AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

CrossMark click for updates

Glycoprotein-Specific Antibodies Produced by DNA Vaccination Protect Guinea Pigs from Lethal Argentine and Venezuelan Hemorrhagic Fever

Joseph W. Golden,[a] Piet Maes,[b] Steven A. Kwilas,[a] John Ballantyne,[c] Jay W. Hooper[a]

Department of Molecular Virology, Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, USA[a]; Department of Microbiology and Immunology, Laboratory of Clinical and Epidemiological Virology, Rega Institute for Medical Research, KU Leuven, Leuven, Belgium[b]; Aldevron, LLC, Fargo, North Dakota, USA[c]

ABSTRACT

Several members of the *Arenaviridae* can cause acute febrile diseases in humans, often resulting in lethality. The use of convalescent-phase human plasma is an effective treatment in humans infected with arenaviruses, particularly species found in South America. Despite this, little work has focused on developing potent and defined immunotherapeutics against arenaviruses. In the present study, we produced arenavirus neutralizing antibodies by DNA vaccination of rabbits with plasmids encoding the full-length glycoprotein precursors of Junín virus (JUNV), Machupo virus (MACV), and Guanarito virus (GTOV). Geometric mean neutralizing antibody titers, as measured by the 50% plaque reduction neutralization test ($PRNT_{50}$), exceeded 5,000 against homologous viruses. Antisera against each targeted virus exhibited limited cross-species binding and, to a lesser extent, cross-neutralization. Anti-JUNV glycoprotein rabbit antiserum protected Hartley guinea pigs from lethal intraperitoneal infection with JUNV strain Romero when the antiserum was administered 2 days after challenge and provided some protection (~30%) when administered 4 days after challenge. Treatment starting on day 6 did not protect animals. We further formulated an IgG antibody cocktail by combining anti-JUNV, -MACV, and -GTOV antibodies produced in DNA-vaccinated rabbits. This cocktail protected 100% of guinea pigs against JUNV and GTOV lethal disease. We then expanded on this cocktail approach by simultaneously vaccinating rabbits with a combination of plasmids encoding glycoproteins from JUNV, MACV, GTOV, and Sabia virus (SABV). Sera collected from rabbits vaccinated with the combination vaccine neutralized all four targets. These findings support the concept of using a DNA vaccine approach to generate a potent pan-arenavirus immunotherapeutic.

IMPORTANCE

Arenaviruses are an important family of emerging viruses. In infected humans, convalescent-phase plasma containing neutralizing antibodies can mitigate the severity of disease caused by arenaviruses, particularly species found in South America. Because of variations in potency of the human-derived product, limited availability, and safety concerns, this treatment option has essentially been abandoned. Accordingly, despite this approach being an effective postinfection treatment option, research on novel approaches to produce potent polyclonal antibody-based therapies have been deficient. Here we show that DNA-based vaccine technology can be used to make potently neutralizing antibodies in rabbits that exclusively target the glycoproteins of several human-pathogenic arenaviruses found in South America, including JUNV, MACV, GTOV, and SABV. These antibodies protected guinea pigs from lethal disease when given post-virus challenge. We also generated a purified antibody cocktail with antibodies targeting three arenaviruses and demonstrated protective efficacy against all three targets. Our findings demonstrate that use of the DNA vaccine technology could be used to produce candidate antiarenavirus neutralizing antibody-based products.

Viruses of the *Arenaviridae* family chronically infect rodents throughout the world (reviewed in reference 1). Many members of this family can cause human diseases through exposure to infected rodent excreta and secreta. Arenaviruses are divided into two complexes, Old World (OW) and New World (NW), based on initial geographical isolation and serological cross-reactivity. OW viruses include the most prominent human arenavirus pathogen, Lassa virus (LASV). NW arenaviruses include a large group of viruses found in North and South America and is further categorized into clades A, B, and C (2). NW arenaviruses pathogenic to humans are exclusively from clade B and include Junín virus (JUNV), Machupo virus (MACV), and Guanarito virus (GTOV). JUNV is the most significant human pathogen of the NW group and is the causative agent of Argentine hemorrhagic fever (AHF) (3). MACV and GTOV are the causative agents of Bolivian HF and Venezuelan HF, respectively. Human infection by JUNV, MACV, or GTOV can result in a febrile disease associated with various degrees of vascular leakage (hemorrhage) and occasional concomitant neurological manifestations (4). Some develop a systemic inflammatory response syndrome that progresses to multiple-organ failure and death in 15 to 30% of cases.

Received 23 November 2015 **Accepted** 23 December 2015

Accepted manuscript posted online 20 January 2016

Citation Golden JW, Maes P, Kwilas SA, Ballantyne J, Hooper JW. 2016. Glycoprotein-specific antibodies produced by DNA vaccination protect guinea pigs from lethal Argentine and Venezuelan hemorrhagic fever. J Virol 90:3515–3529. doi:10.1128/JVI.02969-15.

Editor: T. S. Dermody

Address correspondence to Jay W. Hooper, jay.w.hooper.civ@mail.mil.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

In addition to JUNV, MACV, and GTOV, other human-pathogenic HF arenaviruses, Sabia virus (SABV) and Chapare virus (CHPV), have emerged more recently in South America (5, 6). Likewise, White Water arroyo virus has also recently been associated with HF in humans residing in North America (7). Accordingly, arenaviruses are an important group of emerging and reemerging human pathogens in the Americas.

No postexposure countermeasures are currently licensed to prevent arenavirus disease in infected humans. Passive transfer of human plasma-derived antibodies has been shown to protect humans against disease caused by LASV, JUNV, and MACV in a postexposure setting (8–10). Passive protection against LASV, JUNV, and MACV has also been demonstrated using hyperimmune serum targeting the whole virus in guinea pig and nonhuman primate lethal disease animal models (11–13). The most clear evidence that the use of antibodies is a viable treatment option has been demonstrated in humans infected with JUNV, in whom immune plasma has served as a routine postexposure antiviral since the 1970s (14). Implementation of this treatment reduced AHF case fatality rates from 15 to 30% to less than 1%. Antibody-based therapeutic intervention against AHF is extremely successful even if initiated 8 days after symptoms appear (8). Antibody treatment has few adverse effects, the most severe of which is that 10% of immune plasma-treated patients develop late-stage neurological sequelae several weeks posttreatment that subsequently resolve (8). Immune plasma was procured initially from AHF survivors and subsequently from persons vaccinated with the JUNV vaccine, Candid#1. A major limitation to the use of antibodies for treatment of arenavirus HF is significant variation in the potency of the human-derived product. Enria et al. developed a standardized method for the dosage of therapeutic antiarenavirus antibodies (15). Using this standardized method, they reported that a minimal neutralizing antibody titer of 3,000 therapeutic units (TU) was needed to protect infected humans and that concentrations below this provided protection that was not statistically significantly different from that in untreated patients. Other hurdles in the use of therapeutic antibodies to treat arenavirus HF are limited availability and safety concerns with the use of human products. These concerns led to the abandonment of this option for treatment of JUNV infections, and it was replaced with an attenuated vaccine termed Candid#1 (16). Currently, Candid#1 is used to prevent JUNV infection in Argentina. However, this vaccine is unlikely to function as a postexposure therapeutic, and its use is limited to high-risk populations. Accordingly, to mitigate the threat of arenavirus zoonoses, the development of platforms that consistently produce potently neutralizing antibodies against multiple arenavirus targets while minimizing safety concerns with the use of human products are warranted.

Arenaviruses carry a bisegmented ambisense RNA genome consisting of large (L) and small (S) segments that encode five distinct proteins (1). The L segment encodes both the matrix-like Z protein and the RNA-dependent RNA polymerase, termed the L protein. The S segment encodes the nucleoprotein (NP) and the glycoproteins, GP1 and GP2. The glycoproteins are initially expressed as a precursor polypeptide, GPc, which is proteolytically cleaved within the Golgi apparatus by SK1/S1P, forming GP1 and GP2 (17). Unique among viral glycoproteins, the GPc precursor also carries a stable secretion signal (SSP) which remains part of the glycoprotein complex and plays a role in trafficking and viral fusion (18). Processing of GPc results in a tripartite complex where the outermost molecule, GP1, caps GP2 on the envelope surface while held in place by ionic bond interactions with the SSP, localized in close association with GP2 (19). GP1 is the virus attachment protein and, for clade B NW arenaviruses, binds to target cells via transferrin receptor 1 (20). GP2 mediates acidic pH-facilitated viral fusion upon internalization into the endosomal pathway (19). During viral replication, NP is the most abundant protein produced and is a major target of the humoral response (1). Antibodies also target both GP1 and GP2 (21–23). Owing to its location as the outermost virion protein and its involvement in receptor binding, GP1 is the sole target of known neutralizing antibodies (23, 24).

DNA vaccination involves the delivery of genetic information encoding a target antigen directly into host cells (25). Subsequent expression of the gene product(s) within the host leads to adaptive immune responses, including both cellular and humoral responses. This technology has been used in the development of candidate vaccines against a multitude of viruses, including coronaviruses (26), orthopoxviruses (27), and hantaviruses (28). DNA vaccine technology can produce potently neutralizing antibodies that are effective immunotherapeutics and can protect in lethal disease models (26, 29, 30). Here we exploited DNA vaccine technology exclusively targeting South American arenavirus glycoproteins to generate highly potent neutralizing antibodies in rabbits. Using these antibodies, we explored their protective efficacy against infection by JUNV, MACV, and GTOV in the Hartley guinea pig disease model.

MATERIALS AND METHODS

Viruses and cells. The attenuated JUNV strain Candid#1 was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID). Early-passage stocks of virulent JUNV strain Romero, MACV strain Carvallo, GTOV strain IHD95551, and SABV strain SPH114202 were obtained from the USAMRIID select agent inventory. All strains of arenaviruses were propagated in Vero cell monolayers (ATCC CRL-1587) in Eagle minimal essential medium containing 10% heat-inactivated fetal bovine serum (FBS), 1% antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), and 10 mM HEPES (cEMEM). HEK293T (293T) cells were used for all transfection assays and were also maintained in cEMEM. Work with virulent arenaviruses was performed in biosafety level 4 (BSL4) containment areas.

Cloning. The codon-optimized full-length arenavirus glycoprotein (GPc) genes were synthesized *de novo* (GeneWiz; South Plainfield, NJ). With the exception of the JUNV sequence, the sequences of the glycoprotein genes were derived from NCBI reference sequences (MACV strain Carvallo, accession no. NP_899212.1; GTOV strain INH95551, accession no. NP_899210.1; and SABV strain SPH114202, accession no. YP_089665). For the JUNV glycoprotein, the XJ13 sequence (accession no. NP_899218.1) was modified to include a P208L amino acid change. The JUNV nucleoprotein (NP) (accession no. AY746353) was also synthesized *de novo*. Genes were cloned into the NotI and BglII sites of the pWRG7077 vector and verified by sequence analysis. The constructs were named pWRG/JUN-GPc(opt), pWRG/MAC-GPc(opt), pWRG/GTO-GPc(opt), and pWRG/SAB-GPc(opt). The JUNV NP DNA plasmid was designated pWRG/JUN-NP(opt).

Constructs encoding the GP1 (nucleotides 174 to 718) and GP2 (nucleotides 718 to 1456) portions of the full-length JUNV GPc were created by PCR. GP1 was generated using the 5′ primer GGGGGCTAGCATGG AGGCCTTCAAAATCGGACTCCACAC and the 3′ primer GGGGAGAT CTTCATCACCGGGTGAGAAAGTGGAGTG. GP2 was created using the 5′ primer GGGGCTAGCGGCAAAAACATTCAGCTGCCTAGGA GAT and the 3′ primer GGGGAGATCTTCATCAATGCCCCCTCCGC CAC. For both GP1 and GP2, the forward primers generated an NheI site

and the reverse primers created a BglII site and also included a stop codon. PCR products were cloned into the NheI and BglII sites of a modified pWRG7077 (pWRG/CBDfuse) vector containing the cell-binding domain (CBD) of the orthopoxvirus type I interferon binding protein. Fusion of gene products in frame with the CBD (C-terminal fusion) allows the cell surface localization of exogenously expressed fusion products (31; data not shown).

DNA vaccination. Rabbits were vaccinated by either intramuscular electroporation (i.m. EP) (monovalent vaccine study) or i.m. disposable syringe jet injection (DSJI) (multivalent vaccine study). i.m. EP (Ichor Medical Systems Inc., San Diego, CA) vaccinations consisted of injection of 1 mg/ml plasmid DNA per vaccination in a 0.4-ml volume. Vaccinations using i.m. EP were performed at Aldevron LLC under internally approved animal protocols separate from those of USAMRIID. Rabbits used in the multivalent vaccine study were vaccinated with an i.m. needle-free DSJI device (Pharmajet). Plasmids carrying the glycoprotein genes from JUNV, MACV, GTOV, and SABV were combined at a concentration of 0.125 mg per plasmid (0.5 mg total) in phosphate-buffered saline (PBS) in a total volume of 0.5 ml per injection. Sera were collected from animals after days 0, 42, 56, and 70. For some studies, purified IgG antibodies were used instead of antisera. IgG fractions were isolated from antisera by using protein G monoclonal antibody (MAb) trap columns (GE Healthcare) in accordance with the manufacturer's protocol.

Flow cytometry. 293T cell monolayers in T25 flasks were transfected with the indicated constructs by use of Fugene 6 (Promega). Transfected cells were incubated for 48 to 72 h in a 37°C incubator with 5% CO_2. Cells were detached by gentle tapping, pelleted by centrifugation at 750 $\times$ *g*, and resuspended in 200 μl of fluorescence-activated cell sorter (FACS) buffer (PBS, 5% FBS). Murine MAbs targeting JUNV GP (MAb-GB03 and MAb-QC03), a negative-control MAb (MAb-2G11; targets a poxvirus protein), or rabbit antiserum (1:100 dilution) was added and incubated with cells for 1 h at room temperature. The anti-JUNV murine MAbs were obtained through BEI Resources. MAb-2G11 was obtained from the USAMIID hybridoma collection. Cells were then pelleted by centrifugation at 750 $\times$ *g* and washed three times with FACS buffer. Cells were then incubated with anti-rabbit–Alexa Fluor 488 (Invitrogen) (1:500) for 30 min at room temperature, washed three times, and resuspended in 1 ml of FACS buffer. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson). Data were collected and analyzed using FlowJo software (Tree Star Inc., Ashland, OR). A total of 10,000 cells were analyzed for each sample by using live gating. For GP1 and GP2 surface staining, 293T cells transfected with CBP-GP1 and CBP-GP2 were incubated with fresh 293T cells for 3 h and washed with FACS buffer. Cells were then incubated with rabbit anti-GPc antiserum from vaccinated animals (1:100 dilution) and processed for flow cytometry as described above, with the exception that an anti-rabbit secondary antibody was used.

PRNTs. Plaque reduction and neutralization tests (PRNTs) were performed essentially as previously described (32), with some variation. Briefly, all serum samples were heat inactivated at 56°C for 30 min. The sera were serially diluted 2-fold in 96-well titer tube racks (Bio-Rad). The indicated arenavirus was diluted in cEMEM to obtain ~75 to 100 plaques per well in a 6-well plate and added to serum dilutions at a 1:1 ratio. Antibody-virus mixtures were incubated overnight at 4°C. Subsequently, 180 μl of sample was adsorbed to confluent Vero cell monolayers in 6-well plates for 1 h in a 37°C incubator with 5% CO_2 and rocked for ~15 min. Following adsorption, a 2-ml solid overlay (Earle's basal minimal essential medium [EBME], 0.5% agarose, 5% heat-inactivated FBS, antibiotics [100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin) was added to each well. Plates were incubated for 6 days in a 37°C incubator with 5% CO_2 and 80 to 85% humidity and then stained with 2 ml of solid overlay mixture that also included 5% neutral red (Gibco). Cells were incubated for an additional 24 h in a 37°C incubator with 5% CO_2 before plaque counting. The percent neutralization was calculated relative to that of no-antibody controls. The titer represents the reciprocal of the highest dilution resulting in a 50% reduction in the number of plaques. Data were plotted using Prism software. Virus titers were determined using the PRNT protocol, except that virus was not incubated with serum prior to being adsorbed onto Vero cell monolayers. For titer determinations, virus was diluted 10-fold, starting at a 1:10 dilution.

Arenavirus PsV production. Pseudovirions (PsV) were produced as previously described (33, 34). Briefly, 293T cells were transfected with pWRG/JUN-GPc(opt), pWRG/MAC-GPc(opt), or pWRG/GTO-GPc(opt) by use of Fugene 6 (Promega) at ~70% confluence. The plasmids produced JUN PsV, MAC PsV, and GTO PsV, respectively. After ~18 h, the cells were infected with vesicular stomatitis virus glycoprotein expressing renilla luciferase (VSVΔG*rLuc) at a multiplicity of infection of ~0.02 for 1 h at 37°C. The medium was removed, and fresh medium was added. After 72 h at 37°C, the supernatant from infected cells was collected and clarified by low-speed centrifugation and filtration through a 0.22-μm filter. PsV were concentrated further by pelleting the virus through a 30% sucrose cushion prepared in TNE buffer (10 mM Tris, 135 mM NaCl, 2 mM EDTA, pH 8.0). The pellet was resuspended and stored at −70°C. The number of focus-forming units (FFU)/ml was determined for the PsV as described previously (33).

Pseudovirion enzyme-linked immunosorbent assay (ELISA). Individual PsV were used to coat 96-well high-binding microtiter plates from stock preparations at a dilution of 1:250 in sodium carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed and blocked for 1 h at 37°C with PBS containing 0.05% Tween 20 and 5% nonfat milk. Rabbit serum samples were initially diluted 1:100 and were then serially diluted 10-fold and incubated for 1 h at 37°C. After incubation, the plates were washed and then incubated with a 1:2,000 dilution of a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary. After incubation for 1 h at 37°C, the plates were washed and the substrate tetramethylbenzidine (TMB) was added. The reaction was stopped by the addition of TMB stop solution after color was observed. The endpoint titer was determined as the highest dilution that had an optical density greater than the mean optical density for serum samples from negative-control wells plus 3 standard deviations.

PsVNA. The pseudovirion neutralization assay (PsVNA) used to detect neutralizing antibodies in sera was described previously (33). Briefly, heat-inactivated sera were diluted 1:10, followed by 5-fold serial dilutions. These samples were then mixed with an equal volume of cEMEM containing 10^5 FFU/ml of the specific pseudovirion of interest and 10% guinea pig complement (Cedarlane). This mixture was incubated overnight at 4°C, and then 50 μl was inoculated onto Vero cell monolayers in a clear-bottom, black-walled 96-well plate (Corning). Plates were incubated at 37°C for 18 to 24 h. The medium was discarded, and cells were lysed according to the protocol of a luciferase kit (Promega). A Tecan M200 Pro machine was used to acquire the raw luciferase data. The values were graphed using GraphPad Prism software and used to calculate the percent neutralization normalized to cells alone and pseudovirions alone as the minimum and maximum signals, respectively. The percent neutralization values for triplicate serial dilutions were plotted. Fifty percent PsVNA ($PsVNA_{50}$) titers were interpolated from 4-parameter curves, and geometric mean titers (GMTs) were calculated.

Passive protection studies. Female Hartley guinea pigs (300 to 400 g) were tagged and monitored for temperature by using IPTT-3000 implants (BMDS Inc., Seaford, DE). Implants were placed at least 1 week prior to challenge studies. Animals were challenged with 2,000 PFU of JUNV, MACV, or GTOV, as indicated, by intraperitoneal (i.p.) injection of virus diluted in a total volume of 0.5 ml PBS. To standardized antibody dosages with those in previous studies, antibody doses were calculated using the equation TU/kg = (milliliters of antibody) $\times$ (reciprocal $PRNT_{80}$ titer/average animal weight [in kilograms]) (15). Antiserum or purified antibody at the indicated concentrations was injected subcutaneously (s.c.) at the time points indicated in a total volume of 1 ml buffered with PBS. Animals were weighed and monitored for fever daily. All challenges were

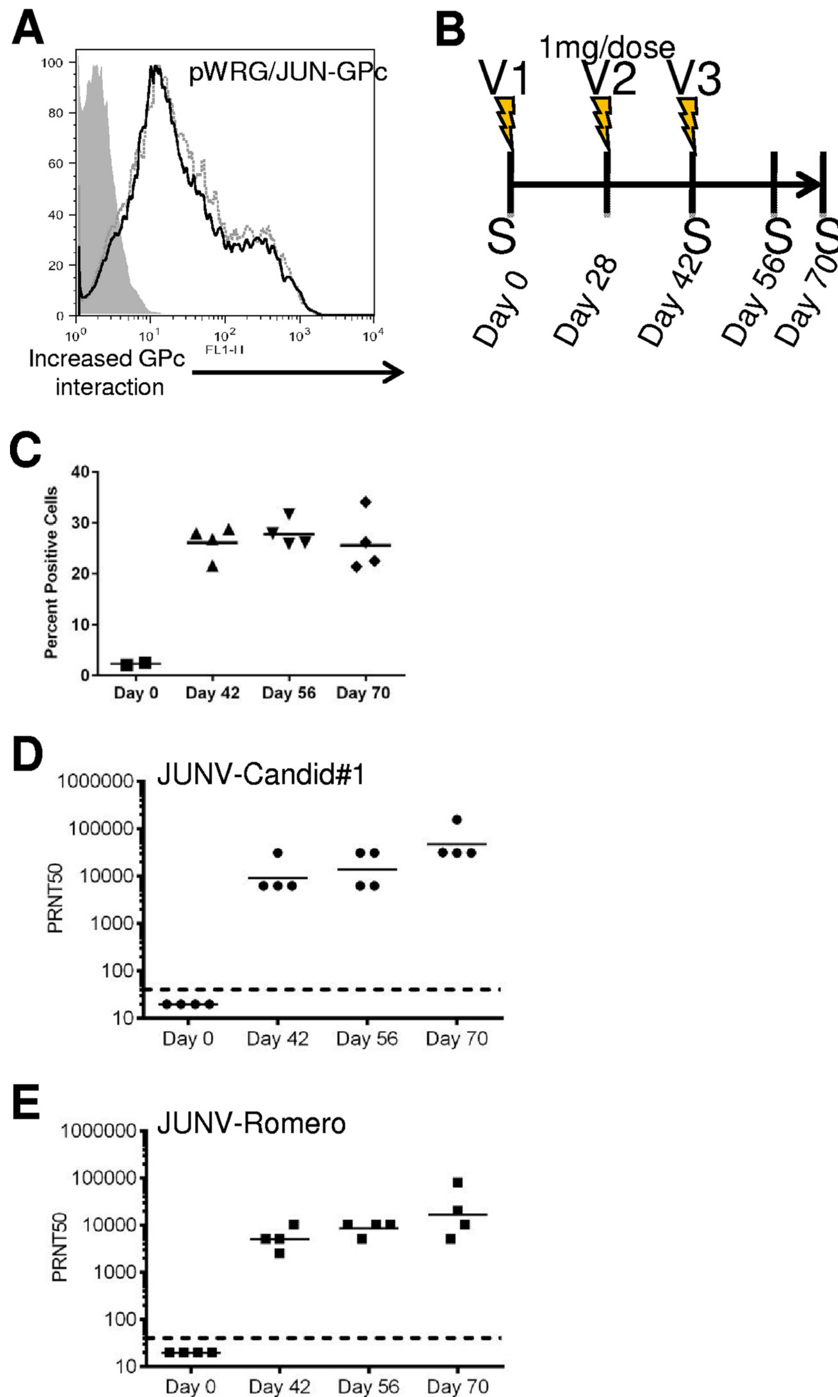


FIG 1 Generation of anti-JUNV glycoprotein antibodies in rabbits by using i.m. EP. (A) 293T cells were transfected with pWRG/JUN-GPc(opt) and then incubated with anti-JUNV GPc antibody MAb-GB03 (solid black line) or MAb-QC03 (dashed gray line) or the nonspecific control antibody MAb-2G11 (gray shaded area). Cells were subsequently stained with an anti-mouse–Alexa Fluor 488 secondary antibody and analyzed on a flow cytometer. Ten thousand cells were counted per sample, and data were plotted with FlowJo software. (B) Schematic outlining the vaccination of rabbits with pWRG/JUN-GPc(opt). Sera were collected from vaccinated rabbits on days marked with an "S." Lightning bolts indicate days of vaccination. (C) 293T cells were transfected as described for panel A, except that cells were incubated with rabbit serum collected before (day 0) or after (day 42, 56, or 70) vaccination. The percentages of positive cells were calculated based on values obtained using negative-control serum. (D) JUNV strain Candid#1 was incubated with serially diluted rabbit antisera, and plaque formation was assayed on Vero cell monolayers by neutral red staining. $PRNT_{50}$ GMTs were calculated based on the plaque formation of virus incubated with the negative-control rabbit antibody. (E) JUNV strain Romero $PRNT_{50}$ GMTs were determined as described for panel D. Dashed lines indicate the limit of detection for the PRNT assay.

performed in CDC-certified BSL4 containment facilities. Animals meeting criteria were humanely euthanized.

Ethics. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals* (35). The facilities where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Statistical analysis. Weight loss significance was determined using two-way analysis of variance (ANOVA) with the Bonferroni correction. Survival statistics utilized the log rank test. The statistical significance of PRNT titers was determined using unpaired two-tailed Student's *t* test. Significance levels were set at P values of <0.05. All analyses were performed using Prism software.

RESULTS

Rabbits vaccinated by intramuscular EP, using plasmid DNA encoding JUNV GPc, produce neutralizing antibodies. An optimized full-length glycoprotein gene based on the JUNV XJ13 sequence with a change at position 208 (P $\rightarrow$ L) was synthesized *de novo* and cloned into the pWRG7077 DNA vaccine expression vector to make pWRG/JUN-GPc(opt). Prior to vaccination studies, protein expression from pWRG/JUN-GPc(opt) was confirmed by flow cytometry. Anti-JUNV glycoprotein-specific MAbs (21) interacted with the surfaces of transfected 293T cells compared to a nonspecific control MAb, demonstrating that pWRG/JUN-GPc(opt) produced an authentic JUNV glycoprotein (Fig. 1A). Next, four rabbits were vaccinated with pWRG/JUN-GPc(opt) three times at 4-week intervals, using 1 mg of plasmid DNA per vaccination, by use of an i.m. EP device (Fig. 1B). Sera were all positive for anti-GP antibodies after the second (day 42) and third (days 56 and 70) vaccinations, as assessed by flow cytometry (Fig. 1C). Rabbit glycoprotein-specific antisera (1:100 dilution) also interacted with Vero cells infected with JUNV strains Candid#1, XJ13, and Romero by immunofluorescence assay (data not shown). The capacity of antibodies to neutralize JUNV was assessed by PRNT. Antisera from all four rabbits neutralized JUNV strain Candid#1. After the second vaccination, the $PRNT_{50}$ GMT was 13,975 (Fig. 1D). This titer increased after the third vaccination, and by day 70, the GMT was 46,915, with one animal (animal 98) producing a $PRNT_{50}$ response of $>$100,000. Serum from each rabbit also neutralized JUNV strain Romero, with a $PRNT_{50}$ GMT of 17,222 and $PRNT_{80}$ GMT of 5,120 on day 70 (Fig. 1E). These findings demonstrated that it was possible to produce high-titer neutralizing antibodies against JUNV in rabbits by using pWRG/JUN-GPc(opt) delivered by i.m. EP.

Production of GP1- and GP2-specific antibodies in rabbits vaccinated with the precursor JUNV glycoprotein. We were interested in determining if antibodies targeting both GP1 and GP2 were produced in rabbits vaccinated with pWRG/JUN-GPc(opt). To this end, we designed a flow-based assay by individually fusing the JUNV GP1 and GP2 molecules to the C-terminal end of a cell-binding protein (CBP) derived from the orthopoxvirus type I interferon decoy receptor (31; J. W. Golden, unpublished findings). This novel technique circumvents the need to incorporate transmembrane anchoring domains because target antigens are secreted and this exogenous protein binds to the surfaces of mammalian cells. When cells are mixed with culture supernatant containing the target antigen fused to CBP, there is nearly 100% labeling of cell populations with the target antigen, which in this case was JUNV GP1 and GP2 molecules. Clarified medium from 293T cells transfected with a gene encoding the chimeric protein CBP-GP1 or CBP-GP2 was incubated with nontransfected 293T cells. Cells decorated with either CBP-GP1 or CBP-GP2 were then incubated with antisera from all four vaccinated rabbits or with prevaccination sera (negative control), stained with secondary anti-rabbit fluorescent antibodies, and analyzed by flow cytometry. All four rabbits developed antibodies that reacted with both the CBP-GP1 and CBP-GP2 proteins (Fig. 2), indicating that the polyclonal pool from each rabbit contained antibodies to both GP1 and GP2.

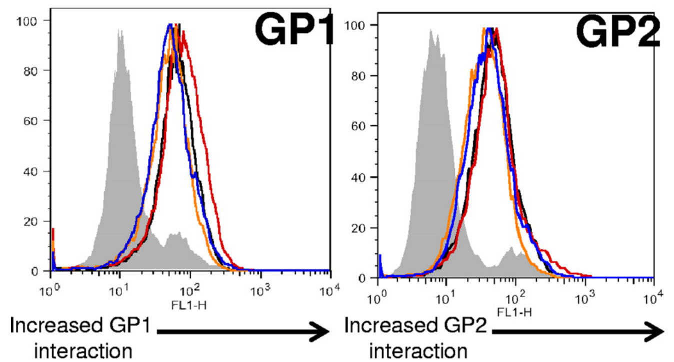
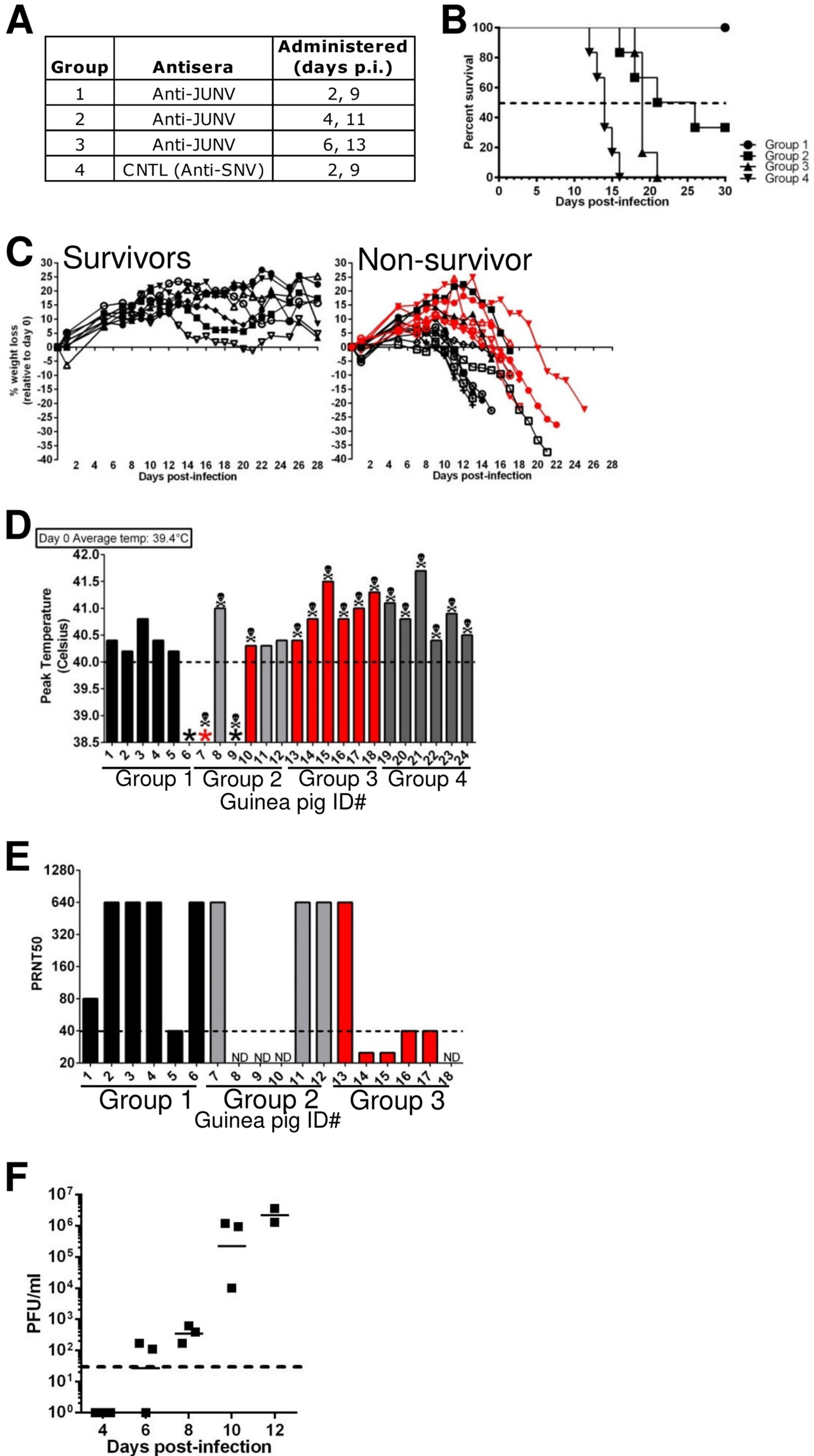


FIG 2 Determining the presence of GP1- and GP2-specific antibodies in sera from vaccinated rabbits. Soluble CBP-GP1 and CBP-GP2, produced by transfection of 293T cells, were bound to the surfaces of fresh 293T cells. These GP1- and GP2-decorated cells were then incubated with sera from vaccinated rabbits. To detect anti-GP1 and anti-GP2 antibodies, the cells were stained with an anti-rabbit–Alexa Fluor 488 secondary antibody and analyzed on a flow cytometer. Ten thousand cells were counted per sample, and data were plotted with FlowJo software. Each colored line represents one of the four rabbits. Normal rabbit serum functioned as a negative control (gray shaded area).

Anti-JUNV glycoprotein antiserum administered postchallenge protects guinea pigs from JUNV lethal challenge. The protective efficacy of DNA vaccine-produced antibodies administered postchallenge was assessed in the Hartley guinea pig model. Prior to infection studies, the half-life of rabbit anti-JUNV GPc antisera in guinea pigs was determined to be 7 days (data not shown). Next, 24 guinea pigs randomized into four groups of six were infected with JUNV strain Romero by the i.p. route. Groups 1 to 3 were injected s.c. with 15,000 TU/kg of rabbit anti-JUNV GPc antiserum, starting on days 2, 4, and 6 postchallenge, respectively. Seven days after the initial dose (day 9, 11, or 13), a second dose of antiserum was administered to the respective groups. As a negative control, group 4 animals were injected with rabbit antiserum targeting Sin Nombre virus (SNV). This control antiserum was produced using methods identical to those that produced the anti-JUNV antiserum (28). All negative-control animals became febrile (temperature of $>$40.0°C) by day 7 and started losing weight after day 8, succumbing to infection with a mean time to death (MTD) of 14.0 days (Fig. 3). In marked contrast, no weight loss occurred in group 1 animals receiving immune sera starting on day 2, and all survived infection. Differences in weight loss for group 1 were statistically significant versus control animals starting on day 10 (two-way ANOVA; $P < 0.05$). Group 2 animals receiving antiserum starting on day 4 lost weight starting between days 10 and 14. Compared to that of the control group, group 2 weight loss was delayed and was statistically different starting on day 9 (two-way ANOVA; $P < 0.05$). Two animals in group 2 developed late-stage paralysis, starting on days 18 and 21, and



Group	Antisera	Administered (days p.i.)
1	Anti-JUNV	2, 9
2	Anti-JUNV	4, 11
3	Anti-JUNV	6, 13
4	CNTL (Anti-SNV)	2, 9

TABLE 1 Summary of results for animals given anti-JUNV antiserum following JUNV challenge[c]

Animal no.	Start day of antiserum treatment	Day of death	Serum titer (PFU/ml)	NP positive[a]	GP positive[a]	Paralysis (day of paralysis onset)[b]
1	2	NA	<10	++	+	N
2	2	NA	<10	++	+	N
3	2	NA	<10	+++	+	N
4	2	NA	<10	++	+	N
5	2	NA	<10	++	+	N
6	2	NA	<10	++	+	N
7	4	21	<10	+++	+	Y (21)
8	4	18	ND	ND	ND	N
9	4	16	ND	ND	ND	N
10	4	26	ND	ND	ND	Y (18)
11	4	NA	<10	++	+	N
12	4	NA	<10	++	+	N
13	6	18	<10	+	+	Y (16)
14	6	18	2.0×10^2	−	−	Y (18)
15	6	21	1.4×10^3	++	+	Y (21)
16	6	18	<10	++	+	Y (18)
17	6	18	<10	+	+	Y (18)
18	6	18	ND	ND	ND	Y (16)
19	CNTL	15	ND	ND	ND	N
20	CNTL	12	ND	ND	ND	N
21	CNTL	14	ND	ND	ND	N
22	CNTL	14	ND	ND	ND	N
23	CNTL	16	ND	ND	ND	N
24	CNTL	14	ND	ND	ND	N

[a] 293 cells were transfected with either an NP- or GP-expressing plasmid and incubated with the indicated guinea pig serum followed by an anti-guinea pig secondary antibody conjugated to Alexa Fluor 488. The percentage of positive cells was determined using flow cytometry and FlowJo software. +, 2 times the background; ++, 3 times the background; +++, >4 times the background; −, negative result.
[b] Paralysis onset began uniformly with hind limb paralysis. Y, paralysis present; N, no paralysis.
[c] $PRNT_{50}$ assays were performed using JUNV strain Romero (challenge virus). NA, not applicable; ND, not done because the animal succumbed to disease before samples could be taken; CNTL, control.

were euthanized (Table 1). The MTD for this group was 23.5 days, and only two animals (33%) survived infection. This delay to death was significant compared to the MTD for control animals (log rank test; $P = 0.0012$). Treatment starting on day 6 (group 3) failed to protect animals from lethality but did extend the MTD to day 19.0, which was statistically significant versus that for control animals (log rank test; $P = 0.0005$). Each animal in group 3 developed paralysis beginning between days 16 and 21 (Table 1). One animal died (day 18) and five were euthanized between days 16 and 21. Similar to the group 2 result, there was a significant delay in weight loss compared to that of control animals starting on day 12 (two-way ANOVA; $P < 0.05$). Hyperthermia was observed in all guinea pigs treated with JUNV-specific antiserum. However, peak fever levels were delayed in animals receiving antibody compared to control guinea pigs (Fig. 3D and data not shown).

Sera from euthanized (see Table 1 for time points) or surviving (day 30) animals were analyzed for anti-nucleoprotein antibody. With one exception (animal 14), animals that survived challenge or were euthanized due to paralysis had antibodies against JUNV NP (Table 1). We also investigated if neutralizing and/or binding anti-glycoprotein antibody was produced in infected guinea pigs. Neutralizing antibodies were detected in the sera of most surviving or euthanized animals, with the exception of two animals from group 3 (animals 14 and 15) (Fig. 3E). The highest neutralizing titers were observed in the three surviving animals from group 3 ($PRNT_{50}$ GMT of 640). $PRNT_{50}$ GMTs for groups 1 and 3 were 285 and 40, respectively. Because the neutralizing antibody could have been residual passively transferred rabbit antibody, we investigated if guinea pigs had guinea pig-specific anti-glycoprotein antibodies by flow cytometry. Guinea pig-specific anti-glycoprotein antibodies were detectable in all samples, with the exception of one (from animal 14) (Table 1). We next investigated if any infected guinea pigs had detectable viremia. Indeed, the two animals in group 3 lacking $PRNT_{50}$ titers had detectable viremia, with titers of 2×10^2 PFU/ml and 1.4×10^3 PFU/ml on days 18 and 21, respectively. Viremia was undetectable in all other animals. Overall, these findings indicate that 15,000 TU/kg of DNA-vaccinated rabbit antiserum targeting JUNV glycoprotein, delivered starting between 2 and 6 days after challenge, can prevent or delay lethal disease in guinea pigs but that complete protection occurs only when treatment is initiated within 2 days after challenge.

We investigated the kinetics of viremia in guinea pigs infected with JUNV. Fifteen animals were challenged with JUNV strain Romero by the i.p. route. On days 4, 6, 8, 10, and 12, three animals were bled and serum viremia was determined by plaque assay. Viremia was undetectable until day 6, when titers were detected in two of three animals, with a GMT of 27 PFU/ml (Fig. 3F). Titers rose precipitously thereafter, with a day 12 GMT of 2.2×10^6 PFU/ml (one animal in the day 12 group died before serum could be collected). Thus, in guinea pigs, JUNV viremia is detectable starting on day 6 and peaks at the MTD, at $\sim 10^6$ PFU/ml.

Generation of neutralizing antibodies against other human-pathogenic South American arenaviruses by using i.m. EP in rabbits. We next produced neutralizing antibodies against two other South American arenaviruses. DNAs encoding the full-length glycoproteins of MACV strain Carvallo [plasmid pWRG/MAC-GPc(opt)] and GTOV strain INH9555 [plasmid pWRG/

FIG 3 Rabbit antiserum protects guinea pigs from lethal disease when administered postinfection. (A) Experimental layout. Guinea pigs were infected i.p. with 2,000 PFU of JUNV. Animals were injected s.c. with anti-JUNV or negative-control antiserum diluted in PBS at the indicated time points. (B) Survival was plotted for up to 30 days postinfection by using Prism software. The dashed line indicates 50% survival. (C) Individual weights were calculated based on day 0 starting weights. The left panel shows weights of surviving animals. The right panel shows weights of nonsurvivors. (D) Maximum temperatures were graphed over a 28-day period. A skull-and-crossbones symbol indicates an infection nonsurvivor. An asterisk represents an animal whose temperature implant was reading erroneously. The dashed line indicates the normal maximum temperature for guinea pigs. (E) $PRNT_{50}$ titers against JUNV strain Romero of sera from euthanized (see Table 1 for days of death) and surviving (day 30) animals postchallenge were analyzed as described in the legend to Fig. 1. The dashed line indicates the limit of detection of the PRNT assay. For all panels, red data indicate an animal that developed paralysis. ND, not done because serum was unavailable for analysis. (F) Viremia in infected guinea pigs was determined at the indicated time points by plaque formation on Vero cell monolayers. The dashed line indicates the limit of detection of the assay. Three guinea pigs were examined per time point, with the exception of day 12, when only two animals were screened for viremia. Note that the graph is on a log scale.

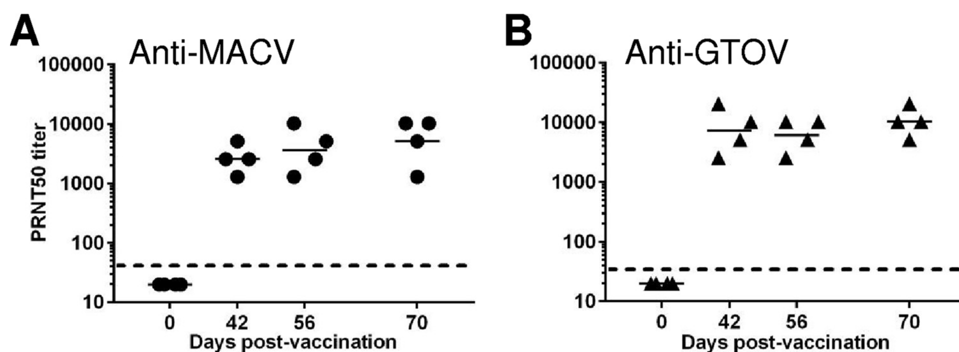


FIG 4 Generation of anti-glycoprotein antibodies targeting MACV and GTOV. Neutralization of MACV (A) and GTOV (B) was tested and graphed in the same manner as that described in the legend to Fig. 1. The dashed lines indicate the limit of detection of the PRNT assay.

GTO-GPc(opt)] were constructed. Two groups of four rabbits per target were vaccinated three times at 4-week intervals by i.m. EP with the MACV or GTOV GPc DNA vaccine. Anti-MACV antiserum neutralized MACV by the second vaccination, and the titer increased slightly after the third vaccination, with a final $PRNT_{50}$ GMT of 5,120 (Fig. 4A). Similarly, anti-GTOV antiserum neutralized GTOV after the second vaccination, with a final $PRNT_{50}$ GMT of 10,240 (Fig. 4B). These data demonstrated that DNA vaccination can be used to produce highly potent neutralizing antibodies against multiple NW arenaviruses.

Cross-binding and neutralization of glycoprotein-specific antibodies targeting South American arenavirus GPs. We evaluated antibodies produced against JUNV, MACV, and GTOV to determine the levels of cross-binding and cross-neutralization. Cross-binding of antibodies targeting the glycoproteins of JUNV, MACV, and GTOV was examined by ELISA, using VSVΔG particles pseudotyped with glycoproteins from the targeted arenaviruses (JUNV, MACV, and GTOV) as antigens. Antisera from all four rabbits vaccinated with MACV-GPc but not GTOV-GPc bound the JUNV glycoprotein, with a $\log_{10}$ GMT of 3.2 (Fig. 5A). This titer was lower than the $\log_{10}$ GMT of 4.7 for homologous antisera targeting JUNV. Anti-JUNV glycoprotein antisera from all four rabbits interacted with the MACV antigen, with a $\log_{10}$ GMT of 2.0. This was 2 orders of magnitude lower than that for homologous antisera targeting MACV, which had a $\log_{10}$ GMT of 4.0. Anti-GTOV glycoprotein antibodies from a single rabbit cross-reacted with the MACV glycoprotein, with a $\log_{10}$ titer of 2.0, but the overall $\log_{10}$ GMT for this group was 1.2. All JUNV and two MACV glycoprotein antiserum samples cross-reacted with the GTOV antigen, with $\log_{10}$ GMTs of 2.0 and 1.5, respectively. These titers were much lower than that of homologous GTOV antiserum, which produced a $\log_{10}$ titer of 5.0. These data revealed that some cross-antibody binding existed between antibodies from JUNV, MACV, and GTOV DNA-vaccinated rabbits. This cross-binding was highest between MACV and JUNV samples.

To determine if cross-binding corresponded with cross-neutralization, we investigated the ability of antibodies to cross-neutralize VSVΔG particles pseudotyped with glycoproteins from JUNV, MACV, and GTOV. All rabbits showed a detectable neutralization response against pseudotyped particles expressing the homologous glycoprotein (Fig. 5B). The $PsVNA_{50}$ GMTs were similar to those against authentic virus (Fig. 1C and 4). Of the four anti-MACV glycoprotein samples that cross-bound with the JUNV glycoprotein, only those from two rabbits (animals 99 and 101) neutralized particles pseudotyped with the JUNV glycoprotein, with $PSVNA_{50}$ titers of 2,171 and 419, respectively. These titers were notably lower (>10-fold) than those of anti-JUNV antibodies. No cross-neutralizing responses were detected using anti-GTOV antiserum against particles pseudotyped with the JUNV glycoprotein. Only one rabbit with antibodies targeting JUNV had a detectable neutralizing response against particles pseudotyped with the MACV glycoprotein. The $PSVNA_{50}$ titer for this rabbit was 148, which is >10-fold lower than that of anti-MACV antibodies. Anti-GTOV glycoprotein antiserum failed to cross-neutralize particles pseudotyped with the MACV glycoprotein. There was no cross-neutralization observed with anti-JUNV or anti-MACV antiserum against particles pseudotyped with the GTOV glycoprotein. Because the highly sensitive pseudovirion neutralization assay revealed some cross-neutralization between anti-JUNV and anti-MACV samples, the capacity of these serum samples to neutralize authentic virus was tested by PRNT (Fig. 5C). The two rabbit samples from animals vaccinated against the MACV glycoprotein that neutralized JUNV pseudotyped particles were also able to cross-neutralize JUNV strain Candid#1, with $PRNT_{50}$ titers of 40 and 160. None of the serum samples targeting the JUNV glycoprotein were able to neutralize MACV. These findings demonstrated that very limited cross-neutralization occurred between anti-glycoprotein antibodies targeting JUNV, MACV, and GTOV.

Purified IgGs targeting the GPc polypeptides of JUNV, MACV, and GTOV protect guinea pigs from lethal JUNV and GTOV challenge. A long-term goal of this research is to develop proof of concept for a neutralizing antibody-based product capable of protecting against multiple NW arenaviruses. Because little cross-neutralization existed between rabbit antibodies targeting JUNV, MACV, and GTOV glycoproteins, we produced a cocktail of purified IgG antibodies derived from rabbits vaccinated with the three different DNA vaccines and determined if it could protect guinea pigs against JUNV, MACV, and GTOV. The $PRNT_{80}$ titers of the purified IgG fractions were 10,240, 10,240, and 40,960 against JUNV, MACV, and GTOV, respectively. The antibodies were combined such that the cocktail could be administered at a dose of 7,500 TU/kg/target in PBS. The protective efficacy of this cocktail was tested in guinea pigs infected with JUNV strain Romero, GTOV, or MACV at 2,000 PFU/animal by the i.p. route. Infected guinea pigs were treated with the cocktail on day 2 and day 7 postinfection. For JUNV, a negative control was used that

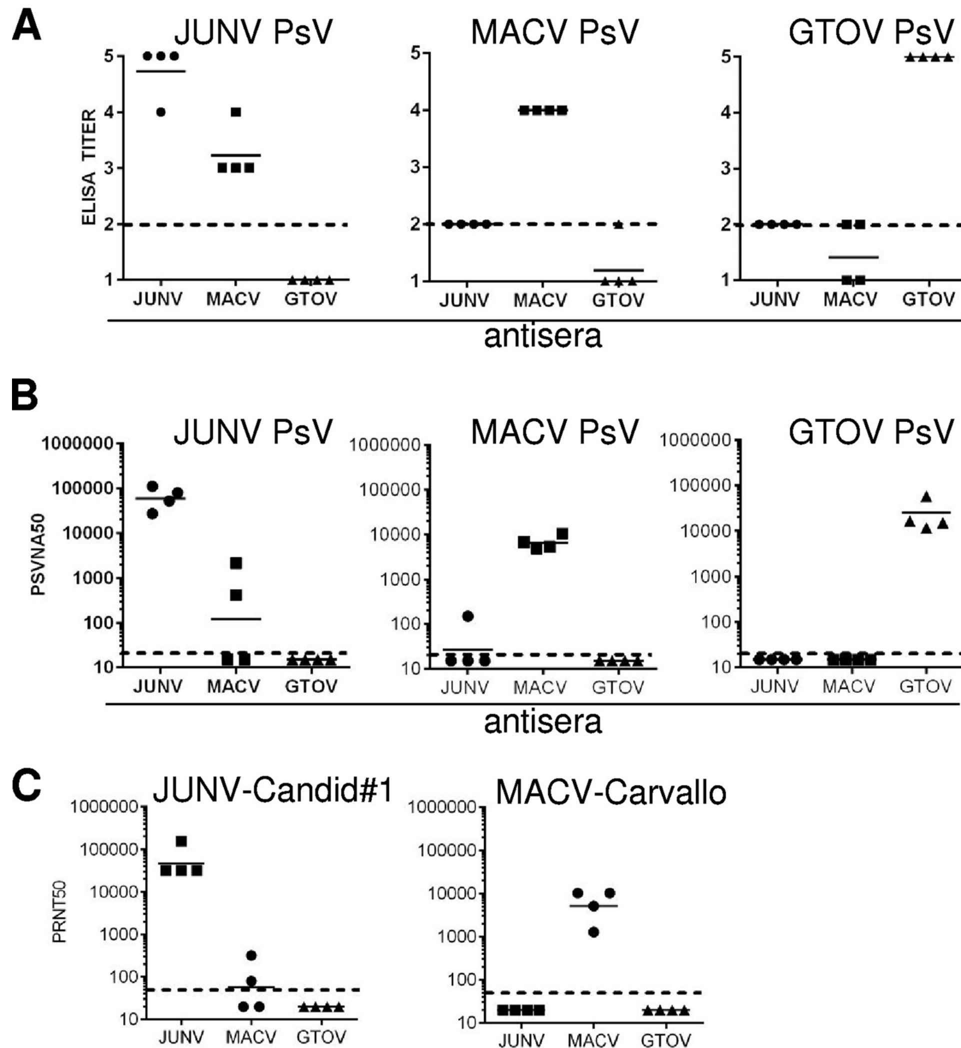


FIG 5 Cross-binding and -neutralization of glycoprotein-specific antibodies targeting South American arenavirus GPs. (A) The indicated PsV were incubated with serially diluted antiserum samples, followed by an HRP-conjugated goat anti-rabbit secondary antibody and TMB substrate. The endpoint titer was determined as the highest dilution with an optical density greater than the mean optical density for serum samples from negative-control wells plus 3 standard deviations. (B) Sera from rabbits vaccinated with either pWRG/JUN-GPc(opt), pWRG/MAC-GPc(opt), or pWRG/GTO-GPc(opt) were incubated with VSVΔG particles pseudotyped with the indicated glycoproteins, and neutralization was determined as described in Materials and Methods. (C) Cross-neutralization of sera targeting pWRG/JUN-GPc(opt) or pWRG/MAC-GPc(opt) were tested in the same manner as that described in the legend to Fig. 1. $PRNT_{50}$ titers were graphed using Prism. For all panels, the dashed line indicates the limit of detection for the respective assay.

consisted of IgG antibody purified from a rabbit vaccinated with an SNV DNA vaccine (28). For GTOV and MACV, PBS was used as the negative control. Control animals infected with JUNV strain Romero became febrile by day 8 (data not shown) and began to lose weight starting on day 9 (Fig. 6A). All animals in the control group developed lethal disease by day 15, with an MTD of 14 days. In sharp contrast, no JUNV-challenged animals receiving the cocktail showed evidence of weight loss or fever. Differences in weight loss between the treated and control groups were statistically significant starting on day 11 (two-way ANOVA; $P < 0.05$). Sera taken on day 25 from guinea pigs receiving the cocktail of antibodies had a JUNV neutralizing antibody $PRNT_{50}$ GMT of 259, suggesting that they produced immune responses against the virus.

Negative-control guinea pigs infected with GTOV began to lose weight starting on day 7, and this weight loss increased on day 9 (Fig. 6B). Hyperthermia was observed in all control animals by day 9 (data not shown), and all animals in this group succumbed to infection by day 17, with an MTD of 15 days. In contrast, GTOV-infected guinea pigs treated with the cocktail did not lose weight, and differences in weight loss were statistically significant versus the control group starting on day 10 (two-way ANOVA; $P < 0.05$). Three of six guinea pigs receiving the cocktail developed an elevated temperature (~40.0°C) on day 7 that dropped to a normal level on day 8 (data not shown). In two of these animals, elevated temperatures returned on days 21 and 22 and subsided to normal on day 23. We were unable to detect postchallenge GTOV neutralizing antibodies in challenged guinea pigs that received the antibody cocktail (data not shown).

Guinea pigs infected with MACV strain Carvallo did not display signs of illness, including elevated temperature or weight loss (data not shown). However, sera taken at 30 days postchallenge

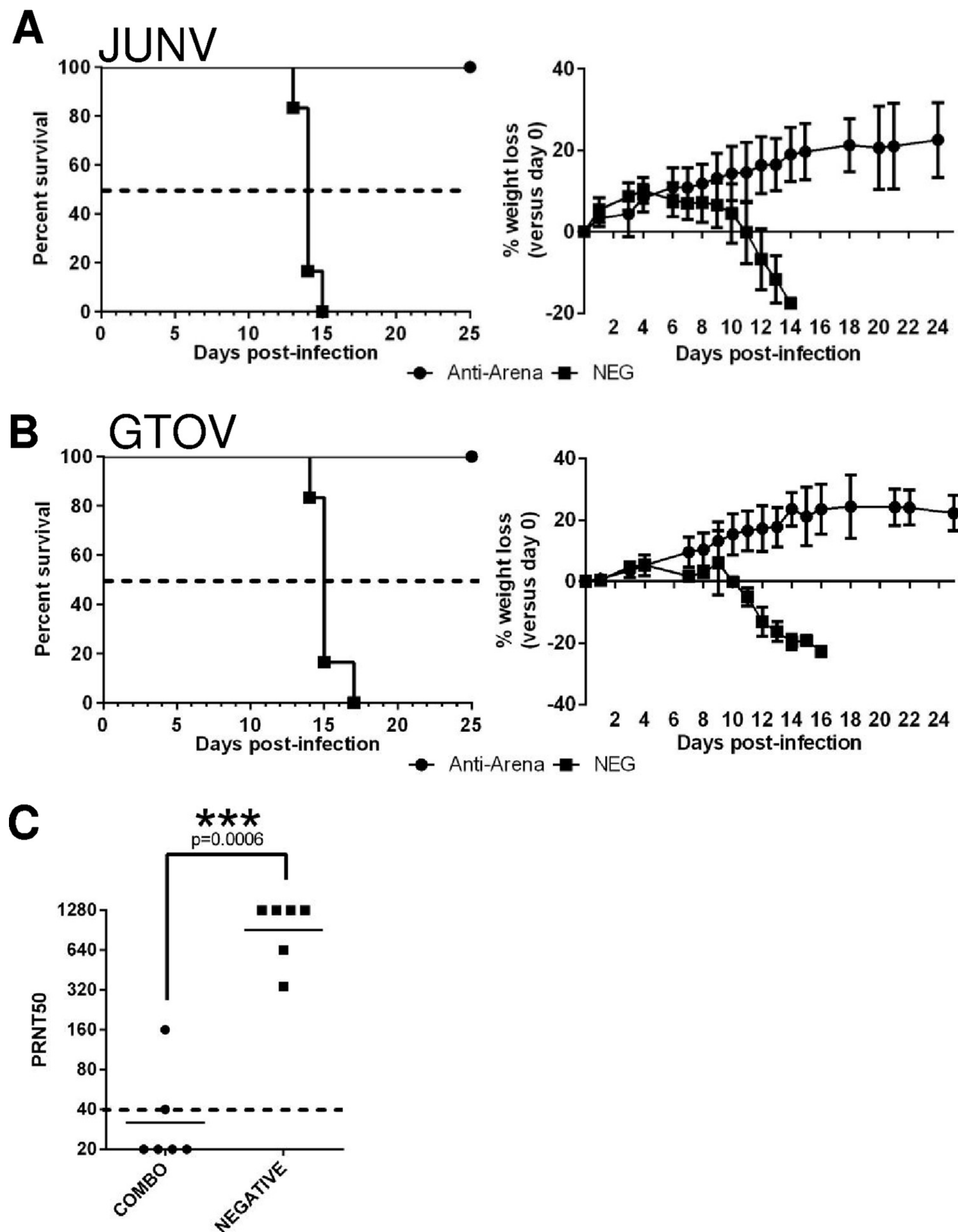


FIG 6 A cocktail of purified IgG antibodies targeting JUNV, MACV, and GTOV glycoproteins protects guinea pigs from infection. (A) Guinea pigs were injected i.p. with 2,000 PFU of JUNV. On days 2 and 7, the indicated animals were injected s.c. with the antibody cocktail (7,500 TU/kg/target) diluted in PBS. The control group (NEG) received a purified anti-SNV glycoprotein IgG antibody. Survival and weights were plotted as described in the legend to Fig. 3. (B) Same as panel A. However, GTOV was used as the challenge virus. The control in this experiment was PBS. The dashed lines indicate the 50% survival mark. (C) MACV was used as the challenge virus. The control in this experiment was PBS. Sera from 30 days postchallenge were analyzed for anti-MACV neutralizing activity. $PRNT_{50}$ titers were calculated as described in the legend to Fig. 1. Statistical significance ($P < 0.05$) was calculated using the *t* test and Prism software. The dashed line indicates the limit of detection for the PRNT assay.

revealed a significant difference in postchallenge anti-MACV neutralizing antibody responses. Specifically, control animals had significantly (*t* test; $P = 0.006$) higher levels of MACV neutralizing antibodies than those that received the cocktail (Fig. 6C). The GMTs for the group receiving the cocktail and the control group were 32 and 900, respectively. This result was confirmed by PsV assay, with $PSVNA_{50}$ GMTs of 23 and 812 for irradiated sera from challenged guinea pigs. These results demonstrated that the cocktail was capable of protecting against JUNV and GTOV lethality. In addition, the cocktail suppressed neutralizing antibody responses against MACV, suggesting that it neutralized the virus before it could effectively stimulate a humoral response.

Vaccination of rabbits with a combination DNA vaccine targeting glycoproteins from JUNV, MACV, GTOV, and SABV produces neutralizing antibodies against each target. We next examined if a combination vaccine targeting multiple arenaviruses could be used to produce a neutralizing antibody response against each virus. A group of eight rabbits were vaccinated with a

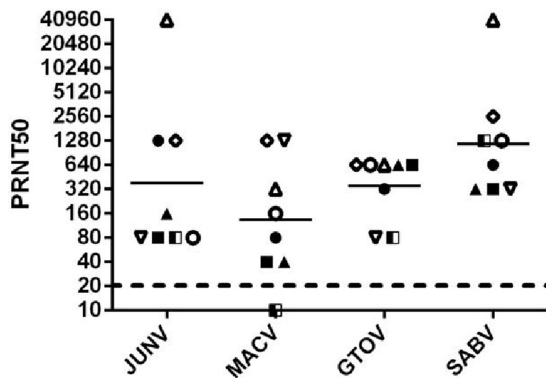


FIG 7 Vaccination of rabbits with DNAs encoding the GPc polypeptides from JUNV, MACV, GTOV, and SABV. Neutralization of JUNV (strain Romero), MACV, GTOV, and SABV was tested in the same manner as that described in the legend to Fig. 1C. The dashed line indicates the limit of detection for the PRNT assay.

combination of the JUNV, MACV, and GTOV GPc DNA vaccines and an optimized construct encoding the SABV GPc [pWRG/SAB-GPc(opt)]. Rabbits were vaccinated twice at 3-week intervals with a needle-free DSJI device by the i.m. route, using 0.5 mg of DNA per dose (0.125 mg/target) in 0.5 ml of PBS. Sera from vaccinated animals were analyzed for neutralizing antibodies 5 weeks after the second vaccination. Vaccinated rabbits developed neutralizing antibody responses against each target (Fig. 7). The highest responses were against SABV, with a $PRNT_{50}$ GMT of 1,174. The weakest responses were against MACV, with a $PRNT_{50}$ GMT of 135. Responses against JUNV and GTOV were 380 and 320, respectively. However, no neutralizing responses were statistically significantly different (one-way ANOVA; $P < 0.05$), indicating that no targets were more immunogenic than the others. These findings demonstrate that combined vaccines targeting multiple glycoproteins can elicit a neutralizing antibody response against each target.

DISCUSSION

Production of neutralizing antibodies targeting arenavirus glycoproteins by using DNA vaccine technology. Arenaviruses are an important family of emerging and reemerging zoonotic agents. In infected humans, convalescent-phase serum is a potent means of postexposure treatment (8, 15). Despite this being an effective treatment option, research on novel approaches to produce potent polyclonal antibody-based therapies have been lacking. In this study, we demonstrated that DNA vaccine technology can be used to produce potently neutralizing anti-arenavirus antibodies that are protective in animal models and exclusively target the full-length GPc precursor gene product. The prototype virus used in these studies was JUNV. There are no serotypes among JUNV strains; however, strain variability in neutralizing titers is common, and antibodies neutralize homologous viruses best, with various degrees of potency against heterologous strains (36). Accordingly, we purposefully generated a full-length JUNV GPc gene encoding a slightly modified XJ13 sequence not completely homologous to that of either JUNV strain Candid#1 (five amino acid differences) or strain Romero (two amino acid differences). Indeed, we observed differences in neutralizing titers when we examined different JUNV strains. The highest titers were against Candid#1 ($PRNT_{50}$ titer = 46,915), which remarkably had the most amino acid differences compared to the vaccine immunogen. For comparison, titers against strain Romero were ~3-fold lower ($PRNT_{50}$ titer = 17,222). Several studies have investigated the potency of anti-JUNV neutralizing antibodies and allow for a comparison with our findings (13, 21, 36–39). These studies mainly employed whole virus as an antigen, generally via a vaccination with an attenuated virus followed by infection with a virulent virus in the same animals. Our study compares well with that of Candurra et al., in which anti-JUNV neutralizing antibody responses were extensively studied in rabbits (36). The highest $PRNT_{50}$ titer generated in that study was 11,171, against the targeted (homologous) JUNV strain IV4454. Neutralizing titers against heterologous strains varied widely, from 2,021 to 8,681. Studies exclusively targeting the JUNV glycoprotein have also been performed by using a fractionated envelope protein or vaccinia virus and Venezuelan equine encephalitis virus vectors (40, 41). However, most of these studies used guinea pigs as target animals and were focused on producing active vaccines. In each of the aforementioned studies, lower neutralizing antibody titers were produced than those achieved in our study. For example, in one recent study, the $PRNT_{50}$ GMT against JUNV strain Candid#1 was ~30 (41), compared to 46,915 in our study (Fig. 1C). We cannot rule out the possibility that the >3-order-of-magnitude difference in titers was due to species-specific factors pertaining to vaccine responses in rabbits versus guinea pigs.

We further demonstrated that neutralizing antibodies could also be produced by vaccinating rabbits with plasmid DNAs encoding MACV and GTOV full-length GPc polypeptides. A paucity of published data regarding neutralizing titers against these viruses prevents any comparisons. $PRNT_{50}$ titers against MACV and GTOV were lower than that against JUNV, by 3.4- and 1.6-fold, respectively. This may reflect differences in immunogenicity among the three glycoproteins in rabbits. Indeed, a recent study showed that differences in neutralizing antibody responses against NW and OW arenaviruses correlate with the glycosylation status of GP1 (42). Consistent with this finding, JUNV GP1 is the least glycosylated protein compared to those of GTOV and MACV, and it produces the highest neutralizing antibody response. We also produced a multivalent antibody product that neutralized four NW arenaviruses (JUNV, MACV, GTOV, and SABV) by combining plasmids encoding the GPc from each target and vaccinating rabbits i.m. with a needle-free DSJI device. PRNT titers produced by the combination vaccine were lower than those for animals vaccinated with the individual targets by i.m. EP. This was likely due to the lower dose of DNA used per target in the combination vaccines (0.125 versus 1 mg/target) and/or the enhanced efficacy of i.m. EP. Importantly, there was no evidence of immune interference between the four arenavirus DNA vaccines, nor was the dominance of a response against any one of the four viruses observed. It is possible that if higher neutralization titers were generated by additional boosts or the use of i.m. EP, the dominance of a particular response might be observed, possibly correlating with the aforementioned differences in arenavirus glycoprotein glycosylation. Overall, this is the first demonstration that a multivalent vaccine can produce antibodies neutralizing four distinct arenaviruses, and it supports the concept of a multivalent arenavirus DNA vaccine.

Anti-glycoprotein antibodies poorly cross-neutralize different South American arenaviruses. The serological relatedness of

arenaviruses has been investigated extensively through the use of panels of monoclonal antibodies (21, 43–45). The findings indicate that antibodies targeting NP are most broadly cross-reactive, with interactions reported between the more distant NW and OW complexes (46). Owing to a highly conserved epitope at positions 368 to 382, GP2 antibodies targeting lymphocytic choriomeningitis virus (LCMV) and LASV also cross-interact with NW arenavirus species (44, 45). In contrast, GP1 is more polymorphic (47), and as a result, antibodies targeting GP1 are less cross-reactive. No cross-neutralizing activity has been observed between NW and OW arenaviruses (48). Only limited neutralizing activity has been reported between NW species, in particular between the closely related JUNV and Tacaribe virus (TACV) species (39, 48). Consistent with this, our study demonstrated that more cross-binding than cross-neutralization existed between rabbit anti-GPc antibodies (Fig. 5). We suspect that the ability of anti-JUNV, -MACV, and -GTOV GPc antibodies to bind heterologous GPc in ELISA mostly correlates with the highly conserved nature of the GP2 epitope. Two amino acid differences (R374K and I378V) distinguish this epitope between GTOV and MACV/JUNV (data not shown). These differences may explain the lack of binding in ELISA between anti-GTOV GPc antibodies and JUNV and the limited binding of anti-MACV GPc with GTOV GP. Antibodies from some rabbits vaccinated against MACV GPc could cross-neutralize JUNV, albeit to a limited extent. The development of cross-neutralizing antibodies against some NW arenaviruses has been observed previously. Nonhuman primates vaccinated with high doses, but not low doses, of JUNV vaccine strain Candid#1 develop antibodies that cross-neutralize MACV (49). Interestingly, animals receiving low and high doses of Candid#1 are equally protected against MACV, suggesting that detectable levels of prechallenge neutralizing antibodies are not a prerequisite for vaccine-mediated protection. The ability of antibodies targeting the MACV glycoprotein to cross-neutralize JUNV directly correlated with the ability of the polyclonal antisera to bind GP1 (data not shown). Because the amino acid sequences show little homology, it is unclear which region(s) of JUNV GP1 is bound by cross-neutralizing anti-MACV antibodies. We assume that cross-neutralizing antibodies interact with heterologous GP1 based on the tertiary structure of the neutralizing epitope, not on amino acid homology. Unfortunately, titers associated with cross-neutralizing polyclonal sera are too low for these to be useful as a cross-protective therapeutic. Thus, any pan-arenavirus immunotherapeutic will have to be formulated with antibodies specifically targeting each human pathogen of interest.

Protective efficacy of DNA vaccine-produced anti-glycoprotein neutralizing antibody. Human disease caused by South American arenaviruses can be recapitulated in guinea pig and nonhuman primate animal models (50, 51). We used outbred strain Hartley guinea pigs to determine if DNA vaccine-produced neutralizing antibodies could protect a heterogeneous population against lethal viral disease caused by JUNV strain Romero. Although the 50% lethal dose (LD_{50}) in this model is below 1 PFU (13; Golden, unpublished result), we chose a high challenge dose (2,000 PFU) for all arenavirus strains tested to remain consistent with previous studies (13, 41). Complete protection by postchallenge administration of rabbit-produced antibodies was possible only if treatment was initiated by day 2. This time point is before the onset of viremia, which begins on day 6 (Fig. 3F). Antibody therapy did not prevent a host humoral immune response, as surviving animals developed guinea pig-specific anti-NP responses as indicated by flow cytometry (Table 1). These animals also produced guinea pig-specific neutralizing antibody responses against challenge virus (Table 1). Sera from three of six animals receiving antibody starting on day 6 had detectable neutralizing antibody titers, but overall neutralizing responses were much lower than those for animals treated on day 2 or 4. Of the two animals that had no detectable neutralizing antibody responses, one animal had no detectable anti-NP response. We hypothesize that this was related to higher levels of virus replication and/or concomitant impairment of adaptive immune responses due to higher levels of inflammation in animals receiving therapeutic antibody late. This is supported by our finding that viremia started on day 6 and increased substantially in subsequent days (Fig. 3F).

Our findings are consistent with those of Kenyon et al., who extensively examined antibody-mediated protection against JUNV in guinea pigs (13). In their study, sera from vaccinated (attenuated virus) and subsequently infected (virulent virus) guinea pigs were used to protect naive guinea pigs from JUNV strain Romero. In our study, the survival of guinea pigs treated postinfection with 15,000 TU/kg of antibody was nearly identical to that in the Kenyon study on days 2 (100% versus 100%), 4 (33% versus 40%), and 6 (no survival) (data are for our study versus the Kenyon study, respectively). As the antiserum used in the Kenyon study was produced against whole virus, it targeted multiple arenavirus proteins, including NP, GP1, and GP2. Our results expand these findings and demonstrate that anti-GP1 and/or -GP2 antibodies are sufficient for protection against JUNV in the guinea pig model. Studies with LCMV suggested that anti-GP2 antibodies do not contribute to immunoprotection (52). However, it is unclear if antibodies targeting both glycoproteins are critical for protection against NW arenaviruses or if anti-GP1 neutralizing antibodies are indeed the most critical. Nevertheless, this is the first study demonstrating that glycoprotein-specific antibodies can protect against NW arenavirus challenge.

Late-stage encephalitis in antibody-treated guinea pigs was observed in the Kenyon study (13). It is therefore not surprising that some animals in our study also developed a neuropathology, particularly those receiving antibody at late time points. Others have speculated that late-stage neurological symptoms are not antibody-mediated pathologies *per se*, but rather occur because the antibody eliminates virus systemically but fails to block replication in the brain due to a limited capacity to cross the blood-brain barrier (13). Late-stage encephalitis has been observed in JUNV-infected humans receiving antibody therapy (8). However, these symptoms resolve after several weeks, without long-term sequelae. It will be of interest to study guinea pigs that develop encephalitis in this model to more completely understand the molecular mechanism(s) that governs this enigmatic pathology.

A cocktail of neutralizing antibodies protects guinea pigs against multiple arenaviruses. The neutralizing IgG antibody cocktail targeting JUNV, MACV, and GTOV protected 100% of guinea pigs from lethal challenge with JUNV when the animals were treated on day 2, similar to the results for anti-JUNV serum administered alone (Fig. 3 and 6A). JUNV-challenged animals treated with the antiserum or the cocktail of purified antibodies both developed similar convalescent-phase $PRNT_{50}$ antibody titers, suggesting that similar levels of protection were afforded by the cocktail and the antiserum. The dose in the cocktail (7,500 TU/kg) was half that in the antiserum (15,000 TU/kg), indicating

that lower doses of antibody are sufficient for protection against JUNV when treatment is started on day 2. This is further supported by the fact that a dose of 7,500 TU/kg can also protect guinea pigs when given on day −1 (Golden, unpublished observation). However, because the anti-MACV IgG present in the cocktail cross-neutralized JUNV to a limited extent ($PRNT_{80}$ titer = 640), the ability of lower doses of anti-JUNV neutralizing antibody will require further exploration.

The cocktail of purified IgGs also protected guinea pigs against two other South American arenaviruses: GTOV and MACV. Our GTOV findings are consistent with previous studies demonstrating this virus to be 100% lethal in Hartley guinea pigs, with death occurring at 12 to 18 days (53). We expanded on this study and showed that antibodies targeting GTOV can protect against lethality when given by day 2 postchallenge (Fig. 6B). We did not detect convalescent-phase neutralizing antibodies in animals receiving antibodies (data not shown), suggesting that the cocktail provided more complete protection against GTOV than against JUNV. MACV strain Carvallo was not lethal in Hartley guinea pigs, and animals showed no signs of illness (e.g., fever or weight loss). This finding was unexpected given that previous studies have shown the Carvallo strain to be >60% lethal in guinea pigs (39, 54). Despite the lack of lethality, guinea pigs receiving the negative control developed significantly higher levels of convalescent-phase neutralizing antibodies than animals receiving the cocktail (*t* test; $P = 0.006$). This suggests that the cocktail limited MACV infection in guinea pigs, thus limiting the humoral response. Studies are under way to determine the cause of the attenuation. Recent studies have identified another strain of MACV, strain Chicava, to be 100% lethal in Hartley guinea pigs (55). Ongoing studies are examining the protective effect of DNA vaccine-derived antibodies against strain Chicava.

DNA vaccine technology as a means to produce potent immunotherapeutics. Novel arenaviruses pathogenic to humans emerge approximately every 3 years (1), with the most recent being Lujo virus (LUJV) in Africa (56). The development of countermeasures to mitigate the threat of emerging and reemerging arenaviruses would improve public health. Locations of arenavirus outbreaks vary, making it difficult to target relevant populations with a preventative vaccine (57). Because immunotherapeutics are effective against human-pathogenic arenaviruses in a postexposure setting, they may represent the best means of controlling these emerging threats (8–10). Here we demonstrated that DNA vaccine technology can be used to produce anti-arenavirus neutralizing antibodies capable of protection in models of lethal Argentine and Venezuelan hemorrhagic fever. Although we used rabbits in these initial studies, the same approach could be used in standard animal systems used to produce animal-derived polyclonal antibodies to treat intoxication, envenomization, or infection. Traditionally, these animal systems have involved purification of polyclonal antibodies from equine or ovine species hyperimmunized with purified proteins. The major drawback of products made in animal systems is that the antibodies are from a heterologous species. To prevent toxicities associated with administering heterologous species antigens, the antibodies produced in those systems are "despeciated" by removal of the Fc portion. Recently, DNA vaccine technology was combined with transchromosomal (Tc) bovines that produce human IgG (58) to generate candidate immunotherapeutic products targeting hantaviruses (59) and Ebola virus (60). Despeciation of the Tc bovine-derived neutralizing antibodies is not necessary because the product is human IgG. It is notable that development of these candidate human IgG products did not involve the use of human donors or infectious agents. This Tc bovine technology could be used to produce human polyclonal antibodies or, with additional steps, human monoclonal antibodies targeting arenaviruses. For products targeting infectious agents, it will likely be necessary to formulate antibody cocktails rather than single monoclonal antibodies. The use of monoclonal antibody cocktails enhances product potency and mitigates the chance that the infectious agent will evolve around the product. However, the emergence of Ebola virus antibody escape mutants in animals treated with monoclonal antibody cocktails (61) suggests that even combinations of monoclonal antibodies might be insufficient to contain infectious agents with high mutation rates, such as RNA viruses. It is possible that the use of polyclonal antibodies targeting multiple epitopes on the viral glycoproteins might help to prevent the emergence of viruses capable of escaping the inhibitory effects of the product. This may be particularly important for arenaviruses, as the development of neutralizing antibody escape mutants is well documented (62). Our findings in the current study indicate that DNA vaccine technology combined with traditional systems, or novel approaches such as the Tc bovine system, could be used to produce candidate anti-arenavirus neutralizing antibody-based products.

ACKNOWLEDGMENTS

We thank Chris Hammerbeck, Becky Brocato, and the Veterinary Medicine Division for technical assistance.

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army or the Department of Defense.

FUNDING INFORMATION

The Joint Program Executive Office for Chemical and Biological Defense, Chemical Biological Medical Systems, provided funding to John Ballantyne under grant number W9113M-08-1-0008. The Military Infectious Disease Research Program provided a Program Area T grant to Joseph W. Golden, Piet Maes, Steven A. Kwilas, and Jay W. Hooper. The Assistant Secretary of the Army for Acquisition, Logistics, and Technology provided an In-House Laboratory Independent Research grant to Joseph W. Golden. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

1. **Buchmeier MJ, de la Torre JC, Peters CJ.** 2013. *Arenaviridae*, p 1283–1303. *In* Knipe DM, Howley PM, Cohen JI, Griffin DE, Lamb RA, Martin MA, Racaniello VR, Roizman B (ed), Fields virology, 6th ed. Lippincott Williams & Wilkins, Philadelphia, PA.
2. **Charrel RN, Feldmann H, Fulhorst CF, Khelifa R, de Chesse R, de Lamballerie X.** 2002. Phylogeny of New World arenaviruses based on the complete coding sequences of the small genomic segment identified an evolutionary lineage produced by intrasegmental recombination. Biochem Biophys Res Commun **296:**1118–1124. http://dx.doi.org/10.1016/S0006-291X(02)02053-3.
3. **Charrel RN, de Lamballerie X.** 2003. Arenaviruses other than Lassa virus. Antiviral Res **57:**89–100. http://dx.doi.org/10.1016/S0166-3542(02)00202-4.
4. **Peters CJ.** 2002. Human infection with arenaviruses in the Americas. Curr Top Microbiol Immunol **262:**65–74.
5. **Delgado S, Erickson BR, Agudo R, Blair PJ, Vallejo E, Albarino CG, Vargas J, Comer JA, Rollin PE, Ksiazek TG, Olson JG, Nichol ST.** 2008. Chapare virus, a newly discovered arenavirus isolated from a fatal hemorrhagic fever case in Bolivia. PLoS Pathog **4:**e1000047. http://dx.doi.org/10.1371/journal.ppat.1000047.

6. **Gonzalez JP, Bowen MD, Nichol ST, Rico-Hesse R.** 1996. Genetic characterization and phylogeny of Sabia virus, an emergent pathogen in Brazil. Virology **221:**318–324. http://dx.doi.org/10.1006/viro.1996.0381.
7. **Centers for Disease Control and Prevention.** 2000. Fatal illnesses associated with a new world arenavirus—California, 1999–2000. MMWR Morb Mortal Wkly Rep **49:**709–711.
8. **Maiztegui JI, Fernandez NJ, de Damilano AJ.** 1979. Efficacy of immune plasma in treatment of Argentine haemorrhagic fever and association between treatment and a late neurological syndrome. Lancet **ii:**1216–1217.
9. **Monath TP.** 1975. Lassa fever: review of epidemiology and epizootiology. Bull World Health Organ **52:**577–592.
10. **Stinebaugh BJ, Schloeder FX, Johnson KM, Mackenzie RB, Entwisle G, De Alba E.** 1966. Bolivian hemorrhagic fever. A report of four cases. Am J Med **40:**217–230.
11. **Eddy GA, Wagner FS, Scott SK, Mahlandt BJ.** 1975. Protection of monkeys against Machupo virus by the passive administration of Bolivian haemorrhagic fever immunoglobulin (human origin). Bull World Health Organ **52:**723–727.
12. **Jahrling PB, Peters CJ.** 1984. Passive antibody therapy of Lassa fever in cynomolgus monkeys: importance of neutralizing antibody and Lassa virus strain. Infect Immun **44:**528–533.
13. **Kenyon RH, Green DE, Eddy GA, Peters CJ.** 1986. Treatment of Junin virus-infected guinea pigs with immune serum: development of late neurological disease. J Med Virol **20:**207–218. http://dx.doi.org/10.1002/jmv.1890200303.
14. **Enria DA, Briggiler AM, Sanchez Z.** 2008. Treatment of Argentine hemorrhagic fever. Antiviral Res **78:**132–139. http://dx.doi.org/10.1016/j.antiviral.2007.10.010.
15. **Enria DA, Briggiler AM, Fernandez NJ, Levis SC, Maiztegui JI.** 1984. Importance of dose of neutralising antibodies in treatment of Argentine haemorrhagic fever with immune plasma. Lancet **ii:**255–256.
16. **Maiztegui JI, McKee KT, Jr, Barrera Oro JG, Harrison LH, Gibbs PH, Feuillade MR, Enria DA, Briggiler AM, Levis SC, Ambrosio AM, Halsey NA, Peters CJ.** 1998. Protective efficacy of a live attenuated vaccine against Argentine hemorrhagic fever. AHF Study Group. J Infect Dis **177:**277–283. http://dx.doi.org/10.1086/514211.
17. **Lenz O, ter Meulen J, Klenk HD, Seidah NG, Garten W.** 2001. The Lassa virus glycoprotein precursor GP-C is proteolytically processed by subtilase SKI-1/S1P. Proc Natl Acad Sci U S A **98:**12701–12705. http://dx.doi.org/10.1073/pnas.221447598.
18. **York J, Romanowski V, Lu M, Nunberg JH.** 2004. The signal peptide of the Junin arenavirus envelope glycoprotein is myristoylated and forms an essential subunit of the mature G1-G2 complex. J Virol **78:**10783–10792. http://dx.doi.org/10.1128/JVI.78.19.10783-10792.2004.
19. **Burri DJ, da Palma JR, Kunz S, Pasquato A.** 2012. Envelope glycoprotein of arenaviruses. Viruses **4:**2162–2181. http://dx.doi.org/10.3390/v4102162.
20. **Radoshitzky SR, Abraham J, Spiropoulou CF, Kuhn JH, Nguyen D, Li W, Nagel J, Schmidt PJ, Nunberg JH, Andrews NC, Farzan M, Choe H.** 2007. Transferrin receptor 1 is a cellular receptor for New World haemorrhagic fever arenaviruses. Nature **446:**92–96. http://dx.doi.org/10.1038/nature05539.
21. **Sanchez A, Pifat DY, Kenyon RH, Peters CJ, McCormick JB, Kiley MP.** 1989. Junin virus monoclonal antibodies: characterization and cross-reactivity with other arenaviruses. J Gen Virol **70:**1125–1132. http://dx.doi.org/10.1099/0022-1317-70-5-1125.
22. **Parekh BS, Buchmeier MJ.** 1986. Proteins of lymphocytic choriomeningitis virus: antigenic topography of the viral glycoproteins. Virology **153:**168–178. http://dx.doi.org/10.1016/0042-6822(86)90020-6.
23. **Buchmeier MJ, Lewicki HA, Tomori O, Oldstone MB.** 1981. Monoclonal antibodies to lymphocytic choriomeningitis and Pichinde viruses: generation, characterization, and cross-reactivity with other arenaviruses. Virology **113:**73–85. http://dx.doi.org/10.1016/0042-6822(81)90137-9.
24. **Bruns M, Cihak J, Muller G, Lehmann-Grube F.** 1983. Lymphocytic choriomeningitis virus. VI. Isolation of a glycoprotein mediating neutralization. Virology **130:**247–251.
25. **Kutzler MA, Weiner DB.** 2008. DNA vaccines: ready for prime time? Nat Rev Genet **9:**776–788. http://dx.doi.org/10.1038/nrg2432.
26. **Yang ZY, Kong WP, Huang Y, Roberts A, Murphy BR, Subbarao K, Nabel GJ.** 2004. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature **428:**561–564. http://dx.doi.org/10.1038/nature02463.
27. **Hooper JW, Ferro AM, Golden JW, Silvera P, Dudek J, Alterson K, Custer M, Rivers B, Morris J, Owens G, Smith JF, Kamrud KI.** 2009. Molecular smallpox vaccine delivered by alphavirus replicons elicits protective immunity in mice and non-human primates. Vaccine **28:**494–511. http://dx.doi.org/10.1016/j.vaccine.2009.09.133.
28. **Hooper JW, Josleyn M, Ballantyne J, Brocato R.** 2013. A novel Sin Nombre virus DNA vaccine and its inclusion in a candidate pan-hantavirus vaccine against hantavirus pulmonary syndrome (HPS) and hemorrhagic fever with renal syndrome (HFRS). Vaccine **31:**4314–4321. http://dx.doi.org/10.1016/j.vaccine.2013.07.025.
29. **Golden JW, Zaitseva M, Kapnick S, Fisher RW, Mikolajczyk MG, Ballantyne J, Golding H, Hooper JW.** 2011. Polyclonal antibody cocktails generated using DNA vaccine technology protect in murine models of orthopoxvirus disease. Virol J **8:**441. http://dx.doi.org/10.1186/1743-422X-8-441.
30. **Hooper JW, Ferro AM, Wahl-Jensen V.** 2008. Immune serum produced by DNA vaccination protects hamsters against lethal respiratory challenge with Andes virus. J Virol **82:**1332–1338. http://dx.doi.org/10.1128/JVI.01822-07.
31. **Golden JW, Hooper JW.** 2010. Evaluating the orthopoxvirus type I interferon-binding molecule as a vaccine target in the vaccinia virus intranasal murine challenge model. Clin Vaccine Immunol **17:**1656–1665. http://dx.doi.org/10.1128/CVI.00235-10.
32. **Webb PA, Johnson KM, Mackenzie RB.** 1969. The measurement of specific antibodies in Bolivian hemorrhagic fever by neutralization of virus plaques. Proc Soc Exp Biol Med **130:**1013–1019. http://dx.doi.org/10.3181/00379727-130-33711.
33. **Kwilas S, Kishimori JM, Josleyn M, Jerke K, Ballantyne J, Royals M, Hooper JW.** 2014. A hantavirus pulmonary syndrome (HPS) DNA vaccine delivered using a spring-powered jet injector elicits a potent neutralizing antibody response in rabbits and nonhuman primates. Curr Gene Ther **14:**200–210. http://dx.doi.org/10.2174/1566523214666140522122633.
34. **Ray N, Whidby J, Stewart S, Hooper JW, Bertolotti-Ciarlet A.** 2010. Study of Andes virus entry and neutralization using a pseudovirion system. J Virol Methods **163:**416–423. http://dx.doi.org/10.1016/j.jviromet.2009.11.004.
35. **National Research Council.** 2011. Guide for the care and use of laboratory animals, 8th ed. National Academies Press, Washington, DC.
36. **Candurra NA, Damonte EB, Coto CE.** 1989. Antigenic relationships between attenuated and pathogenic strains of Junin virus. J Med Virol **27:**145–150. http://dx.doi.org/10.1002/jmv.1890270215.
37. **Cresta B, Padula P, de Martinez Segovia M.** 1980. Biological properties of Junin virus proteins. I. Identification of the immunogenic glycoprotein. Intervirology **13:**284–288.
38. **Kenyon RH, Condie RM, Jahrling PB, Peters CJ.** 1990. Protection of guinea pigs against experimental Argentine hemorrhagic fever by purified human IgG: importance of elimination of infected cells. Microb Pathog **9:**219–226. http://dx.doi.org/10.1016/0882-4010(90)90010-N.
39. **Weissenbacher MC, Coto CE, Calello MA.** 1975. Cross-protection between Tacaribe complex viruses. Presence of neutralizing antibodies against Junin virus (Argentine hemorrhagic fever) in guinea pigs infected with Tacaribe virus. Intervirology **6:**42–49.
40. **Lopez N, Scolaro L, Rossi C, Jacamo R, Candurra N, Pujol C, Damonte EB, Franze-Fernandez MT.** 2000. Homologous and heterologous glycoproteins induce protection against Junin virus challenge in guinea pigs. J Gen Virol **81:**1273–1281. http://dx.doi.org/10.1099/0022-1317-81-5-1273.
41. **Seregin AV, Yun NE, Poussard AL, Peng BH, Smith JK, Smith JN, Salazar M, Paessler S.** 2010. TC83 replicon vectored vaccine provides protection against Junin virus in guinea pigs. Vaccine **28:**4713–4718. http://dx.doi.org/10.1016/j.vaccine.2010.04.077.
42. **Sommerstein R, Flatz L, Remy MM, Malinge P, Magistrelli G, Fischer N, Sahin M, Bergthaler A, Igonet S, Ter Meulen J, Rigo D, Meda P, Rabah N, Coutard B, Bowden TA, Lambert PH, Siegrist CA, Pinschewer DD.** 2015. Arenavirus glycan shield promotes neutralizing antibody evasion and protracted infection. PLoS Pathog **11:**e1005276. http://dx.doi.org/10.1371/journal.ppat.1005276.
43. **Howard CR, Lewicki H, Allison L, Salter M, Buchmeier MJ.** 1985. Properties and characterization of monoclonal antibodies to Tacaribe virus. J Gen Virol **66:**1383–1395. http://dx.doi.org/10.1099/0022-1317-66-7-1383.
44. **Ruo SL, Mitchell SW, Kiley MP, Roumillat LF, Fisher-Hoch SP, McCormick JB.** 1991. Antigenic relatedness between arenaviruses defined at

the epitope level by monoclonal antibodies. J Gen Virol **72:**549–555. http://dx.doi.org/10.1099/0022-1317-72-3-549.

45. **Weber EL, Buchmeier MJ.** 1988. Fine mapping of a peptide sequence containing an antigenic site conserved among arenaviruses. Virology **164:**30–38. http://dx.doi.org/10.1016/0042-6822(88)90616-2.
46. **Buchmeier MJ, Lewicki HA, Tomori O, Johnson KM.** 1980. Monoclonal antibodies to lymphocytic choriomeningitis virus react with pathogenic arenaviruses. Nature **288:**486–487. http://dx.doi.org/10.1038/288486a0.
47. **Buchmeier MJ, Elder JH, Oldstone MB.** 1978. Protein structure of lymphocytic choriomeningitis virus: identification of the virus structural and cell associated polypeptides. Virology **89:**133–145. http://dx.doi.org/10.1016/0042-6822(78)90047-8.
48. **Howard CR.** 1987. Neutralization of arenaviruses by antibody. Curr Top Microbiol Immunol **134:**117–130.
49. **Martinez Peralta LA, Coto CE, Weissenbacher MC.** 1993. The Tacaribe complex, p 281–298. *In* Salvato M (ed), The Arenaviridae. Plenum Press, New York, NY.
50. **Peters CJ, Jahrling PB, Liu CT, Kenyon RH, McKee KT, Jr, Barrera Oro JG.** 1987. Experimental studies of arenaviral hemorrhagic fevers. Curr Top Microbiol Immunol **134:**5–68.
51. **Golden JW, Hammerbeck CD, Mucker EM, Brocato RL.** 2015. Animal models for the study of rodent-borne hemorrhagic fever viruses: arenaviruses and hantaviruses. Biomed Res Int **2015:**793257. http://dx.doi.org/10.1155/2015/793257.
52. **Wright KE, Buchmeier MJ.** 1991. Antiviral antibodies attenuate T-cell-mediated immunopathology following acute lymphocytic choriomeningitis virus infection. J Virol **65:**3001–3006.
53. **Hall WC, Geisbert TW, Huggins JW, Jahrling PB.** 1996. Experimental infection of guinea pigs with Venezuelan hemorrhagic fever virus (Guanarito): a model of human disease. Am J Trop Med Hyg **55:**81–88.
54. **Peters CJ, Kuehne RW, Mercado RR, Le Bow RH, Spertzel RO, Webb PA.** 1974. Hemorrhagic fever in Cochabamba, Bolivia, 1971. Am J Epidemiol **99:**425–433.
55. **Bell TM, Bunton TE, Shaia CI, Raymond JW, Honnold SP, Donnelly GC, Shamblin JD, Wilkinson ER, Cashman KA.** 2016. Pathogenesis of Bolivian hemorrhagic fever in guinea pigs. Vet Pathol **53:**190–199. http://dx.doi.org/10.1177/0300985815588609.
56. **Briese T, Paweska JT, McMullan LK, Hutchison SK, Street C, Palacios G, Khristova ML, Weyer J, Swanepoel R, Egholm M, Nichol ST, Lipkin WI.** 2009. Genetic detection and characterization of Lujo virus, a new hemorrhagic fever-associated arenavirus from southern Africa. PLoS Pathog **5:**e1000455. http://dx.doi.org/10.1371/journal.ppat.1000455.
57. **Kenyon RH, Canonico PG, Green DE, Peters CJ.** 1986. Effect of ribavirin and tributylribavirin on Argentine hemorrhagic fever (Junin virus) in guinea pigs. Antimicrob Agents Chemother **29:**521–523. http://dx.doi.org/10.1128/AAC.29.3.521.
58. **Matsushita H, Sano A, Wu H, Jiao JA, Kasinathan P, Sullivan EJ, Wang Z, Kuroiwa Y.** 2014. Triple immunoglobulin gene knockout transchromosomic cattle: bovine lambda cluster deletion and its effect on fully human polyclonal antibody production. PLoS One **9:**e90383. http://dx.doi.org/10.1371/journal.pone.0090383.
59. **Hooper JW, Brocato RL, Kwilas SA, Hammerbeck CD, Josleyn MD, Royals M, Ballantyne J, Wu H, Jiao JA, Matsushita H, Sullivan EJ.** 2014. DNA vaccine-derived human IgG produced in transchromosomal bovines protect in lethal models of hantavirus pulmonary syndrome. Sci Transl Med **6:**264ra162. http://dx.doi.org/10.1126/scitranslmed.3010082.
60. **Bounds CE, Kwilas SA, Kuehne AI, Brannan JM, Bakken RR, Dye JM, Hooper JW, Dupuy LC, Ellefsen B, Hannaman D, Wu H, Jiao JA, Sullivan EJ, Schmaljohn CS.** 2015. Human polyclonal antibodies produced through DNA vaccination of transchromosomal cattle provide mice with post-exposure protection against lethal Zaire and Sudan ebolaviruses. PLoS One **10:**e0137786. http://dx.doi.org/10.1371/journal.pone.0137786.
61. **Kugelman JR, Kugelman-Tonos J, Ladner JT, Pettit J, Keeton CM, Nagle ER, Garcia KY, Froude JW, Kuehne AI, Kuhn JH, Bavari S, Zeitlin L, Dye JM, Olinger GG, Sanchez-Lockhart M, Palacios GF.** 2015. Emergence of Ebola virus escape variants in infected nonhuman primates treated with the MB-003 antibody cocktail. Cell Rep **12:**2111–2120. http://dx.doi.org/10.1016/j.celrep.2015.08.038.
62. **Alche LE, Coto CE.** 1988. Differentiation of Junin virus and antigenic variants isolated in vivo by kinetic neutralization assays. J Gen Virol **69:**2123–2127. http://dx.doi.org/10.1099/0022-1317-69-8-2123.