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Development of horse neutralizing immunoglobulin and immunoglobulin fragments against Junín virus

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ABSTRACT

Argentine haemorrhagic fever (AHF) is a rodent-borne disease with a lethality as high as ~30%, which is caused by the New World arenavirus, Junín virus (JUNV). It was once a major epidemic in South America and puts millions of people in Argentina at risk. Here, we aimed to develop horse antibodies or antibody fragments against JUNV. Before preparing the horse antibodies, a strategy to efficiently generate horse antisera was established based on comparisons among immunogens and immunization methods in both mice and horses. Antisera against JUNV were finally obtained by vaccinating horses with vesicular stomatitis virus pseudotypes bearing JUNV GP. The horse antibodies IgG and F(ab’)2 were subsequently demonstrated to effectively neutralize vesicular stomatitis virus pseudotypes bearing JUNV GP and to show some cross-neutralization against pathogenic New World arenaviruses.

1. Introduction

Enveloped RNA viruses that cause human viral haemorrhagic fevers include mainly filo-, flavi-, bunya-, and arenaviruses (Paessler and Walker, 2013). These viruses are prevalent worldwide and infect people in areas with poor public health (Gonzalez et al., 2018). New World (NW) arenaviruses, such as Junín virus (JUNV), Machupo virus (MACHV), Guanarito virus (GTOV), Sabia virus (SBAV), and Chapare virus (CHAPV), uninterruptedly break out in South American countries, such as Argentina, Bolivia, Venezuela, and Brazil (Gonzalez et al., 2018). They asymptptomatically reside in rodents but are highly pathogenic in humans after occasional infection through the skin, respiratory tract or gastrointestinal mucosa (Radoshitzky et al., 2008). Due to their high fatality rates, as high as ~30%, and limited prophylactics or therapeutics, they are identified as biosafety level (BSL) 4 pathogens in most countries, except that JUNV can be handled at BSL 3 lab in certain circumstances (Numberg and York, 2012).

JUNV, one of the most important NW arenaviruses, is the aetiologic agent of Argentine haemorrhagic fever (AHF), for which almost five million people in Argentina are at risk (Enria et al., 2018). As we know, an attenuated live-virus vaccine (Candid#1) licensed in Argentina markedly reduces the mortality rate of AHF (Maiztegui et al., 1998). Human immunoglobulin G (IgG) from convalescent individuals is currently the main therapeutic option for AHF, while the risk of viral transmission between humans and concern regarding a shortage of plasma may exist (Enria et al., 2008; Kenyon et al., 1990; Ruggiero et al., 1986). Hence, additional drugs are urgently needed.

Several monoclonal antibodies (mAbs) against JUNV have been reported in recent years, including human-mouse chimaera mAbs modified by Zeitlin et al. (2016), vaccine-elicited human mAbs discovered by Clark et al. (2018), and a series of mouse mAbs identified by our group (Pan et al., 2018). These mAbs target the envelope glycoprotein complex (GPC) for neutralization, which consists of trimeric noncovalently bound subunits: receptor recognition subunit glycoprotein 1 (GP1), membrane-fusion subunit glycoprotein 2 (GP2), and stable signal peptide (SSP) (Radoshitzky et al., 2007; Wang et al., 2016). Although one single-domain antibody targeting nucleoprotein was also reported to neutralize Candid#1 in vitro (Linero et al., 2018), most reported neutralizing antibodies (nAbs) for JUNV were demonstrated to target GP1. In addition to mAbs, polyclonal antibodies also play a role in combating JUNV infection. Polyclonal antibodies purified from DNA-
immunized rabbits have been demonstrated to protect guinea pigs from a lethal JUNV challenge (Golden et al., 2016). In fact, horse antiserum-derived polyclonal antibodies that demonstrated good efficacy against infectious diseases caused by highly pathogenic agents, such as Ebola virus (Wang et al., 2018a; Zheng et al., 2016), West Nile virus (Cui et al., 2016), H5N1 influenza virus (Lu et al., 2006), severe acute respiratory syndrome coronavirus (Zhao et al., 2017), have been extensively reported.

Thus, we studied polyclonal antibodies against JUNV by preparing horse antiserum and antibodies. To achieve this goal, different immunogens and immunization strategies were first tested in mice and then in horses. Antiserum prepared from horses vaccinated with vesicular stomatitis virus (VSV) pseudotypes bearing JUNV GP were subsequently demonstrated to contain abundant nAbs against JUNV. Then, total IgG was purified, and F(ab')2 was obtained by removing Fc from IgG by pepsin digestion. Specific IgG and F(ab')2 were prepared through affinity chromatography against recombinant JUNV GP1. Finally, the neutralizing activity was verified with both VSV pseudotypes bearing JUNV GP and recombinant VSV expressing JUNV GP along with four other pathogenic NW arenaviruses. Antibodies specific to GP1 were finally found to bind an important site, Asp123, on GP1. This study presents an efficient strategy to prepare horse nAbs against JUNV and provides a foundation for developing potential therapeutics for AHF.

2. Materials and methods

2.1. Cells and viruses

HEK293T, Vero-E6, HeLa, and BHK-21 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% foetal bovine serum (FBS, Gibco) at 37 °C with 5% CO2. HEK293F cells were cultured in Freestyle 293 expression medium (Invitrogen, Carlsbad, CA) supplemented with 10% foetal bovine serum (FBS, Gibco) at 37 °C with 5% CO2 while shaking at 180 rpm/min.

VSV pseudovirus packaging was performed as described below. First, GPC genes of JUNV (GenBank: NC_005081.1), MACV (GenBank: NC_005078.1), GTOV (GenBank: NC_005077.1), SBAV (GenBank: U41071.1), and CHAPV (GenBank: NC_010562.1) were synthesized by GenScript Co., Ltd. (Nanjing, China) and then separately inserted into restriction sites of the eukaryotic expression vector pCAGGS to generate pCAGGS-JUNV GPC, pCAGGS-MACV GPC, pCAGGS-GTOV GPC, pCAGGS-SBAV GPC, and pCAGGS-CHAPV GPC, respectively. HEK293T cells seeded in dishes were transfected with the pCAGGS-JUNV GPC plasmid for 24 h and then infected with VSVAG-GFP/VSV G or VSVAG-Rluc/VSV G (Whitt, 2010) (packaged by our laboratory members) for 1 h. Subsequently, the cell supernatant was completely removed, and culture supernatants containing pseudovirus (VSVAG-GFP/JUNV GPC or VSVAG-Rluc/JUNV GPC) were collected after 36 h. VSV pseudotypes bearing MACV, GTOV, SBAV or CHAPV GPCs were packaged in the same way as VSV pseudotypes bearing JUNV GP. These pseudoviruses were restricted to a single round of replication, thus enabling them to be handled in a BSL 2 lab.

The packaging of recombinant VSV expressing JUNV GP was performed as described previously (Garbutt et al., 2004; Wang et al., 2018b). Briefly, the JUNV GPC gene was cloned into the plasmid pVSVGAG-eGFP (Addgene 31842) at the ΔG site to generate pVSVGAG-eGFP-JUNV GPC. BHK-21 cells were seeded in 6-plate wells overnight. Culture supernatants were removed, and cells were infected with recombinant vaccinia virus (vTF7-3) encoding T7 RNA polymerase at an MOI = 5. Forty-five minutes later, the infectious mixture was completely removed, and the cells were washed with DMEM. Then, a total of 11 μg of plasmids (pBS-N, pBS-P, pBS-S, pBS-L and pVSVGAG-eGFP-JUNV GPC) at a ratio of 3:5:8:1:5 were transfected into cells using Lipofectamine 3000 (Invitrogen). After 48 h, supernatants were collected, flowed through a 0.22 μm filter to remove residual vaccinia virus, and inoculated into BHK-21 cells that had been transfected with pCAGGS-VSV G 24 h previously. Four days later, supernatants were collected and passed on BHK-21 cells to obtain the third-generation virus (rVSVAG-eGFP/JUNV GPC), which exclusively expresses JUNV GP. The viral titre was measured on Vero-E6 cells by calculating fluorescent plaques.

2.2. Immunogen preparation

Codon-optimized JUNV GPC was synthesized by GenScript Co., Ltd. Plasmid pCAGGS-JUNV GPC was generated as usual and purified from E. coli (DH5α) with an endotoxin-free plasmid extraction kit (Invitrogen, Carlsbad, CA). VSVAG-GFP/JUNV GPC collected from HEK293T culture supernatants was subjected to ultracentrifugation with a 20% sucrose solution to obtain pure viral particles.

GP1 sequences (87–232 AA) were amplified from JUNV GPC to generate pcDNA3.1-CDS-GP1-his. The pcDNA3.1-CDS-GP1-his plasmid was transfected into HEK293F cells with polyethylenimine (PEI) (Polysciences, Warrington, PA), and sodium valproate (2 mM) was added to control the overgrowth of cells. Cell culture supernatants were collected 7 days later, dialysed with phosphate buffer (0.5 M NaCl, 20 mM NaH2PO4, pH 8.0) and then subjected to Ni-sepharose purification. Recombinant GP1 was eluted with 300 mM imidazole and dialysed before use.

2.3. Ethics statements

All animal experiments were performed strictly according to the Regulations for the Administration of Affairs Concerning Experimental Animals in China, and the protocols were approved by the Laboratory Animal Care and Use Committee of Wuhan Institute of Virology, Chinese Academy of Sciences (Wuhan, China).

2.4. Mouse immunizations

Female BALB/c mice aged 6–8 weeks were housed in specific pathogen-free animal care facilities. The mice were divided into four groups (n = 3 in each group), and a homogeneous prime-boost-boost protocol was applied to all the groups. Mice receiving recombinant GP1, pCAGGS-JUNV GPC, and VSVAG-GFP/JUNV GPC were vaccinated via different routes, and phosphate-buffered saline (PBS) was used as a control. Each vaccination was performed three times in total with a two-week interval.

In detail, recombinant GP1 or pseudoviral particles were mixed with Freund’s complete adjuvant (Sigma-Aldrich, St. Louis, MO) for priming and mixed with Freund’s incomplete adjuvant (Sigma-Aldrich) for boosting. Protein (50 μg per mouse) or pseudoviral particles (107 focus-forming units, FFU) were mixed with adjuvant up to a volume of 100 μl and then used for intradermal, subcutaneous, intraperitoneal and lymphatic injection in each mouse. The pCAGGS-JUNV GPC plasmid was diluted with PBS to 1.0 mg/ml, and a volume of 100 μl was equally injected into two legs of each mouse. Then, the mice were subjected to electroporation with an electroporator (BTX, San Diego, CA) with setting parameters of 100 V and 50 ms.

2.5. Horse immunizations

Six healthy horses aged 3–10 years old were divided into three groups. Horses homogenously received subcutaneous and intramuscular multipoint injections with DNA (5 mg) in aluminium phosphate (0.45%) or pseudoviral particles (1.5 × 107 FFU) in aluminium hydroxide (0.45%). Immunizations were implemented at weeks 0, 2, 4, and 6, and serum samples were collected from the jugular vein at weeks 1, 3, 5, and 7 for monitoring the variation in antibody response.
2.6. Preparation of horse IgG and F(ab′)2

After immunization, horse plasma was collected with a plasma collection machine and preserved at 4°C for further analysis. For purification of total IgG, plasma was first diluted with PBS (50 mM NaCl, 20 mM Na2HPO4, pH 8.0) at a ratio of 1:5 and then filtered with a 0.8 μm filter before being subjected to affinity chromatography with Protein A-conjugated resin. For preparation of total F(ab′)2, pepsin (Sigma-Aldrich) was used to remove Fc from IgG. In detail, total IgG was first diluted with sodium acetate buffer (0.2 M, pH 3.0) to 1.0 mg/ml, and pepsin was then added in a mass proportion of 1:50. The digestion reaction was performed at 37°C for 1 h and then terminated by adding Tris-base buffer (2 M, pH 9.0) to adjust the pH to 7.0. The digestion products were subjected to a Protein A column to remove residual IgG and Fc fragments, followed by concentration with a 50 kDa ultrafiltration device to remove small proteins.

For purification of GP1-specific IgG, recombinant GP1 expressed by HEK293F cells, described above, was coupled on preactivated resin (PabPurSulfolink Beads, SMART Life Sciences, Changzhou) through an amino reaction, and the GP1-coupled resin was then used to purify GP1-specific IgG from total IgG. Eluted component (GP1-specific IgG) and flow-through component (residual IgG) from affinity chromatography against GP1 were collected, respectively. GP1-specific F(ab′)2 was acquired by purifying F(ab′)2 from total F(ab′)2 in the same manner as GP1-specific IgG. These antibodies were maintained in PBS before use.

2.7. Nonreducing and reducing SDS-PAGE

To verify the purities and sizes of IgG and F(ab′)2 prepared in our study, nonreducing and reducing SDS-PAGE were employed. Nonreduced samples were prepared by mixing 5 μg of protein with loading buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 0.1% bromophenol blue), adding neither DTT nor 2-β-mercaptoethanol. Reduced samples were prepared by mixing 5 μg of protein with loading buffer, adding 2-β-mercaptoethanol, and then boiling in water for 10 min. Both nonreduced and reduced samples were concentrated with 4% SDS-PAGE and separated with 10% SDS-PAGE. Finally, SDS-PAGE gels were stained with Coomassie Brilliant Blue. An image of the gel after decolorization was captured with a ChemiDoc MP Imaging system (Bio-Rad).

2.8. Enzyme-linked immunosorbent assay (ELISA)

For the GP1-captured or peptide ELISA, recombinant GP1 or peptide was diluted to 10 μg/ml with coating buffer (0.05 M NaHCO3, pH 9.5). For the pseudovirus-captured ELISA, pseudoviral particles were diluted with coating buffer to 1012 FFU/ml. Antigen diluents were added to a 96-well polystyrene plate at 100 μl/well, and the plate was incubated at 4°C overnight to complete the antigen coating. For the cell-based ELISA, pCAGGS-JUNV GPC was transfected into HeLa cells at 250 ng/well in 96-well plates, and culture supernatants were discarded 24 h later; the cells were then fixed with 4% paraformaldehyde. After coating, the plates were washed three times with PBS-Tween 20 (0.05%) and blocked with 2% FBS at room temperature for 1 h. Subsequently, the liquid in the plates was aspirated, the wells were washed 3 times, and samples (diluted sera or antibodies, 100 μl volume) were added to each well. PBS or irrelevant antibodies were used as controls. The plates were placed at room temperature for 1 h to implement a binding step. Then, the liquid in the plates was aspirated, and the wells were washed and incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) (catalogue number: SA00001, Proteintech, China) at room temperature for another hour. After washing, 3, 3′, 5, 5′-tetramethylbenzidine was added, the plates were placed in the dark for approximately 20 min, and the chromogenic reaction was terminated by adding H2SO4. Finally, the absorbance at 450 nm was measured with a microplate reader (TECAN, Swiss), and values greater than twice those of the controls were considered positive.

2.9. Virus neutralization test

Vero-E6 cells were seeded in 96-well plates overnight. The pseudovirus VSVA-G-Rluc/JUNV GPC (JUNpv), VSVA-G-Rluc/MACV GPC (MACpv), VSVA-G-Rluc/GTOV GPC (GTOpv), VSVA-G-Rluc/SBAV GPC (SBAvpv), VSVA-G-Rluc/CHAPV GPC (CHAPpv) and VSVA-G-FP/JUNV GPC diluted at the same titre or recombinant VSV expressing JUNV GPC (rVSVA-eGFP/JUNV GPC) were incubated with gradient-diluted sera or antibodies at 37°C for 1 h. The mixture was added to Vero-E6 cells to implement infection at 37°C for 1 h. Then the supernatant was completely removed, and cells were cultured for 24 or 48 h. Finally, the cells were subjected to luminescence detection using a Luciferase Assay Kit (catalogue number: E2810, Promega, WI, USA) according to the manufacturer's instructions. Images of GFP-expressing cells were captured with a fluorescence microscope (Olympus, Japan). The numbers of fluorescent dots or foci of infection were counted by ImageJ (National Institutes of Health).

2.10. Biomolecular interaction analysis (BLIA)

The affinities of IgG and F(ab′)2 to GP1 were monitored by biolayer interferometry (BLI) using an Octet-Red 96 device (Pall ForteBio LLC., CA) according to previously described protocols (Pan et al., 2018). Briefly, recombinant GP1 was biotinylated at room temperature for 0.5 h by incubating with biotin at a molar ratio of 1:3. Residual biotin was removed by dialysis with PBS. Biotinylated GP1 at 10 μg/ml was loaded onto streptavidin biosensors (ForteBio) until saturation, and IgG and F(ab′)2 diluted to different concentrations were then loaded. The kinetics of association (K_a) and dissociation (K_d) were measured, and the data were processed by an Octet data analysis system.

2.11. Epitope mapping

Sixteen peptides (15-mers) with 5-mer overlaps were synthesized by GenScript Co., Ltd. according to the GP1 sequence (81–135, with mutations at 122 AA (Tyr to Ala), 123 AA (Asp to Ala), and 135, with mutations at 122 AA (Tyr to Ala), 123 AA (Asp to Ala), and 123 AA (Tyr to Ala) & 123 AA (Asp to Ala), were also synthesized. Recombinant GP1 with mutations at 122 AA (Tyr to Ala), 123 AA (Asp to Ala), and 122 AA (Tyr to Ala) & 123 AA (Asp to Ala) were expressed and purified as described for the wild-type one. The corresponding mutational pcDNA3.1-CDS-GP1-his was generated by PCR site-directed mutagenesis. ELISAs for mutational peptides and GP1s were performed as described in section 2.8.

2.12. Statistical analysis

Data were analysed using GraphPad Prism 8.0 software (San Diego, CA, USA), as presented as the mean ± SD based on at least three independent experiments except where specifically noted in the figure legends. The statistical analysis was performed using one-way ANOVA with Dunnett's test. P values were defined as **p < 0.01 and ***p < 0.001.

3. Results

3.1. Evaluation of immunogens in mice

A suitable immunogen is the key to stimulating nAbs in vivo. GPC mediates the entry of JUNV, and GP1 is responsible for receptor recognition. To systematically analyse the effectiveness of GPC and GP1 in stimulating nAbs in mice, we employed recombinant GP1 expressed by HEK293F cells, a full-length GPC gene vectored by a eukaryotic expression plasmid and intact GPC displayed on VSV pseudovirus as...
Fig. 1. Comparison of immunogens in mice. A Sera from each mouse were collected one week after the third vaccination. Titres of specific antibodies in sera from the recombinant GP1 group, pCAGGS-JUNV GPC group, and pseudovirus group were correspondingly detected by recombinant GP1-captured, cell-based, and pseudovirus-captured ELISAs, respectively. B, C and D Sera from the recombinant GP1 group, pCAGGS-JUNV GPC group, and pseudovirus group were collected one week after each vaccination, and sera from each group were pooled. Their PVNT\textsubscript{50}s were characterized with VSV\textDelta\textsubscript{G}-Rluc/JUNV GPC on Vero-E6 cells, and the diluted ratios of sera ranged from 1:20 to 1:2560. Titres of 50% and 80% neutralization (NT\textsubscript{50} and NT\textsubscript{80}) were marked.

| Group | Horse # | Amount of antigen |
|-------|---------|-------------------|
|       |         | Prime | Boost 1 | Boost 2 | Boost 3 |
| 1     | 1859    | 1.5\texttimes\textsuperscript{10} FFU | 1.5\texttimes\textsuperscript{10} FFU | 1.5\texttimes\textsuperscript{10} FFU | 1.5\texttimes\textsuperscript{10} FFU |
| 1     | 1863    | 1.5\texttimes\textsuperscript{10} FFU | 1.5\texttimes\textsuperscript{10} FFU | 1.5\texttimes\textsuperscript{10} FFU | 1.5\texttimes\textsuperscript{10} FFU |
| 2     | 1871    | 5 mg  | 5 mg    | 1.5\texttimes\textsuperscript{10} FFU | 1.5\texttimes\textsuperscript{10} FFU |
| 2     | 1886    | 5 mg  | 5 mg    | 1.5\texttimes\textsuperscript{10} FFU | 1.5\texttimes\textsuperscript{10} FFU |
| 3     | 1887    | 5 mg  | 5 mg    | 5 mg    | 5 mg    |
| 3     | 1888    | 5 mg  | 5 mg    | 5 mg    | 5 mg    |

Fig. 2. Comparison of immunization strategies in horses. A Six horses were divided into three groups with two horses in each group. Horses #1859 and #1863 were homogeneously vaccinated with VSV pseudoviral particles bearing JUNV GP, horses #1871 and #1886 were vaccinated with DNA the first two times and with VSV pseudotypes bearing JUNV GP the last two times, and horses #1887 and #1888 were homogeneously vaccinated with DNA. The corresponding amounts of pseudovirus and DNA are listed. B Horses were vaccinated four times in total with a two-week interval. The three groups followed the same immunization schedule. Serum samples were collected one week after each vaccination. C and D Titres of specific antibodies in sera were detected by both cell-based and pseudovirus-captured ELISAs. E PVNT\textsubscript{50}s of sera from horses during immunization were characterized with VSV\textDelta\textsubscript{G}-Rluc/JUNV GPC. Data in each group are from two horses.
immunogens. Mice in each group were subjected to continuous homogenous vaccination, as described in the Methods section, and sera were collected at different time points for monitoring the antibody response. In Fig. 1A, sera from the recombinant GP1, pCAGGS-JUNV GPC, and pseudovirus groups collected after the last vaccination were detected by GP1 protein-captured, cell-based, and pseudovirus-captured ELISAs, respectively. Titres of antigen-specific antibodies from the three groups were all as high as ~10⁶, indicating that the three kinds of immunogens employed in our study were able to stimulate a strong antibody response in mice.

Variation in nAbs was monitored by a pseudovirus neutralization test (PVNT) after each vaccination. As shown in Fig. 1B, the 50% neutralization titre (PVNT₅₀) of sera from the GP1 group was approximately 40–80 after three vaccinations. However, the PVNT₅₀ of sera from the DNA and pseudovirus groups were both as high as ~2560 (Fig. 1C and 1D), and the PVNT₈₀ were obviously elevated by the second boosting. Although the GP1 protein, DNA-JUNV GPC, and pseudovirus exhibited similar abilities to trigger antigen-specific antibody production in mice, DNA-JUNV GPC and VSV pseudotypes bearing JUNV GP triggered nAbs better than GP1 protein.

### 3.2. Comparison of immunization strategies in horses

Thus, the immunogens DNA-JUNV GPC and pseudovirus were employed to vaccinate horses. Six horses in three groups either homogenously received pseudovirus or DNA, or heterogeneously received DNA and pseudovirus (Fig. 2A). The three groups were maintained on the same schedule and were vaccinated four times in total with a two-week interval (Fig. 2B). Sera from the six horses after each vaccination were collected for monitoring antigen-specific antibodies by either cell-based ELISA or pseudovirus-captured ELISA. As shown in Fig. 2C, the titres of specific antibodies of sera from horses (#1859, #1863, #1887, and #1888) in groups 1 and 3 markedly increased with the number of vaccinations, while the titres of sera from horses (#1871 and #1886) in group 2 barely changed. Compared with the titres determined by the cell-based ELISA shown in Fig. 2C, the titres of specific antibodies detected by the pseudovirus-captured ELISA shown in Fig. 2D were different. In which, only titres of specific antibodies of sera from group 1 (#1859 and #1863) were remarkably elevated by boosting, while those from other horses remained unchanged. These results demonstrated that either homogenous vaccination with pseudovirus or DNA could effectively stimulate antigen-specific antibodies in horses, while the combined vaccination with DNA and pseudovirus could not achieve the desired goal. Additionally, antibodies triggered by pseudovirus could recognize not only GPC displayed on the virus but also GPC expressed on the cell surface.

Then, variations in nAbs from the six horses were monitored by PVNTs during immunization. As shown in Fig. 2E, the PVNT₅₀ of sera from horses #1859 and #1863 in group 1 substantially increased with the number of vaccinations, while those from the other four horses barely changed. Based on these results, we can conclude that homogenous vaccination with pseudovirus could successfully generate nAbs with high titres in horses, which was superior to the other two immunization strategies.

### 3.3. Neutralization by sera from horses #1859 and #1863

Sera from horses #1859 and #1863 were taken for further analysis, and their neutralizing activities were further characterized with VSV pseudotypes bearing JUNV GP. As shown in Fig. 3A, sera from horses #1859 and #1863 neutralized VSV pseudotypes bearing JUNV GP in a dose-dependent manner, and the PVNT₅₀ and PVNT₈₀ were as high as 1280–2560 and 160–320, respectively. Their inhibition on the VSV pseudotypes bearing JUNV GP carrying the GFP reporter gene is shown in Fig. 3B, and the number of GFP-expressing cells shown in the images was significantly reduced by treatment with gradient-diluted sera. Specifically, approximately 50% of VSV pseudotypes bearing JUNV GP were inhibited when sera were diluted 1:2560, and over 90% of VSV pseudotypes bearing JUNV GP were inhibited when sera were diluted 1:160. The result shown in Fig. 3B was consistent with that shown in Fig. 3A.

Subsequently, we explored cross-neutralization against the five pathogenic NW arenaviruses, JUNV, MACV, GTOV, SBAV, and CHAPV by horse sera. As shown in Fig. 3C, mixed sera from horses #1859 and #1863 showed moderate neutralization against MACpv, GTOpv, SBApv, and CHAPpv, and the PVNT₅₀ on the four pathogenic NW arenaviruses ranged from 160 to 320 and the PVNT₈₀ were around 40, except that neutralization on MACpv didn't reach 80% using a dilution factor of 1:2560. These results illustrated that the horse antisera prepared via our established strategy possessed cross-neutralization capabilities against the five pathogenic NW arenaviruses to some extent, while the strongest neutralization was against JUNV.

### 3.4. Preparation and characterization of IgG and F(ab')₂

A bulk of antisera were collected from horses #1859 and #1863 for purification, and antibodies as total IgG and F(ab')₂ along with GP1-
specific IgG and F(ab')2 were prepared as described in detail in the Methods section. GP1-specific IgG and GP1-specific F(ab')2 were yielded one fiftieth of total IgG and total F(ab')2, respectively, through affinity chromatography against eukaryotic GP1. IgG and F(ab')2 were detected by both nonreducing and reducing SDS-PAGE, and their actual sizes were consistent with the theoretical sizes, as shown in Fig. 4A.

After a set of PVNTs, the 50% and 80% inhibitory concentrations (IC50 and IC80) of these antibodies were obtained. As presented in Fig. 4B, the IC50 values of total IgG and total F(ab')2 were 13.9 and 7.7 μg/ml, respectively. GP1-specific IgG and GP1-specific F(ab')2 had lower IC50s than total IgG and total F(ab')2, which were 0.8 and 2.0 μg/ml, respectively. Additionally, the IC80s of total IgG, total F(ab')2, GP1-specific IgG, and GP1-specific F(ab')2 were 72.1, 27.6, 3.5, and 9.5 μg/ml, respectively. Furthermore, residual IgG collected from the flow-through fractions of GP1-affinity chromatography hardly neutralized VSV pseudotypes bearing JUNV GP even at a concentration of 100 μg/ml. These results collectively demonstrated that purification against GP1 could significantly enrich nAbs from total IgG and F(ab')2 and, in turn, confirmed GP1 as an important target of nAbs in sera prepared in our study.

We further verified the neutralization abilities of the antibodies with recombinant VSV expressing JUNV GP. rVSVΔG-eGFP/JUNV GPC permanently carrying eGFP as a reporter was recombined by replacing VSV G with JUNV GPC, which could form fluorescent plaques on Vero-E6 cells with rSVAG-eGFP/JUNV GPC. Residual IgG was used as a control. The corresponding IC50s and IC80s were calculated and are listed below.

Cross-neutralization of JUNV GP1-specific IgG against the pathogenic NW arenaviruses JUNV, MACV, GTOV, SBAV, and CHAPV was characterized with pseudoviruses, and VSV pseudotypes bearing VSV G was used as a control. The IC50s and IC80s are listed below.
3.5. Key site on JUNV GP1 bound by nAbs

Since GP1-specific purification significantly enriched the neutralizing activity of IgG and F(ab')2, we tried to analyse their binding to GP1. Using a set of BiAs, the kinetics of binding to and dissociating from recombinant GP1 were determined. As shown in Fig. 5A and B, the \( K_d \) of total IgG and F(ab')2 were 187 nM and 194 nM, respectively, and the \( K_d \) of GP1-specific IgG and GP1-specific F(ab')2 were 2.84 nM and 9.43 nM, respectively (Fig. 5C and D), reflecting that affinity chromatography against GP1 successfully selected high-affinity binders from the total fractions.

To locate their preferential binding sites on GP1, the binding of GP1-specific IgG to sixteen peptides derived from GP1 was detected by ELISA. In Fig. 6A, the fifth peptide (GP1 121–135 AA) was most strongly bound by GP1-specific antibodies among the sixteen peptides; GP1 was used as a positive control. In comparison, residual IgG weakly bound to these peptides, reflecting little nonspecific binding. In fact, P5 overlapped with the neutralizing epitopes of GD01 reported by Mahmutovic et al. (2015) and with those of EOD01 reported by Zeltina et al. (2017) at Y122 and D123 on GP1 (Fig. 6B). To verify the importance of Y122 and D123 to neutralization with the GP1-specific antibodies prepared in our study, we employed both mutated peptides and GP1s as antigens in another two sets of ELISAs. As a result, mutations at Y122A, D123A, and both sites on P5 partially abolished the binding of antibodies to similar degrees (Fig. 6C). We further validated this result with recombinant GP1, and mutation at D123A and mutations at Y122A&D123A impaired the binding to GP1, while mutation at only Y122A did not (Fig. 6D). Thus, we speculated that Asp123 on P5 is an important site on GP1 for the binding of these GP1-specific nAbs.

4. Discussion

Highly pathogenic arenaviruses, such as the five clade B NW arenaviruses JUNV, MACV, GTOV, SBAV, and CHAPV, which share an analogous envelope glycoprotein structure and employ the same receptor, transferrin receptor 1, for entry into human cells, cause similar symptoms in humans. However, reported nAbs rarely possess cross-neutralization against them. MAbs targeting sequence-conserved GP2 are considered very promising, while antibodies that cross-react with both NW and Old World arenaviruses neutralize neither (Amanat et al., 2018). NAbs for JUNV simply targeting GP1 have not been reported to have cross-neutralization capabilities against the five arenaviruses except for one elicited by Candid#1 that neutralizes both JUNV and MACV with varying efficiency in vitro (Clark et al., 2018).

In contrast to mAbs, polyclonal antibodies that were reported to neutralize JUNV, MACV, GTOV, and SBAV were achieved by vaccinating rabbits with mixed plasmids encoding GPCs of the four NW arenaviruses (Golden et al., 2016). In our study, both antisera and JUNV GP1-specific IgG purified from antisera had some cross-neutralizing activity on the five NW clade B arenaviruses, although the results were only validated with VSV pseudotypes bearing arenaviral GPs, which may be different from those validated with actual viruses. Beyond that, the exact cross-neutralizing mechanism is not clear. As one clue, antisera produced by vaccinating horses with pseudovirus...
expressing GPC reacted with not only pseudovirus-expressed GPC but also with cell-expressed GPC. This result implies that antisera and JUNV GP1-specific IgG purified from antisera might target diversiform or flexible structures of GPC to work, thus escaping hindrance from tiny structural differences among the five viruses. Another clue may be that they bound the key site Asp123 on GP1, which is conserved across the five NW arenaviruses. Even so, studies on cross-nAbs for pathogenic NW arenaviruses are at an early stage and require further validation or exploration. Additionally, cross-neutralization against the five NW pathogenic arenaviruses remains a major challenge, and more attention should be paid to their structural similarities rather than their sequence similarities.

Currently, the commercial process of obtaining horse antiserum and its derivatives is mature in most countries. The removal of Fc from IgG enables horse F(ab')₂ to be an acceptable immune therapeutic with reduced side effects for complex and intractable diseases, especially snakebites and highly pathogenic infectious diseases (Guidolin et al., 2016; Ratanabanangkoon et al., 2016; Wang et al., 2018a). Even so, some disadvantages limit the wide application of horse F(ab')₂. As a fragment of full-length IgG, horse F(ab')₂ cannot function through antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and crystallisable receptor-mediated cross-presentation, which are contributed by Fc and are considered important in protection in vivo (Chung et al., 2006; Mehlhop et al., 2009; Yu et al., 2017). As a heterologous protein to human immune system, horse F(ab')₂ may be susceptible to immune resistance, thus a large therapeutic dose should be administered when applied in clinic. However, horse antibodies advantageously have a multitarget mode of action, which differentiates them from other antibodies. Multitarget actions are contributed by Fc and are considered important in protection in vivo (Chung et al., 2006; Mehlhop et al., 2009; Yu et al., 2017). As a heterologous protein to human immune system, horse F(ab')₂ may be susceptible to immune resistance, thus a large therapeutic dose should be administered when applied in clinic. However, horse antibodies advantageously have a multitarget mode of action, which differentiates them from other antibodies.

In this study, based on substantial exploration of immunogens and immune strategies, we finally proved that pseudoviral particles decorated with mature GPCs were able to stimulate abundant nAbs in both mice and horses. This finding provides a good immunogen for preparing horse antiserum against JUNV. In addition, the eukaryotic expression vector inserted with the full-length GPC gene, which was vaccinated through electroporation to realize expression in situ, exhibited a capacity to stimulate nAbs similar to that of the pseudoviral particle in mice. DNA immunization has been confirmed to be a good way to stimulate antibodies in mice and guinea pigs by both our group (Pan et al., 2018) and by Golden et al. (Golden et al. (2016). However, DNA immunization in horses did not exhibit the same efficacy as that in mice according to our results. We vaccinated horses through multipoint injection with aluminium phosphate as an adjuvant while employing electroporation to vaccinate mice without adjuvant. Hence, we speculate that electroporation is a necessary means for using DNA immunization to stimulate the antibody response in horses. This conclusion is also confirmed by other reports (Grunwald and Ulbert, 2015; Saade and Petrovsky, 2012).

It is worth mentioning that the affinity chromatography method against GP1 employed in our study substantially enriched the neutralizing activities of the horse antibodies. Specifically, the neutralizing activity of GP1-specific IgG (IC₅₀ = 0.9 μg/ml) was almost 17 fold higher than that of total IgG (IC₅₀ = 13.9 μg/ml). Hence, we deem GP1 to be an important target of nAbs in horse antisera despite that the immunogen employed by our study possessed full-length GPC. Through peptide epitope mapping, we found that P121-135 was strongly bound by GP1-specific IgG and that Asp123 on GP1 was an important site for antibody binding. Even though P121-135 shows significance, there can be other potential highly reactive epitopes in GP1 which are not presented in the peptide mapping scheme. These findings further proved that GP1, as a receptor recognition subunit, is a pivotal target of
nAbs for JUNV. Despite that, antibodies targeting GP2 or GP1/GP2 interface may also exist in the horse antisera prepared by our study. Actually, antibodies targeting GP1/GP2 interface for blocking structural rearrangements that are required for fusion, have been demonstrated effectively neutralize Lassa virus (Yu et al., 2016). Thus antibodies in horse antisera targeting more epitopes are worth to be further studied.

5. Conclusions

In summary, we herein successfully obtained potential nAbs against JUNV by vaccinating horses with pseudovirus. The strategy to produce and purify neutralizing IgG and F(ab')2 could be an example for developing horse immune products against pathogens such as pathogenic arenaviruses, and the GP1-specific antibodies produced herein was highlighted as a potential therapeutic for AHF.

Authors’ contributions

X.-Y. P drafted the manuscript and did most experiments, Y. W. prepared antigens and purified horse immunoglobulin, W. W. and L.-K. Z helped analyse data, G.-F. X. revised the manuscript and supervised the project process.

Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

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