Antiviral Research 163 (2019) 106–116

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Assessing cross-reactivity of Junín virus-directed neutralizing antibodies

Anne Leske[a], Irke Waßmann[a], Kevin Schnepel[a,1], Kyle Shifflett[b,2], Julia Holzerland[a], Linus Bostedt[a], Patrick Bohn[a], Thomas C. Mettenleiter[c], Ana M. Briggiler[d], Julia Brignone[d], Delia Enria[d], Sandra M. Cordo[e], Thomas Hoenen[b,c], Allison Groseth[a,b,*]

[a] Junior Research Group Arenavirus Biology, Friedrich-Loeffler-Institut, Greifswald, Insel Riems, Germany
[b] Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Hamilton, MT, USA
[c] Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, Greifswald, Insel Riems, Germany
[d] Instituto Nacional de Enfermedades Virales Humanas, ANLIS, Pergamino, Argentina
[e] Laboratory of Virology, IQUIBICEN-Department of Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Transcription and replication competent virus-like particle (trVLP) assay
Neutralization assay
Arenavirus
JunÍn virus
Neutralizing antibodies
Antibody cross-reactivity

ABSTRACT

Arenaviruses cause several viral hemorrhagic fevers endemic to Africa and South America. The respective causative agents are classified as biosafety level (BSL) 4 pathogens. Unlike for most other BSL4 agents, for the New World arenavirus Junín virus (JUNV) both a highly effective vaccination (Candid#1) and a post-exposure treatment, based on convalescent plasma transfer, are available. In particular, neutralizing antibodies (nAbs) represent a key protective determinant in JUNV infection, which is supported by the correlation between successful passive antibody therapy and the levels of nAbs administered. Unfortunately, comparable resources for the management of other closely related arenavirus infections are not available. Given the significant challenges inherent in studying BSL4 pathogens, our goal was to first assess the suitability of a JUNV transcription and replication-competent virus-like particle (trVLP) system for measuring virus neutralization under BSL1/2 conditions. Indeed, we could show that infection with JUNV trVLPs is glycoprotein (GP) dependent, that trVLP input has a direct correlation to reporter readout, and that these trVLPs can be neutralized by human serum with kinetics similar to those obtained using authentic virus. These properties make trVLPs suitable for use as a proxy for virus in neutralization assays. Using this platform we then evaluated the potential of JUNV nAbs to cross-neutralize entry mediated by GPs from other arenaviruses using JUNV (strain Romero)-based trVLPs bearing GPs either from other JUNV strains, other closely related New World arenaviruses (e.g. Tacaribe, Machupo, Sabiá), or the distantly related Lassa virus. While nAbs against the JUNV vaccine strain are also active against a range of other JUNV strains, they appear to have little or no capacity to neutralize other arenavirus species, suggesting that therapy with whole plasma directed against another species is unlikely to be successful and that the targeted development of cross-specific monoclonal antibody-based resources is likely needed. Such efforts will be supported by the availability of this BSL1/2 screening platform which provides a rapid and easy means to characterize the potency and reactivity of anti-arenavirus neutralizing antibodies against a range of arenavirus species.

1. Introduction

Rodent-borne arenaviruses include a number of significant zoonotic pathogens that can cause serious human infections and are classified as BSL4 pathogens. The virus lineage found primarily in Africa, known as Old World (OW) arenaviruses, includes the well-known Lassa virus (LASV), which despite the relatively rare occurrence of severe disease and a correspondingly modest case fatality rate has a major public

* Corresponding author. Friedrich-Loeffler-Institut Südufer 10, Greifswald, Insel Riems, 17493, Germany.
E-mail addresses: anne.leske@fli.de (A. Leske), iw142102@uni-greifswald.de (I. Waßmann), kevin.schnepel@uni-rostock.de (K. Schnepel), kyle.shifflett@umconnect.umt.edu (K. Shifflett), julia.holzerland@fli.de (J. Holzerland), linus.bostedt@fli.de (L. Bostedt), pb132684@uni-greifswald.de (P. Bohn), thomasc.mettenleiter@fli.de (T.C. Mettenleiter), abriggiler@hotmail.com (A.M. Briggiler), jbrignone@anlis.gov.ar (J. Brignone), deliaenria@gmail.com (D. Enria), scordo@qb.fcen.uba.ar (S.M. Cordo), thomas.hoenen@fli.de (T. Hoenen), allison.groseth@fli.de (A. Groseth).
[1] Present address: University of Rostock, Rostock, Germany.
[2] Present address: Montana State University, Department of Microbiology and Immunology, Bozeman, MT, USA.

https://doi.org/10.1016/j.antiviral.2019.01.006
Received 2 October 2018; Received in revised form 21 December 2018; Accepted 11 January 2019
Available online 19 January 2019
0166-3542/

health impact due to the large number of cases (estimated 300,000–500,000) that occur annually (McCormick et al., 1987). In contrast, among the viruses found in the Americas, New World (NW) arenaviruses, 5 virus species are so far known to cause severe human infections (i.e. Junín virus (JUNV), Machupo virus (MACV), Guanarito virus, Sabiá virus (SABV) and Chapare virus). While these viruses cause much more modest case numbers, infection frequently results in severe disease characterized by hemorrhagic and/or neurological manifestations with a 15–30% case-fatality rate if untreated (Charrel and de Lamballerie, 2003).

While prophylactic and therapeutic measures for most BSL4 pathogens are very limited and often experimental, JUNV, which is the causative agent of Argentine hemorrhagic fever (AHF), is a rare example of a BSL4 pathogen for which both effective vaccination and therapeutic options are available. Indeed, both measures have been broadly implemented within target populations in the endemic region of Argentina, where they have resulted in a significant reduction in case numbers and improved individual patient outcomes (Enria et al., 2008). To date, over 250,000 individuals have received the vaccine, which is based on a live-attenuated virus strain known as Candid#1, without evidence of serious side effects (Enria et al., 2008; Maiztegui et al., 1998). Successful vaccination is determined by the development of neutralizing antibody (nAb) titres (Maiztegui et al., 1998), which while modest appear to be maintained in the majority of vaccinees for many years, if not lifelong (Maiztegui et al., 1998; Ruggiero et al., 1981). Unfortunately, to date no vaccines have been generated for related VHF-causing New World arenaviruses, and it remains unclear how well Candid#1 would protect against human infection with these agents, although studies from animal models suggest the possibility of some cross-protection (Golden et al., 2017; Koma et al., 2016; Weissenbacher et al., 1975, 1982).

For those who contract AHF, timely administration of convalescent plasma, within 8d of symptom onset, has been shown to have strong protective effects (Maiztegui et al., 1979). Importantly, the success of this approach is also directly dependent on delivery of an adequate quantity of neutralizing antibody (> 3500 TU/kg) (Enria et al., 1984, 1986). To facilitate efficient therapeutic intervention, a national stockpile of high-titre convalescent donor plasma has been established in Argentina, although maintaining it has become increasingly challenging in light of the decreasing number of cases of natural infection. Similar resources do not currently exist for other New World arenavirus infections and it remains unclear to what extent such convalescent plasma-based resources for JUNV could be re-purposed for treatment of related viruses. However, based on the experience with JUNV plasma dosing, any therapeutic value would likely be dependent on robust cross-neutralization activity. Indeed significant effort is now being invested both at the academic and industry levels in identifying targets and modes of action of antibody neutralization with the goal of identifying monoclonal antibodies, whether mono-specific or broadly neutralizing, with therapeutic potential (Brouillette et al., 2017; Clark et al., 2018; Mahmutovic et al., 2015; Pan et al., 2018; Zeitlin et al., 2016; Zeltina et al., 2017). Improved BSL1/2 alternatives for conducting neutralization assays for arenaviruses are urgently needed to facilitate this type of research.

We have recently reported on the development of a JUNV transcription-replication competent virus-like particle system (trVLP) system (Dunham et al., 2018) to generate VLPs in which the virus genome is replaced with a genome analogue (minigenome) encoding only a reporter protein such as GFP or luciferase. This not only makes them safe to use under BSL1/2 conditions, but also provides a convenient means to monitor virus infection using reporter expression as the readout. Since these particles are formed only by authentic arenavirus components, they would be expected to not only authentically recapitulate arenavirus particle structure, but also mimic the mechanics of arenavirus entry. Therefore, we considered whether they might prove an ideal substrate for the evaluation of neutralizing antibody activity. Indeed, we successfully developed protocols to screen for neutralizing antibody responses against JUNV, which are known to be a key determinant for protection. Further, we could show that trVLPs with a JUNV internal structure could also efficiently incorporate glycoproteins (GPs) from other arenaviruses allowing us to assess cross-neutralization of GPs from various other NW arenaviruses, as well as LASV, by antibodies in the serum of human Candid#1 vaccinees.

Table 1
Virus strains used for GP-expression and generation of heterologous transcription and replication-competent virus-like particles (trVLPs).

Virus	Strain	Country	Year	Accession Number	
				S segment	L segment
Junín	Romero (P3235)	Argentina	1986	AY619641	AY619640
	Espindola (P3790)	Argentina	1986	JN714129	JN714130
	P3551	Argentina	1977	–	–
	XJ Clone 3	-[a]	-[a]	–	–
	XJ 13	-[a]	-[a]	NC_005081	NC_005080
Machupo	Carvallo	Bolivia	1963	NC_005078	NC_005079
	Chicava	Bolivia	1993	AY624355	AY624354
Tacaribe	TRVL 11573	Trinidad	1956	NC_004293	NC_004292
Guanarito	INH95551	Venezuela	1990	NC_005077	NC_005082
Sabiá	SPH114202	Brazil	1990	NC_006317	NC_006313
Lassa virus	Josiah	Sierra Leone	1976	NC_004296	NC_004297

[a] Passaging adapted variant of the original XJ strain isolated in Argentina in 1958.

2. Materials and methods

2.1. Viruses and cell lines

The arenavirus strains listed in Table 1 were used for construction of heterologous arenavirus GP constructs.

HEK293T (CCLV-RIE1018) were grown in MEM with Hanks' Salts or Earle's Salts (1:1 mixture) and 1× non-essential amino acids. Huh7 (CCLV-RIE1079) were grown in Ham's F12 and Iscove's Modified Dulbecco's Medium (1:1 mixture). BSR-T7/5 cells (kindly provided by Stefan Finke, Friedrich-Loeffler-Institut, Germany; CCLV-RIE0583 (Buchholz et al., 1999)) were grown in MEM Glasgow with 1 mg/ml Geneticin added every other passage. These cell lines were supplemented with 2 mM L-glutamine (Q), penicillin (100 U/mL)/streptomycin (100 µg/mL; PS), and 10% fetal calf serum (FCS), except BSR-T7/5 cells, for which 10% newborn calf serum (NCS) was used. Vero cells (ATCC CCL 81) were grown in Minimum Essential Medium (MEM) containing 5% FCS and supplemented with 50 µg/ml gentamicin. Cells were cultured at 37 °C with 5% CO_2.

2.2. Plasmids

The optimized T7-driven JUNV minigenome was based on the S segment from the Romero strain of JUNV with both open reading frames (ORFs) removed and NanoLuciferase (nLuc) cloned into the GP locus (Dunham et al., 2018). All other plasmids for the trVLP assay (i.e. pCAGGS-NP, pCAGGS-L, pCAGGS-T7, pCAGGS-Firefly (FF), pCAGGS-Z, pCAGGS-GP) are also based on the Romero strain and have been previously described (Groseth et al., 2010; Watt et al., 2014). For cloning of the additional arenavirus GP ORFs, RNA was extracted from the virus stocks listed in Table 1 using the QIAamp Viral RNA Mini Kit (Qiagen) and the GP ORFs were amplified by RT-PCR using virus-specific primers. Reverse primers contained an additional C-terminal FLAG tag to facilitate detection. GP amplicons were cloned into pCAGGS using standard molecular cloning techniques. Primers and details of the cloning strategies are available upon request.

2.3. Candid#1 sera

Positive control sera for this study were collected from two healthy human vaccinees without previous history of JUNV infection who had confirmed nAb titres 1 month and 5 months after a single Candid#1 vaccination administered by the clinical staff of *Instituto Nacional de Enfermedades Virales Humanas* in Argentina in May 2009 (Supplemental Table 1). Serum was isolated from whole blood using Monovette S serum collection tubes according to the manufacturer's direction. Control pooled human AB serum was obtained commercially (Sigma). All sera were decomplemented by heat inactivation at 56 °C for 30 min. The serum collection from human volunteers in this study was approved by the ethics committee of the University of Greifswald and was conducted with informed consent of the participants and without remuneration.

2.4. Analysis of heterologous GP expression

The pCAGGS-GP-cFLAG constructs described above were transfected (1ug) into HEK293T cells in 12-well plates using TransIT-LT1 (Mirus) with complex formation in OptiMEM using 3 µl TransIT-LT1/µg DNA. After 48 h cells were harvested in 4× SDS gel loading buffer containing β-mercaptoethanol and boiled for 5 min at 99 °C. Samples were then separated on a 12% polyacrylamide gel and transferred to PVDF for Western blotting using a mouse anti-FLAG, Clone M2 primary antibody (1:1000, Sigma) and a horse anti-mouse-HRP (1:5000, CST) secondary antibody. Blots were subsequently stripped and reanalyzed using a rabbit anti-Tubulin (1:500; CST) primary antibody and a goat anti-rabbit-HRP (1:2000; CST) secondary antibody.

2.5. JUNV transcription/replication competent virus-like particle (trVLP) assays

JUNV trVLP assays were conducted as previously described using BSR-T7/5 cells in 6-well plates as p0 cells and Huh7 cells in 96-well plates as p1 cells (Dunham et al., 2018). Transfected plasmid amounts are shown in Supplemental Table 2. In addition to control samples prepared without pCAGGS-L, controls were also run with or without Z and/or GP, using empty pCAGGS to maintain equal plasmid mass. For the generation of heterologous trVLPs, an equal mass of pCAGGS-GP plasmids encoding GP from different virus strains or species (Table 1) was substituted.

2.6. Correlation of reporter activity and trVLP input

Positive control trVLP preparations (produced with Z and GP from strain Romero) were diluted 1:2 to 1:128 or left undiluted and the resulting reporter activity in p1 cells was measured as described above. To exclude a dramatic dilution effect on assay dynamic range we further diluted both positive (with Z and GP) and negative control (no Z or GP) trVLPs 1:10 and analyzed reporter expression.

2.7. trVLP stability during storage

Positive (with Z and GP) and negative control (no Z or GP) trVLPs were either analyzed fresh or stored at −20 °C for 1 year before being analyzed for reporter activity. Additional aliquots of positive (with Z and GP) trVLP preparations were freeze-thawed at either −20 °C or −80 °C one or four times before reporter activity was analyzed.

2.8. trVLP-based neutralization assay

To assess trVLP neutralization, trVLP preparations produced with Z and GP from JUNV strain Romero were incubated 1:1 with two-fold serial dilutions of heat-inactivated human serum either from Candid#1 vaccinees or a control pooled donor serum (final serum dilutions from 1:2 to 1:256). Samples were incubated for 2 h at 37 °C in a 96-well cell culture plate before 100 µl of the trVLP/serum mixture was applied to Huh7 p1 cells pre-transfected with pCAGGS-NP and pCAGGS-L in 96-well plates, prepared as previously described (Dunham et al., 2018). Reporter activity (nLuc) was measured after 48 h, also as previously described (Dunham et al., 2018). To further establish the effect of variation in absolute trVLP titre on virus neutralization, trVLP preparations produced with Z and GP from JUNV strain Romero were diluted 1:4 before being subject to neutralization with undiluted control serum of the serum from human Candid#1 vaccinees (final serum dilution 1:2). To assess neutralization of heterologous arenavirus GP proteins, trVLP preparations containing GP from various different arenavirus isolates (Table 1) were incubated 1:1 with undiluted control or human Candid#1 vaccinee serum (i.e. final serum dilution 1:2) before being used to infect p1 cells, as described above.

2.9. Plaque-reduction neutralization assay

To assess virus neutralization, a JUNV stock was diluted to 10^3 pfu/ml and incubated 1:1 with a two-fold dilution series of heat-inactivated human serum either from Candid#1 vaccinees or a control pooled donor serum (final serum dilutions from 1:10 to 1:160). Samples were incubated for 1 h at 37 °C before 100 µl of the virus/serum mixture was applied to Vero cells grown in 24-well microplates. Cells were incubated for a further 1 h at 37 °C before the inoculum was replaced with 1 ml of semi-solid medium (1.4% methylcellulose + 2× MEM with 3% FCS) and plates were incubated for 7 days before being fixed in 10% formalin and stained with 1% crystal violet.

2.10. Statistical analysis

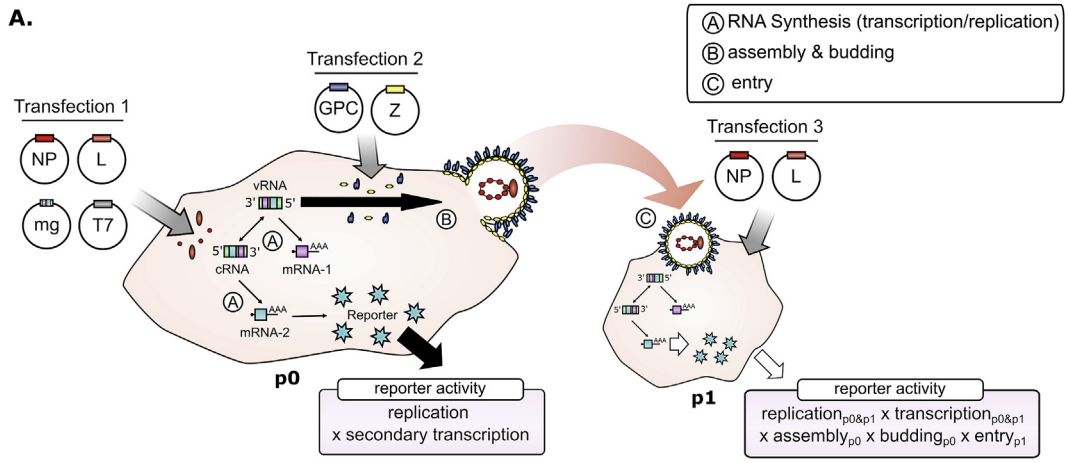
T-tests (2 sample groups) or one-way ANOVA (> 2 sample groups) were performed in GraphPad Prism v6 (GraphPad). Significance cut-offs: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001. Post hoc tests were performed for ANOVA analyses using Dunnett's (comparison to a control), Sidak's (comparison of selected pairings) and/or Tukey's (multiple pairwise comparisons) test.

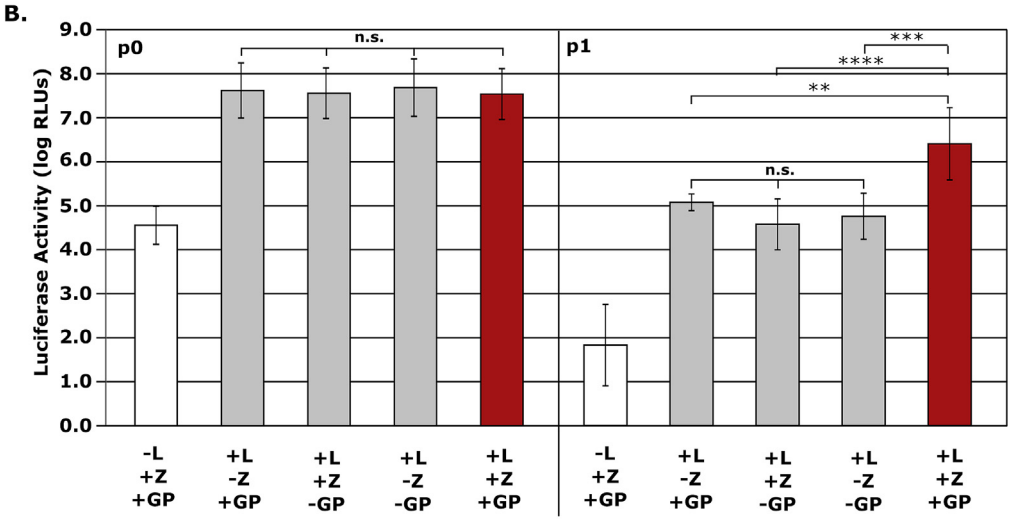
3. Results

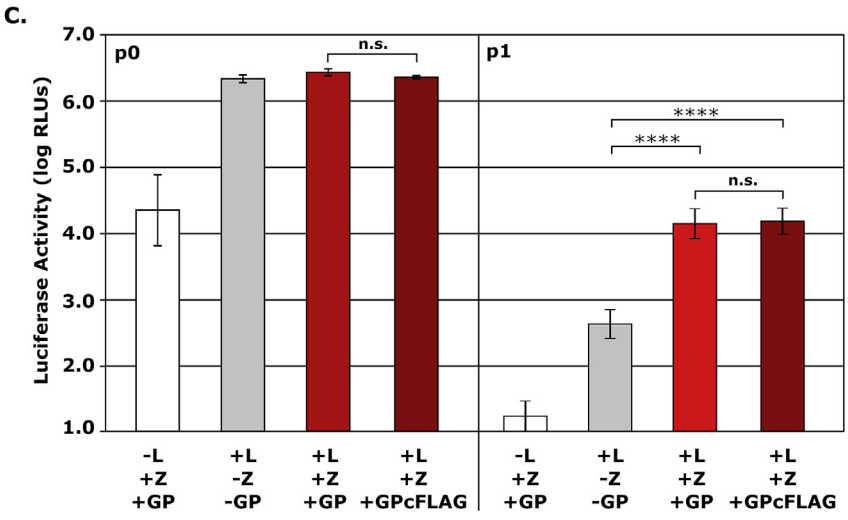
3.1. Characterization of JUNV trVLP production, reporter expression and stability

The successful generation of trVLPs has previously been shown to rely on the presence of the viral matrix protein (Z) and GP (Dunham et al., 2018) and, presumably due to the activity of Z as a viral polymerase inhibitor, is critically dependent on these components being provided in a separate transfection after the minigenome has had a chance to be replicated and transcribed (Fig. 1A). However, the individual contribution of these two components has not been assessed. Therefore, we compared the reporter activities of trVLPs containing, in addition to the minigenome, NP and L (supplied in transfection 1; Fig. 1A), 1) GP only, 2) Z only, 3) Z + GP, or 4) no Z or GP (supplied in transfection 2; Fig. 1A). As expected, both Z and GP are required for trVLP activity (i.e. successful particle production and subsequent infection of target cells) (Fig. 1B). Further, by substituting GP from strain Romero with an equivalent construct containing a C-terminal FLAG tag, we could show that tagging of the C-terminus of GP2 is well tolerated (Fig. 1C). The use of such FLAG tagged constructs for various arenavirus strains and species provided us with a possibility to accurately compare their expression without hampering subsequent trVLP production/activity.

To clearly establish whether a relationship exists between the amount of trVLP input and the resulting reporter activity measured, functional trVLPs (containing both Z and GP) were diluted from 1:2 to 1:128 and used to infect target cells. The reporter activities in these







(caption on next page)

cells 48 h post transfection (p.t.) showed a strong direct correlation between trVLP dilution and reduction of reporter activity across a wide range of dilutions ($R^2 = 0.997$; Fig. 2A). Importantly, when positive (with Z and GP) and negative control (without Z or GP) trVLPs were diluted 1:10 there was no significant difference in the assay dynamic range (Fig. 2B), further indicating that absolute reporter activity levels do not affect assay performance.

In order to reduce assay variability and increase the reproducibility and comparability of results, it would be highly desirable to be able to generate large standardized trVLP stocks that can be used for entire sets of experiments. To explore the feasibility of such an approach we analyzed trVLP stability by testing freshly prepared control trVLPs, with or without Z and GP, and comparing the results to samples that were stored for 1 year at −20 °C. We observed no significant difference in the reporter activity of these preparations during this time-frame. Similarly, positive control trVLPs were freeze-thawed from −20 °C or −80 °C

Fig. 1. Characterization of the JUNV trVLP system. (A) Schematic diagram of JUNV trVLP assay system. Producer cells (p0 cells) were first transfected with a JUNV minigenome along with expression plasmids encoding the T7 polymerase and the JUNV nucleoprotein (NP), with or without the JUNV polymerase (L). Following initial transfection of the p0 cells, T7 directs initial transcription of the minigenome RNA, which is autocatalytically processed by HDV ribozyme cleavage to generate an RNA containing authentic JUNV leader and trailer sequences. This genome analogue can then be encapsidated by NP and transcribed and replicated by L. The transcribed mRNA from the GP gene encodes the assayable NanoLuciferase (nLuc) reporter protein. The same p0 cells are subsequently transfected with plasmids encoding the JUNV glycoprotein precursor (GP) and the matrix protein (Z). This results in inhibition of further viral RNA synthesis and budding of transcription and replication competent virus-like particles (trVLPs) that have a structure analogous to that of virus particles, but instead contain only a minigenome as their genetic material. These particles can be used to infect new target cells (p1 cells) that have been pre-transfected with pCAGGS-NP and pCAGGS-L, and thus are able to further transcribe and replicate the viral minigenome. Reporter activity in p0 cells reflects viral RNA synthesis (i.e. transcription and replication) taking place in these cells, while reporter activity in p1 cells reflects the cumulative RNA synthesis in p0 and p1 cells, as well as trVLP production and entry. **(B) JUNV trVLP assay for detection of nLuc expression.** BSR-T7/5 cells (p0 cells) were transfected as described in (A) either with (+L) or without JUNV L (-L). A control plasmid pCAGGS-Firefly (FF) was also transfected in all cells as a measure of cellular RNA synthesis. After 24 h cells were further transfected with pCAGGS-Z only (-GP), pCAGGS-GP only (-Z), with both pCAGGS-Z and pCAGGS-GP (+Z, +GP), or with pCAGGS as a control (-Z, -GP). These p0 cells were harvested 48 h later and measured for both nLuc (viral RNA synthesis) and FF (host cell RNA synthesis) activity. In addition, the supernatants from these p0 cells were transferred onto Huh7 cells that had been pre-transfected with pCAGGS-NP and pCAGGS-L (p1 cells) as described in (A) 24 h previously. After 48 h the p1 cells were harvested and measured for nLuc activity (viral RNA synthesis). The means and standard deviations of normalized reporter levels (nLuc/FF, p0 cells) or nLuc activity alone (p1 cells) are shown and represent data from three independent experiments. Results of a one-way ANOVA to compare the activity of trVLPs with and without Z and/or GP are shown. **(C) Effect of C-terminal FLAG tagging on GP function.** trVLP assays were performed as described in (B) except that GP was substituted in replicate samples for an equivalent amount of GP encoding a C-terminal FLAG tag. The means and standard deviations of normalized reporter levels (nLuc/FF, p0 cells) or nLuc activity alone (p1 cells) are shown and represent data from three independent experiments. Results of a one-way ANOVA to compare the activity of GP with and without a C-terminal tag are shown.

either one or four times in succession, or stored for a comparable period at +4 °C. Short-term storage (6 h) at 4 °C had no effect on trVLP activity, nor did a single freeze-thaw cycle from either temperature. Even multiple cycles of freezing and thawing had only a minimal effect on the performance of trVLP preparations, which was not statistically significant (Fig. 2C). These results suggest that trVLP preparations are highly amenable to batch preparation with subsequent long-term storage prior to use.

3.2. Assessment of JUNV trVLP neutralization using reference sera from known vaccinees

It is known from other trVLP systems that the material produced contains not only trVLPs, but non-infectious VLPs as well, and indeed those data suggest that these non-infectious VLPs in fact represent the vast majority of the total protein content (Spiegelberg et al., 2011; Watt et al., 2014). Since it is not possible to accurately determine the absolute proportion of trVLPs to VLPs in such preparations, this raises the possibility that excess VLP material in these preparations might bind available Abs, leaving them unavailable for the neutralization of trVLPs. To control for this, trVLP preparations were left undiluted or diluted 1:4, and neutralization of trVLPs (Fig. 3A) by undiluted vaccinee serum was assessed. Input trVLP dilution resulted in the expected linear decrease in absolute signal levels predicted by the relationship established in Fig. 2A, regardless of whether the samples were neutralized with control or Candid-1 specific antisera (i.e. control: 1.7×10^5 RLU vs. 4.1×10^4 RLU, Donor 1: 5.3×10^4 RLU vs. 8.4×10^3 RLU, Donor 2: 4.5×10^4 RLU vs. 1.3×10^4 RLU). More importantly, even with the sera used in this study, which contained only modest nAb levels, the total input amount of trVLP-containing material did not impact the magnitude of the observed neutralization (Fig. 3B), suggesting that extraneous VLP production does not appreciably affect the ability of trVLP preparations to undergo neutralization.

In order to determine whether trVLP neutralization would show a dose-response and generate inhibition values comparable to those obtained using classical plaque-reduction techniques, we further incubated trVLPs containing JUNV GP with serum dilutions (final serum dilutions from 1:2 to 1:256) of either a human pooled control serum or one of two serum samples from human Candid#1 vaccinees (Donor #1 and Donor #2). Both vaccinee sera showed clear neutralizing activity at modest dilutions (Fig. 3C). Closer analysis of these data using the relationship between trVLP input and reporter activity established above (section 3.1) generated trVLP 50% neutralization values of 1:16 and 80% neutralization values of 1:4 for both donors. Data obtained with a classical JUNV-based PRNT assay closely matched these observations, producing PRNT50 values of 1:10, and PRNT80 values of < 1:5, for both sera (Fig. 3D), demonstrating that trVLPs can indeed serve as a proxy for infectious virus in assays assessing serum neutralization.

3.3. Generation of trVLPs containing heterologous arenavirus glycoproteins and assessment of cross-neutralization by JUNV nAb-containing sera

Finally, it was of interest to assess the extent to which human serum with neutralizing antibodies against JUNV might be capable of blocking entry mediated by GPs from other closely related arenaviruses. To accomplish this we first generated vectors for the expression of GP both from a range of different JUNV strains (i.e. Espindola, P3551, Clone 3 and XJ13), MACV (strains Carvallo and Chicava), Tacaribe virus (TCRV) and SABV, as well as LASV GP. All of these GPs were well-expressed from their respective plasmids and most showed clear evidence of processing of GPC to the mature glycoprotein (represented by GP2; Fig. 4A). Surprisingly, we were unable to detect trVLP release into the supernatants of transfected p0 cells by Western Blot, even when samples were concentrated by ultracentrifugation using approaches that have been previously applied to examine New World arenavirus budding in the context of standard VLP assays (Groseth et al., 2010) (data not shown). However, this may be due to the much lower amounts of transfected plasmid used in the trVLP assay (125 ng vs. 1 μg). Importantly though, all heterologous GPs tested resulted in readily detectable trVLP activity when substituted against the plasmid encoding JUNV GP (strain Romero) in our trVLP assay (Fig. 4B). Interestingly, no clear correlation was observed between trVLP activity and either the observed efficiency of GP processing or the genetic distance between the donor species of the GP and JUNV species that is the source of the internal viral components and minigenome (Fig. 4B). When such heterologous GP-containing trVLPs were analyzed in a neutralization assay, we clearly saw that human Candid#1 vaccinee sera were able to efficiently neutralize entry based on the GP of a range of different JUNV strains. However, no neutralization was observed using other New World arenavirus species, even for the most closely related species, MACV and TCRV (Fig. 4C). As expected, no neutralization was observed for the distantly related LASV GP.

4. Discussion

The generation of nAbs is key in the defense against many viral diseases. In the case of JUNV this relationship is particularly clear given the direct relationship established between patient outcome and the delivery of an adequate dose of neutralizing antibodies as part of convalescent antibody therapy (Enria et al., 1984, 1986). Similarly, the

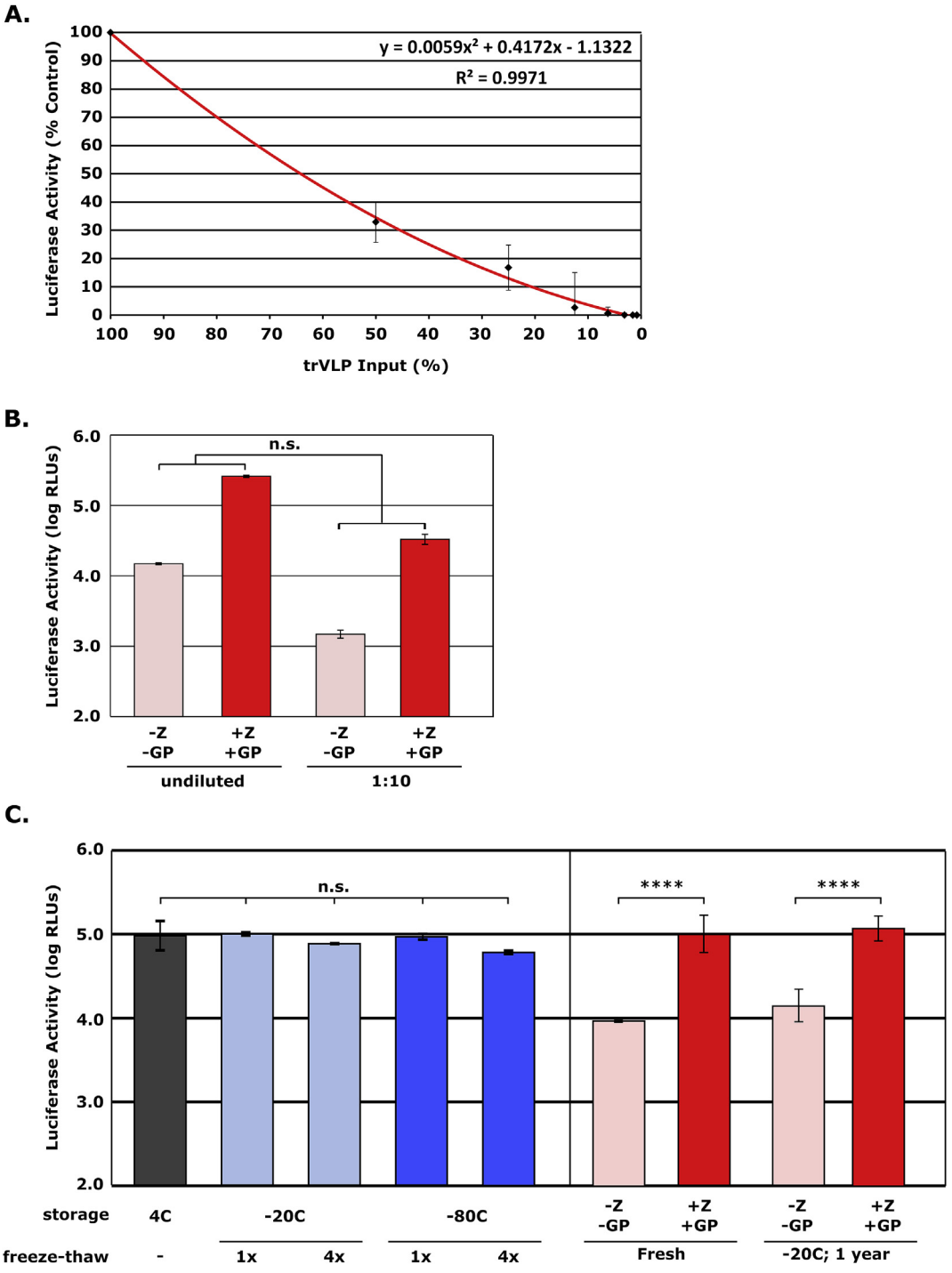
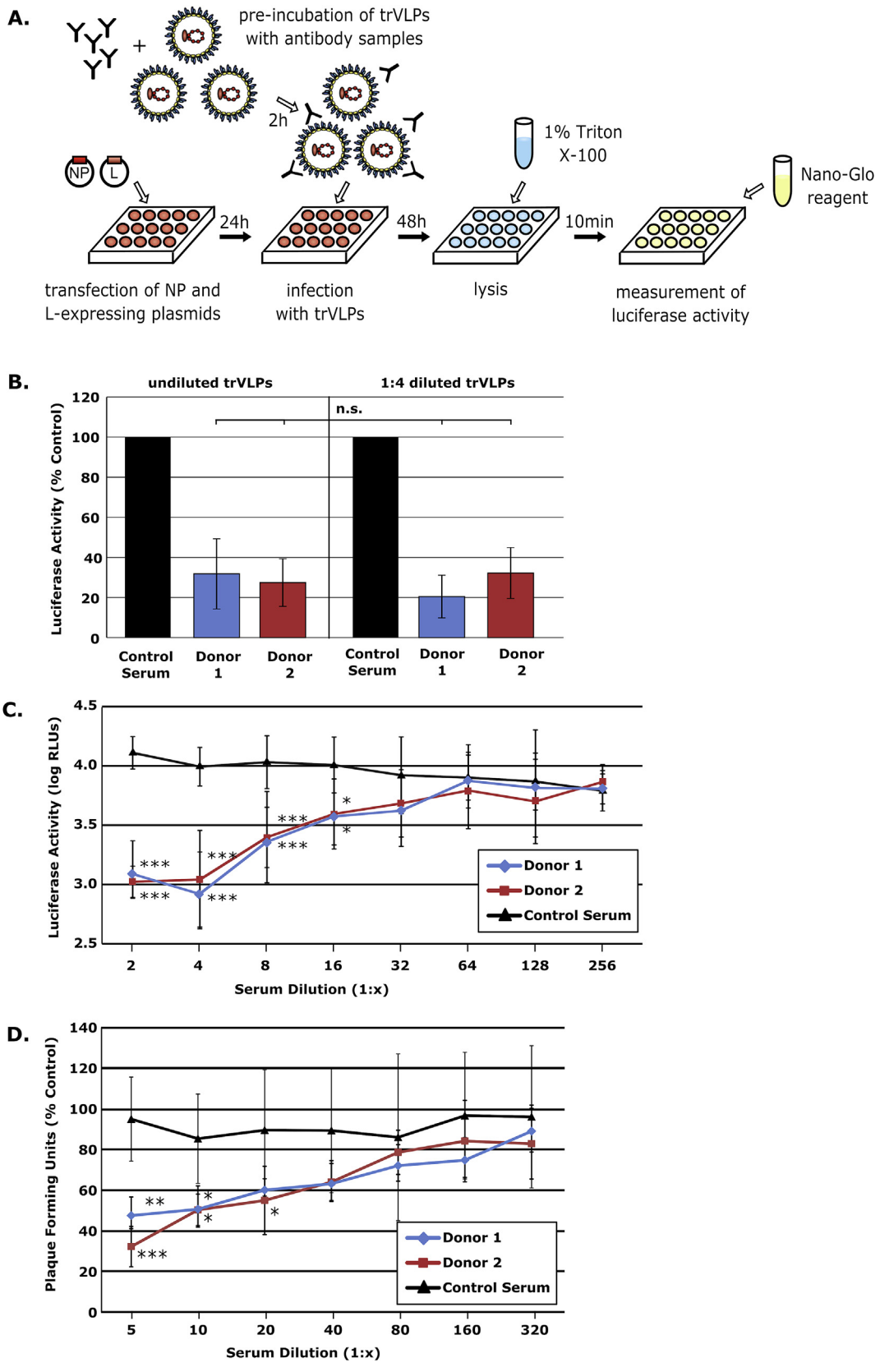


Fig. 2. Evaluation of trVLP input/reporter activity relationship and particle stability. (A) Quantification of the relationship between trVLP input and reporter activity. To establish the relationship between trVLP input and reporter activity, trVLP preparations were diluted in a two-fold series before being used to infect p1 cells. The means and standard deviations of NanoLuciferase (nLuc) activity are shown and represent data from three independent experiments. The equation and coefficient of determination (R^2) of the best-fitting model (i.e. nonlinear regression - quadratic) are indicated. **(B) Effect of trVLP dilution on assay dynamic range.** To assess the effect of total trVLP activity on assay dynamic range (i.e. the difference between negative and positive control samples), trVLP preparations with and without Z and GP were diluted 10 fold before being used to infect p1 cells and the reporter activity in the respective samples was compared. The means and standard deviations of nLuc activity are shown and represent data from four independent experiments. Results of a T-test comparing assay dynamic range (mean positive control – mean negative control) are shown. **(C) Stability of frozen trVLP preparations and resistance to freeze-thaw.** trVLP preparations were either stored briefly at 4 °C or subjected to freezing and thawing at either −20 °C or −80 °C, as indicated, before being used for infection of p1 cells (right panel). Alternatively, samples of trVLPs produced either with or without Z and GP were tested directly after preparation or stored for a year at −20 °C before being used to infect p1 cells. To control for differences between these assays, the mean luminescence values for the respective assays were normalized. The means and standard deviations of nLuc activity are shown and represent data from two independent experiments. Results of a one-way ANOVA to compare the activity of trVLPs before and after storage are shown.



(caption on next page)

generation of nAb titres is the measure by which successful vaccination against JUNV is established. As such, assessment of nAb reactivity is clearly of key clinical importance for the evaluation of approaches aimed at vaccination as well as therapy for AHF.

Traditionally neutralizing antibody activity and specificity are measured using PRNT assays. While such assays have been used

Figure 3. trVLP-based neutralization assay optimization. (A) Schematic diagram of trVLP neutralization assay. To assess neutralizing activity of a serum sample, supernatants of p0 cells containing trVLPs containing JUNV GP (strain Romero) that had been generated using the trVLP assay were incubated with serum at 37 °C. These trVLP:serum complexes were the used to infect target cells (p1 cells) that had been pre-transfected with JUNV NP and L. After 48 h the cells were lysed and measured for reporter activity, reflecting trVLP entry. **(B) Effect of total trVLP content on neutralization.** trVLPs preparations were either left undiluted or diluted 1:4 before being incubated with control pooled human AB serum or Candid#1-specific sera. Subsequent infection of p1 cells was performed as described in (A). The means and standard deviations of nLuc activity are shown and represent data from two independent experiments. Results of a one-way ANOVA to compare diluted and undiluted trVLP samples are shown. **(C) Titration of Candid#1 vaccinee control sera in a trVLP-based neutralization assay.** Serum samples from known Candid#1 vaccinees or a control pooled Ab serum were prepared as a 2-fold dilution series, before incubation with JUNV (Romero) trVLPs at 37 °C for 2 h. Afterwards trVLP:serum complexes were used to infect Huh7 cells (p1 cells) that had been pre-transfected 24 h previously with pCAGGS-NP and pCAGGS-L (p1 cells). After 48 h the p1 cells were harvested and measured for nLuc (viral RNA synthesis). The means and standard deviations of Nanoluciferase (nLuc) activity are shown and represent data from four independent experiments. Results of a one-way ANOVA to compare samples incubated with control or Candid#1-specific sera are shown. **(D) Titration of Candid#1 vaccinee control sera in a virus-based neutralization assay.** Heat-inactivated serum samples from known Candid#1 vaccinees or a control pooled Ab serum were prepared as a 2-fold dilution series (straining from an initial 1:2.5 dilution), before incubation with approximately 100 pfu of JUNV (Cba IV4454) at 37 °C for 1 h. Afterwards virus:serum complexes were used to infect Vero cells for 1 h at 37 °C before the inoculum was replaced with a semi-solid overlay. Plaques were allowed to form for 7 days at 37 °C before being fixed and stained for counting. The means and standard deviations of the resulting percent titre reduction values are shown and represent data from three independent experiments. Results of a one-way ANOVA to compare samples incubated with control or Candid#1-specific sera are shown.

successfully for many decades, and exhibit high sensitivity and specificity with few technical requirements and low cost, they also have a number of drawbacks. The first and often significant limitation is that the virus in question must form large well-resolved plaques. However, even where this is the case, the ability to distinguish individual plaques in a sample limits the dynamic range of such PRNT assays. The small starting virus input makes sample-to-sample variation problematic and the need to resolve individual plaques means that miniaturization of such conventional PRNTs is challenging. Perhaps the biggest issue is that the evaluation of PRNT assays is extremely labor intensive, being dependent on manual analysis, and thus limiting sample throughput. Of course, since PRNT assays are based on work with the infectious pathogens, biosafety requirements are an additional consideration depending on the virus in question.

Plaque-reduction neutralization remains the gold standard for assessing neutralizing antibody activity. However, a number of different approaches have been reported in recent years for different pathogens that have tried to address some of its limitations, e.g. (Koishi et al., 2018; Li et al., 2017; Putnak et al., 2008; Shan et al., 2017; Taketa-Graham et al., 2010). To date the main alternative to plaque-based neutralization with authentic arenaviruses has been the use of low virulence recombinant/pseudotyped viruses (e.g. Murine Leukemia virus, Vesicular Stomatitis virus) expressing the specific virus GP of interest. This allows work with reduced biocontainment requirements and reduces assay time (depending on the vector virus background), and such systems have recently been used to successfully screen neutralizing antibodies against JUNV (Clark et al., 2018; Pan et al., 2018). However, they also have obvious limitations with respect to fundamental differences in particle structure and composition compared to the authentic virus they are meant to mimic. Certainly for filoviruses these kinds of differences in particle shape complicated recombinant/pseudotype-based studies of the viral entry mechanism for many years, until work with VLPs/trVLPs could finally help to clarify this issue (Aleksandrowicz et al., 2011; Nanbo et al., 2010; Saeed et al., 2010). Thus, while for arenaviruses where these trVLP-based systems are just coming into use it is not yet clear to what extent this represents an issue, particularly given the unusual ability of arenavirus particles to extensively incorporate cellular content, up to and including intact ribosomes, this would appear to be a factor worthy of future consideration. However, at the same time the contribution of studies using such recombinant/pseudotyped virus platforms clearly cannot be overlooked, and especially with the well-established resources available for their study and their general ease of use they will likely also continue to remain a front-line approach for many applications in the future. Further, in the case of JUNV, this issue can in many cases be circumvented by using the attenuated Candid#1 strain, although the classification of this strain as BSL2 is not universal. However, similar options for other arenaviruses are not currently available. Indeed, the need for technical advancements to support antibody screening for arenaviruses is highlighted by the recent WHO R&D roadmap for LASV which specifically addresses the need for assays to assess vaccine immunogenicity and highlights the extent to which the need for BSL4 containment still restricts the development and testing of therapeutic approaches for these agents (World Health Organization, 2018).

Here we show that using JUNV trVLPs as a proxy for virus in a classical neutralization assay produced serum titre values comparable to those obtained using infectious JUNV while having several notable advantages: 1) the assay can be performed under BSL2 conditions, 2) the assay has an objective automated read-out, and 3) the assay time is much shorter (48 h vs 7 days). In addition, the smaller plate format (96-well vs. 24-well) may be more practical when analyzing a large number of samples. Further, while it remains necessary to first generate trVLP preparations for testing using this method (somewhat analogous to the need to prepare and titre virus stocks before use in a traditional PRNT assay), the observed long-term stability of arenavirus trVLPs at sub-zero temperatures allows large batches to be produced and frozen for future use, thereby eliminating a major potential source of assay-to-assay variation and simplifying their use to something comparable to the ease of infectious virus. Thus, our data suggest that the analysis of trVLP neutralization may indeed offer a more efficient method for determining neutralizing antibody titres, particularly in the context of larger scale analyses (e.g. vaccination studies or antibody screening studies). Unfortunately, the assay still requires the availability of a basic BSL2 laboratory facility with cell culture capabilities and is, therefore, not suitable for implementation in highly resource limited settings or in the field – although similar limitations of course also apply to conventional virus neutralization assays as well.

An exciting feature of trVLPs is that the glycoprotein can be easily substituted for that of other arenaviruses, allowing not only analysis of homotypic neutralization, but of cross-neutralization of related virus species as well. Indeed, in this study we used this approach to examine cross-neutralization of other New World arenaviruses by human anti-JUNV antibodies. We found that JUNV GP from a range of virus isolates could be effectively neutralized by human sera, consistent with the long-term clinical success of both the Candid#1 vaccine and plasma therapy. In contrast, when we produced trVLPs expressing the GP from different New World arenavirus species there was no neutralization, even for the most closely related virus species, MACV and TCRV. Interestingly, GPC processing to the mature GP1/GP2 appeared to vary between arenavirus species, and even between JUNV strains, with the Clone 3 and XJ13 constructs producing especially low levels of mature GP2. However, the functionality of the trVLP preparations produced using these constructs suggest that even such modest levels of processing are sufficient for particle infectivity, which for arenaviruses is known to depend on mature GP (Rojek et al., 2008). However, this variability in GP processing, coupled with limitations in standardizing

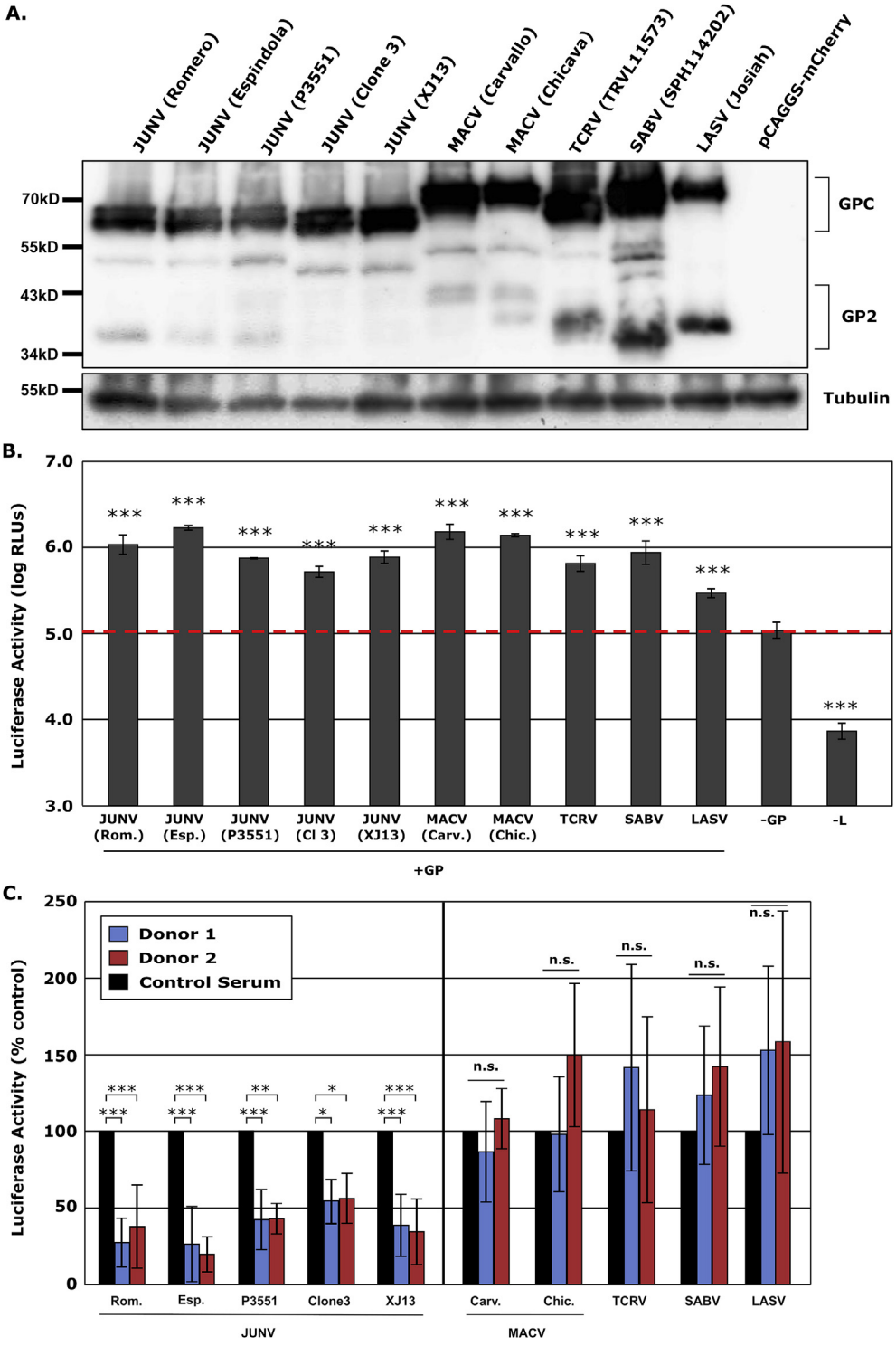


Fig. 4. Generation and neutralization of heterologous trVLPs. (A) Expression of C-terminally flag tagged arenavirus glycoproteins. Glycoprotein (GP) open reading frames for the arenavirus species indicated were cloned together with a C-terminal FLAG tag in pCAGGS. Constructs were transfected into 293T cells and lysates harvested 48 h post-transfection for analysis by Western Blot using a mouse anti-FLAG primary antibody and an anti-mouse-HRP conjugated secondary antibody. **(B) Generation of trVLPs expressing heterologous GPs.** JUNV trVLPs expressing heterologous GP proteins from different arenaviruses were generated as described in Fig. 1, but the plasmid encoding JUNV (strain Romero) GP was substituted for pCAGGS constructs encoding GPs from different JUNV strains, or other arenavirus species, as indicated. These trVLP preparations were used to infect p1 cells (Huh7) pre-transfected with JUNV NP and L. After 48 h the p1 cells were harvested and measured for Nanoluciferase (nLuc; viral RNA synthesis). The means and standard deviations of nLuc activity are shown and represent data from three independent experiments. Results of a one-way ANOVA to compare samples to a control without GP are shown. **(C) Neutralization of heterologous trVLPs by Candid#1 vaccinee sera.** trVLPs produced as described in (B) containing the GPs of various arenaviruses were incubated 1:1 with serum from human Candid#1 vaccinees or with a control pooled human AB serum for 2 h at 37 °C before being used to infect p1 cells (Huh7) pre-transfected with JUNV NP and L. After 48 h the p1 cells were harvested and measured for nLuc (viral RNA synthesis). The means and standard deviations of nLuc activity relative to the data obtained with control sera are shown and represent data from at least two independent experiments. Results of a one-way ANOVA comparison to control antibody treated samples are shown.

different trVLP preparation for GP content (due to the mixture of trVLPs and non-infectious VLPs that are present in samples) make it challenging to directly compare between different samples, and rather each sample can only reliably be compared to its own untreated control. Surprisingly, despite the widespread application of neutralization assays in both laboratory and diagnostic settings, the relationship between infectious and non-infectious particle content in stock preparations (be they of virus or trVLPs) is in fact rarely, if ever, considered. However, the lack of attention to this issue during decades of successful application of conventional virus-based neutralization assays, coupled with our own data indicating that antibody saturation by excessive amounts of non-infectious material does not occur (Fig. 2B), seem to suggest that this is likely not a major issue in either assay platform.

While at first glance our observations regarding cross-species neutralization appear to conflict with reports of successful cross-protection in animal models following vaccination with various attenuated New World arenaviruses (Golden et al., 2017; Koma et al., 2016; Weissenbacher et al., 1975, 1982), where these studies have specifically looked at neutralization, they also observed a lack of cross-neutralization (Golden et al., 2017; Koma et al., 2016), suggesting that cross-protection is driven by aspects of the immune response unrelated to nAb response. Thus our data support the available evidence from

animal models in suggesting that, also in humans, neutralizing antibodies generally display little cross-reactivity between different virus species. As a consequence, convalescent plasma therapy using donations from JUNV patients would likely exhibit little clinical utility against infection with other related arenaviruses, even if it would be technically feasible to perform such treatments. Thus it appears that specific efforts to develop therapeutic antibody-based resources will likely be necessary for each of the pathogenic New World arenavirus species. Alternatively, it may be possible to identify individual monoclonal antibodies with adequate cross-reactivity, and indeed some limited recent success has already been reported in this respect (Clark et al., 2018).

As research on New World arenaviruses moves ahead, and hopefully continues to expand to include species other than JUNV, the availability of an assay to examine neutralizing antibody responses against a range of arenaviruses that can be performed in small format with high-throughput and with automated readout under BSL2 conditions will provide significant benefits. In particular we believe this approach will prove especially useful for those looking to demonstrate protective responses elicited by various novel vaccination platforms that are now being developed and/or those seeking to characterize cross-neutralization by monoclonal Abs.

5. Conclusions

We demonstrate here that trVLPs can be effectively used as a substitute for infectious virus in the analysis of antibody-mediated neutralization. Given their non-infectious nature, combined with the shorter assay time, smaller format, higher dynamic range and more objective readout this assay will provide a valuable tool, especially for the analysis of large sample sets. Further, the trVLP system offers the opportunity to easily exchange surface glycoproteins among arenaviruses, thereby facilitating studies of neutralizing antibody cross-reactivity between virus species. Using precisely this approach we demonstrated that crude human serum against JUNV is unlikely to contain sufficient cross-reactive neutralizing antibodies to demonstrate significant therapeutic potential, and thus efforts to identify novel monoclonal antibodies against other arenavirus species and/or cross-reactive monoclonal antibodies are very much needed.

Acknowledgements

The authors thank Claudia Drabent for her technical assistance with this study, Stefan Finke for providing the BSR-T7/5 cells, and the Friedrich-Loeffler-Institut Cell Bank for their assistance in supplying the other cell lines used in this work. This work was funded by the Friedrich-Loeffler-Institut, the Deutsche Forschungsgemeinschaft (DFG; grant number 324484564), the Division of Intramural Research, NIAID, NIH, and by the University of Buenos Aires (UBACyT 20020160100091BA). The funding sources had no involvement in the study design, the collection, analysis or interpretation of data, in the writing of the report, or in the decision to submit the article for publication. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the NIH.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.01.006.

References

Aleksandrowicz, P., Marzi, A., Biedenkopf, N., Beimforde, N., Becker, S., Hoenen, T., Feldmann, H., Schnittler, H.J., 2011. Ebola virus enters host cells by macropinocytosis and clathrin-mediated endocytosis. J. Infect. Dis. 204 (Suppl. 3), S957–S967.

Brouillette, R.B., Phillips, E.K., Ayithan, N., Maury, W., 2017. Differences in glycoprotein complex receptor binding site accessibility prompt poor cross-reactivity of neutralizing antibodies between closely related arenaviruses. J. Virol. 91.

Buchholz, U.J., Finke, S., Conzelmann, K.K., 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J. Virol. 73, 251–259.

Charrel, R.N., de Lamballerie, X., 2003. Arenaviruses other than Lassa virus. Antivir. Res. 57, 89–100.

Clark, L.E., Mahmutovic, S., Raymond, D.D., Dilanyan, T., Koma, T., Manning, J.T., Shankar, S., Levis, S.C., Briggiler, A.M., Enria, D.A., Wucherpfennig, K.W., Paessler, S., Abraham, J., 2018. Vaccine-elicited receptor-binding site antibodies neutralize two New World hemorrhagic fever arenaviruses. Nat. Commun. 9, 1884.

Dunham, E.C., Leske, A., Shifflett, K., Watt, A., Feldmann, H., Hoenen, T., Groseth, A., 2018. Lifecycle modelling systems support inosine monophosphate dehydrogenase (IMPDH) as a pro-viral factor and antiviral target for New World arenaviruses. Antivir. Res. 157, 140–150.

Enria, D., Franco, S.G., Ambrosio, A., Vallejos, D., Levis, S., Maiztegui, J., 1986. Current status of the treatment of Argentine hemorrhagic fever. Med. Microbiol. Immunol. 175, 173–176.

Enria, D.A., Briggiler, A.M., Fernandez, N.J., Levis, S.C., Maiztegui, J.I., 1984. Importance of dose of neutralising antibodies in treatment of Argentine haemorrhagic fever with immune plasma. Lancet 2, 255–256.

Enria, D.A., Briggiler, A.M., Sanchez, Z., 2008. Treatment of Argentine hemorrhagic fever. Antivir. Res. 78, 132–139.

Golden, J.W., Beitzel, B., Ladner, J.T., Mucker, E.M., Kwilas, S.A., Palacios, G., Hooper, J.W., 2017. An attenuated Machupo virus with a disrupted L-segment intergenic region protects Guinea pigs against lethal Guanarito virus infection. Sci. Rep. 7, 4679.

Groseth, A., Wolff, S., Strecker, T., Hoenen, T., Becker, S., 2010. Efficient budding of the tacaribe virus matrix protein z requires the nucleoprotein. J. Virol. 84, 3603–3611.

Koishi, A.C., Suzukawa, A.A., Zanluca, C., Camacho, D.E., Comach, G., Duarte Dos Santos, C.N., 2018. Development and evaluation of a novel high-throughput image-based fluorescent neutralization test for detection of Zika virus infection. PLoS Neglected Trop. Dis. 12, e0006342.

Koma, T., Patterson, M., Huang, C., Seregin, A.V., Maharaj, P.D., Miller, M., Smith, J.N., Walker, A.G., Hallam, S., Paessler, S., 2016. Machupo virus expressing GPC of the Candid#1 vaccine strain of junin virus is highly attenuated and immunogenic. J. Virol. 90, 1290–1297.

Li, W., Cao, S., Zhang, Q., Li, J., Zhang, S., Wu, W., Qu, J., Li, C., Liang, M., Li, D., 2017. Comparison of serological assays to titrate Hantaan and Seoul hantavirus-specific antibodies. Virol. J. 14, 133.

Mahmutovic, S., Clark, L., Levis, S.C., Briggiler, A.M., Enria, D.A., Harrison, S.C., Abraham, J., 2015. Molecular basis for antibody-mediated neutralization of new World hemorrhagic fever mammarenaviruses. Cell Host Microbe 18, 705–713.

Maiztegui, J.I., Fernandez, N.J., de Damilano, A.J., 1979. Efficacy of immune plasma in treatment of Argentine haemorrhagic fever and association between treatment and a late neurological syndrome. Lancet 2, 1216–1217.

Maiztegui, J.I., McKee Jr., K.T., Barrera Oro, J.G., Harrison, L.H., Gibbs, P.H., Feuillade, M.R., Enria, D.A., Briggiler, A.M., Levis, S.C., Ambrosio, A.M., Halsey, N.A., Peters, C.J., 1998. Protective efficacy of a live attenuated vaccine against Argentine hemorrhagic fever. AHF Study Group. J. Infect. Dis. 177, 277–283.

McCormick, J.B., Webb, P.A., Krebs, J.W., Johnson, K.M., Smith, E.S., 1987. A prospective study of the epidemiology and ecology of Lassa fever. J. Infect. Dis. 155, 437–444.

Nanbo, A., Imai, M., Watanabe, S., Noda, T., Takahashi, K., Neumann, G., Halfmann, P., Kawaoka, Y., 2010. Ebolavirus is internalized into host cells via macropinocytosis in a viral glycoprotein-dependent manner. PLoS Pathog. 6, e1001121.

Pan, X., Wu, Y., Wang, W., Zhang, L., Xiao, G., 2018. Novel neutralizing monoclonal antibodies against Junin virus. Antivir. Res. 156, 21–28.

Putnak, J.R., de la Barrera, R., Burgess, T., Pardo, J., Dessy, F., Gheysen, D., Lobet, Y., Green, S., Endy, T.P., Thomas, S.J., Eckels, K.H., Innis, B.L., Sun, W., 2008. Comparative evaluation of three assays for measurement of dengue virus neutralizing antibodies. Am. J. Trop. Med. Hyg. 79, 115–122.

Rojek, J.M., Lee, A.M., Nguyen, N., Spiropoulou, C.F., Kunz, S., 2008. Site 1 protease is required for proteolytic processing of the glycoproteins of the South American hemorrhagic fever viruses Junin, Machupo, and Guanarito. J. Virol. 82, 6045–6051.

Ruggiero, H.A., Magnoni, C., de Guerrero, L.B., Milani, H.A., Izquierdo, F.P., Milani, H.L., Weber, E.L., 1981. Persistence of antibodies and clinical evaluation in volunteers 7 to 9 Years following the vaccination against Argentine hemorrhagic fever. J. Med. Virol. 7, 227–232.

Saeed, M.F., Kolokoltsov, A.A., Albrecht, T., Davey, R.A., 2010. Cellular entry of ebola virus involves uptake by a macropinocytosis-like mechanism and subsequent trafficking through early and late endosomes. PLoS Pathog. 6, e1001110.

Shan, C., Ortiz, D.A., Yang, Y., Wong, S.J., Kramer, L.D., Shi, P.Y., Loeffelholz, M.J., Ren, P., 2017. Evaluation of a novel reporter virus neutralization test for serological diagnosis of zika and dengue virus infection. J. Clin. Microbiol. 55, 3028–3036.

Spiegelberg, L., Wahl-Jensen, V., Kolesnikova, L., Feldmann, H., Becker, S., Hoenen, T., 2011. Genus-specific recruitment of filovirus ribonucleoprotein complexes into budding particles. J. Gen. Virol. 92, 2900–2905.

Taketa-Graham, M., Powell Pereira, J.L., Baylis, E., Cossen, C., Oceguera, L., Patiris, P., Chiles, R., Hanson, C.V., Forghani, B., 2010. High throughput quantitative colorimetric microneutralization assay for the confirmation and differentiation of West Nile Virus and St. Louis encephalitis virus. Am. J. Trop. Med. Hyg. 82, 501–504.

Watt, A., Moukambi, F., Banadyga, L., Groseth, A., Callison, J., Herwig, A., Ebihara, H., Feldmann, H., Hoenen, T., 2014. A novel life cycle modeling system for Ebola virus shows a genome length-dependent role of VP24 in virus infectivity. J. Virol. 88, 10511–10524.

Weissenbacher, M.C., Coto, C.E., Calello, M.A., 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.

Weissenbacher, M.C., Coto, C.E., Calello, M.A., Rondinone, S.N., Damonte, E.B., Frigerio, M.J., 1982. Cross-protection in nonhuman primates against Argentine hemorrhagic fever. Infect. Immun. 35, 425–430.

World Health Organization, 2018. Lassa Fever R&D Roadmap. http://www.who.int/blueprint/priority-diseases/key-action/lassa-fever/en.

Zeitlin, L., Geisbert, J.B., Deer, D.J., Fenton, K.A., Bohorov, O., Bohorova, N., Goodman, C., Kim, D., Hiatt, A., Pauly, M.H., Velasco, J., Whaley, K.J., Altmann, F., Gruber, C., Steinkellner, H., Honko, A.N., Kuehne, A.I., Aman, M.J., Sahandi, S., Enterlein, S., Zhan, X., Enria, D., Geisbert, T.W., 2016. Monoclonal antibody therapy for Junin virus infection. Proc. Natl. Acad. Sci. U. S. A. 113, 4458–4463.

Zeltina, A., Krumm, S.A., Sahin, M., Struwe, W.B., Harlos, K., Nunberg, J.H., Crispin, M., Pinschewer, D.D., Doores, K.J., Bowden, T.A., 2017. Convergent immunological solutions to Argentine hemorrhagic fever virus neutralization. Proc. Natl. Acad. Sci. U. S. A. 114, 7031–7036.