean weight change after virus challenge is shown. Error bars represent SEM. Statistical significance was determined using an ANOVA with Sidak's multiple comparisons test (****p < 0.0001).

(C) Serum was collected at 2 dpi and measured for viremia by qRT-PCR. Median viral titers are shown. Statistical significance was determined by Mann-Whitney test (****p < 0.0001).

(D–F) Serum was collected at 2 and 21 days post-challenge and assayed for neutralization activity by RVP assay.

(D) Neutralization titers are shown. Bars indicate median values. Statistical significance was determined by paired t test.

(E and F) Representative dose response curves are shown of sera collective at day 2 (E) and day 21 (F) post-challenge.

Error bars represent ± SEM from two technical replicates. Serum samples were collected over two independent experiments and assayed once each for neutralization potency.

signal sequence of JEV (JEV$_{sig}$); the latter heterologous JEV signal sequence was tested because for some flaviviruses, it results in enhanced secretion of SVPs (Davis et al., 2001; Dowd et al., 2016). Six-week-old WT C57BL/6 mice were immunized with 10 μg of POWV$_{sig}$, JEV$_{sig}$, or placebo LNPs by intramuscular inoculation, followed by a second 10 μg booster dose 28 days later (Figure 2A). Serum was collected preboost on day 27 and postboost on day 42, and neutralizing activity was assessed by reporter virus particle (RVP) assay using the C-prM-E proteins of the POWV lineage II strain P0375 and Raji-SIGNR cells (Mukherjee et al., 2014). RVP technology has been used extensively in the preclinical and clinical evaluation of flavivirus vaccine candidates (Dowd et al., 2016; Emanuel et al., 2018; Gaudinski et al., 2018; Pardi et al., 2017; Richner et al., 2017a). Following the first dose, the reciprocal mean titers that reduced RVP infection by 50% (50% maximal effective inhibitory concentration [EC$_{50}$]) values were 13,683 ± 2,671 (n = 20) and 9,476 ± 1,271 (n = 20) for POWV$_{sig}$ and JEV$_{sig}$, respectively, and were not statistically different between the two formulations (p > 0.9). Neutralization titers were boosted approximately 15-fold following the second dose for both formulations, with EC$_{50}$ values of 200,402 ± 20,414 (n = 20) for POWV$_{sig}$ and 225,380 ± 21,112 (n = 20) for JEV$_{sig}$. Despite the high EC$_{50}$ values after the first dose, a neutralization-resistant fraction was observed for both formulations.

However, this fraction was reduced following the booster dose from 20% to 7% (p < 0.0001 for both vaccines) (Figures 2C–2H).

A subset of post-boost sera (day 42) was tested for neutralization potency by a second assay, a focus-reduction neutralization test (FRNT) in Vero cells. Generally, lower EC$_{50}$ values were observed by FRNT than by RVP neutralization assays, as was reported with ZIKV (Dowd et al., 2016). Nonetheless, both POWV$_{sig}$ and JEV$_{sig}$ induced robust FRNT titers of 3,373 ± 629 (n = 17) and 3,738 ± 635 (n = 18), respectively, against POWV Spooner (Figures S1A–S1C). Furthermore, virtually no resistant fraction was observed by FRNT, with EC90 values of 544 ± 204 (n = 17) and 392 ± 92 (n = 18) for POWV$_{sig}$ and JEV$_{sig}$, respectively (Figures S1A and S1C). Two other contemporary lineage II virus strains (POWV MA5/12-#40 and FA5/12-#40), isolated from ticks collected in Wisconsin and passaged once on BHK-21 cells, as well as the lineage I POWV LB strain, also were neutralized efficiently by serum from both POWV$_{sig}$- and JEV$_{sig}$-vaccinated mice, as measured by FRNT (Figures S1D–S1I).

mRNA Vaccines Protect against Lethal Challenge with Lineage I and II POWV Strains

Following the two-dose vaccination schedule, mice were challenged via subcutaneous inoculation with 10^2 FFU of POWV Spooner or POWV LB at 28 days post-boost (Figure 2A).

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determined using an ANOVA with Dunnett's multiple comparisons test to compare vaccine groups to the placebo group (ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). Serum was collected at 2 dpi and measured for viremia (C, F, and I) by qRT-PCR. Bars indicate median values. Statistical significance was determined using the Kruskal-Wallis test with Dunn's multiple comparisons test to compare vaccine groups to the placebo group (*p < 0.05; ****p < 0.0001). Data in each panel were collected over two independent experiments; n = 9–10 mice per group.

Table 1. Comparison of Neutralization Titers of Serum Collected on Day 2 or Day 21 Post-challenge

EC_{50} Day 2	EC_{50} Day 21	Day 21/Day 2 Fold Change
22213	32989	1.5
13443	45702	3.4
19565	30234	1.5
32503	25243	0.8
22913	19910	0.9
11619	11871	1.0
9921	22770	2.3
30521	29327	1.0
19043	30857	1.6
18334	18706	1.0
20008[a]	26761[a]	1.5[a]

Shown are the EC_{50} values of serum collected at day 2 or day 21 post-POWV challenge from mice receiving a single dose of $POWV_{sig}$ mRNA vaccine. The fold change of day 21 EC_{50}/day 2 EC_{50} is shown for each sample. Serum samples were collected from two independent experiments.
[a]Mean

POWV Spooner-challenged mice were treated one day before infection with 0.5 mg anti-Ifnar1 mAb to achieve high mortality rates in placebo-treated mice. Challenged mice were monitored for survival and weight loss for 21 days, and serum samples were collected two days post-challenge and assayed for viremia. Following POWV Spooner challenge in anti-Ifnar1 mAb-treated mice, and as expected, 100% of placebo-vaccinated mice succumbed to infection. In comparison, all $POWV_{sig}$- and JEV_{sig}-vaccinated mice survived the challenge (Figure 3A). Furthermore, both $POWV_{sig}$- and JEV_{sig}-vaccinated mice maintained body weight, whereas placebo-vaccinated mice lost 30%–40% of their weight (Figure 3B). Consistent with these data, high levels of viremia were measured in placebo-vaccinated mice, but not in $POWV_{sig}$- and JEV_{sig}-vaccinated mice (Figure 3C). Similar results were observed following POWV LB challenge in the absence of anti-Ifnar1 mAb treatment. Whereas 80% of placebo-vaccinated mice succumbed following LB infection, 100% of $POWV_{sig}$- and JEV_{sig}-vaccinated mice survived infection (Figure 3D). Placebo-vaccinated mice lost between 20% and 40% of body weight, whereas $POWV_{sig}$- and JEV_{sig}-vaccinated mice maintained weight following virus challenge (Figure 3E). In addition, viremia was undetectable in $POWV_{sig}$- or JEV_{sig}-vaccinated mice but present in placebo-vaccinated mice (Figure 3F). Thus, both mRNA vaccine formulations protect against lethal POWV challenge with strains from both lineages.

Passive Transfer of Serum from POWV mRNA-Vaccinated Animals Protects against Lethal POWV Challenge

To assess the role of the humoral immune response in mediating protection from POWV challenge, we passively transferred serum from $POWV_{sig}$-vaccinated (post-boost, day 42) or placebo-treated mice to naive C57BL/6 mice one day before inoculation with 10^2 FFU of POWV LB. Whereas 8 of 9 (89%)

mice that received 10 μL of serum from placebo-treated mice succumbed to POWV LB challenge, 3 of 9 (33%) and 0 of 9 (0%) mice that received 3 and 10 μL of serum, respectively, from $POWV_{sig}$-vaccinated mice succumbed to viral challenge (Figure 3G). Furthermore, the 67% and 100% of mice that survived viral challenge following passive transfer of 3 and 10 μL of $POWV_{sig}$ serum, respectively, exhibited no weight loss following viral challenge, whereas all mice receiving serum from placebo-treated mice lost between 15% and 30% of their body weight. Passive transfer of serum from vaccinated mice resulted in significant reductions in viremia at 2 dpi: mice receiving 3 μL of $POWV_{sig}$ serum had more than a 500-fold reduction (p < 0.05) in viremia following challenge compared to mice receiving serum from placebo-treated mice, and viremia was undetectable in mice that received 10 μL of $POWV_{sig}$ serum. These data indicate that the humoral immune response to $POWV_{sig}$ vaccination is sufficient to protect mice from lethal POWV challenge.

A Single Dose of POWV mRNA Vaccine Induces Protection

We next tested whether a single dose of the POWV mRNA vaccine was sufficient for protection. For this study, we immunized 10-week-old WT C57BL/6 mice with 10 μg of $POWV_{sig}$ or placebo vaccines. Twenty-eight days later, mice were treated with anti-Ifnar1 mAb and then challenged via subcutaneous inoculation with 10^2 FFU of POWV Spooner. As observed previously, 100% of placebo-immunized mice succumbed to infection and lost body weight (Figures 4A and 4B). In contrast, $POWV_{sig}$-immunized mice uniformly survived challenge and maintained body weight. Furthermore, no viremia was detected in $POWV_{sig}$-immunized mice at 2 dpi, compared to high levels in placebo-immunized mice (Figure 4C). Serum samples were collected on days 2 and 21 post-challenge for evaluation of neutralization titers. EC_{50} values were not significantly different between the two time points (p > 0.07) (Figure 4D), with most mice having less than a two-fold change in titer (Table 1), indicating that little or no boost in antibody response occurred following viral challenge in $POWV_{sig}$-immunized mice. These data suggest that one dose of mRNA vaccine was sufficient to induce protective, if not sterilizing, immunity against POWV challenge.

Cross-Neutralizing Activity of Serum from POWV mRNA-Vaccinated Mice against Other TBFVs

POWV shares 70%–78% amino acid identity of its E protein with other mammalian TBFVs and 55%–60% identity with the seabird TBFV clade (Figure 5A). To determine the ability of serum from POWV-vaccinated mice to inhibit other TBFVs, we tested sera from $POWV_{sig}$- or JEV_{sig}-vaccinated (two-dose) mice for their capacity to inhibit TBEV, LGTV, and GGYV RVPs (Figures 5B–5D; Figure S2). TBEV was chosen because it overlaps geographically with POWV in parts of far eastern Russia (Leonova et al., 2009, 2017) and has a large public health impact, infecting more than 10,000 people annually in Europe and Asia (Gritsun et al., 2003). LGTV was selected due to its relatedness to TBEV and prior use as a TBEV vaccine (Mandl et al., 1991). GGYV is one of the most divergent viruses of the mammalian
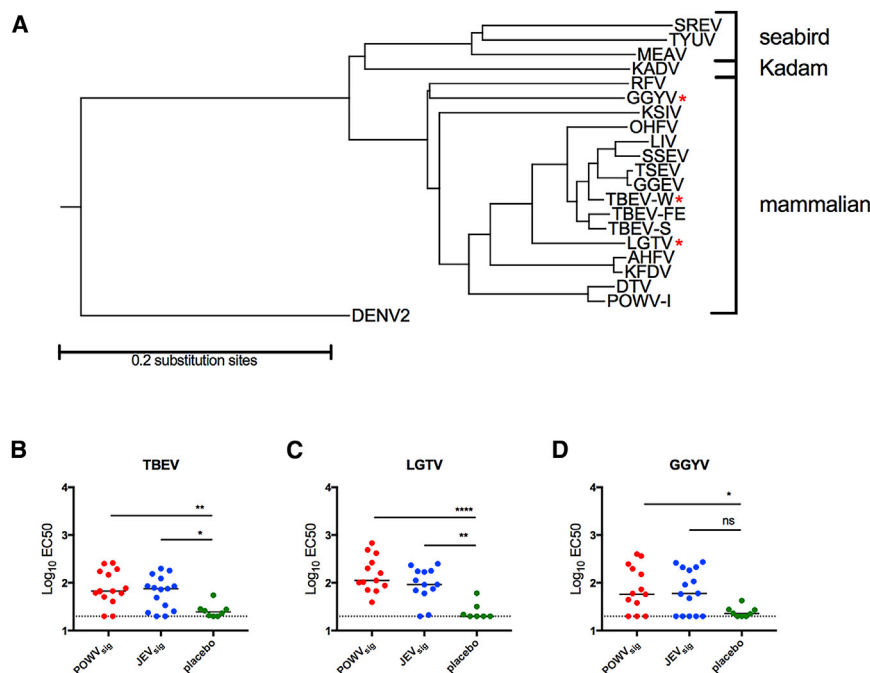
Figure 5. POWV mRNA Vaccine Induces Cross-Neutralizing Antibodies against Other Tick-Borne Flaviviruses

(A) Dendrogram depicting the relatedness of tick-borne flavivirus E proteins (scale bar represents 0.2 amino acid substitutions per site).

(B–D) Serum samples collected post-boost in Figure 2 were evaluated by RVP assay for neutralizing activity against (B) TBEV (POWV$_{sig}$ and JEV$_{sig}$, n = 15; placebo, n = 8), (C) LGTV (POWV$_{sig}$ and JEV$_{sig}$, n = 13; placebo, n = 7), and (D) GGYV (POWV$_{sig}$ and JEV$_{sig}$, n = 15; placebo, n = 8).

Median EC$_{50}$ values are shown. Statistical significance was determined using an ANOVA with Dunnett's multiple comparisons test to compare vaccine and placebo group (ns, not significant; *p < 0.05; **p < 0.01; ****p < 0.0001). Serum samples were collected over four independent experiments and assayed once for neutralization potency. See also Figures S1 and S2.

group of TBFVs (Grard et al., 2007) and thus can demonstrate the breadth of cross-reactive response of the POWV vaccine sera. POWV shares about 77% amino acid identity in E protein sequence with LGTV and TBEV and 72% with GGYV. Most vaccine sera neutralized infection by RVPs bearing the E proteins of the other TBFVs, although some samples neutralized GGYV, whereas others did not (Figures 5B–5D). The variable neutralization of GGYV by POWV vaccine sera warrants further study but may be due to the greater sequence dissimilarity between the viruses (Figure 5A) and the resultant differential display of neutralizing epitopes. Sera from POWV$_{sig}$-vaccinated mice exhibited cross-neutralizing activity with mean EC$_{50}$ values against TBEV, LGTV, and GGYV of 104 ± 21 (n = 15), 215 ± 112 (n = 13), and 113 ± 57 (n = 15), respectively. JEV$_{sig}$ induced similar cross-neutralizing titers against TBEV (82 ± 75, n = 15), LGTV (119 ± 92, n = 23), and GGYV (107 ± 60, n = 15).

A POWV mRNA Vaccine Protects against LGTV Infection *In Vivo*

Because both POWV mRNA vaccines showed a capacity for *in vitro* neutralization of other TBFVs, we tested the ability of the POWV$_{sig}$ vaccine to protect against LGTV challenge *in vivo*. We tested the efficacy of the POWV$_{sig}$ vaccine against LGTV, but not TBEV or GGYV due to the requirement of an A-BSL4 facility or lack of an existing challenge model, respectively. Following the two-dose vaccination schedule (Figure 2A), mice were challenged via subcutaneous inoculation with 10^2 FFU of LGTV at 28 days post-boost. Because LGTV is pathogenic in *Ifnar1$^{-/-}$* mice, but not WT mice (Weber et al., 2014), we treated animals with 0.5 mg of anti-Ifnar1 mAb one day before virus challenge. Following LGTV challenge, 60% of placebo-treated mice lost 15% to 35% body weight, whereas only 7% of POWV$_{sig}$-vacci-

nated mice lost more than 10% body weight and 87% of vaccinated mice maintained their weight (Figure 6A). POWV$_{sig}$-vaccinated mice were fully protected from clinical disease, whereas ~50% of placebo-treated mice exhibited signs of paralysis, with one mouse succumbing to LGTV challenge (Figure 6B). POWV$_{sig}$-vaccinated mice had significantly less viremia than placebo-treated mice at 2 dpi, with ~6,000-fold reduction in viral titer (p < 0.0001) (Figure 6C). POWV$_{sig}$-vaccinated mice also had substantially less viral RNA in the spleen, brain, and spinal cord at 15 days post-LGTV challenge (p < 0.0001 for all comparisons) (Figure 5D). Thus, the POWV$_{sig}$ vaccine induces an immune response that can protect against heterologous challenge with LGTV.

DISCUSSION

The frequency of epidemics caused by emerging and reemerging viruses, such as ZIKV, chikungunya virus, Ebola virus, and Middle East respiratory syndrome coronavirus and the lack of preparedness for these public health crises highlight the need for the development of versatile vaccine platforms that can be rapidly manufactured and deployed against various viral threats. An advantage of the LNP-encapsulated modified mRNA platform is that it allows rapid development of vaccine candidates by insertion of the desired viral mRNA sequence into the coding region. In this study, we used the mRNA vaccine platform previously shown to be effective in mice against ZIKV (Pardi et al., 2018; Richner et al., 2017a) to develop a countermeasure against POWV, another emerging flavivirus (Hermance and Thangamani, 2017). Vaccination of mice with LNP-encapsulated mRNA-encoding POWV structural genes induced potently neutralizing antibody responses and protection against challenge by POWV strains from both lineages. Furthermore, one dose of the POWV mRNA vaccine was sufficient to induce robust immunity, because no viremia or significant anamnestic antibody response was observed following POWV challenge.
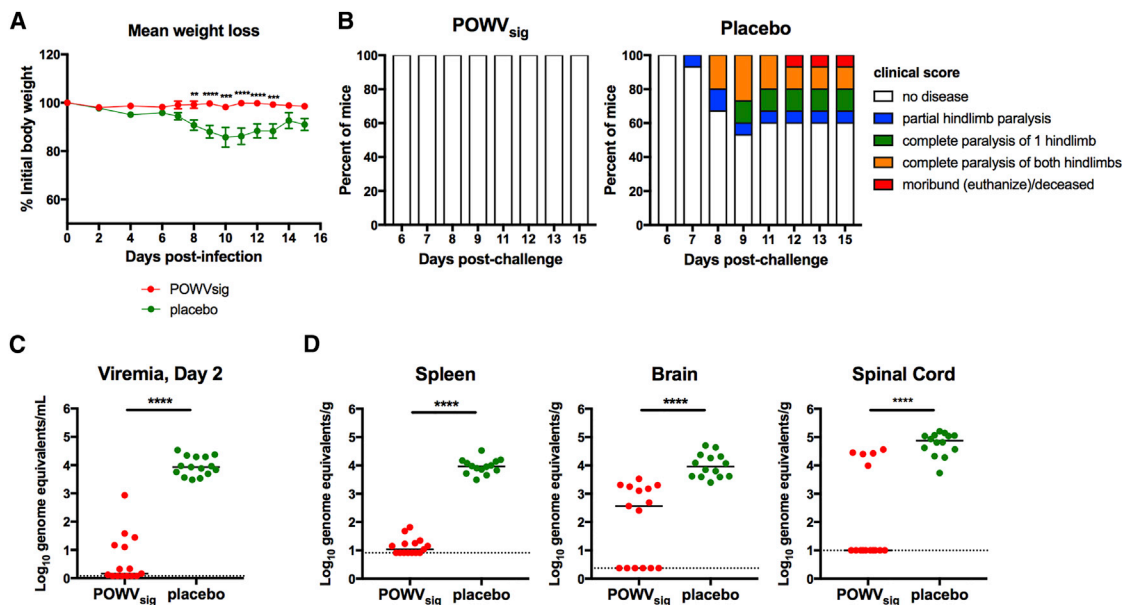
Figure 6. POWV mRNA Vaccine Cross-Protects against LGTV Challenge

C57BL/6 mice receiving two doses of the POWV$_{sig}$ (n = 15) or placebo (n = 15) vaccines were challenged 4 weeks after boosting with 10^2 FFU of LGTV (one day post-anti-Ifnar1 mAb treatment, 0.5 mg).

(A) Mice were monitored for weight change for 15 days following viral challenge. Mean weight change is shown. Error bars represent SEM. Statistical significance was determined using an ANOVA with Sidak's multiple comparisons test (**p < 0.01; ***p < 0.001; ****p < 0.0001).

(B) Mice were scored for signs of paralysis following viral challenge.

(C) Serum was collected at 2 dpi and measured for viremia by qRT-PCR.

(D) At day 15 post-viral challenge, the indicated tissues were harvested, and viral load was determined by qRT-PCR.

(C and D) Median viral titers are shown. Statistical significance was determined by Mann-Whitney test (****p < 0.0001). Data are from three independent experiments.

The antibody response induced by the POWV mRNA vaccines broadly neutralized infection of a panel of TBFVs that included LGTV, TBEV, and GGYV. These TBFVs differ from POWV E protein sequence by approximately 20% to 30%. The neutralization titers were lowest against GGYV, which is the more distantly related TBFV of those tested. Furthermore, the POWV mRNA vaccine protected mice from disease following LGTV challenge, indicating that this vaccine has inhibitory activity against multiple TBFV, although the durability of the cross-protective response warrants further testing.

Our results contrast with a prior study that observed little to no cross-reactivity of sera from TBEV-infected or TBEV-vaccinated humans against POWV (McAuley et al., 2017). This apparent disparity in results may have several reasons. The antibody repertoire produced in mice versus humans in response to TBFVs may be more cross-reactive, because mouse and human antibody specificities to TBEV immunization can be different (Jarmer et al., 2014). Notwithstanding these data, mouse immune ascites fluid raised against some TBFVs (TBEV, OHFV, and KFDV) was not neutralizing against POWV (McAuley et al., 2017). Furthermore, a separate study evaluating a TBEV vaccine in mice observed little cross-neutralization of POWV (a 1:8 antibody titer) and no cross-protection against POWV challenge *in vivo* (Chernokhaeva et al., 2016). It remains possible that there is a directionality to the cross-reactive response, such that POWV induces more

cross-reactive antibodies than TBEV or other TBFVs due to differential display of conserved epitopes. Alternatively, the SVPs induced by the mRNA vaccine may display more cross-reactive epitopes than inactivated or fully infectious TBEV virions due to differences in the arrangement of E proteins on SVPs compared to virions (Kiermayr et al., 2009; Kuhn et al., 2015). Studies comparing the antibody repertoires induced by LNP-mRNA vaccines to those generated after live virus infections or inactivated virus vaccination could address these questions. In addition, the RVP-based assay we used to assess neutralization titers against TBEV may be more sensitive than the plaque-reduction neutralization test (PRNT) used by others. We observed increased sensitivity of the RVP-based neutralization assay compared to FRNT for POWV; however, the reciprocal result was true for LGTV in our study.

Another explanation for the difference in cross-neutralizing responses could be the timing of serum sampling. We evaluated the neutralization potency of sera collected 28 days post-vaccination, whereas McAuley and colleagues tested sera collected 3 to 204 months post-vaccination or 1.5 to 120 months post-infection (McAuley et al., 2017). Although a previous study showed the anti-ZIKV antibody responses to an LNP-encapsulated mRNA vaccine endure for at least five months post-vaccination (Pardi et al., 2018), the durability and breadth of the POWV LNP-mRNA antibody response

warrants further study. This may apply particularly to the cross-reactive antibody response, because anti-flavivirus antibody responses generated by long-lived plasma cells in the memory B cell compartment in the context of West Nile virus (WNV) infection had a more type-specific repertoire (Purtha et al., 2011). Moreover, previous studies with ZIKV and dengue virus (DENV) found the cross-neutralizing antibody response to flavivirus infections is greatest during the early-convalescent stage (<6 months) and then wanes over time (Collins et al., 2017; Montoya et al., 2018). Chernokhaeva and colleagues challenged mice with POWV four weeks following immunization with TBEV and observed no cross-protection (Chernokhaeva et al., 2016).

A neutralizing antibody response is an established correlate of protection following vaccination against several flaviviruses, including yellow fever virus, Japanese encephalitis virus, and TBEV (Belmusto-Worn et al., 2005; Heinz et al., 2007; Mason et al., 1973; Monath et al., 2002), and likely contributes to protection following immunization with the POWV LNP-mRNA vaccine. Passive transfer of serum from POWV LNP-mRNA-vaccinated mice was sufficient for protection against lethal POWV challenge. However, an LNP-mRNA ZIKV vaccine described by Pardi and colleagues induced strong, antigen-specific T cell responses, in addition to neutralizing antibodies (Pardi et al., 2018). Although we did not characterize the T cell response to the POWV mRNA-LNP vaccine, because insight into the immunodominance hierarchy and tetramer-specific reagents are lacking, if a similar such T cell response is generated, it could contribute to protection against POWV or LGTV challenge.

In summary, we show that the LNP-mRNA vaccine platform is readily adaptable for development of a vaccine against another flavivirus, POWV, with epidemic potential. This highlights the utility of this platform for development of vaccines against various flaviviruses, from both the mosquito-borne and the tick-borne groups, and points to the potential for optimizing adaptive immune responses so that they are broadly protective against multiple viruses.

STAR★METHODS

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AUTHOR CONTRIBUTIONS

Conceptualization, L.A.V., M.S.D., and S.H.; Methodology, L.A.V. and M.S.D.; Investigation, L.A.V. and B.M.F.; Resources, S.H., G.D.E., T.C.P., and B.M.F.; Supervision, M.S.D.; Writing – Original Draft, L.A.V. and M.S.D.; Writing – Review & Editing, all authors.

DECLARATION OF INTERESTS

M.S.D. is a consultant for Inbios and on the scientific advisory board of Moderna. S.H. is an employee of Moderna.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ZV-13	Diamond laboratory	Zhao et al., 2016
MAR1-5A3 (anti-IFNAR1)	Leinco	Cat#: I-401; RRID: AM_2737538
Bacterial and Virus Strains		
POWV strain LB	Ebel laboratory	Mandl et al., 1993
POWV-DTV strain Spooner	Ebel laboratory	Ebel et al., 1999
LGTV strain E5	Ebel laboratory	Campbell and Pletnev, 2000
POWV-DTV strain MA5/12-#40	Ebel laboratory	Brackney et al., 2008
POWV-DTV strain FA5/12-#40	Ebel laboratory	Brackney et al., 2008
Experimental Models: Cell Lines		
Vero	ATCC	CVCL_0059
HEK293T	ATCC	CRL-3216
Raji-DCSIGNR	Pierson laboratory	N/A
Experimental Models: Organisms/Strains		
C57BL/6J mice	The Jackson Laboratory	000664
WNVrepG/Z	Pierson laboratory	N/A
POWV CprME (strain P0375)	Pierson laboratory	N/A
LGTV CprME strain TP21	Pierson laboratory	N/A
TBEV CprME (strain Neudoerfl)	Diamond laboratory, this paper	N/A
GGYV CprME (strain Macquarie Island)	Diamond laboratory, this paper	N/A
Oligonucleotides		
POWV Spooner qPCR primer 5′- GCAGCACCAT AGGTAGAATGT-3	IDT	N/A
POWV Spooner qPCR primer 5′- CCACCCACTGAACCAAAGT-3′	IDT	N/A
POWV Spooner qPCR probe 5′-/56-FAM/ TCTCAGTGG/Zen/ TTGGAGAACACGCAT/3IABkFQ-3′	IDT	N/A
POWV LB qPCR primer 5′- GGCTGCAAATGAGACCAATTC-3′	IDT	N/A
POWV LB qPCR primer 5′- CAGCGACACATCTCCATAGTC-3′	IDT	N/A
POWV LB qPCR probe 5′-/56-FAM/TGGCATCCG/Zen/ AGAAAGTGATCCTGC/3IABkFQ/-3′	IDT	N/A
LGTV qPCR primer 5′- GGAACTAGGCCTTGCAGAAT-3′	IDT	N/A
LGTV qPCR primer 5′- TGTTCTCCATTGTCGGGTTAG-3′	IDT	N/A
LGTV qPCR probe 5′-/56-FAM/TGAGGTTAA/Zen/ CGTGGCCATGCTCAT/3IABkF-3′	IDT	N/A
Other		
mRNA LNP vaccines (POWV and controls)	Moderna (current paper)	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Michael S. Diamond (diamond@wusm.wustl.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells

Cell lines were maintained at 37°C in the presence of 5% CO2. HEK293T cells were passaged in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific) and 100 U/mL penicillin-streptomycin (PS)

(Invitrogen). Vero cells were passaged in DMEM supplemented with 5% FBS and 100 U/mL P/S. Raji B lymphoblast cells stably expressing the C-type lectin DC-SIGNR (Raji-DCSIGNR) were cultured in RPMI 1640 medium (Invitrogen) supplemented with 7% FBS and 100 U/mL P/S.

Mouse Studies
Experiments were approved and performed in accordance with Washington University Animal Studies Committee. C57BL/6J mice were purchased from Jackson Laboratories and housed in a pathogen-free animal facility at Washington University in St. Louis. Both male and female mice were vaccinated at 6 and 10 weeks of age (two dose studies) or 10 weeks of age (one dose study), and were infected with POWV or LGTV at 14 weeks of age, or 7 weeks where indicated.

METHOD DETAILS

Generation of modified mRNA and LNP
The mRNA was synthesized *in vitro* using DNA-dependent T7 RNA polymerase-mediated transcription where UTP was substituted with N1-methylpseudoUTP. A linearized DNA template was used, which incorporates 5′ and 3′ untranslated regions (UTRs) and includes a poly-A tail (5′-UTR: GGGAAAUAAGAGAGAAAAGAAGAGUAAGAAGAAAUAUAAGAGCCACC; and 3′-UTR: GCUGGAGC CUCGGUGGCCUAGCUUCUUGCCCCUUGGGGCCUCCCCCCCAGCCCCUCCUCCCCUUCCUGCACCCGUACCCCCGUGGUCUUU GAAUAAAGUCUGAGUGGGCGGC). The final mRNA utilized a cap 1 structure modification to increase mRNA translation efficiency. Encoded signal sequences were from POWV prM (SGVDWTWTFLVMALMT) or JEV prM (MWLVSLAIVTACAGA) and the prM and E genes were derived from POWV Spooner.

   LNP formulations were prepared by ethanol drop nanoprecipitation on a microfluidic mixer (Precision Nanosystems). Briefly, lipids were dissolved in ethanol at molar ratios of 50:10:38.5:1.5 (ionizable lipid: structural lipid: sterol: PEG-lipid) and mixed with mRNA in 50 mM citrate buffer, pH 4.0, at a ratio of 3:1 (aqueous:ethanol). Formulations were dialyzed against 20 mM Tris-Cl, pH 7.4, containing sucrose (8%) in dialysis cassettes for at least 18 h. Formulations were concentrated using Amicon Ultra Centrifugal Filters (EMD Millipore) and sterile filtered through 0.22-μm filters. All formulations were tested for particle size, RNA encapsulation, and endotoxin and were between 80 to 100 nm in size, with greater than 90% encapsulation and < 10 EU/ml of endotoxin.

Viruses
POWV lineage I strain LB (Mandl et al., 1993) was isolated originally from a human brain sample. POWV lineage II (DTV) strain Spooner was isolated originally from adult deer ticks (Ebel et al., 1999). LGTV strain E5 was derived from strain TP21 (Campbell and Pletnev, 2000). POWV LB, POWV Spooner, and LGTV were obtained from World Reference Center for Emerging Viruses and Arboviruses (R. Tesh and S. Weaver, University of Texas Medical Branch). POWV strains MA5/12-#40 and FA5/12-#40 were isolated from ticks collected in Wisconsin as described (Brackney et al., 2008). Virus was isolated by inoculating BHK-21 cells with 100 μl of the homogenate of POWV RNA positive ticks and harvesting virus supernatant when CPE was approximately 50%. Virus stocks were propagated on Vero cells and used at passage 3 (POWV LB and POWV Spooner) or passage 4 (LGTV). Viral titer was determined by focus-forming assay (FFA) on Vero cells as described previously (Brien et al., 2013) with the following modifications: FFA infections were fixed after 72 (POWV LB and POWV Spooner) or 48 (LGTV) h and stained with ZV-13, a flavivirus cross-reactive mouse mAb (Zhao et al., 2016).

Plasmids
The plasmid encoding a sub-genomic replicon of the WNV lineage II strain 956 that expresses GFP (WNVrepG/Z) has been reported (Ansarah-Sobrinho et al., 2008; Pierson et al., 2006). Plasmids that express the C-prM-E structural genes of POWV strain P0375 (GenBank accession number KU886216), TBEV strain Neudoerfl (GenBank accession number U27495), LGTV strain TP21 (GenBank accession number NC_003690), and GGYV strain Macquarie Island (GenBank: DQ235145) were cloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). All plasmid propagation and cloning procedures were performed using Stbl2 bacteria grown at 30°C (Invitrogen, Carlsbad, CA).

Reporter virus particles
Reporter virus particles (RVPs) were produced by the genetic complementation of a DNA-launched, sub-genomic replicon with a C-prM-E expression plasmid, as described previously (Ansarah-Sobrinho et al., 2008; Pierson et al., 2006). Briefly, pre-plated HEK293T cells were co-transfected with WNVrepG/Z, a WNV replicon expressing a GFP reporter gene, and the C-prM-E expression plasmids described above using FugeneHD (Promega) in accordance with the manufacturer's instructions. Transfected cells were incubated at 37°C, and supernatant was harvested at 48 h post-transfection, filtered using a 0.22 μm syringe filter, and stored at −80°C. The infectious titer of RVPs was assayed using Raji-DC-SIGNR cells, as described previously (Ansarah-Sobrinho et al., 2008; Pierson et al., 2006).

Neutralization assays
(a) RVP neutralization assays were performed as described (Mukherjee et al., 2014). Briefly, RVP stocks were diluted and incubated with serial dilutions of serum for 1 h at 37°C prior to the addition of Raji-DCSIGNR cells. Infections were carried out at 37°C for 48 h,

and infectivity was scored as the percentage of GFP-expressing cells determined by flow cytometry. (b) Focus-reduction neutralization test (FRNT) experiments were performed as described for other flaviviruses (Richner et al., 2017a). Briefly, serial dilutions of serum were incubated with 200 FFU of POWV Spooner for 1 h at 37°C. Serum/virus mixtures were added to Vero cell monolayers and incubated for 1 h at 37°C prior to the addition of 1% (w/v) methylcellulose in MEM. FRNT experiments proceeded as described for FFA experiments above. For both RVP neutralization assays and FRNT, antibody-dose response curves were analyzed using non-linear regression analysis (with a variable slope) (GraphPad Software). Data are expressed as the serum dilution required to reduce infection by half (EC_{50}) or, where indicated, by 90% (EC90). To obtain EC_{50} values when a resistant fraction was present, the bottom of the curve was constrained to zero.

Mouse experiments

Immunizations with the LNP mRNA vaccines were administered via intramuscular injection in a 50 μL volume. For passive transfer studies, serum was diluted in PBS and administered to mice via intraperitoneal injection in a 100 μL total volume; mice received a dose of 3 μL or 10 μL of serum from $POWV_{sig}$-vaccinated mice or 10 μL of serum from placebo-treated mice. Viral infections with POWV or LGTV were performed via subcutaneous inoculation in the footpad with 10^2 FFU of virus. For LGTV and POWV Spooner challenge, 0.5 mg of anti-Ifnar1 blocking mAb MAR1-5A3 (Sheehan et al., 2006) was administered one day prior to infection via intraperitoneal injection. Animals were monitored for mortality, weight loss, and clinical score. To evaluate clinical disease following POWV infection, mice were assigned to one of the following categories each day: (A) no disease, (B) ruffled fur, (C) ruffled fur with hunched posture, (D) lethargy or decreased scurrying activity, (E) sick with slow to no movement, or (F) moribund or dead; moribund mice were euthanized. To evaluate clinical disease following LGTV challenge, cages were blinded and mice were assigned to one of the following categories each day: (A) no disease, (B) partial hindlimb paralysis, (C) complete paralysis of one hindlimb, (D) complete paralysis of both hindlimbs, or (E) moribund or dead; moribund mice were euthanized.

Measurement of viral burden

On indicated days post-infection, mice were sacrificed and organs were collected following extensive perfusion with PBS. Organs were weighed and homogenized using a MagNA Lyser (Roche). Viral RNA from homogenized organs or serum was isolated using the MagMAX Viral RNA Isolation Kit (ThermoFisher) and measured by TaqMan one-step quantitative reverse-transcription PCR (RT-qPCR) on an ABI 7500 Fast Instrument. Viral RNA levels are expressed on a log10 scale as viral RNA equivalents per gram or milliliter after comparison with a standard curve produced using serial 10-fold dilutions of viral RNA from known quantities of infectious virus in order to estimate viral burden. POWV Spooner primers, which have been described (Platt et al., 2018), were 5′- GCAGCACCATAGG TAGAATGT-3′, 5′- CCACCCACTGAACCAAAGT-3′, and probe 5′-/56-FAM/ TCTCAGTGG/Zen/TTGGAGAACACGCAT/3IABkFQ-3′. POWV LB primers were 5′- GGCTGCAAATGAGACCAATTC-3′, 5′- CAGCGACACATCTCCATAGTC-3′, and probe 5′-/56-FAM/ TGGCATCCG/Zen/AGAAAGTGATCCTGC/3IABkFQ/-3′. LGTV primers were 5′- GGAACTAGGCCTTGCAGAAT-3′, 5′- TGTT CTCCATTGTCGGGTTAG-3′, and probe 5′-/56-FAM/TGAGGTTAA/Zen/CGTGGCCATGCTCAT/3IABkF-3′.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using Prism software Version 7.0 for Mac OS X (GraphPad Software). Log EC_{50} or Log EC90 values were compared by one-way ANOVA followed by Tukey's multiple comparisons test. For survival analysis, Kaplan-Meier curves were plotted and analyzed by the log rank test with a Bonferroni correction for multiple comparisons. Weight loss experiments were compared by an ANOVA followed by Dunnett's multiple comparisons test to compare vaccine groups to the placebo group, or by Sidak's multiple comparisons test when two groups were compared. Viremia and viral burden in tissues were compared by Mann-Whitney test when two groups were compared, or by Kruskal Wallis test followed by Dunn's multiple comparisons test when more than two groups were compared.