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# Recombinant lentogenic Newcastle disease virus expressing Ebola virus GP infects cells independently of exogenous trypsin and uses macropinocytosis as the major pathway for cell entry

Zhiyuan Wen<sup>†</sup>, Bolin Zhao<sup>†</sup>, Kun Song, Xule Hu, Weiye Chen, Dongni Kong, Jinying Ge and Zhigao Bu<sup>\*</sup>

## Abstract

**Background:** Using reverse genetics, we generated a recombinant low-pathogenic LaSota strain Newcastle disease virus (NDV) expressing the glycoprotein (GP) of Ebola virus (EBOV), designated rLa-EBOVGP, and evaluated its biological characteristic in vivo and in vitro.

**Results:** The introduction and expression of the EBOV GP gene did not increase the virulence of the NDV vector in poultry or mice. EBOV GP was incorporated into the particle of the vector virus and the recombinant virus rLa-EBOVGP infected cells and spread within them independently of exogenous trypsin. rLa-EBOVGP is more resistant to NDV antiserum than the vector NDV and is moderately sensitive to EBOV GP antiserum. More importantly, infection with rLa-EBOVGP was markedly inhibited by IPA3, indicating that rLa-EBOVGP uses macropinocytosis as the major internalization pathway for cell entry.

**Conclusions:** The results demonstrate that EBOV GP in recombinant NDV particles functions independently to mediate the viral infection of the host cells and alters the cell-entry pathway.

**Keywords:** Recombinant Newcastle disease virus, Ebola virus, Glycoprotein, Virus entry, Macropinocytosis

## Background

Ebola virus (EBOV) causes severe hemorrhagic fever in humans, with a case fatality rate of up to 90% [1,2]. Its high fatality rate and human-to-human spread renders the virus a potential bioterrorism weapon. Currently, there are no licensed vaccines or therapeutic regimens for the disease. A safe and efficient vaccine for EBOV is yet to be developed. EBOV is an enveloped single-stranded negative-sense RNA virus belonging to the family *Filoviridae* [3]. The envelope glycoprotein (GP) of EBOV is an important virulence factor and mediates cell receptor binding and virus–cell membrane fusion [2,4–10]. GP protein also plays a central role in inducing

protective neutralizing antibodies in the host [11,12]. Several recombinant GP-expressing viruses have been developed, including replication-defective adenovirus-5 (rAd5) [13,14], replication-competent vesicular stomatitis virus (VSV) [15,16], Newcastle diseases virus (NDV) [17], rabies virus (RV) [18] and human parainfluenza virus type 3 (HPAIV) (Bukreyev et al., 2007).

Newcastle disease virus (NDV) is a member of the genus *Avulavirus* of the family *Paramyxoviridae*. NDV strains are classified as nonvirulent (lentogenic), moderately virulent (mesogenic), or highly virulent (velogenic) in poultry [19]. NDV has two envelope glycoproteins, hemagglutinin-neuraminidase (HN) and fusion protein (F). HN functions sialic acid receptor binding and F induces fusion during cell entry of NDV [20]. Viral

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virulence is mainly determined by the amino acid sequence at the protease cleavage site of the F precursor [20]. Lentogenic strains contain fewer basic amino acids at this site and can only be cleaved by trypsin-like extracellular proteases, which are largely confined to the respiratory tract, whereas highly virulent strains are cleaved by ubiquitous intracellular proteases, potentially resulting in systemic infections [21]. The attractions of NDV as a vaccine vector for emerging human infectious diseases include: preexisting immunity and maternal antibodies to mammalian paramyxoviruses do not interfere with the infection or replication of NDV because NDV is antigenically distinct from the mammalian paramyxoviruses [22,23]; lentogenic NDV usually shows limited replication in mammalian host cells because it requires a trypsin-like proteinase for the cleavage of the F glycoprotein [20,21,24,25]. Currently, lentogenic NDV strains, such as the LaSota strain, are used as live attenuated vaccines against NDV in poultry [26] and have been actively developed and evaluated as vaccine vectors for the control of human and animal infectious diseases, including influenza [27,28], severe acute respiratory syndrome [29], human parainfluenza [30], highly pathogenic H5N1 [13,31,32], human immunodeficiency virus [33,34], rabies [35], Nipah disease [36], and Rift Valley fever [37]. The safety and efficacy of NDV has been demonstrated in mice [32,36], dogs [35], pigs [36], cattle [38,39], sheep [37], African green and rhesus monkeys [17,30], and humans [40-43]. Recently, a study by DiNapoli et al. showed that a recombinant NDV expressing EBOV GP was immunogenic and caused no abnormalities or disease symptoms after its inoculation into rhesus monkeys [17]. Their study also showed that EBOV GP was incorporated into the recombinant NDV particles, which raised a serious question. Does EBOV GP function biologically normally in the virus particle during cell entry? If so, this entails biosafety concerns regarding the candidate vaccine vector.

In this study, we generated a recombinant lentogenic NDV, based on the LaSota strain, that expresses the EBOV GP protein. Its safety for poultry and mice *in vivo*, its infection and spreadability among cells *in vitro*, its sensitivity to anti-NDV and anti-EBOV neutralizing antibodies, and the internalization pathway of this recombinant virus were characterized.

## Results

### Expression of EBOV GP does not increase the pathogenicity of the NDV vector in poultry or mice

Recombinant NDV expressing the Zaire EBOV GP (rLa-EBOVGP) was generated by inserting the EBOV GP gene between the P and M genes in the genomic cDNA of the NDV LaSota strain (rLa) (Figure 1A). Expression of the GP gene was confirmed by indirect confocal

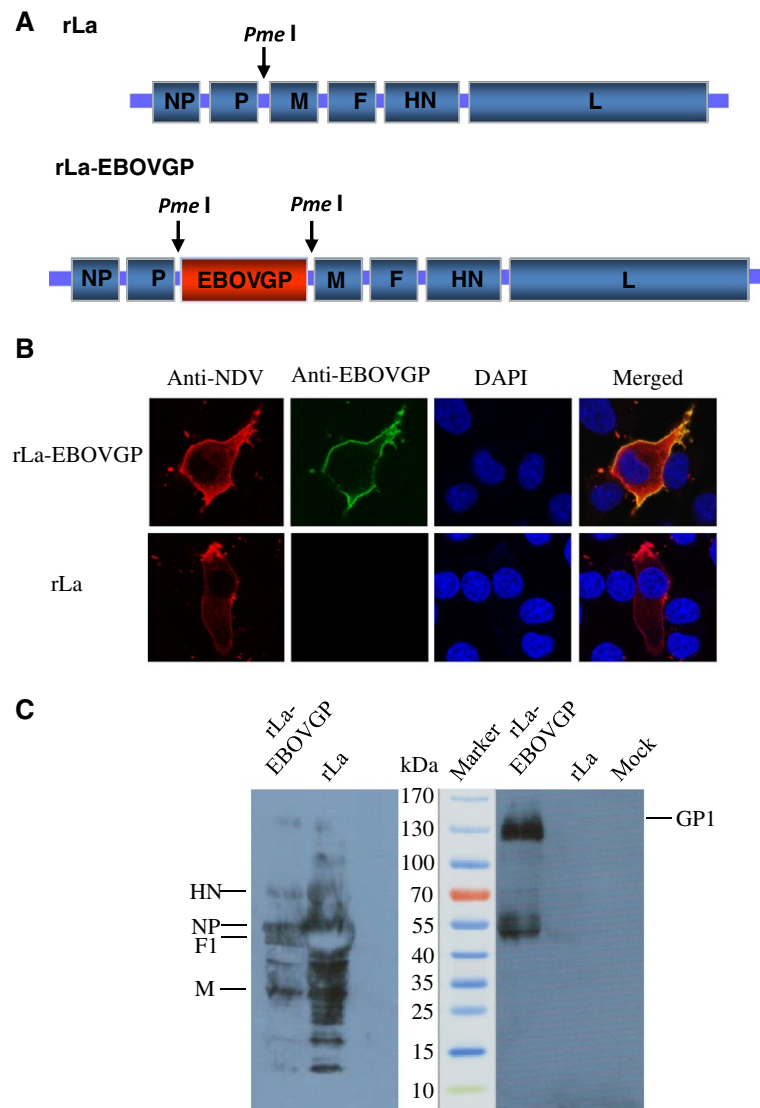
immunofluorescent staining of rLa-EBOVGP-infected BHK-21 cells. rLa-EBOVGP-infected BHK-21 cells were stained with mouse anti-EBOV GP antiserum, whereas rLa-infected BHK-21 cells were not stained with the antiserum (Figure 1B). NDV antigens and EBOV GP protein colocalized on the surfaces of the BHK-21 cells, confirming the surface expression of the EBOV GP protein in the rLa-EBOVGP-infected BHK-21 cells (Figure 1B).

rLa-EBOVGP showed similar growth properties to those of rLa, with a maximum titer of  $10^{9.6} \times 50\%$  embryo infectious doses (EID<sub>50</sub>) at 72 h after inoculation in specific-pathogen-free (SPF) chicken eggs. rLa-EBOVGP retained its low pathogenicity, as a lentogenic strain, in eggs and chickens [44], with a mean death time (MDT) > 140 h, an intracerebral pathogenicity index (ICPI) of 0, and an intravenous pathogenicity index (IVPI) of 0. During the three-week observation period after the mice were inoculated either intramuscularly (*i.m.*) or intranasally (*i.n.*) with a high dose of rLa-EBOVGP, they showed no signs of sickness or death, and did not differ in weight gain from the mice inoculated with rLa. These results suggest that the expression of EBOV GP does not increase the pathogenicity of the NDV vector in poultry or mice.

### EBOV GP is incorporated into the vector virus particles and the recombinant virus rLa-EBOVGP infects cells and spreads among them independently of exogenous trypsin

Previous studies have reported that the envelope glycoproteins of heterogeneous viruses can be expressed by recombinant NDV and incorporated into the viral vector particles [17,35]. To investigate whether the EBOV GP expressed by the recombinant NDV is incorporated into the viral vector particles, the viral particles of rLa-EBOVGP and rLa were purified by sucrose gradient centrifugation and subjected to immunoblotting analysis with mouse anti-EBOV GP antiserum as the primary antibody. A clear GP1 band of ~130 kDa was apparent in the rLa-EBOVGP sample but not in the rLa sample. This indicates that the GP protein was expressed by rLa-EBOVGP and incorporated into the recombinant NDV particles, which is consistent with a previous report [17].

The cleavage of the F glycoprotein is a prerequisite for the infectivity of NDV. Because it is a lentogenic NDV strain, the infectivity of rLa depends on exogenous trypsin-like extracellular proteases [35,36]. To investigate whether the incorporation of EBOV GP alters the infectivity of the recombinant NDV, rLa and rLa-EBOVGP were propagated in eggs (rLa-egg and rLa-EBOVGP-egg, respectively) and in BHK-21 cells with or without TPCK-trypsin (Sigma) in the medium (rLa-cell/TPCK, rLa-EBOVGP-cell/TPCK, rLa-cell, and rLa-EBOVGP-cell respectively). These prepared viruses were then used to infect BHK-21 cells at a multiplicity of infection

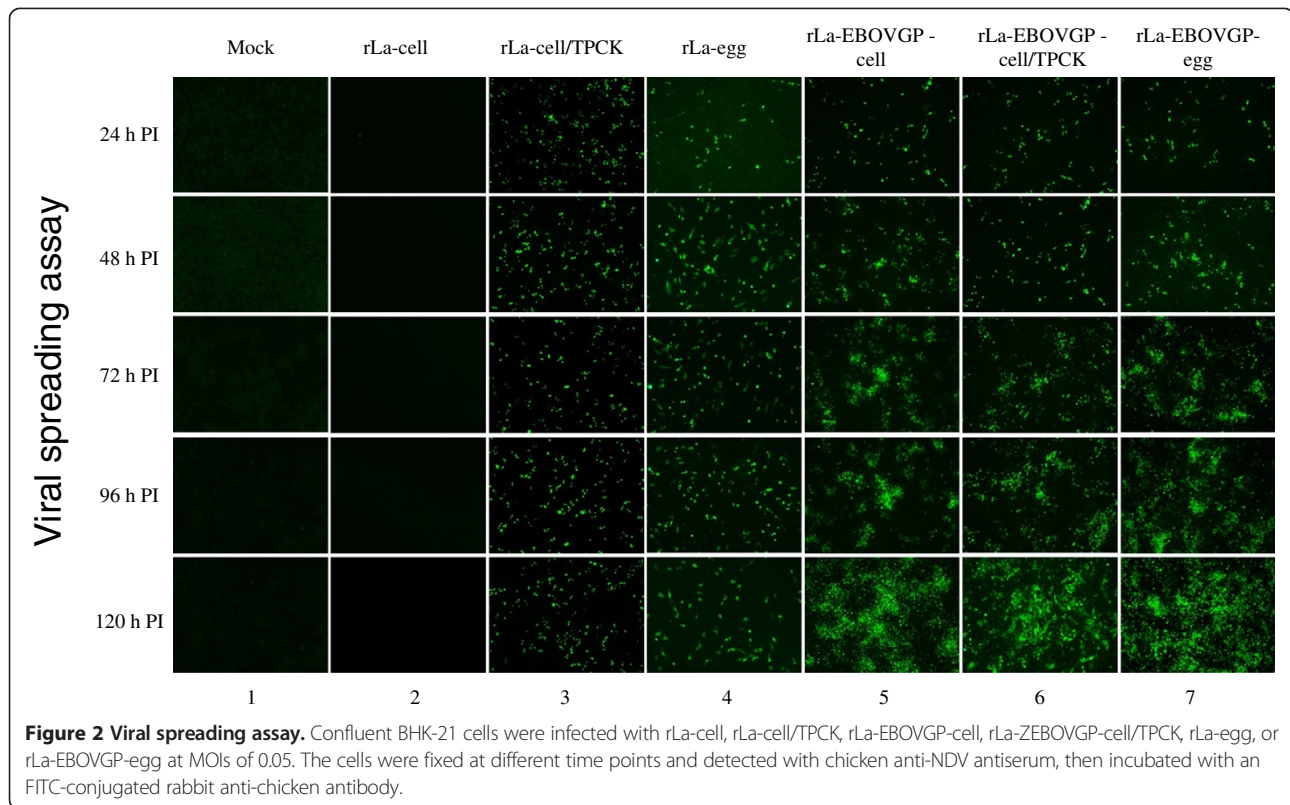


**Figure 1** Generation of recombinant NDV expressing the EBOV GP gene. **(A)** Schematic representation of the rLa genome, showing the restriction site for endonuclease *Pme*I, introduced between the *P* and *M* genes, and the EBOV GP gene inserted into the *Pme*I site. **(B)** Indirect immunofluorescent staining of rLa-EBOVGP-infected BHK-21 cells with chicken anti-NDV antiserum and mouse anti-EBOV GP antiserum, observed with confocal laser microscopy. **(C)** Western blotting of sucrose-gradient-purified rLa and rLa-EBOVGP, hybridized with chicken anti-NDV antiserum and mouse anti-EBOV GP antiserum.

(MOI) of 0.02–0.05 with no exogenous trypsin in the medium. As expected, rLa-cell did not infect any cells (Figure 2, column 2), whereas rLa-egg and rLa-cell/TPCK infected individual cells but did not spread between the cells. However, rLa-EBOVGP-egg, rLa-EBOVGP-cell, and rLa-EBOVGP-cell/TPCK showed similar infectivity and spreadability in the cells. At 120 h postinfection (PI), more than 90% of cells were infected by all the differently prepared rLa-EBOVGP viruses (Figure 2, columns 3 and 4). These results suggest that the expression and incorporation of EBOV GP allow the vector NDV to infect cells and to spread among the cells independently of exogenous trypsin.

#### rLa-EBOVGP is more resistant to NDV antiserum than vector NDV and partially sensitive to EBOV antiserum

The two envelope glycoproteins of NDV, HN and F, are indispensable for cell entry, which includes receptor binding and virus-cell membrane fusion. This is the first step in infection and a prerequisite for viral replication [26]. EBOV has only one envelope glycoprotein, which functions in receptor binding and membrane fusion [4–8]. To understand the impact of EBOV GP on the infectivity of the NDV vector, the sensitivities of rLa and rLa-EBOVGP to NDV antiserum and EBOV GP antiserum were evaluated and compared (Figure 3). As expected, both rLa-egg and rLa-cell were resistant to



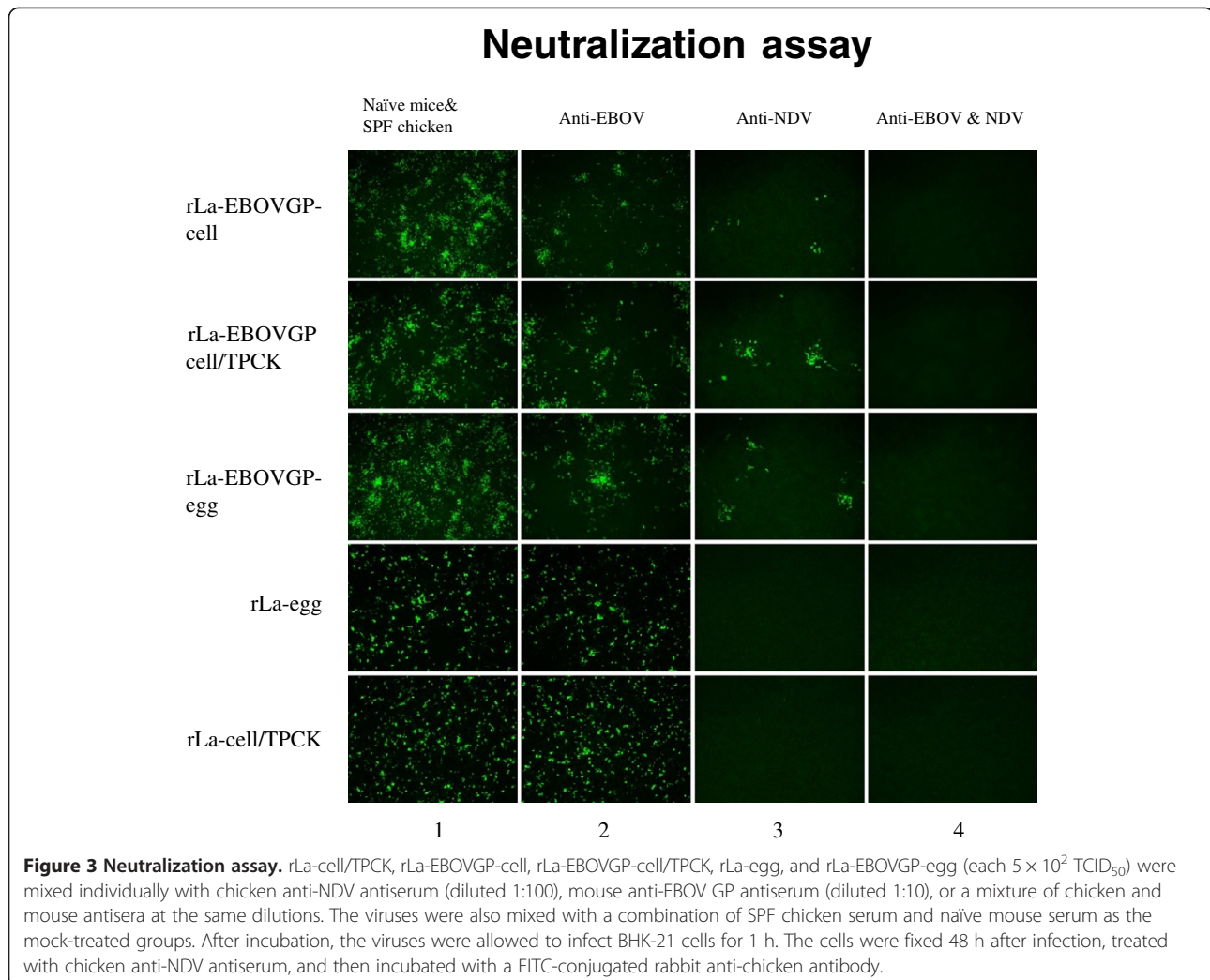
mouse anti-EBOV GP antiserum (diluted 1:10) but were completely neutralized by chicken anti-NDV antiserum (diluted 1:100). However, both anti-NDV antiserum (diluted 1:100) and anti-EBOV GP antiserum (diluted 1:10) only partially neutralized rLa-EBOVGP-cell, rLa-EBOVGP-cell/TPCK, and rLa-EBOVGP-egg (Figure 3, column 2). The anti-NDV antiserum and anti-EBOV GP antiserum reduced the infection with each rLa-EBOVGP virus by about 90% and 60%, respectively (Figure 3, column 3). When the anti-NDV antiserum (diluted 1:100) and anti-EBOV GP antiserum (diluted 1:10) were mixed, infection with rLa-EBOVGP-cell, rLa-EBOVGP-cell/TPCK, or rLa-EBOVGP-cell was completely blocked (Figure 3, column 4). The same dilution of SPF chicken0020-serum, naïve mouse serum, or a mixture of SPF chicken serum and naïve mouse serum, used as the controls, showed no neutralization activity against any of the differently prepared rLa or rLa-EBOVGP viruses. These results suggest that the incorporation of EBOV GP into the recombinant viral particle made the NDV vector more resistant to anti-NDV antiserum and more sensitive to anti-EBOV antiserum.

#### rLa-EBOVGP uses macropinocytosis as the major internalization pathway for cell entry

NDV envelope glycoproteins HN and F usually bind to receptor and induce virus-cell membrane fusion at

neutral pH. A previous study reported that NDV can also partially enter the host cells by caveolae-mediated endocytosis [45], whereas EBOV mainly enters cells via macropinocytosis [46-48] in a GP-dependent manner [46,49]. Because EBOV GP is incorporated into the vector NDV particles and independently mediates the cell entry of rLa-EBOVGP, it is necessary to determine whether the incorporation of the GP protein alters the endocytosis pattern of rLa-EBOVGP during infection. To address this question, we used two chemicals, IPA3 and methyl  $\beta$ -cyclodextrin (M $\beta$ CD) to treat BHK-21 cells before their infection with the rLa or rLa-EBOV virus. IPA3 inhibits the activation of PAK1 kinase, which is required for macropinocytosis [50,51] and M $\beta$ CD sequesters cholesterol from the cell membrane, thus inhibiting clathrin- and caveolae-mediated endocytosis [52,53]. As shown in Figure 4, the infection of IPA3-pretreated BHK-21 cells with rLa-EBOVGP-cell, rLa-EBOVGP-cell/TPCK, or rLa-EBOVGP-egg was greatly reduced, whereas IPA3 had no inhibitory effect on the infectivity of rLa-egg or rLa-cell/TPCK. Fewer than 10% of IPA3-pretreated cells were infected with rLa-EBOVGP-cell, rLa-EBOVGP-cell/TPCK, or rLa-EBOVGP-egg compared with the untreated cells. Infection by rLa-EBOVGP-cell, rLa-EBOVGP-cell/TPCK, or rLa-EBOVGP-egg was not inhibited in cells pretreated with M $\beta$ CD, but their infection by rLa-cell/TPCK and rLa-egg was moderately reduced. Pretreatment with a





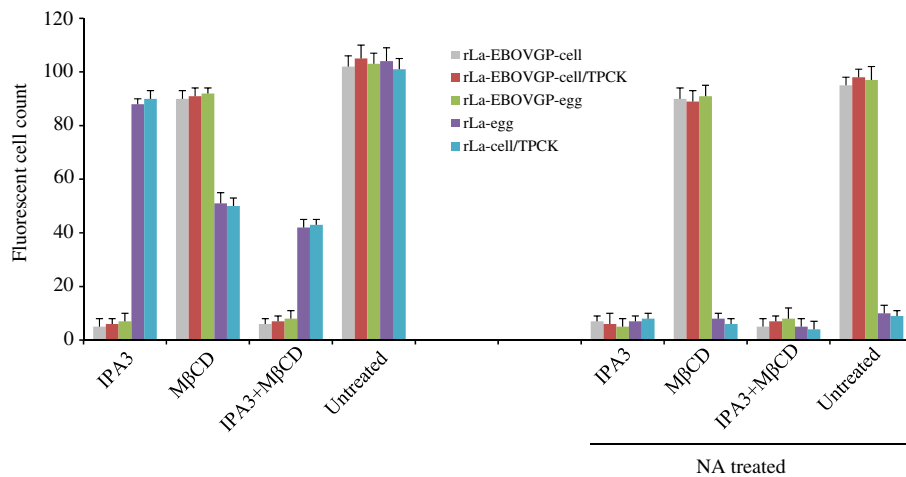
combination of IPA3 and MβCD almost completely blocked the infection of cells by rLa-EBOVGP-cell, rLa-EBOVGP-cell/TPCK, and rLa-EBOVGP-egg, and also mildly inhibited the infection of cells by rLa-cell/TPCK and rLa-egg. To further testify the role of EBOV GP on the internalization of the recombinant virus, we use 800 mU/ml bacterial neuraminidase (NA, Sigma N2876) to treat cells with chemical inhibitors together with IPA3 or alone before infection. Our preliminary data showed use 800 mU/ml NA to treat cells could block over 90% of NDV infection, thus in this assay we used NA to exclude the influence of HN protein in the internalization of rLa-EBOVGP-egg and rLa-EBOVGP-cell/TPCK. Also shown in Figure 4 (right panel), NA treatment reduced over 90% of rLa-egg and rLa-cell/TPCK infection, while it had almost no reduction on the infection of rLa-EBOVGP-egg, rLa-EBOVGP-cell/TPCK and rLa-EBOVGP-cell. The NA + IPA3 treatment could block 90% of the recombinant viruses from infection, while NA + MβCD had no inhibition on these viruses. These results further explicated the role

of GP on the macropinocytotic internalization of the recombinant viruses. In conclusion, Our results indicate that recombinant rLa-EBOVGP uses macropinocytosis as its major internalization pathway for cell entry, rather than the direct fusion at the cell plasma membrane at neutral pH like NDV.

### Discussion

Using reverse genetics, we generated a recombinant low-pathogenic LaSota NDV that expresses GP of EBOV, designated rLa-EBOVGP, and evaluated its biological characteristics in vivo and in vitro. The introduction and expression of the EBOV GP gene did not increase the virulence of the NDV vector in poultry or mice, which is consistent with the results of a previous study in monkeys [17]. EBOV GP was incorporated into the viral particles of rLa-EBOVGP and allowed the NDV vector to infect mammalian cells independently of exogenous trypsin.

The restriction of NDV replication in mammalian host cells is one of the most attractive properties of



**Figure 4 Chemical inhibition of virus internalization pathways.** BHK-21 cells were pretreated with (right panel) or without (left panel) NA alone or together with inhibitor 10  $\mu$ M IPA3 or 10 mM M $\beta$ CD or a mixture of the two chemicals at 37°C for 1 h. The cells were washed and infected with  $5 \times 10^2$  TCID<sub>50</sub> of rLa-egg, rLa-EBOVGP-egg, rLa-cell/TPCK rLa-EBOVGP-cell, or rLa-EBOVGP-cell/TPCK at 4°C for 1 h in the presence of the chemical(s). The cells were then washed and incubated at 37°C. At 7 h after infection, the cells were washed, fixed, permeabilized, and immunostained with chicken anti-NDV antiserum, then incubated with a FITC-conjugated rabbit anti-chicken antibody.

lentogenic NDV in terms of its safety when used as a live vaccine vector in animals and humans, as is also the case for fowlpox virus [54,55] and a modified vaccinia virus Ankara [56,57]. The V protein encoded by the NDV *P* gene functions as an interferon antagonist and is usually less efficient in mammalian cells [58-60]; NDV is usually a strong inducer of the interferon response in mammalian cells and is highly sensitive to the interferon induced in these cells [61,62]. The limited replication in mammalian cells of low-pathogenic NDV, like the LaSota strain, is also determined by its trypsin-dependent infectivity. The trypsin-independent infectivity acquired by rLa-EBOVGP means that this virus behaves like a velogenic NDV in mammalian host cells. Its restricted replication may have to depend on the only defense line in host, the native immunity.

The ability of foreign envelope proteins to function as new cell-entry proteins has also been demonstrated in other enveloped negative-strand RNA viruses [63]. The function of EBOV GP in mediating the cell entry of VSV when the *G* gene had been deleted from the VSV genome could be compensated by several foreign envelope glycoproteins from different viruses in trans or with recombinant expression, including Ebola virus, Marburg virus, Lassa fever virus, Hantaan virus, and Nipah virus [4,6,7,64-67]. Therefore, it is reasonable to infer that the incorporation of EBOV GP into the viral particle may cause the trypsin-independent infectivity of rL-EBOVGP.

So far, very few studies have examined the biological functions of native and foreign envelope glycoproteins when they are incorporated into the same viral particle. Our previous study showed that the *G* proteins of the

rabies virus were incorporated on the surface of a recombinant NDV LaSota particle. An anti-rabies virus antiserum did not reduce the infectivity of the recombinant NDV [35]. The *G* proteins on the virion surface of the rabies virus did not mediate the infection of cells by the recombinant NDV particle [35]. The reasons for these phenomena are unclear. Another study showed that EBOV GP was incorporated into the viral particle of recombinant human parainfluenza virus 3 (hPIV3) and that the recombinant hPIV3 became more sensitive to neutralizing antibody directed against EBOV than to neutralizing antibody directed against hPIV3 [63]. Because the biological functions of the hPIV3 envelope glycoproteins could not be abolished, it was difficult to clarify whether EBOV GP on the virion surface functioned independently to mediate the infection of cells by the recombinant hPIV3 particle. In the present study, the recombinant NDV rLa-EBOVGP-cell was prepared in BHK-21 cells in the absence of trypsin, so the membrane fusion function of the *F* protein, essential for viral entry, was completely abolished. Therefore, we can confidently conclude that the EBOV GP that was incorporated into the viral particle independently mediated the cell entry of the recombinant NDV.

As a prototype member of the paramyxoviruses, NDV usually enters host cells by direct fusion at the plasma membrane via a pH-independent mechanism [68-70]. However, NDV can also enter host cells by an endocytic pathway [45]. It had been shown that the cellular entry of EBOV involves a macropinocytosis-like mechanism and subsequent trafficking through early and late endosomes [46,47,71]. Recently, Niemann-Pick C1, a protein

involved in the endocytic pathways, has been identified as an important host factor in the cell-entry process [72,73]. In this study, the neutralization assay showed that the mixture of anti-NDV antiserum and anti-EBOV GP antiserum completely blocked the infectivity of rLa-EBOVGP, whereas either anti-NDV antiserum or anti-EBOV GP antiserum only partially blocked the infectivity of this virus. Therefore, we infer that both the NDV envelope proteins F/HN and EBOV GP contribute to the cell entry of rLa-EBOVGP. It is surprising that the inhibition of macropinocytosis almost completely abolished the infectivity of rLa-EBOVGP, whereas it did not markedly reduce the infectivity of rLa. These results indicate that the direct fusion between rLa-EBOVGP and the plasma membrane facilitated by F/HN may not allow viral entry into the cell. EBOV GP plays a major role in the cell-entry process of rLa-EBOVGP, and may predominate over NDV F/HN in the cell-entry function of this recombinant virus. The exact mechanism underlying this predominance requires further investigation. The recombinant virus rLa-EBOVGP provides an interesting model with which to explore the functional interactions between native and foreign envelope glycoproteins in one viral particle.

Although animal tests have shown that recombinant NDV expressing EBOV GP is safe for monkeys [17] and for poultry and mice (this study), safety concerns remain. Because the incorporation of EBOV GP protein into NDV particles significantly alters the behavior of the vector virus, the use of an NDV-vectored EBOV vaccine should be investigated with caution and evaluated rigorously.

## Methods

### Cells and viruses

The BSR-T7/5 cells for virus rescue and the BHK-21 cells for virus growth and titration were maintained in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The NDV strains were propagated and titrated in 9-day-old SPF embryonated chicken eggs [44] or BHK-21 cells in the presence or absence of TPCK-trypsin (Sigma).

### Plasmid construction and virus rescue

To construct a full-length recombinant genomic cDNA, the cDNA of the GP gene of Zaire EBOV was amplified from synthesized cDNA (GenBank accession no. AF086833.2) using the following primers: 5'-GACTGTTTAAACtgaaaaaaTaccggtagaaCgccaccATGGGCGTTACAGGAATA TTGCAG-3' and 5'-CTGAGTTTAAACGCTAAAAAGCAAATTTGCATATACAG-3'. The GP gene was flanked by the restriction site for endonuclease *PmeI* (boldface letters); the NDV gene start sequence (GS, 5'-acgggtgaa-3') and gene end sequence (GE, 5'-ttagaaaaa-3')

are included before the optimal Kozak sequence (*italic lowercase letters*) and the GP sequence (*italic uppercase letters*). The amplified fragment was digested with *PmeI* and then inserted into the P-M intergenic region at nucleotide position 3165 of the NDV genome, as described previously [32]. The resultant plasmid was used for recombinant NDV rescue, as described previously [32]. The expression of EBOV GP was confirmed with an immunofluorescence assay (IFA) and western blotting. The resultant recombinant virus was designated "rLa-EBOVGP".

### Immunofluorescence assay

NDV infection was detected in cells with IFA with chicken anti-NDV antiserum, as previously described [32]. For the confocal assays, BHK-21 cells were grown in 24-well plates or plated on cover slips in dishes (35 mm diameter) and infected with rLa or rLa-EBOVGP. At 24 h after infection, the cells were fixed in prechilled 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, washed three times with PBS, and then blocked with PBS containing 1% (wt/vol) bovine serum albumin at room temperature for 1 h. The cells were then incubated with mouse anti-EBOV GP antiserum (the antiserum was prepared in mice immunized with two doses of recombinant VSV expressing EBOV GP, which was generated in our laboratory) or chicken anti-NDV antiserum for 1 h at room temperature. The cells were then washed three times with PBS containing 0.05% Tween 20 and stained with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma) or a tetramethylrhodamine isothiocyanate-conjugated rabbit anti-chicken antibody (Sigma) for 30 min. The cells were washed three times with PBS, stained with DAPI, and then analyzed with fluorescence microscopy or confocal laser microscopy. The images were acquired with a Zeiss (Thornwood, NY) Axioskop microscope equipped for epifluorescence with a Sensys charge-coupled device camera (Photometrics, Tucson, AZ) using the IPLab software (Scanalytics, Vienna, VA).

### Western blotting

Egg-propagated rLa and rLa-EBOVGP were purified by sucrose gradient ultracentrifugation. The total protein (5 µg) of each purified virus was subjected to SDS-PAGE under denaturing conditions. After the proteins were transferred from the gel to nitrocellulose membrane, the target band (s) were detected with chicken anti-NDV antiserum or mouse anti-EBOV GP antiserum as the primary antibody and the corresponding horseradish-peroxidase-conjugated goat anti-chicken or goat anti-mouse immunoglobulin G as the secondary antibody. The bands were

visualized with ECL Plus Western Blotting Detection Reagents (GE Health Science) on Kodak X-ray film.

#### Virus neutralization

Each recombinant virus ( $5 \times 10^2$  TCID<sub>50</sub>) was mixed with chicken anti-NDV antiserum (diluted 1:100) or mouse anti-EBOV GP antiserum (diluted 1:10) or with a mixture of chicken and mouse antisera (at the same dilutions). The viruses were also mixed with a mixture of SPF chicken serum and naïve mouse serum as the mock-treated group. The virus-serum mixtures were incubated at 37°C for 1 h and then used to infect monolayers of BHK-21 cells in a 12-well plate. At 1 h after infection, the supernatants were discarded and the cells were washed three times with DMEM. At 48 h after infection, the cells were fixed, and IFA was performed with chicken anti-NDV antiserum as the primary antibody.

#### Assessment of viral pathogenicity

To determine the pathogenicity of rLa-EBOVGP in poultry, MDT, ICPI, and IVPI were determined according to the OIE Manual [44]. To assess the pathogenicity of rLa-EBOVGP in mammalian cells, two groups of 10 six-week-old female Balb/c mice (Vital River, Beijing, China) were inoculated *i.m.* with  $10^8$  EID<sub>50</sub> of rLa-EBOVGP or rLa, and simultaneously *i.n.* with  $3 \times 10^7$  EID<sub>50</sub> of rLa-EBOVGP or rLa. The third group of 10 mice was inoculated *i.m.* with 0.1 mL and *i.n.* with 0.03 mL of PBS as the mock-infection control. The mice were monitored daily to detect any weight changes, signs of illness, or death.

#### Chemical inhibition of virus internalization pathways

BHK-21 cells were pretreated with either the inhibitor 10 μM IPA3 (Sigma) or 10 mM MβCD (Sigma) alone or together at 37°C. To exclude the influence of HN of vector NDV virus on the internalization of the recombinant viruses, another plate of BHK-21 cells were pretreated with 800 mU/ml bacterial neuraminidase (NA, Sigma N2876) alone or together with the inhibitors at 37°C. At 1 h after treatment, the cells were washed three times and infected with  $5 \times 10^2$  TCID<sub>50</sub> of rLa or rLa-EBOVGP at 4°C for 1 h in the presence of the inhibitor (s). The cells were then washed three times on ice. DMEM containing 10% FBS was added and the samples were incubated in 37°C for 7 h. After incubation, the cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% saponin. Cells infected with rLa or rLa-EBOVGP were detected with immunofluorescence assay, as described above. The mean number of fluorescent positive cells of a minimum of 5 fields of view were counted. The data was expressed as means and standard deviations.

#### Ethics statements

The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocol was approved by the Animal Research Ethics Committee of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (approval numbers 20132085 for chickens and 20132138 for mice).

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

ZB designed and oversaw the experiments. ZB and ZW wrote the manuscript. BZ and JG rescued the recombinant viruses and characterized the viruses. ZW carried out the neutralization assay and in vitro cell entry assay. KS, XH, WC and DK carried out the plasmids construction and animal studies. All authors have read and approved the submitted manuscript.

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