



Heterologous prime-boost regimens with HAdV-5 and NDV vectors elicit stronger immune responses to Ebola virus than homologous regimens in mice

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Abstract

The 2014 Ebola outbreak in West Africa resulted in more than 11,000 deaths, highlighting the need for a vaccine. A phase I clinical trial of a human adenovirus type 5 vector-based Ebola virus (EBOV) vaccine (HAdV-5-MakGP) showed that a homologous prime-boost regimen with HAdV-5 vaccine elicited a robust humoral response but a weak cellular immune response. Due to pre-existing anti-vector immunity, boosting with the same vaccine did not increase the magnitude of the cellular immune response, which contributes significantly to protection against EBOV infection. Here, we generated a recombinant Newcastle disease virus (NDV), based on the LaSota vaccine strain, expressing the GP protein of the EBOV variant Makona (rLS/EB-GP) using reverse genetics technology. The humoral and cellular immune responses to this vaccine candidate in mice immunized using a homologous prime-boost regimen or a heterologous prime-boost regimen with the HAdV-5-vectored Ebola vaccine were assessed using ELISA and ELISPOT assays. The ELISA and ELISPOT results showed that mice primed with rLS/EB-GP and boosted with HAdV-5-MakGP (NDV+HAdV-5) or, reversed, primed with HAdV-5-MakGP and boosted with rLS/EB-GP (HAdV-5+NDV) exhibited more-potent EBOV GP-specific antibody and cellular immune responses than those receiving the same vaccine twice. The most robust EBOV GP-specific antibody and T-cell responses were detected in the HAdV-5-MakGP-primed and rLS/EB-GP-boosted (HAdV-5+NDV) mice. These results suggest that the HAdV-5 prime-NDV boost regimen is more effective in stimulating EBOV-specific immunity than homologous regimens alone, indicating the potential boosting ability of the NDV vector in human vaccine use.

Introduction

The 2013-16 Ebola outbreak in West Africa was the most devastating human infectious disease witnessed in recent times, causing over 28,000 reported cases and more than 11,000 deaths [7]. Recent studies have revealed that the Ebola virus (EBOV) spreads by human-to-human

transmission through contact with body fluids of infected people; even the recovered can still transmit the virus via their semen for up to 7 weeks after recovery from illness [5]. Due to the ease of transmission and lack of approved antiviral therapy, further small outbreaks could occur at any time. This threat highlights the need for a vaccine to prevent infection and avoid repetition.

In 2017, the China FDA approved an Ebola vaccine based on a human adenovirus serotype 5 (HAdV-5) vector. A phase I clinical trial of the HAdV-5-vectored Ebola vaccine in China has shown that two doses of the homologous HAdV-5 Ebola vaccine with a six-month interval elicit a robust humoral response but only a weak cellular immune response [17]. Unlike the induction of antibodies, repeated homologous boosting with the same vaccine does not increase the magnitude of the cellular immune response, as pre-existing anti-vector immunity inhibits antigen processing [13]. The suppression of immune responses caused by HAdV-5 pre-existing immunity (PEI) to the homologous vaccine boost has also been extensively reported in animal

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models and clinical trials [3, 27]. To address this concern, researchers have attempted to develop a heterologous prime-boost immunization strategy. Several pre-clinical and clinical studies have shown that heterologous prime-boost regimens generate stronger and broader immune responses and provide significant durability benefits when compared to homologous boosting [21, 24, 25]. These advantages of heterologous prime-boost regimens over homologous regimens might be due to their ability to avoid the consequences of pre-existing anti-vector immunity. The heterologous prime-boost strategy delivers the same or similar antigens using different vaccine vectors, which redirects the immune response to the antigens rather than the vectors. Because of the highlighted potential mentioned above, such a strategy represents a new way of immunization and has been applied to the development of vaccines against a wide range of pathogens [13].

Newcastle disease virus (NDV) is a non-segmented, single-stranded negative-sense RNA virus belonging to the genus *Orthoavulavirus*, family *Paramyxoviridae* [23]. Several characteristics make NDV a promising vaccine vector against human and veterinary pathogens. NDV is safe for administration to humans, due to a natural host range restriction [8]. In clinical trials for cancer treatment, NDV as an oncolytic agent was well tolerated without causing adverse effects [11]. Since NDV is an avian virus that is antigenically distinct from human pathogens, there is no preexisting immunity in humans that would limit the utility of an NDV vaccine vector [1]. Furthermore, NDV replicates in the respiratory tract and induces strong humoral and cellular immune responses, both at the mucosal and systemic levels, in murine and nonhuman primate models, thus making this vector an attractive candidate for vaccine development [1, 15].

In the present study, we generated an infectious clone of the NDV LaSota strain as a vaccine vector, inserted the surface glycoprotein (GP) gene of EBOV into the infectious clone, and rescued a recombinant NDV that expresses the GP protein of the EBOV variant Makona. Evaluation of the NDV-vectored Ebola vaccine in mice through different immunization regimens with the HAAdV-5-vectored Ebola vaccine allowed us to better understand the immune responses elicited after heterologous and homologous prime-boost regimens.

Materials and methods

Cell lines, virus, and RNA preparation

HEp-2, DF-1, and Vero cell lines, obtained from the American Type Culture Collection (Manassas, VA), were maintained in Dulbecco's minimal essential medium (DMEM)

supplemented with 10% fetal bovine serum (FBS). The modified vaccinia virus vTF7-3, expressing the T7 RNA polymerase, was obtained from ATCC (VR-2153; ATCC) and was grown in primary chicken embryo fibroblast cells. The NDV LaSota strain obtained from the China Veterinary Culture Collection Center was propagated in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs as a stock. Total RNA was extracted from the allantoic fluid of the infected eggs at 72 h post-inoculation using TRIzol Reagent (Invitrogen).

Construction of a full-length cDNA clone of the NDV LaSota strain

The construction of a plasmid carrying the full-length antigenomic cDNA of the NDV LaSota strain has been described previously [31]. In brief, RNA was extracted and subjected to RT-PCR with four pairs of specific primers using a SuperscriptTM III One Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen) to generate four overlapping PCR fragments of the entire viral genome (Fig. 1). These four cDNA fragments were sequentially cloned into a modified pBluescript vector using an In-Fusion[®] PCR Cloning Kit (Clontech) to construct a complete genomic cDNA of the LaSota virus. The final plasmid containing the full-length LaSota strain genome, designated as pFLC-LS, was amplified in Stbl2 cells at 30°C and purified using a QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA).

Construction of NP, P, and L gene expression plasmids

Viral rescue from infectious clones for members of the paramyxovirus family requires the co-expression of the viral nucleoprotein (NP), phosphoprotein (P), and polymerase (L) genes [9]. Amplicons containing the open reading frames (ORFs) of the NP, P, and L genes were generated by RT-PCR from the virus genome and cloned into a modified pBluescript vector using an In-Fusion PCR Cloning Kit (Clontech). The resulting plasmid constructs were designated as p-NP, p-P, and p-L, respectively.

Construction of a recombinant cDNA clone containing the GP gene of EBOV

The plasmid containing the GP gene of the EBOV Makona strain was a kind gift from Dr. Xu, Beijing Institute of Biotechnology [28]. The procedure for construction of the recombinant cDNA clone containing the GP gene of EBOV is illustrated in Fig. 1. Briefly, a cDNA fragment representing the ORF encoding the EBOV GP was amplified using paired specific primers into which gene end-intergenic-gene

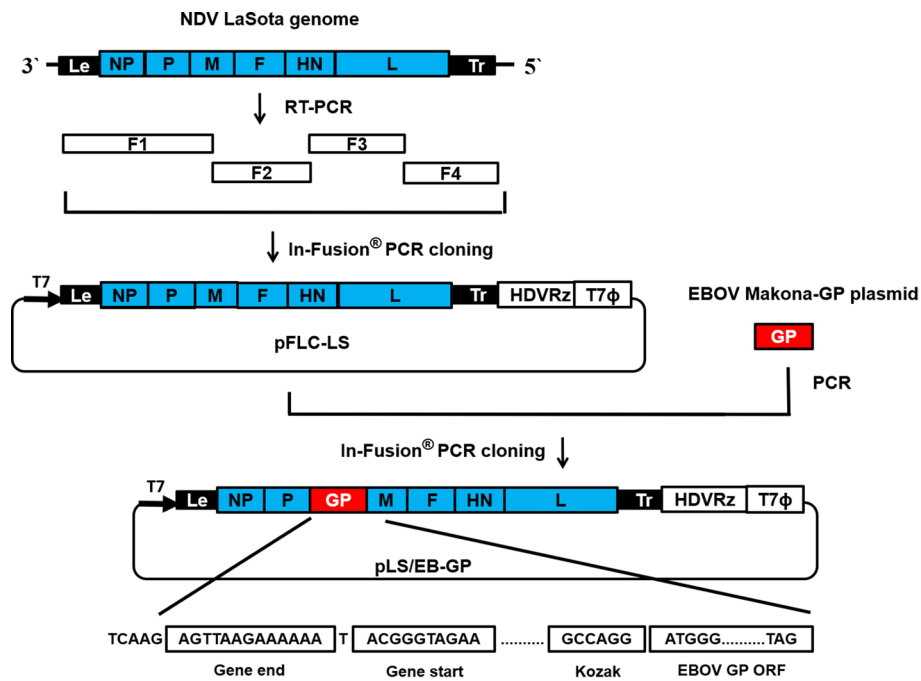


Fig. 1 Schematic representation of the recombinant cDNA clone containing the GP gene of EBOV. Four cDNA fragments covering the complete sequence of the LaSota strain genomic RNA were generated and cloned into a pBlueScript-based modified plasmid. The open reading frame (ORF) of the EBOV GP gene along with the NDV *trans*-acting signal sequences was amplified and inserted into

the pFLC-LS vector in the non-coding region downstream of the P gene, resulting in the pLS/EB-GP clone. The direction of the T7 promoter is indicated by a bold black arrow. HDVRz and T7Φ represent the site of the hepatitis delta virus ribozyme and the T7 terminator sequence, respectively. The NDV gene start and gene end sequences, Kozak sequence, and the EBOV GP ORF are boxed

start motifs sequences were incorporated. An optimal Kozak translation sequence was inserted upstream of the GP gene to enhance foreign gene expression. Subsequently, the PCR product containing the GP ORF along with the NDV *trans*-acting signal sequences was introduced into the P-M intergenic region of the pFLC-LS vector using an In-Fusion[®] PCR Cloning Kit (Clontech). The resulting recombinant cDNA clone was designated as pLS/EB-GP.

Virus rescue and propagation

The procedures for transfection of HEP-2 cells with the full-length cDNA clone, pFLC-LS or pLS/EB-GP, and the supporting plasmids, p-NP, p-P, and p-L, were described previously [32]. HEP-2 cells grown in a six-well plate were infected with vTF7-3 at a multiplicity of infection (MOI) of 3. Subsequently, the cells were cotransfected with 2 μg of pFLC-LS or pLS/EB-GP together with 1 μg of p-NP, 0.5 μg of p-P, and 0.1 μg of p-L using 6 μl of Lipofectamine[™] 2000 (Invitrogen). After 6 h of incubation at 37°C, the medium was replaced with DMEM containing 2% FBS, and the cells were incubated for three days at 37°C. The supernatants were then inoculated into the allantoic cavities of 10-day-old embryonated SPF eggs. After 4 days of incubation, the

allantoic fluid was harvested, and the rescued viruses (rLS and rLS/EB-GP) were screened by hemagglutination assay (HA). The nucleotide sequences of the rescued viruses were determined by sequencing the RT-PCR products amplified from the viral genomes as described previously [12].

Immunofluorescence assay (IFA)

The procedure for IFA was described previously [31]. Vero cells in 6-well plates were infected with rLS or rLS/EB-GP at an MOI of 0.01. The primary antibody was rabbit anti-EBOV GP protein antibody (Sino Biological Inc, Beijing, China). The secondary antibody was fluorescein (FITC)-conjugated goat anti-rabbit IgG (H+L) (Thermo Fisher). Fluorescence images were observed under an inverted fluorescence microscope at 100× magnification with a matching wavelength filter for FITC.

Prime-boost regimens and animal experiments

The replication-defective HAΔV-5-MakGP vaccine was developed and prepared by the Beijing Institute of Biotechnology and Tianjin CanSino Biotechnology as described previously [33].

A group of 4- to 6-week old female BALB/c mice was obtained from Beijing Vital River Laboratory Animal Co. Ltd (Beijing, China) and housed in an animal biosafety level 2 facility. The mice were confirmed by ELISA to be free of EBOV antibodies prior to immunization and then randomly divided into six groups of six mice each and inoculated with the initial dose of vaccine on day 0 and the booster on day 21, as illustrated in Fig. 2. Mice in the HAdV-5 group were vaccinated intramuscularly (i.m.) with 1×10^7 infectious units (IFU) of HAdV-5-MakGP on days 0 and 21. Mice in the NDV group were inoculated intranasally (i.n.) with 1×10^6 plaque-forming units (PFU) of rLS/EB-GP on days 0 and 21. Mice in the HAdV-5+NDV group were primed by i.m. injection with 1×10^7 IFU of HAdV-5-MakGP on day 0, followed by i.n. boosting with 1×10^6 PFU of rLS/EB-GP on day 21. Conversely, mice in the NDV+ HAdV-5 group were primed i.n. with 1×10^6 PFU of rLS/EB-GP and then boosted by i.m. injection with 1×10^7 IFU of HAdV-5-MakGP. Mice in the rLS group were vaccinated i.n. with 1×10^6 PFU of rLS and served as LaSota vector controls. Mice in the PBS group were inoculated with 100 μ l of PBS via the intranasal route and served as unvaccinated controls.

At 21 and 49 days post-immunization (PI), blood was collected from each mouse, and serum was obtained by centrifugation of whole blood. Isolated sera were transferred to labeled tubes and stored at -20°C until analysis. Eighteen mice from each group were humanely euthanized at day 49, and splenocytes were isolated for ELISPOT assay.

EBOV GP ELISA

96-well polystyrene microtiter plates (Costar, Bethesda, MD) were coated overnight at $2-8^\circ\text{C}$ with $2 \mu\text{g}$ of EBOV GP protein (subtype Zaire, strain H. sapiens-wt/GIN/2014/Kissidougou-C15) (Sino Biological Inc, Beijing, China) per ml. Plates were washed with PBS containing 0.05% Tween

(PBST) and blocked with 5% goat serum for 1 h at 37°C . After blocking, twofold serial dilutions of sera were added to EBOV-GP-protein-coated plates in duplicate and incubated for 1 h at 37°C . The bound antibodies were detected using horseradish-peroxidase-conjugated goat anti-Mouse IgG antibody (Thermo Fisher). After a 1-h incubation, color was developed by adding TMB (Solarbio, Beijing, China) peroxidase substrate, and absorbance was read at 450 nm in a Bio-Rad 550 ELISA plate reader (Bio-Rad, Hercules, CA). The antibody titer was expressed as the reciprocal of the highest antibody dilution that yielded greater than or equal to 2.1 times the background absorbance of PBS (the negative control) [19].

ELISPOT assay

A commercial kit (mouse IFN- γ precoated ELISPOT kit, Dakewe Biotech, Shenzhen, China) was used for the ELISPOT assay. Lymphocytes from the spleen were harvested from each mouse, and a single cell suspension (5×10^5 cells/well) was prepared and stimulated with $20 \mu\text{g}$ of the EBOV GP protein described above per ml. After incubation at 37°C for 24 h, the cells were removed and the plates were processed according to the manufacturer's instructions. The number of spots was determined automatically using an automatic CTL Immunospot® Reader (Cellular Technology, Shaker Heights, Ohio).

Statistical analysis

Statistical analysis was performed using GraphPad Prism. All data were shown as the mean and standard deviation. Data were statistically analyzed using the unpaired, two-tailed Student's *t*-test. *p*-values of <0.05 were considered significant.

Results

Generation of the rLS and rLS/EB-GP viruses

A full-length cDNA clone encoding the complete anti-sense genome of the NDV LaSota strain was constructed by assembling four overlapping PCR fragments through PCR and In-Fusion PCR cloning, as illustrated in Fig. 1. The GP gene of EBOV flanked by the NDV *trans*-acting elements was inserted into the P-M intergenic region of the LaSota full-length clone as an additional transcription unit, which increased the length of the recombinant clone by 2232 nucleotides (nt). Thus, the total length of the cDNA clone in the pLS/EB-GP plasmid was 17,418 nt and therefore divisible by 6, conforming to the "Rule of Six" [16]. Then, the NDV LaSota virus and the recombinant virus, designated as rLS

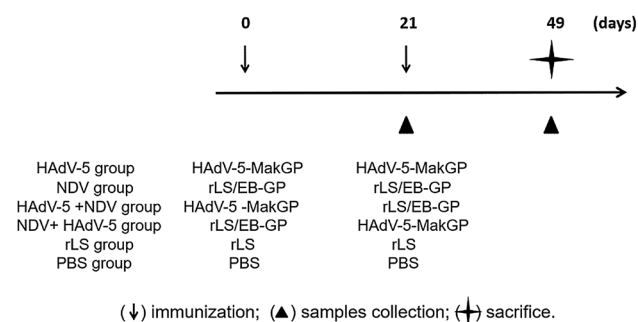


Fig. 2 Schemes for animal immunization and sample collection. BALB/c mice were divided randomly into six groups and were immunized at day 0 (prime) and day 21 (boost). Serum samples were collected at day 21 and 49 PI for ELISA assay. Mice were sacrificed at day 49 PI, and splenocytes were isolated for ELISPOT assay

and rLS/EB-GP, respectively, were rescued from the cDNAs as described in [Materials and methods](#). Sequencing analysis confirmed the nucleotide sequence fidelity of the rescued viruses (Fig. 3).

Expression of the EBOV GP protein by rLS/EB-GP

To examine the expression of EBOV GP protein by rLS/EB-GP, Vero cells were infected with rLS/EB-GP at an MOI of 0.01. After 48 h, the cells were fixed and incubated with a rabbit antiserum directed against the EBOV GP protein, followed by immunostaining with FITC-conjugated goat anti-rabbit IgG. Positive staining was observed in rLS/EB-GP-infected Vero cells using an anti-EBOV GP antibody and an FITC-labeled secondary antibody (Fig. 4a). In contrast, no fluorescence was detected in NDV-LaSota-infected cells after staining with the same antiserum and FITC conjugate (Fig. 4b). This result demonstrated that the EBOV GP protein was expressed from the rLS/EB-GP-recombinant-infected cells.

Heterologous prime-boost regimens elicit more-robust EBOV-GP-specific antibody responses than homologous regimens

To assess the effectiveness of heterologous prime-boost vaccination regimens with rLS/EB-GP and HAdV-5-MakGP for

inducing immune responses, BALB/c mice were immunized on day 0 (prime) and day 21 (boost) with these vectored EBOV vaccines as illustrated in Fig. 2. EBOV-GP-specific antibodies in the collected sera were detected by ELISA. As shown in Fig. 5, although EBOV-GP-specific IgG titers were relatively low in all groups after the first immunization (prime), they increased dramatically after the second immunization (boost). The heterologous prime-boost with HAdV-5 and NDV induced a significantly higher geometric mean titer (GMT) of GP-specific IgG at day 49 post-immunization (PI) than the homologous prime-boost regimens (HAdV-5+NDV group vs. HAdV-5 group, $p < 0.05$; HAdV-5+NDV group vs. NDV group, $p < 0.05$). The mice in the NDV+HAdV-5 group produced approximately 1.5-fold and 3.1-fold higher GP-specific IgG titers than those receiving the NDV and HAdV-5 homologous regimens, respectively. These results demonstrated that the heterologous prime-boost regimens could elicit more-robust EBOV-GP-specific antibody responses than the homologous regimens.

Heterologous prime-boost regimens elicit stronger cellular immune responses than homologous regimens

Splenocytes isolated from the immunized mice at day 49 PI were assayed by IFN- γ -specific ELISPOT to analyze the antigen-specified T-cell responses induced by the different

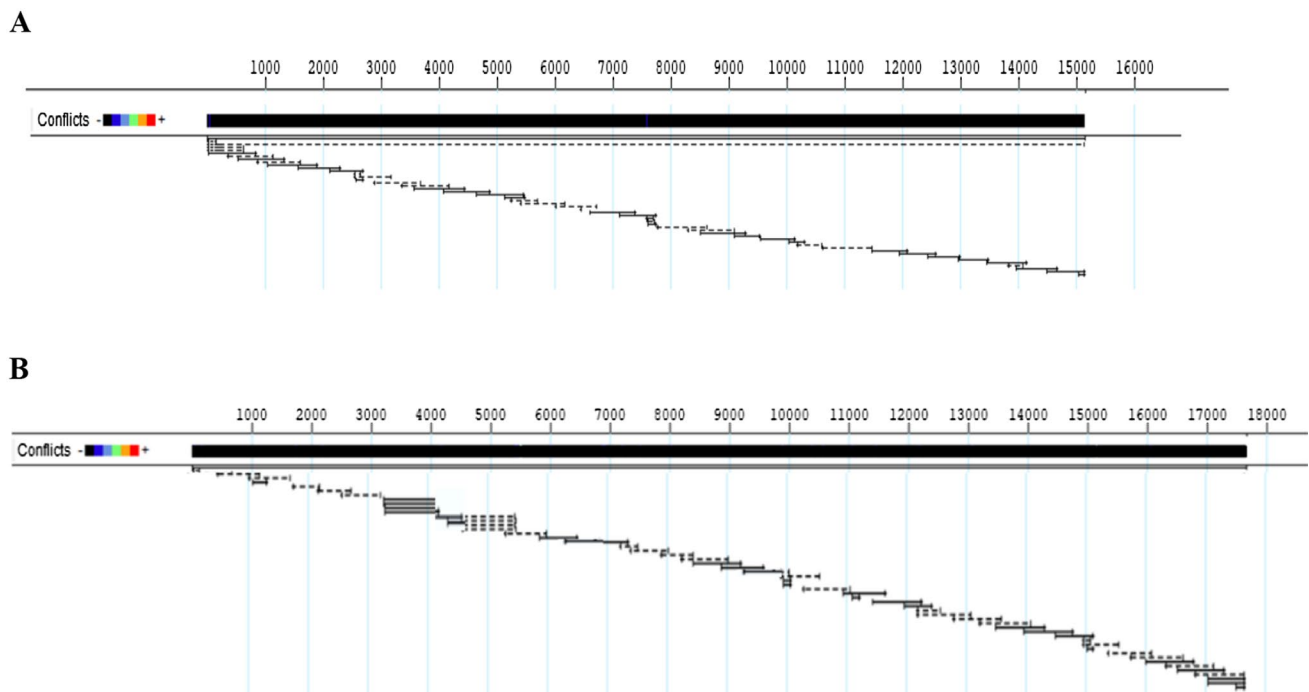


Fig. 3 Sequencing analysis of the rescued viruses. The RT-PCR products generated from the rescued viruses rLS (A) and rLS/EB-GP (B) were sequenced directly with 32 and 37 pairs of gene-specific prim-

ers, respectively. Nucleotide sequence editing, assembly, and comparison analysis were carried out using the DNASTAR Lasergene software (DNASTAR, Madison, WI, USA)

Fig. 4 Detection of EBOV GP protein expression by IFA. Vero cells were infected with rLS or rLS/EB-GP at an MOI of 0.001. At 48 hours postinfection, the cells were fixed and stained with a rabbit anti-EBOV GP protein antibody, followed by an FITC-labeled goat anti-rabbit IgG secondary antibody. Cytopathic effect (CPE) and fluorescence images in the same field of the infected cells were photographed using an inverted fluorescence microscope at 100× magnification under visible light for CPE or a UV light with a matching wavelength filter for FITC

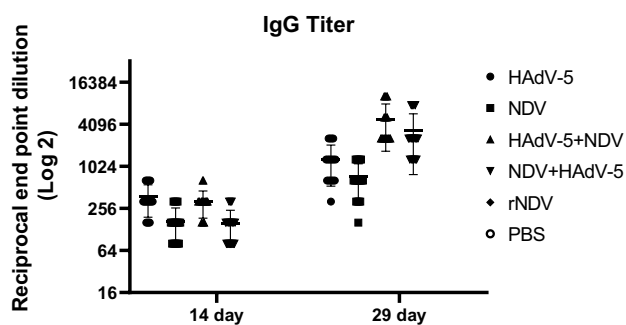
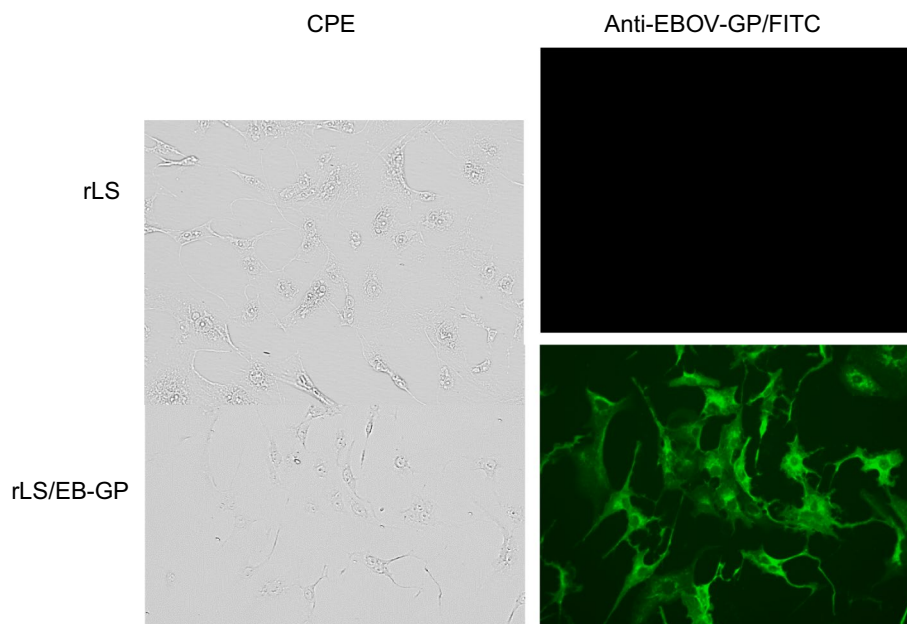


Fig. 5 EBOV-GP-specific antibody responses. Serum samples were collected from each mouse at 21 and 49 days post-inoculation. Serum EBOV-GP-specific IgG antibodies were determined by ELISA and used to calculate the GMT for each group of mice

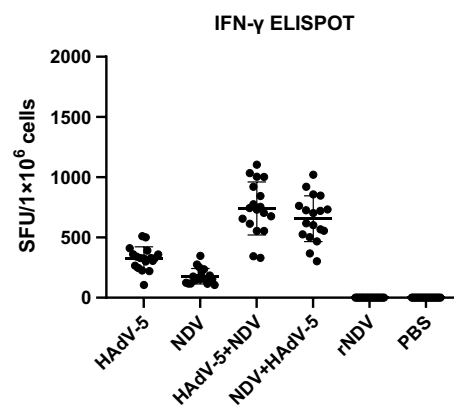


Fig. 6 EBOV-GP-specific IFN- γ responses. Mice were sacrificed at 49 days post-inoculation. The splenocytes were isolated and assayed by ELISPOT for an EBOV-GP-specific IFN- γ response

immunization regimens. Interestingly, as shown in Fig. 6, the magnitude of the IFN- γ -specific ELISPOT responses correlated positively with the antibody responses in all immunization groups. Specifically, splenocytes obtained from mice in the heterologous HAdV-5+NDV and NDV+HAdV-5 regimen groups exhibited significantly higher IFN- γ expression than those from mice in the homologous HAdV-5 regimen ($p < 0.05$) and rLS/EB-GP regimen ($p < 0.05$) groups. The IFN- γ -specific ELISPOT data, together with the antibody response results, demonstrate that mice immunized with the heterologous prime-boost regimens not only elicited stronger humoral responses but also induced stronger cellular responses than those receiving the homologous regimens.

Discussion

In the present study, we successfully generated an NDV LaSota strain-based recombinant virus, rLS/EB-GP, expressing the GP protein of the EBOV variant Makona, as a vaccine candidate, using reverse genetics techniques. The immune responses of mice to this rLS/EB-GP recombinant vaccine and an HAdV-5-based EBOV experimental vaccine were evaluated by prime-boost regimens. The ELISA and ELISPOT results indicated that heterologous prime-boost regimens elicit more-robust immune responses than homologous regimens. The HAdV-5 prime

and rLS/EB-GP boost immunization strategy was found to be the most effective approach for stimulating EBOV-specific immunities among the different regimens.

Human adenovirus has emerged as an ideal vaccine vector because of its relative safety, high transgene expression levels, and its capacity to elicit robust humoral and cellular immune responses to the encoded antigens. [30]. However, serosurveillance has shown that PEI to HAdV-2 and HAdV-5 existed in up to 50% of the American population and up to 95% in people from other countries. This high seroprevalence limits the efficacy of adenovirus (Ad)-vectored vaccines [4]. To circumvent the problem of PEI interference and improve the effectiveness of Ad-based vaccines, researchers have developed non-human primate (e.g., chimpanzee and gorilla) adenovirus vectors [6], explored different immunization routes [22], and, notably, investigated prime-boost regimens with heterologous vectors [18]. Regarding the last of these, studies have demonstrated that heterologous prime-boost regimens employing a DNA prime and an HAdV-5 or ??modified vaccinia virus Ankara?? (MVA) boost could effectively induce humoral and cellular immune responses, and more importantly, improve vaccine efficacy, regardless of pre-existing Ad or MVA immunity [14, 29]. Three EBOV vaccine candidates adopting the heterologous prime-boost strategy have undergone different phases of clinical trials [20]. Results from these investigations indicated that both heterologous vector vaccine regimens of ChAd3-EBOV and Ad26.ZEBOV vaccines boosted with MVA elicited stronger B-cell and T-cell immune responses to EBOV than those induced by single-dose prime vaccination alone [2, 10].

EBOV transmission occurs exclusively through contact with mucosal surfaces. It might therefore be advantageous to use a vaccine vector with a natural tropism for the respiratory tract, which could induce the first line of defense at mucosal points of entry and robust systemic immune responses in the respiratory tract. Numerous researches have shown that NDV, an avian paramyxovirus, is an attractive vaccine vector for human pathogens, inducing humoral and cellular immune responses at both the mucosal and systemic levels in murine and non-human primate models [15]. Therefore, in this study, we adopted NDV as a vaccine vector to express the EBOV GP protein as a heterologous boost vaccine with the hope of avoiding the PEI interference associated with Ad-based primed vaccines. The results presented in this report are in agreement with the findings from the EBOV vaccine trials using the heterologous prime-boost immunization strategy [20], confirming that heterologous prime-boost regimens can elicit more-robust humoral and cellular responses to EBOV than homologous regimens [10, 26].

We observed with the homologous regimens that although two doses of rLS/EB-GP or HAdV-5 induced detectable EBOV-GP-specific antibody and T-cell responses in mice,

the immune responses elicited by HAdV-5 were twofold higher than those induced by NDV. This difference might be due to differences in the amount of antigen expressed/presented by these two vectors or the different immunization routes used to stimulate the immune system. Furthermore, the DNA vector HAdV-5 and the RNA vector NDV undoubtedly stimulate innate pathways differently. Therefore, we adopted the non-uniform but normal dosage of HAdV-5 (1×10^7 IFU) and NDV (1×10^6 PFU) in this study. Further studies may be needed to determine whether a higher dose of NDV could enhance vaccine effectiveness.

In contrast to the homologous regimens, the heterologous prime-boost with HAdV-5 and rLS/EB-GP induced stronger immune responses than the homologous regimens. The most robust EBOV-GP-specific antibody and T-cell responses were detected by ELISA and ELISPOT in the HAdV-5 prime and NDV boost group, while the reversed NDV prime-HAdV-5 boost ranked second. The reason for this difference is still unclear. A possible explanation may be differences in the way stimulating innate immunity is stimulated by an RNA virus (NDV) and a DNA virus (Ad), which would affect the direction and strength of the subsequent adaptive immune response. This hypothesis needs to be tested in future studies by analyzing markers of immune responses.

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Declarations

Conflict of interest No competing financial interests exist.

Ethical approval Animal usage protocols used in this study were approved by the Beijing Center for Disease Prevention and Control (CDC) Institutional Animal Care and Use Committee (IACUC). At the termination of the experiments, all animals were humanely euthanized in accordance with Beijing-CDC-IACUC-approved animal usage protocols.

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