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Use of a Scalable Replicon-Particle Vaccine to Protect Against Lethal Lassa Virus Infection in the Guinea Pig Model

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Abstract

Lassa fever is a viral zoonosis that can be transmitted from person to person, especially in the hospital setting. The disease is endemic to several countries in West Africa and can be a major contributor to morbidity and mortality in affected areas. There are no approved vaccines to prevent Lassa virus infection. In this work, we present a vaccine candidate that combines the scalability and efficacy benefits of a live vaccine with the safety benefits of single-cycle replication. The system consists of Lassa virus replicon particles devoid of the virus essential glycoprotein gene, and a cell line that expresses the glycoprotein products, enabling efficient vaccine propagation. Guinea pigs vaccinated with these particles showed no clinical reaction to the inoculum and were protected against fever, weight loss, and lethality after infection with Lassa virus.

Keywords

Lassa; vaccine; virus replicon particle; VRP; single-cycle replication

Lassa fever was first recognized in Nigeria in 1969, when a nurse working in the town with that name became ill; 2 healthcare workers and a laboratory investigator subsequently also contracted the disease. Infection was fatal in half of these initial cases [1, 2]. The causative agent, Lassa virus (LASV), an arenavirus, was isolated from all 4 patients [3]. Soon after its discovery, 2 critical factors of Lassa fever epidemiology were established: the virus is rodent-borne [4], and there is a broad spectrum of clinical signs and symptoms associated

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Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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with the disease [5]. LASV causes fever with many nonspecific symptoms, such as general malaise and headache; vomiting, diarrhea, abdominal/chest pain, and sore throat are commonly observed, with bleeding and hearing loss developing in a minority of patients [6, 7].

Lassa fever has long been considered endemic in Nigeria, Sierra Leone, Liberia, and Guinea, and recent studies and reports of exported cases suggest that the countries between these 2 West African regions are affected, as well [8]. While there have been calls for more research on Lassa fever distribution and case numbers [9], extrapolation of data from surveys conducted in Sierra Leone suggests that, overall, hundreds of thousands of people may be infected yearly, with the number of fatalities potentially rising to the thousands [10].

Despite the high burden of Lassa fever, currently no vaccines are approved to prevent infection. Here, we describe an efficient system to produce Lassa virus replicon particles (VRPs) that replicate in the first cells they encounter but do not spread and thus do not cause disease. Two VRP vaccines were evaluated based on efforts to capitalize on known immune evasion strategies of LASV. Both VRP-based vaccines demonstrated complete protective efficacy against disease in a lethal guinea pig model.

METHODS

More-detailed descriptions of materials and methods are provided in the Supplementary Materials.

Biosafety

All work with infectious viruses and VRPs was performed in a biosafety level 4 facility at the Centers for Disease Control and Prevention (Atlanta, GA).

Generation of the Vero-LASV-GPCco Cell Line

A cassette for expressing a codon-optimized open reading frame for LASV Josiah glycoprotein precursor (GPC; corresponding to HQ688672) was inserted into the Vero E6 cell line, using the Flp-In system (Invitrogen).

Virus and VRP Rescue

LASV Josiah was rescued as described elsewhere [11]. The rescue constructs were modified to enable rescue of nonspreading VRPs by replacing the GPC open reading frame with the ZsGreen (ZsG) open reading frame (Clontech). For some VRPs, mutations D389A and G392A were made to inactivate the nucleoprotein (NP) exonuclease domain [12, 13], or the LASV Z open reading frame was replaced with that of Mopeia virus (NC_006574). VRPs were rescued on BSR T7/5 cells (a generous gift from Karl-Klaus Conzelmann), using rescue constructs for the L and S segments and a helper construct for LASV GPC.

Antibodies

Antibodies used included an α-LASV NP (703079-Lassa Josiah HMAF; in-house reagent) for immunoblotting; an α-LASV NP (mouse monoclonal 52-129-18) for

immunofluorescence analysis; an α-LASV GP1 (monoclonal antibody 52-74-7) for immunoblotting and immunofluorescence analysis; an α-LASV GP2 (monoclonal antibody 52-85-6) [14] for immunoblotting; an anti–influenza virus hemagglutinin tag, clone HA-7 (Sigma-Aldrich), for immunofluorescence analysis; secondary α-mouse immunoglobulin G (IgG) antibodies coupled to Alexa488, Alexa594, or Alexa633 fluorophores (Molecular Probes), for immunofluorescence analysis; and a horseradish peroxidase–conjugated β-actin antibody (GenSript A00730) for immunoblotting.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Commercial and custom-made assays against the LASV Josiah/VRP NP sequence, as well as guinea pig GAPDH messenger RNA (mRNA), interferon β (IFN-β) mRNA, 18S RNA, and CCL5/RANTES mRNA, were used as described in the Supplementary Materials. For in vitro experiments, the relative abundance of each analyte was calculated using a correction for PCR efficiency [15] and GAPDH as the internal control.

Animal Experiments

Experiments with strain 13/N guinea pigs from our in-house breeding colony were conducted under biosafety level 4 containment as described in the Supplementary Materials. A group size of 5 animals each was chosen so that a statistically significant survival difference (defined as a difference with a *P* value of < .05, by the Fisher exact test) between vaccinated and mock-vaccinated animals would be detected if complete protection was achieved after vaccination and if mortality in the mock-vaccinated group was 80%–100%.

Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma was inactivated using 5×10^4 Gy of gamma irradiation from a ^{60}Co source. For IgG ELISA, lysates from either Vero E6 cells or LASV Josiah–infected Vero E6 cells were used as the coating antigen. To detect immunoglobulin M (IgM) antibodies, a capture ELISA was used. ELISA measurements were repeated twice, and the average titer is reported for samples that yielded positive results on both repeats.

Statistical Analysis

The statistical significance of differences in guinea pig survival was evaluated using the 2-sided Fisher exact test (GraphPad Prism 7.03 software).

RESULTS

Lassa VRPs Exhibit Single-Cycle Infectivity

To produce VRPs that resemble infectious virus but do not spread beyond the initially infected cells, we modified our LASV Josiah strain reverse-genetics system [11] by replacing the gene for the GPC, coded by the S segment, with the gene for the fluorescent protein ZsG (Figure 1A). VRPs could be rescued when the resulting plasmid pLasSΔGPC +ZsG and the native pLasL plasmid were transfected into BSR T7/5 cells together with an expression construct for LASV GPC. When passaged in parallel in Vero E6 cells, VRPs and authentic virus showed similar NP immunofluorescence signals at day 1 of infection,

suggesting comparable replication efficiencies (Figure 1B). Spread of authentic virus in the monolayer was evident on day 2 after infection, with virtually all cells infected by day 7, and the supernatant readily infected fresh cell monolayers. In contrast, VRPs did not spread in the primary culture or pass into secondary cells, confirming the expected single-cycle phenotype.

Establishing a Cell Line for Efficient VRP Propagation

While Lassa VRPs could be rescued in BSR T7/5 cells with the help of a GPC expression construct, low titers were obtained. Therefore, a cell line stably expressing GPC was cloned to enable large-scale VRP production. Vero E6 cells were chosen as the basis of this cell line because they exhibit contact inhibition and, most importantly, lack the gene cluster for type I IFN genes [16]. These characteristics make Vero E6 cells the standard choice for growing virus and VRP stocks free of the potent inducers of antiviral defenses.

The cell line Vero-LASV-GPCco (cloned as described in Methods) showed marked GP1 immunofluorescence staining (Figure 2A). To test whether Vero-LASV-GPCco cells can support VRP production, spread of the ZsG signal in the monolayer was observed by imaging the same areas repeatedly after VRP inoculation. VRPs inoculated at a low multiplicity spread to most cells in the Vero-LASV-GPCco culture within 3 days (Figure 2B). Interestingly, initial VRP infectivity was reduced in Vero-LASV-GPCco cells as compared to Vero E6 cells. Although we did not study the reason for this, the lower infectivity upon GPC expression may have been due to reduced expression of functional α-dystroglycan, a receptor for LASV, on the cell surface [17]. Nonetheless, 1 passage in Vero-LASV-GPCco cells yielded VRP titers approximately 3 logs above those seen after the initial rescue, showing that the stable cell line can be used for efficient VRP propagation (Figure 3A).

To characterize GP expression in more detail, parental Vero E6 cells and Vero-LASV-GPCco cells were inoculated with LASV, VRP, or mock inoculum. The immunoblot pattern of GP1, GP2, and higher-molecular-weight GPC expression in mock-infected Vero-LASV-GPCco cells closely resembled that of virus-infected Vero E6 cells (Figure 3B), indicating that GPC expressed by the stable cell line was processed the same way as during virus infection. As expected, VRP-inoculated Vero E6 cells contained NP but no detectable viral glycoproteins. In contrast, VRP-inoculated Vero-LASV-GPCco cells expressed NP and GP1/2 similarly to virus-infected Vero E6 cells, albeit with somewhat reduced GP1/GP2 levels. Taken together, the results show that the gene encoding GPC can be inserted into a producer cell genome to yield a system in which authentic viral GP processing enables propagation of nonspreading VRPs.

Preventing Evasion of Innate Immune Response by VRPs

The primary target cells of LASV, macrophages and dendritic cells, fail to activate upon infection [18, 19]. LASV NP mediates inhibition of innate immunity recognition [20] via its C-terminal exonuclease domain [12, 13]. Additionally, arenavirus Z protein has been designated as an inhibitor of RIG-I signaling. While some researchers found that this Z protein function is a feature of LASV and other pathogenic arenaviruses regardless of origin

[21], others suggested that the RIG-I inhibitory function is found in New World arenaviruses of South America but not in Old World arenaviruses such as LASV [22]. We reasoned that removing the ability of the VRPs to antagonize the innate immune response could improve vaccine immunogenicity.

To examine this idea, we rescued 4 different VRPs: wild type (WT)–WT, which has no changes in the L and S segments; WT-Exo(N), which has no change in the L segment and the S segment-encoded NP exonuclease made null by mutations D389A/G392A [12, 13]; MopZ-WT, which has no change in the S segment and replaces LASV Z with Mopeia virus Z, which was reported not to inhibit RIG-I signaling [21]; and MopZ-Exo(N), which has the changes in the S segment and Z protein described above. Immunofluorescence staining was used to study IFN regulatory factor 3 (IRF-3) relocalization in VRP-inoculated A549 cells. As seen in Figure 4A, IRF-3 remained in the cytoplasm of cells with WT-WT or MopZ-WT replication. In contrast, frequent nuclear accumulation of the transcription factor was seen in cells with WT-Exo(N) or MopZ-Exo(N) replication. Inactivating the VRPs with UV light prior to inoculation prevented the observed changes in IRF-3 relocalization, showing that VRP replication, not soluble factors in the inocula, trigger the response.

To confirm these results, guinea pig GPC-16 cells were inoculated with the VRPs in parallel with LASV Josiah, and activation of the innate immune response was monitored by IFN-β and CCL5/RANTES qRT-PCR analysis. As seen in Figure 4B, WT-WT–inoculated cells displayed low activation levels comparable to those of the virus control. NP exonuclease mutations enabled a more robust response, as previously reported using recombinant viruses [23]. However, replacing LASV Z with Mopeia virus Z did not enhance the cellular response to VRPs. Based on these results, WT-WT and WT-Exo(N) VRPs were chosen as the vaccine candidates to be tested in the guinea pig model of Lassa fever.

VRP-Conferred Protection From Lethal LASV Infection in the Guinea Pig Model

LASV Josiah causes a lethal infection in inbred strain 13 guinea pigs [24], and this model, recently characterized in detail elsewhere [25], has been used extensively to test LASV vaccine candidates [26–30] and therapeutics [31, 32]. To evaluate the protective efficacy and critically test the scalability of the candidates, we chose a vaccine dose that could be delivered without concentrating the VRPs. Groups of 5 or 4 strain 13 guinea pigs were mock vaccinated or vaccinated subcutaneously with 5×10^5 focus-forming units of WT-WT VRPs or WT-Exo(N) VRPs. No clinical signs, fever, or weight loss were associated with the injections (Figure 5), indicating that the VRPs caused no disease in an animal model susceptible to Lassa fever.

After 28 days, prechallenge blood samples were collected, and the guinea pigs were challenged subcutaneously with 1×10^4 focus-forming units of LASV Josiah. Elevated temperatures and body weight loss were seen from day 11 onward in mock-vaccinated animals, which succumbed to infection 20–23 days after infection (Figure 6). All animals vaccinated with WT-WT or WT-Exo(N) VRPs survived the infection without exhibiting fever, weight loss, or clinical signs during the 42-day observation period ($P < .01$ for the difference in survival between the recipients of each vaccine and the control group).

All tissue samples (ie, blood, liver, lung, spleen, kidney, adrenal glands, heart, ovary, epididymis, vagina, testes, eye, and brain) collected from animals in the mock-vaccinated group after euthanasia revealed LASV RNA (Figure 7A). In contrast, none of the vaccinated animals had detectable LASV RNA in any of the examined tissues at the end of the observation period.

Two of 5 euthanized animals in the mock-vaccinated group had detectable IgM antibodies against LASV at the time of euthanasia, as determined by ELISA (Figure 7B). None of the vaccinated animals exhibited anti-LASV IgM ELISA titers before challenge (ie, 28 days after vaccination) or after challenge (ie, 42 days after asymptomatic infection). No IgG ELISA titers were observed in the mock-vaccinated or WT-WT–vaccinated animals either before or after challenge, but all animals of the WT-Exo(N) group developed IgG ELISA titers against LASV by the end of the postchallenge observation period. A more sensitive immunofluorescence study of the serological responses detected IgG against LASV NP in 2 of 5 WT-WT–vaccinated animals before challenge, with no such prechallenge antibodies in the mock-vaccinated or WT-Exo(N) groups (Supplementary Figure 1). Using this assay, postchallenge seroconversion could be detected in all animals of the study.

DISCUSSION

Several approaches have been taken to develop a vaccine against Lassa fever. A LASV peptide vaccine [33], a nonreplicating LASV particle vaccine [34], a LASV antigen–expressing bacterial vector vaccine [35], and rationally designed live attenuated vaccines [36] have been proposed. Platforms with proven efficacy in animal models include DNA vaccination, which, with an optimized GPC plasmid and delivery method, protects guinea pigs [30] and cynomolgus macaques [37] against Lassa fever. Other vaccine candidates with demonstrated efficacy in animal models include live attenuated arenaviruses, as well as heterologous virus vectors expressing LASV proteins. Delivery of LASV GPC by yellow fever virus vectors protected most vaccinated guinea pigs from lethal disease [28], and complete protection was achieved with 3 doses of Venezuelan equine encephalitis virus particles expressing either LASV NP or GPC [26]. Recombinant vaccinia viruses expressing NP or GPC also protected guinea pigs [38, 39], with NP appearing to be the preferred protective antigen in that model [40] but complete GPC offering better protection in primates [41]. In the vesicular stomatitis virus (VSV) vector context, GPC was chosen over NP after experiments in guinea pigs, and VSVΔG/LASVGPC provided complete protection in cynomolgus macaques [29, 42]. Apart from the vectored approaches, a live attenuated reassortant of the Mopeia virus L segment and the LASV S segment protected strain 13 guinea pigs [27] and marmosets [43] from Lassa fever.

Clearly, the preclinical data accumulated to date show that multiple technologies have the potential to yield protective Lassa fever vaccines. Therefore, the lack of a clinical vaccine almost 50 years after the disease was first described seems to be due to other factors, such as economic considerations and safety concerns. In this article, we have described a system to produce Lassa VRPs that combines the principal potency of live attenuated vaccines, scalability (<100 μL of unconcentrated WT-WT VRP stock was used per animal), and the inherent higher safety of single-cycle replication as compared to fully replicating live

attenuated vaccines. VRPs are stable, since no reversion of the single-cycle phenotype was observed during 10 blind VRP passages. In contrast to vectored approaches, the VRPs contain the complete LASV proteome, encoding 85% of it. GPC was chosen as the portion removed from the genome, since NP and polymerase L are essential for genome replication and since stable expression of Z is known to render cells resistant to LASV infection [44]. We compared the 2 proposed mechanisms by which arenaviruses inhibit the innate immune system by testing VRPs in human and guinea pig cell lines, and we verified the role of the NP exonuclease domain. Disabling the innate immunity antagonism proved nonessential for protection, as guinea pigs vaccinated with either wild-type VRPs or exonuclease-null VRPs showed no clinical reactions to the vaccine yet were protected from lethality and signs of disease when challenged with LASV. Two of 5 animals vaccinated with wild-type VRPs developed low but detectable levels of NP-specific IgG antibodies before challenge, while this was not observed in any of the animals vaccinated with exonuclease-null VRPs. Conversely, LASV challenge caused a more pronounced seroconversion in exonuclease-null–vaccinated animals, implying that while those animals were fully protected, the virus may have replicated more efficiently in them than in animals vaccinated with wild-type VRPs. With full survival and because of limitations in available reagents and assays to measure T-cell activation in guinea pigs, establishing immunological correlates of protection is challenging. However, the lack of detectable antibodies in most animals at the time of challenge suggests involvement of cellular immunity. Importantly, no residual LASV RNA was detected in any vaccinated animal at the end of the experiment.

To conclude, we provide evidence that the VRP platform offers complete protection from lethal Lassa fever in the small-animal model. Our safety and efficacy results obtained so far mirror those of nonspreading Ebola virus [45, 46] and Rift Valley fever virus [47, 48] vaccine candidates generated using the same logic. Combining these results from 3 different virus families suggests that single-cycle systems should be further evaluated as a powerful immunization strategy against negative- strand RNA viruses. Bringing a LASV vaccine to the clinic has recently become a defined, multisector priority [49, 50]. The breadth of described preclinical candidates should aid in developing widely available vaccines against Lassa fever, a disease the burden of which is sometimes forgotten.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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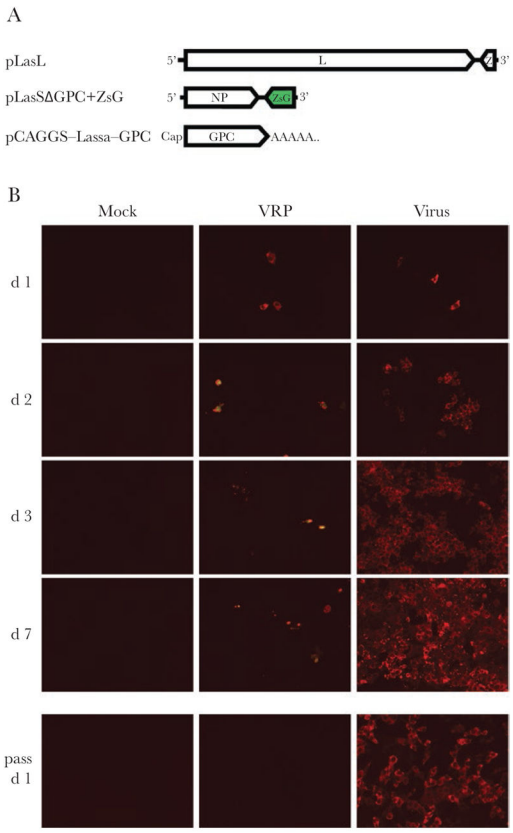

Figure 1.

Schematic representation of the Lassa virus (LASV) replicon particle (VRP) system and single-cycle phenotype. *A*, Transcripts produced from the VRP rescue plasmids pLasL and pLasSΔGPC+ZsG, as well as the helper construct pCAGGS-Lassa- GPC. *B*, Lack of VRP spread in Vero E6 cells. Cells were inoculated with LASV or VRPs at a multiplicity of infection of 0.01 or were mock inoculated. Cells were then fixed on days 1, 2, 3, and 7 after infection. Day 7 supernatants were passed onto fresh Vero E6 cell monolayers, and cells were fixed 1 day later. Green, ZsGreen (ZsG) fluorescence; red, immunostaining against LASV NP.

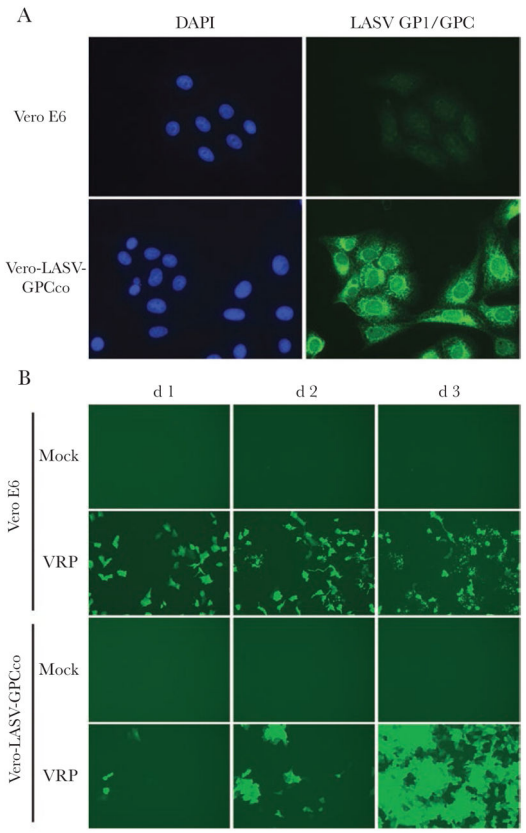

Figure 2.

Establishing a stable cell line for Lassa virus (LASV) replicon particle (VRP) propagation. *A*, Immunofluorescence staining for LASV glycoprotein 1 (GP1) and glycoprotein precursor (GPC) in Vero-LASV-GPCco cells. *B*, Spread of VRPs in Vero-LASV-GPCco cells. Cells were inoculated with VRPs at a multiplicity of infection of 0.05 (as quantified on Vero E6 cells). ZsGreen (ZsG) fluorescence was imaged in a fixed position of live cell monolayers on consecutive days.

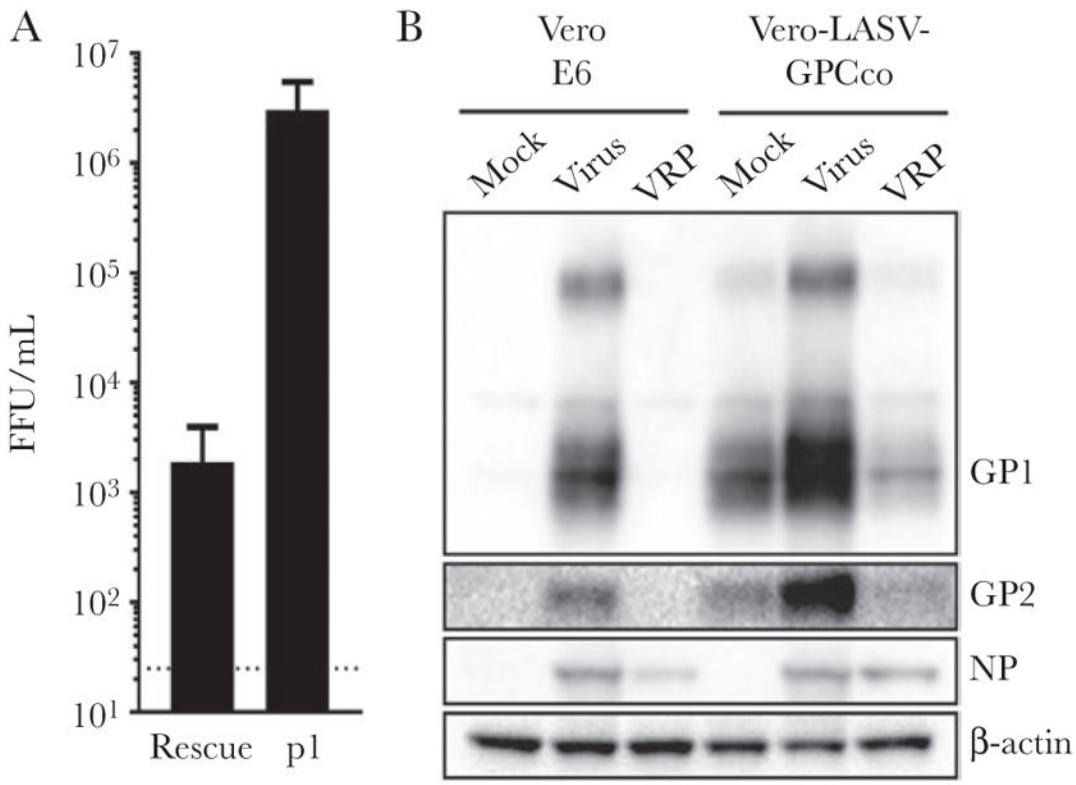

Figure 3.

Vero-LASV-GPCco cells enable efficient Lassa virus (LASV) replicon particle (VRP) amplification with authentic glycoprotein processing. *A*, VRP titers after rescue and after 1 passage in Vero-LASV-GPCco cells. Averages and standard deviations from 3 independent experiments are shown. *B*, Western blot analysis of LASV glycoprotein 1 (GP1) and GP2 and nucleoprotein (NP) in mock-, virus-, or VRP-inoculated Vero E6 and Vero-LASV-GPCco cells. Cells were mock infected or infected at a multiplicity of infection of 0.5 with LASV or VRPs (taking into account the reduced infectivity on cells stably expressing LASV GPC) for 48 hours. FFU, focus-forming units.

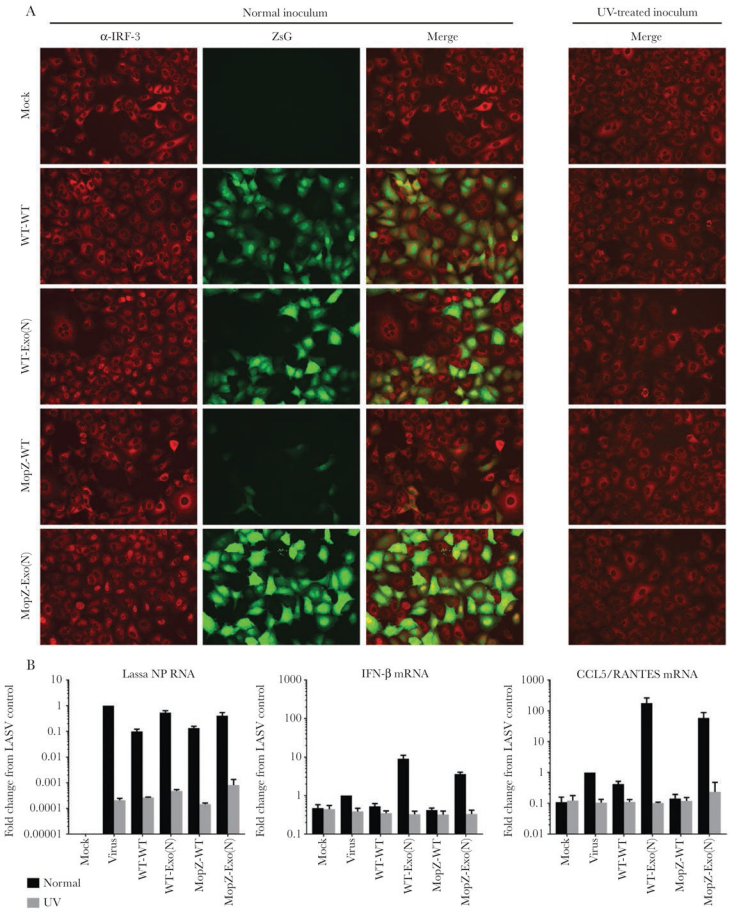

Figure 4.

Evaluating innate immunity antagonism by Lassa virus (LASV) nucleoprotein (NP) and Z protein. *A*, Human A549 cells were inoculated with wild-type (WT) LASV replicon particles (VRPs) with no additional mutations in the L and S segments (WT-WT), VRPs with no change in the L segment and the S segment–encoded NP exonuclease made null by mutations D389A/G392A (WT-Exo[N]), VRPs with no change in the S segment and replacement of LASV Z with Mopeia virus Z (MopZ-WT), or VRPs containing both mutations (MopZ-Exo[N]). Interferon (IFN) regulatory factor 3 (IRF-3) relocalization from the cytoplasm to the nucleus was studied 15 hours after infection. UV-inactivated VRPs were used to verify that replication is necessary for the observed effects. *B*, The guinea pig cell line GPC16 was inoculated with LASV or with VRPs listed in panel *A*, and LASV NP RNA, IFN-β messenger RNA (mRNA), or CCL5/RANTES mRNA was quantified by quantitative reverse-transcription polymerase chain reaction analysis 48 hours after infection. The data were normalized to values obtained with LASV and represent averages and standard deviations from 3 independent experiments. ZsG, ZsGreen.

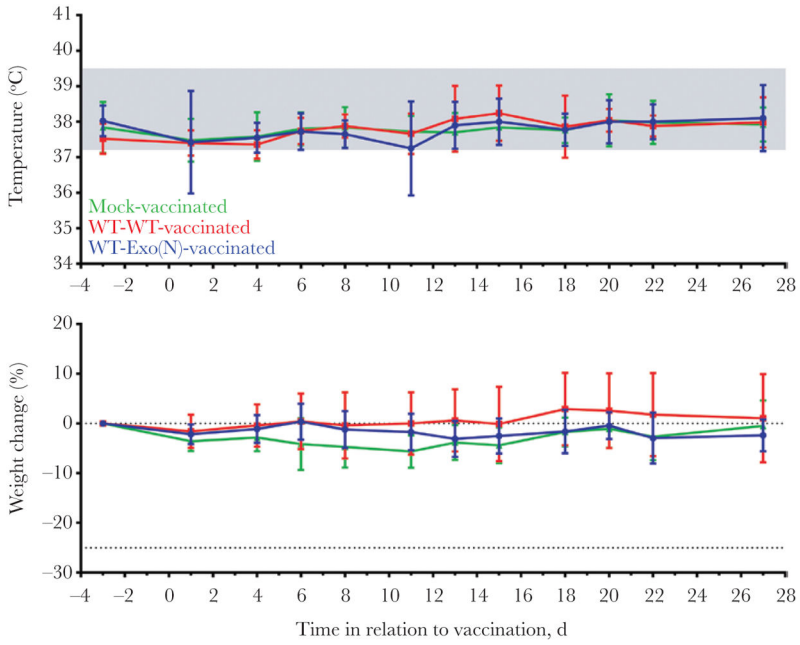

Figure 5.

Guinea pig body temperatures and weight changes after vaccination. Animals were vaccinated subcutaneously with 5×10^5 focus-forming (FFU) units of wildtype virus replicon particles (VRPs) (WT-WT; n = 5), VRPs with the S segment–encoded nucleoprotein exonuclease made null by mutations D389A/G392A (WT-Exo[N]; n = 4; 1 animal was removed from the study after failure to acclimate to the biosafety level 4 laboratory), or Dulbecco's modified Eagle's medium as a vehicle control (n = 5). Averages ± standard deviations are presented.

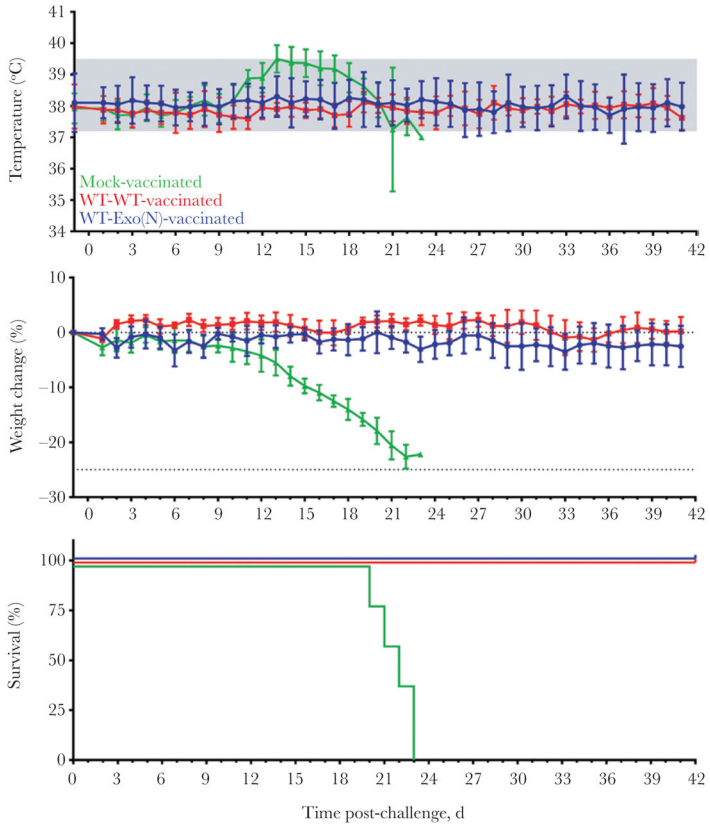

Figure 6.

Lassa virus (LASV) replicon particle (VRP)–based LASV vaccine candidates protect against lethal infection. Guinea pigs from Figure 5 were challenged subcutaneously with 1×10^4 focus-forming units of LASV Josiah 28 days after vaccination. Data on body temperature, weight change (averages ± standard deviations), and survival after challenge are presented.

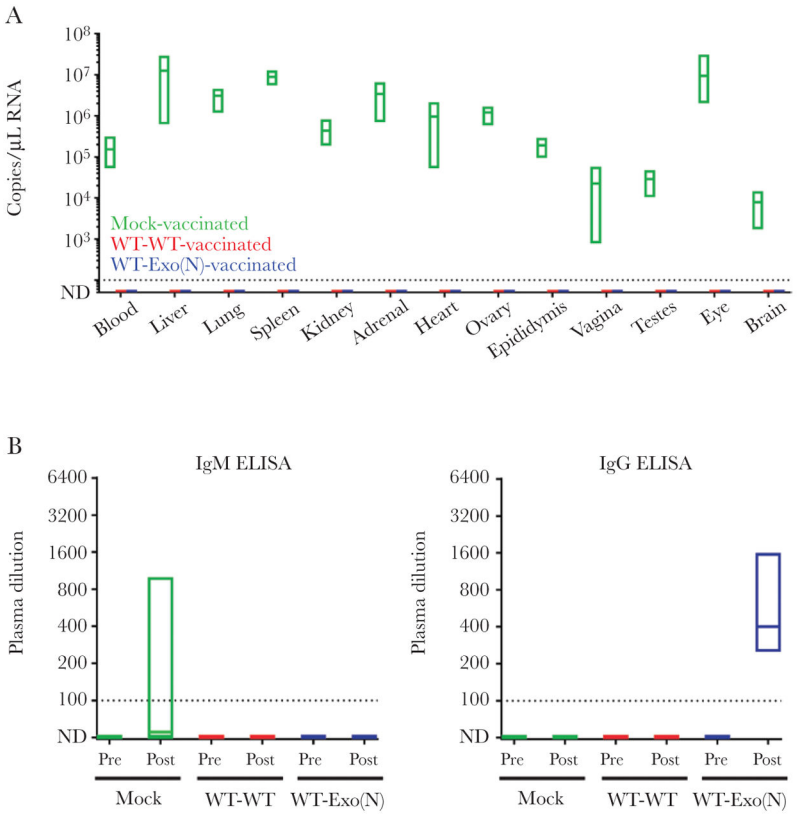

Figure 7.

Viral RNA and antibody titers in vaccinated and challenged guinea pigs. *A*, Quantitative reverse-transcription polymerase chain reaction analysis to detect Lassa virus (LASV) nucleoprotein (NP) RNA in guinea pig tissues at the time of euthanasia (ie, acute disease in the mock-vaccinated group and at the end of the observation period in the VRP-vaccinated groups). Averages and ranges are shown. *B*, Prechallenge and postchallenge immunoglobulin M (IgM) and immunoglobulin G (IgG) antibody titers against LASV as determined by enzyme-linked immunosorbent assay (ELISA). Ranges and medians are shown.