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Electronic Supplementary Material

The Application of a Safe Neutralization Assay for Ebola Virus Using Lentivirus-Based Pseudotyped Virus

Zengguo Cao[1,2] • Hongli Jin[1,2] • Gary Wong[3,4] • Ying Zhang[2] • Cuicui Jiao[1,2] • Na Feng[2,5] • Fangfang Wu[2] • Shengnan Xu[2] • Hang Chi[2,5] • Yongkun Zhao[2,5] • Tiecheng Wang[2,5] • Weiyang Sun[2,5] • Yuwei Gao[2,5] • Songtao Yang[1,2,5] • Xianzhu Xia[1,2,5] • Hualei Wang[1,2,5]✉

1 Key Laboratory of Zoonosis Research, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun 130062, China
2 Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Institute of Military Veterinary, Academy of Military Medical Sciences, Changchun 130122, China
3 Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200031, China
4 Département de microbiologie-infectiologie et d'immunologie, Université Laval, Québec QC G1V 0A6, Canada
5 Jiangsu Co-Innovation Center for the Prevention and Control of Important Animal Infectious Disease and Zoonose, Yangzhou University, Yangzhou 225009, China

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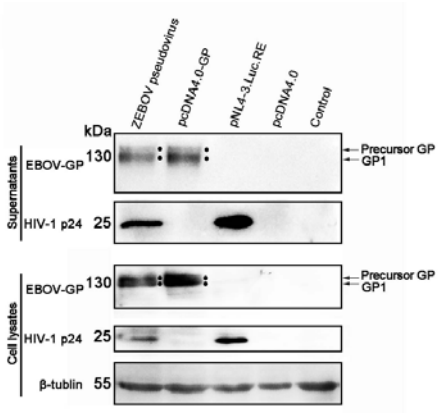

Fig. S1 Western blotting analysis of pseudo-EBOV. HEK293T cells were transfected with the two-plasmid system or with the indicated plasmids. At 30 h.p.t., the supernatants were harvested, and the transfected cells were collected and lysed. Western blotting with the corresponding antibodies was performed for the indicated proteins.

Supplementary Methods

Recombinant plasmid construction and generation of pseudotyped EBOV

The genome of the luciferase-expressing lentivirus plasmid pNL4-3.luc.R-E- is shown in Fig. 1A (bottom). The HIV envelope gene has been deleted from this virus to abolish the infectivity; infectiousness could be restored by co-transfection of cells with other envelope or glycoprotein-expressing plasmids. The full-length G gene of EBOV (GenBank accession no. KJ660346.2) was synthesized by Sangon Biotech Biotechnology Co., Ltd. (Shanghai, China) and cloned into the pcDNA4.0 vector to produce the pcDNA4.0-GP plasmid. The recombinant plasmid was confirmed by restriction endonuclease digestion and DNA sequencing; it is shown in detail in Fig. 1B. Pseudotyped EBOV was produced by transfection of HEK293T cells with a two-plasmid system consisting of pcDNA4.0-G and pNL4-3.luc.R-E-.

Infectivity and titration of pseudotyped EBOV

Pseudotyped EBOV samples were five-fold serially diluted in DMEM without FBS, four wells of each dilutions were replicated. For each dilution, 100 μL samples were added to 96-well plates containing target cells at 90% confluency. The cells were incubated with diluted pseudotyped EBOV-containing samples for 4 h. After the incubation, the inocula were removed, and after two washes with phosphate-buffered saline (PBS), 100 μL DMEM containing 10% FBS was added to each well. The plates were incubated for 48 h at 37 °C under 5% CO_2. After two washes with PBS, the cells were lysed in lysis buffer (Promega, WI, USA) according to the manufacturer's protocol. Relative luciferase units (RLUs) were measured, and the results were normalized to the RLU value of the negative control; every well that shows the ratio more than 3.0 to negative control is considered to be positive. Based on the positive wells in serial dilution of a sample, $TCID_{50}$ (50% tissue culture infective dose) were calculated by the method of Reed and Muench (Reed LJ, 1938). For the detection of infectivity, cells from various species (Huh-7, HEK293T, A549, Vero, BHK-21, BSR, and C6/36) were used as target cells.

Western blot analysis

HEK293T cells were transfected with the two-plasmid system and incubated at 37 °C until the pseudotyped EBOV was harvested. The cells were harvested and lysed. After centrifugation, the lysates were mixed with 4×LDS sample buffer (ThermoFisher Scientific, MA, USA) and denatured at 90 °C for 10 min. The samples were loaded on a 12% SDS-PAGE gel. After electrophoresis, the separated proteins were transferred to nitrocellulose (NC) membranes. The NC membranes were then blocked using SuperBlocking Buffer (ThermoFisher Scientific) at room temperature for 2 h and subsequently incubated with anti-p24 antibody (Sino Biological, Beijing, China) or anti-EBOV GP antibody (serum from a rabbit immunized with purified, truncated EBOV GP expressed in *Escherichia coli*, targeted 1-211 N-terminal residues) for 1 h at room temperature. After three washes with phosphate-buffered saline containing Tween 20 (PBST), the NC membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Bios, China) at room temperature for 1 h. The signals were visualized on a Fujifilm LAS-4000 image reader (Fujifilm, Tokyo, Japan) using the SuperSignal West Dura Extended Duration Substrate Kit (ThermoFisher Scientific).

Microscopy

After staining with 1% sodium phosphotungstate, grids loaded with pseudotyped EBOV were observed using

transmission electron microscopy (TEM). For immunoelectron microscopy (IEM), the pseudotyped EBOV was bound to formvar-coated grids followed by incubating with a murine anti-EBOV GP monoclonal antibody and gold-labeled goat anti-mouse IgG (Sigma-Aldrich, MO, USA). The formvar-coated grids were then stained with 1% sodium phosphotungstate and observed.

Neutralization assays

A pseudotyped EBOV-based neutralization assay was developed to evaluate the neutralizing activity of different samples. Briefly, 100 $TCID_{50}$ (50 μL) of pseudotyped EBOV-containing supernatant was incubated with 50 μL of a five-fold dilution series of samples at 37 °C for 1 h. The pseudotyped EBOV-sample mixtures were then added to cultures of Huh-7 cells seeded in 96-well plates at 80%–90% monolayer density. Four hours later, the Huh-7 cells were washed with PBS and incubated for an additional 48 h. The Huh-7 cells were then lysed in 30 μL/well of cell lysis buffer, and 20 μL of each lysate was used to determine luciferase activity using luciferase substrate (Promega). Live EBOV was also used in the neutralization assay that was described previously (Qiu *et al*., 2014) to provide a comparison with the results of the pseudotyped EBOV-based neutralization assay.

Indirect ELISA

96-well microtiter plates (Corning Costar, AZ, USA) were coated with recombinant truncated EBOV GP (1 μg/well) at 4 °C overnight, followed by blocking with 1% BSA at 37 °C for 2 h; 100 μL of serially diluted serum samples were then added to the wells, and the plates were incubated at 37 °C for 90 min. After five washes, HRP-conjugated goat anti-horse IgG (Bios, Beijing, China) was added, and the plates were incubated for 1 h at 37 °C. Subsequently, 100 μL of the substrate 3,3',3,5'-tetramethyl-benzidine (TMB) (Sigma-Aldrich, USA) was added, followed by incubation for 10 min at room temperature. After five washes, 50 μL of 0.5 mol/L H_2SO_4 was added to stop the reaction. The optical density (OD) values were measured at a wavelength of 450 nm. A positive result was defined as over 2.1 after normalized by OD value compared to the negative control.