Vaccine 37 (2019) 3335–3342

Contents lists available at ScienceDirect

Vaccine

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

Intranasal immunization with recombinant Vaccinia virus Tiantan harboring Zaire Ebola virus *gp* elicited systemic and mucosal neutralizing antibody in mice

Lilan Xie [1], Junjie Zai [1], Kai Yi, Yaoming Li *

Hubei Engineering Research Center of Viral Vector, Applied Biotechnology Research Center, Wuhan University of Bioengineering, Wuhan 430400, China

ARTICLE INFO

Article history:
Received 6 November 2018
Received in revised form 18 March 2019
Accepted 24 April 2019
Available online 7 May 2019

Keywords:
Vaccinia virus vector
Ebola virus
GP
Mucosal sIgA
Neutralization

ABSTRACT

Accumulating literature revealed that human mucosa was likely one of the important routes for EBOV attachment and further infection. Therefore inducing effective mucosal immune responses play key role in preventing the virus infection. Vaccinia virus Tiantan strain (VV) was a remarkably attenuated poxvirus, which has been broadly exploited as a multifunctional vector during the development of genetically recombinant vaccine and cancer therapeutic agent. In this study, we generated a recombinant VV harboring EBOV *gp* (VV-$_{Egp}$) that was used to immunize mice, followed by assessing immune responses, particularly the mucosal immune responses to EBOV GP. A stable and further attenuated VV-$_{Egp}$, in which the VV *ha* gene was replaced with the EBOV *gp*, was generated. In BALB/c mouse model, intranasal immunization with VV-$_{Egp}$ elicited robust humoral and cellular immune responses, including high level of neutralizing serum IgG and IgA against EBOV, and a large amount of GP-specific IFN-γ secreting lymphocytes. More importantly, EBOV GP-specific neutralizing secreted IgA (sIgA) in nasal wash and both sIgA and IgG in vaginal wash were induced. In summary, immunization with a safe and stable recombinant VV carrying a single EBOV *gp* conferred robust systemic immune response and mucosal neutralizing antibodies, indicating that the recombinant virus could be utilized as a viral vector for plug-and-play universal platform in mucosal vaccine development.

1. Introduction

Zaire Ebola virus (EBOV) caused a rapid and severe infection with high fatality rates of up to 90% in infected patients. The disease is becoming a public health-associated issue due to the high morality rate and the lack of both prophylactic vaccines and therapeutic drugs. So far, the precise pattern of natural transmission between humans remains to be addressed. Some recent studies revealed that EBOV were detected in the saliva, semen, vaginal secretions, breast milk, and occasionally in the sweat of infected patients [1–3], suggesting that human mucosa was likely a latent route of the virus transmission.

EBOV is enveloped, non-segmented, negative-stranded RNA virus belonging to the family *Filoviridae*. The EBOV glycoprotein (GP), which interacts with host receptor Niemann-Pick C1 (NPC1), mediates viral attachment and entry into host cells and is considered to be the major inducer for the host immune responses [4]. EBOV GP-based recombinant viruses (VSV-GP and Ad5-GP) that were currently considered as the most promising vaccine candidates have entered phase IV clinical trails [5]. Intramuscular immunization of EBOV GP-based VSV and Ad5 stimulated robust humoral and cellular immune responses, subsequently conferred sterile protection against lethal live virus challenge of animals [6–8]. A clinical study revealed that a monovalent chimpanzee Adenovirus Ebola vaccine expressing Ebola virus GP elicited neutralizing serum IgG while boosted with recombinant MVA [9]. However, the mucosa-associated immune responses have not been intensively evaluated in those studies. Mittal et al. reported that intramuscular administration could rarely elicit mucosal immunity [10]. Needle-free-mediated mucosal immunization offered a more convenient and safer approach and conferred systemic and mucosal protective immune responses [11–13]. Therefore, exploiting novel mucosal immune strategies not only provide sterile immunity but also make the immunization procedure safer and more acceptable.

A Vaccinia virus (VV) Tiantan strain was originally isolated in China and played a crucial role in the eradication of local smallpox epidemic. The VV has been recently developed as a multifunctional vector for vaccines against influenza virus and HIV-1 [14,15]. For

* Corresponding author at: College of Life Science and Technology, Wuhan University of Bioengineering, Wuhan 430400, China.
E-mail address: limeming@webmail.hzau.edu.cn (Y. Li).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.vaccine.2019.04.070
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instance, the replication-competent viral vector-based HIV-1 vaccines that are presently tested in various phases of clinical trials have exhibited immune protection efficacy in non-human primates (NHPs) [14,16,17]. We previously demonstrated that a single anino acid mutation in the structural gene (A34R) enhanced immunogenicity but did not increase its toxicity in mice [18]. More importantly, our earlier study demonstrated that the recombinant VV containing HIV-1 *env* (VV-$_{HIVenv}$) were detectable in upper respiratory trait within a few days after intranasal administration, and elicited substantial mucosal immunity, such as HIV-1 ENV-specific secreted IgA (sIgA) in nasal tract, lung and virginal tract [19]. Therefore recombinant VV has a high potential using as an universal vaccine vector for expressing exogenous immunogens to induce humoral, cellular, and mucosal immunity.

In the present study, we developed a recombinant VV harboring EBOV *gp* gene (VV-$_{Egp}$) and immunized mice with this recombinant virus, followed by assessing those immune responses, particularly for the mucosal immune responses against EBOV. The elevated mucosal immune response indicated that recombinant VV could be used as a promising vaccine candidate preventing mucosal infection of EBOV and other mucosal transmission pathogens.

2. Materials and methods

2.1. Cell lines, Ebola virus GP, antibodies, plasmids, and viruses

The cell lines, including Vero cell (Vero 1008), Caco2, and 293 T were grown in Dulbecco's Modified Eagle's medium (DMEM, Gibco) supplemented with 10% FBS (Gibco). Recombinant Zaire Ebola virus GPdTM (0501-015, IBT BIOSERVICES, USA) was mature recombinant, Zaire Ebola virus Glycoprotein minus the transmembrane domain (rZEBOV GPdTM). The parental wild-type VV was obtained from the Chinese Center for Disease Control and Prevention (China CDC). The viral stocks were prepared as previously described [19]. EBOV-GP-specific antibodies 4G7 and 2G4 were kindly provided by Dr. George F Gao, China CDC. The plasmid pEAK13-GP was a gift from Dr. C Jiang, Tsinghua University, China.

2.2. Recombinant virus

Homologous recombination was used to obtain recombinant viruses as previously described [18]. Briefly, two DNA fragments about 600 bp in length derived from VV *ha* gene were inserted into the shuttle plasmid which harbored two DNA cassettes driving the expression of EBOV *gp* and *gfp* respectively. Then this construct was transfected into the Vero cells which were simultaneously infected by VV (Fig. 1A). The fluorescent recombinant virus was selected under fluorescent microscope and purified with limited dilution approach.

2.3. Western blot

Vero 1008 cells were infected with VV-$_{HIVenv}$, VV-$_{Egp}$, or mock infected. At 48 h postinfection, cells were subject to 12% SDS-PAGE and transferred to PVDF membranes (0.45 μm, Millipore) followed by blocking with 5% nonfat milk in PBST and probed with EBOV-GP-specific mAb 4G7 at room temperature (RT) for 2 h. After washing three times with PBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:8000, SouthernBiotech, USA). The membranes were developed using an enhanced chemiluminescence Western blot detection system (Amersham, Little Chalfont, UK).

To test the stability *gp* gene in recombinant virus, Vero 1008 cells were infected with different passages of viruses, the Western blot was performed as described above.

2.4. Indirect immunofluorescence assay (IFA)

Vero 1008 cells were seeded in 35-mm glass-bottom dishes and infected with VV-$_{HIVenv}$ or VV-$_{Egp}$. At 24 h postinfection, cells were fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After three washes with PBS, cells were blocked in PBS containing 5% BSA at 4℃ overnight. Thereafter, cells were incubated with mAb 4G7 at concentration 1 μg/mL at 37 °C for 1 h, respectively. After three times of washes with PBST, cells were then incubated with FITC-conjugated goat anti-murine IgG and DAPI. Finally, cells were washed and subject to incubation with antifluorescence quenching reagent (Beyotime, CN) and observed under a fluorescence microscope (Olympus IX51).

2.5. Immunization

Twenty-four Six-week-old BALB/c mice were randomly grouped (six mice per group), among which twelve mice were vaccinated with 5×10^6 plaque forming units (pfu) in 10 μL (5 μL each time with an interval of 10 min) of VV-$_{Egp}$ or VV-$_{HIVenv}$ respectively, six mice were immunized with 10 μg of GP in 10 μL, and the rest were mock vaccinated with PBS through intranasal immunization (I.N.) after complete anaesthesia with pentobarbital sodium. All the mice were immunized twice with an interval of 30 days. The mice were sacrificed at day10 after the final immunization, and then the mucosal samples, sera, and splenocytes were collected. The animal study was approved by the ethics committee of the Wuhan University of Bioengineering, China (permit number WUB20170108). All animal studies and methods were confirmed to ARRIVE guidelines.

2.6. IFN-γ and IL-4 ELISPOT assay

The EBOV GP-specific T cell immune responses in mice was determined by ELISPOT assay. The mice were sacrificed at day 10 after the final immunization. The individual splenocyte was prepared with Ficoll-Paque (GE). The IFN-γ-secreting T cells were detected using a mouse IFN-γ pre-coated ELISPOT kit (DAKEWE, China), and the IL-4-secreting T cells were detected by a mouse IL-4 T cell ELISPOT kit (U-CyTech, Netherlands) according to the manufacturers' instructions. Briefly, 2×10^5 splenocytes were added to pre-coated wells in duplicates and incubated with complete RPMI 1640 containing 10% FBS and 5 μg/mL of EBOV-GP for 24 h at 37 °C. Spots were visualized by adding 100 μL of the substrates to the wells after their reaction with Horseradish Peroxidase-labeled avidin and were counted by a MultiSpot Spectrum (AID Diagnostika GmbH, Germany).

2.7. ELISA

The binding antibody responses against EBOV-GP were determined by ELISA. Generally, 96-well ELISA plates were coated with 100 μL of the indicated antigen (2 μg/mL of EBOV-GP) in coating buffer overnight at 4 °C. Sera and mucosa samples from the individual mouse were serially diluted and then analyzed using AP-labeled goat anti-mouse IgG (Southern Biotechnology, USA), followed by substrate p-nitrophenyl phosphate (PNPP) (Sigma-Aldrich, USA). The reciprocal values of the last dilution giving an OD_{405nm} reading that was twice the background of the empty wells were determined as the titer of the antibody.

2.8. Neutralization assay

To generate EBOV-GP-packaged pseudotype virus, 4×10^6 293 T cells were co-transfected with 10 μg of a pNL4-3.Luc.R$^-$E$^-$ [20] and 10 μg of a DNA plasmid encoding entire EBOV-GP (pEAK13-GP) [21]. The efficacy was evaluated by EBOV-specific

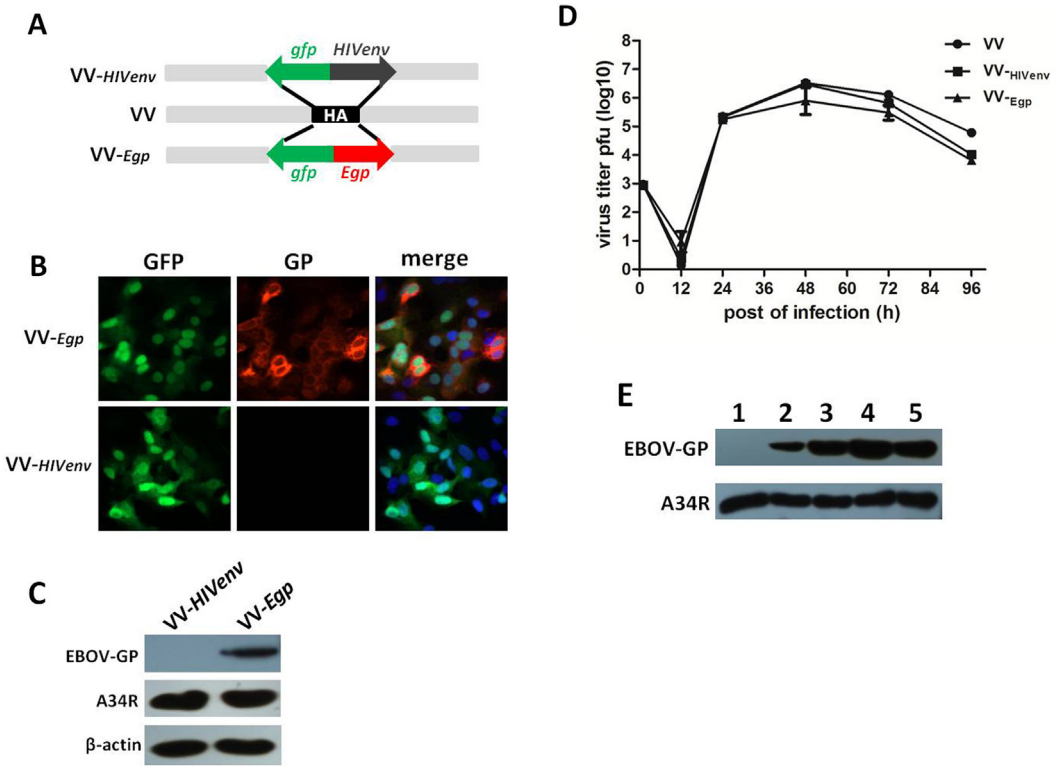

Fig. 1. Development and characterization of the recombinant Vaccinia virus (VV) carrying EBOV *gp*. (A). Schematic diagram of the construction procedure of VV-$_{Egp}$. Expression cascade harboring *gfp* and *Egp* replaced the VV *ha* by using homologous recombination. (B). Exogenous expression of EBOV-GP in VV-$_{Egp}$-infected Vero cells was evaluated by IFA. Mouse 4G7 mAb served as a positive detector antibody for EBOV-GP. (C). EBOV-GP in VV-$_{Egp}$-infected Vero was also evaluated by Western blot. (D). Replication dynamic of VV-$_{Egp}$ was assessed on Vero cells. (E). Expression stability of *Egp* gene in different passage of VV-$_{Egp}$. Line 2–5 represent 1, 4, 7, and 10 passage of progeny virus. 4G7 acted as a detector antibody for GP.

neutralizing mAb 2G4 [22]. Sera were two-fold diluted in 50 μL, and mixed with 10 $TCID_{50}$ pseudovirus in 50 μL. Then the mixture was added to the 96-well plate culturing for 1 h, followed by application 1×10^4 293 T cell each well. At 48 h post infection (hpi), cells were subject to determine the luciferase activity by a Luciferase Assay System according to the manufacturer's instructions (Promega) using Tuner Biosystems Modulus II.

Antibodies in serum or mucosal samples were depleted with corresponding anti-IgG (NAb™ Protein G Spin Kit, Thermo Scientific™) or anti-IgA (Goat Anti-Mouse IgA-BIOT, SouthernBiotech; Streptavidin Agarose, Themorfisher) beads according to the manufacturers' instructions. The antibody depletion efficiency was determined by using sandwich ELISA (Southern Biotechnology, USA).

2.9. Statistics

Data were analyzed using GraphPad Prism software (San Diego, CA). All of the data analysis was performed with one-way ANOVA or two-way ANOVA. NS, $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$.

3. Results

3.1. Construction of recombinant VV containing Zaire EBOV gp gene (VV-$_{Egp}$)

Recombinant VV containing Zaire EBOV complete *gp* gene was generated by homologous recombination, and the recombinant virus was selected as previously described [18] (Fig. 1A). The expression of EBOV *gp* was estimated using IFA and Western blot (Fig. 1B), showing that the green and red fluorescence were co-localized in recombinant virus (VV-$_{Egp}$) infected Vero cells. Whereas only green fluorescent signal was observed in the recombinant VV-$_{HIVenv}$-infected Vero cells. The virus-infected cell lysates were subject to Western blot using the mAb 4G7 antibody. The GP was clearly detected in VV-$_{Egp}$-infected Vero cells, but not in VV-$_{HIVenv}$-infected cells (Fig. 1C). Plaque assay was used to evaluate the VV-$_{Egp}$ replication dynamic. As shown in Fig. 1D, progeny VV-$_{Egp}$ was seen at 24 hpi and the virus titer reached the highest level at 48 hpi (0.9×10^6 pfu/mL). However, VV-$_{Egp}$ replicated slightly slower than both VV-$_{HIVenv}$ and VV in Vero cells at the late stage. To evaluate the stability of recombinant virus VV-$_{Egp,}$ we used the recombinant virus to repeatedly infect Vero cells until the virus achieved at least ten passages in the cultured cells. The EBOV GP after different passages were analysed by Western blot using the mAb 4G7 as detector antibody [22]. As shown in Fig. 1E, EBOV-GP was readily detected in 1, 4, 7, and 10 passage of VV-$_{Egp}$. These results indicated that a recombinant VV expressing EBOV *gp* was successfully selected and could stably express Ebola GP, thereby it has potential for utilizing as a vaccine candidate against Ebola virus infection.

3.2. Intranasal immunization of VV-$_{Egp}$ elicited neutralizing antibodies of both IgG and IgA in serum

Humoral immune responses, especially serum IgG and IgA, play important role in preventing virus reinfection [10]. Thus we first assessed the level of serum antibodies that bound to Ebola GP by using indirect ELISA. As shown in Fig. 2A and B, the total level of serum IgG to GP reached 10000, while the level of serum IgA against GP was about 100. Of note, the ratio of IgG1/IgG2a was close to 0.5 (Fig. 2C), indicating that VV immunization induced

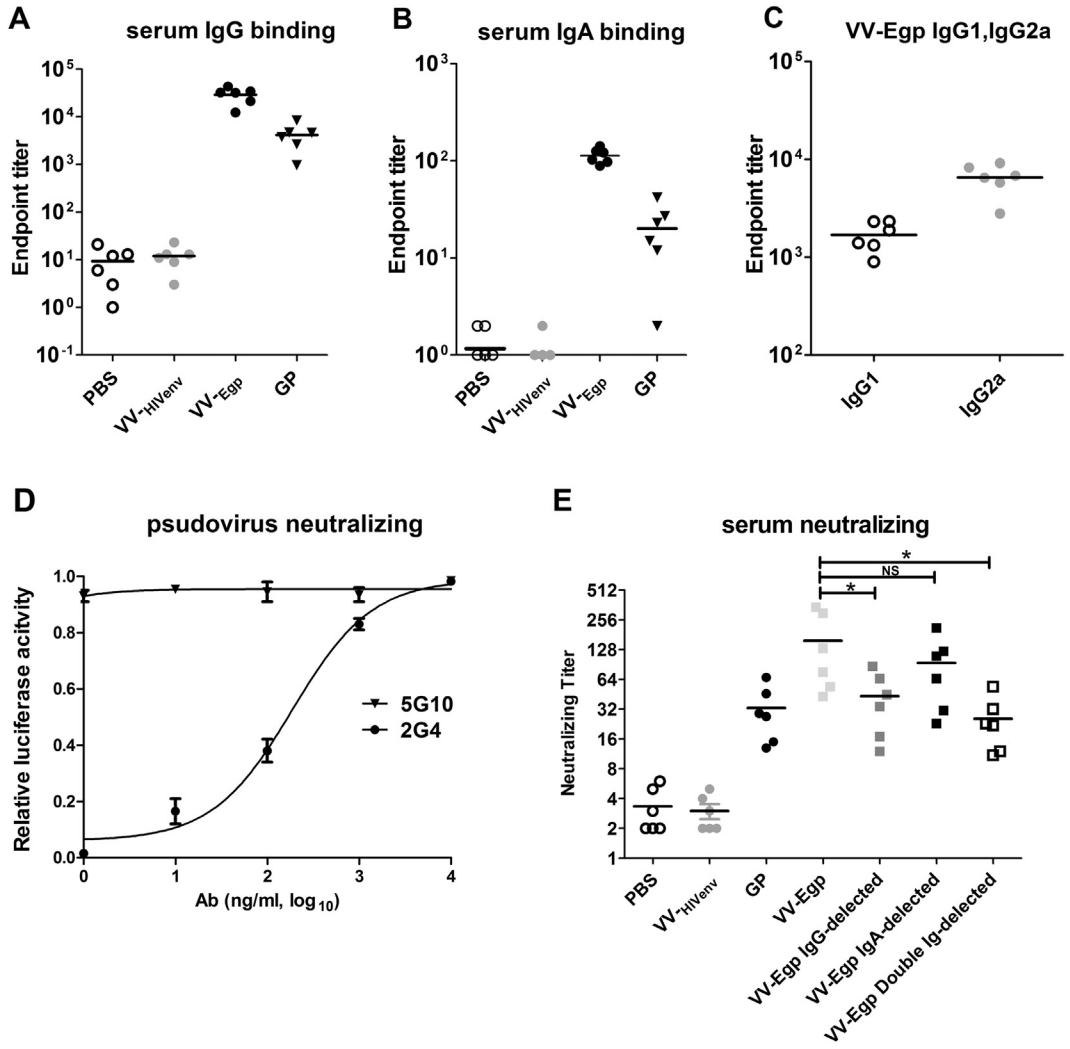

Fig. 2. VV-$_{Egp}$ elicited neutralizing both serum IgG and IgA in mice. (AB). EBOV-GP-specific antibody titers (IgG and IgA) were determined by ELISA. (C). EBOV-GP-specific IgG1 and IgG2a were evaluated by ELISA for VV-$_{Egp}$ -immunized mice. (D). Ebola psudovirus was verified by a known neutralizing antibody 2G4 against EBOV and an isotype antibody control 5G10. (E). Neutralizing antibody in serum were assessed by neutralization assay. Sera taken at day 10 following the last immunization were used in the assay. Sera from VV-$_{Egp}$ -immunized mice significantly neutralized Ebola psudovirus. When single deletion of either IgA or IgG in sera occurred, the antiviral capacity were not completely abolished, whereas the antiviral function decreased significantly when double deletion of both IgA and IgG.

Th1-biased immune responses, despite of significant GP-specific IgG and IgA.

We next investigated the neutralizing capacity of sera derived from the immunized mice. As shown in Fig. 2D, mAb 2G4 could significantly suppressed the pseudovirus (see Materials and methods) attachment, demonstrating the validity of the EBOV pseudovirus system. Two-fold diluted serum samples were mixed with luciferase reporter pseudovirus followed by DLR assay. The results revealed that the sera from VV-$_{Egp}$-immunized mice effectively neutralized the pseudovirus (Fig. 2E). In contrast, the control sera did not show any neutralization effect even at the minimum dilution tested (1:4). These results suggested that antibodies elicited by VV-$_{Egp}$ could neutralize EBOV pseudovirus. We next exploited the crucial factor in sera that neutralized virus infection. Deletion of either serum IgG or IgA did not abolished its antiviral effect (Fig. 2E). However double deletion of IgG and IgA significantly decreased the neutralizing capacity of sera ($p < 0.05$) (Fig. 2E). It was confirmed that corresponding antibody were undetectable following depletion (Fig. S1). Collectively, the results indicated that sera IgG and IgA derived from VV-$_{Egp}$-immunized mice synergistically neutralized EBOV attachment.

3.3. VV-$_{Egp}$ activated more EBOV-GP -specific IFN-γ-secreting T cell responses

Virus-based vaccine generally induced remarkable cellular immune response, thereby we evaluated GP-specific cellular immune responses of immunized mice [23]. The amount of EBOV-GP -specific IFN-γ-secreting T cells detected by ELISPOT reached an average of 500 spot-forming cells (SFC) per million splenocytes (Fig. 3A), indicating that the VV-$_{Egp}$ induced not only a GP-specific T cell response but also the lower level of IL-4-secreting T cells (Fig. 3B). Compared to VV-$_{Egp}$, the GP alone induced relatively lower level of IFN-γ ($p < 0.05$). The VV-$_{Egp}$ immunization also induced a significant Th1-biased immune tendency which was agreement with the results shown in Fig. 2C.

3.4. VV-$_{Egp}$ elicited GP-specific mucosal sIgA

Mucosal antibodies, especially secreted IgA (sIgA), were considered as crucial protective elements for the transmission of respiratory viruses [24]. Some recent studies reported that mucosal GP-specific antibody conferred substantial protection for EBOV

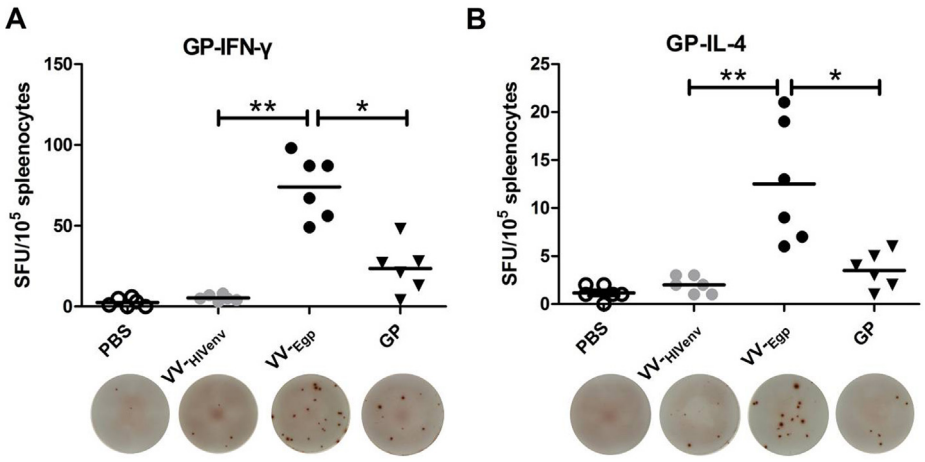

Fig. 3. Cellular immune responses. EBOV-GP-specific IFN-γ-secreting T cells (A) or IL-4-secreting T cells (B) were analyzed by ELISPOT. At day 10 after the final immunization, mice splenocytes were isolated with Ficoll-Paque, followed by stimulation with GP in 96-well plate. Cytokine-secreting cells forming or accounting according to the manufacturers' instructions.

infection [12,25]. To investigate whether GP-specific mucosal binding antibodies were produced, including sIgA and IgG, their antiviral capacities were evaluated *in vitro*. We found that only mucosal sIgA against GP were presented in nasal wash (Fig. 4AB) and no GP-specific mucosal antibody was detected in lung extract (Fig. 4CD). Whereas both GP-specific mucosal IgG and sIgA were detectable in vaginal wash (Fig. 4EF). And both nasal wash and vaginal wash possessed the properties of neutralizing pseudovirus of EBOV (Fig. 4GH). Compared to VV-$_{Egp,}$ the GP induced relatively lower level of sIgA ($p < 0.05$). To demonstrate whether sIgA or IgG was the crucial antiviral factor, antibody deletion assay was performed. The corresponding antibody were undetectable following depletion (Fig. S2). Mucosal IgG was undetectable in nasal tract (Fig. 4G). Following deletion of IgG, sIgA depletion completely abolished the antiviral capacity of nasal wash, indicating sIgA was the major antiviral factor in nose tract (Fig. 4G). The antiviral capacity with single deletion of either sIgA or IgG in vaginal wash was not completely abolished ($p < 0.05$), but it occurred with double deletion of both sIgA and IgG ($p < 0.01$), indicating that both sIgA and IgG synergistically conferred neutralizing role of vaginal wash (Fig. 4H).

3.5. No significant side-effects of VV-$_{Egp}$ was observed in infected cells and mice

To investigate whether the VV-$_{Egp}$ cause the cytotoxicity [21], we analyzed the side-effect of VV-$_{Egp}$. -infected Vero 1008 cells and Caco2 cells by MTT assay, no significant pathogenesis was observed following infection of VV-$_{Egp}$ compared with VV (Fig. 5A). ($p > 0.05$).

We next evaluated whether intranasal immunization would make side-effects to mice. Mice were inoculated by intranasal immunization with 5×10^6 pfu of VV or VV-$_{Egp}$ in 10 μL, followed by continuous monitoring for body weight of immunized mice. As shown in Fig. 5B, body weight of mice in the group with VV-$_{Egp}$ slightly decreased at around day 4 post inoculation and then recovered rapidly. However the body weight in the VV inoculated group decreased significantly compared with other groups.

4. Discussion

In the present study, we constructed and evaluated a genetically recombinant virus vaccine candidate derived from VV, which contained an entire Zaire EBOV *gp* (VV-$_{Egp}$). Intranasal administration of recombinant VV-$_{Egp}$ induced not only robust systemic humoral and cellular immune response but also high level of mucosal immune responses.

The epidemic of EBOV spread significantly in western Africa, threatening all over the world due to its high morbidity and morality. No approved vaccine and drug specifically against EBOV are available currently, despite vaccine candidates that elicited significantly protective immune responses for challenged animal models [5]. Intramuscular administration of recombinant VSV harboring EBOV *gp* induced remarkable systemic immune responses in NHPs, including high level neutralizing serum IgG and significant EBOV GP-specific IFN-γ secreting lymphocytes [7]. Intradermal inoculation of VV Ankara strain also elicited protective immune responses in NHPs. Of note, mucosal immunization induces both systemic and local mucosal immune responses [24]. In our present study, vaccination with attenuated replication-competent VV-$_{Egp}$ successfully induced systemic immune responses, e.g. high level of neutralizing IgG and serum IgA (Fig. 2), as well as remarkable GP-specific IFN-γ secreting lymphocytes (Fig. 3), which was agreement with our earlier study [18,19]. Therefore mucosal administration of replication-competent VV induced systemic immune responses comparable with that of intramuscular injection of other immunogens, such as adenovirus-based GP, recombinant VSV-GP, and the truncated GP protein [5,12].

Despite those ongoing vaccine candidates induced robust systemic GP-specific immune responses, mucosa-associated immune responses have not been intensively evaluated. Generally, the routine muscular immunization typically only stimulates systemic immune responses, whereas mucosal immunization could stimulate efficacious mucosal and systemic immune responses [25]. Takada et al. reported that EBOV could be found not only in the blood but also in mucosa such as saliva, semen, vaginal secretions, breast milk of infected patients [4], indicating that mucosa was likely associated with EBOV infection and transmission. Thus, it is possible that the robust mucosal immune responses are elicited to help to prevent viral invasion at the first step. Our earlier study showed that the self-limited recombinant VV containing HIV-1 *env* (VV-$_{HIVenv}$) replicated in upper respiratory trait after intranasal immunization and elicited substantial mucosal immunity, such as HIV-1 ENV-specific sIgA in nasal tract, lung and virginal tract [19]. In this study we also showed that intranasal vaccination of VV-$_{Egp}$ induced significant systemic immune responses (Fig. 2), and especially protective mucosal antibodies such as sIgA and IgG (Fig. 4). Deletion of antibody significantly decreased the antiviral capacity of mucosa samples, which further confirmed that the mucosal sIgA in upper airway tract played a key role in preventing virus infection. sIgA and IgG in

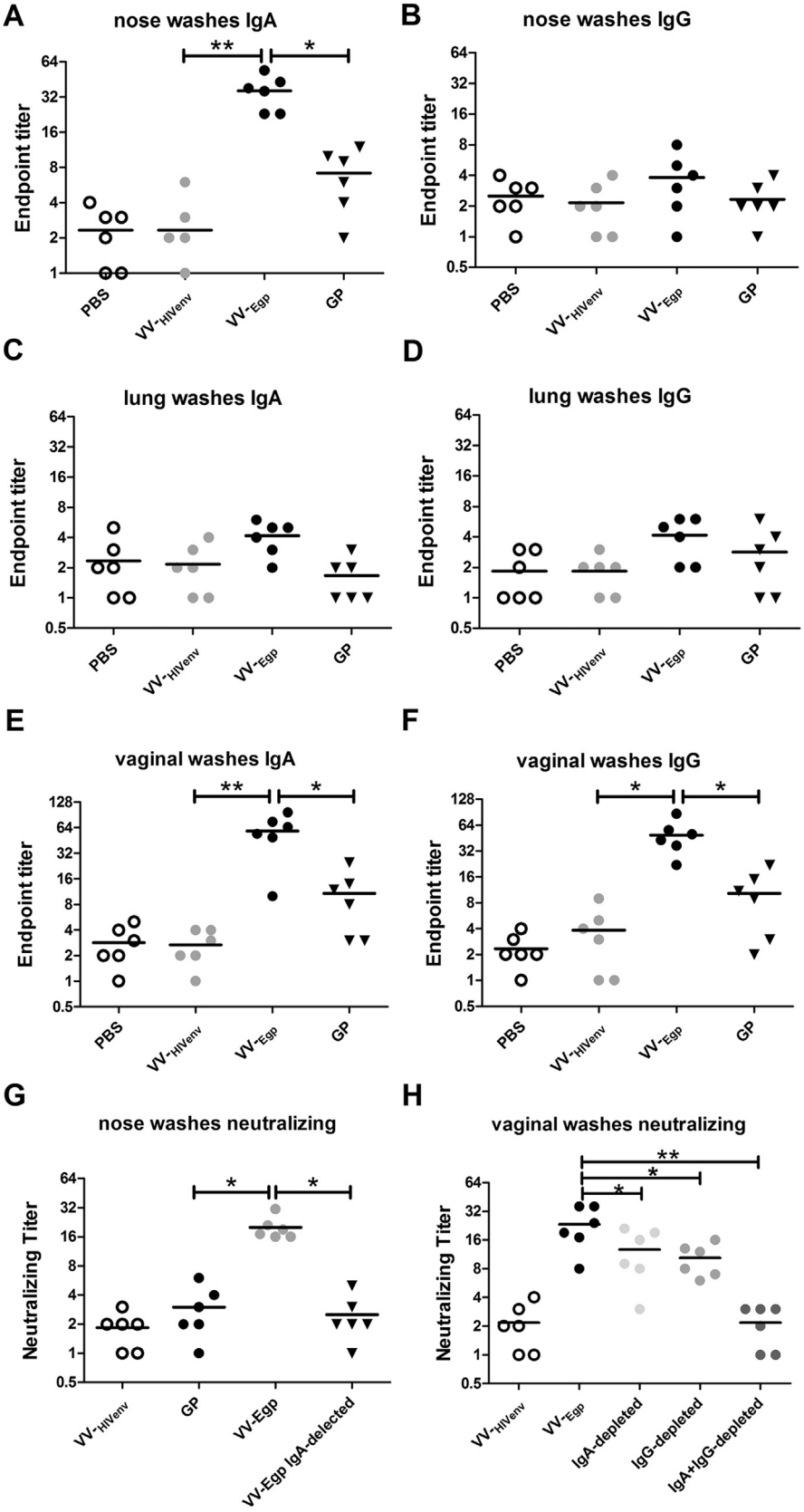

Fig. 4. Mucosal responses. At day 10 after the final immunization, mouse mucosa samples were collected, and the mucosal IgG and sIgA were determined by ELISA. (AB). sIgA and IgG were detected in nose wash. (CD). Neither sIgA nor IgG was detectable in lung extract. (EF). Both sIgA and IgG appeared in vaginal wash. Protein-G/A was used to delete the IgG. Anti-IgA-BIOT and Streptavidin Agarose were applied to depleted sIgA. (G). Without IgG, sIgA depletion completely abolished the antiviral capacity of nasal wash. (H). When single deletion of either sIgA or IgG in vaginal wash occurred, the antiviral capacity were not completely abolished, whereas the antiviral function disappeared when double deletion of both sIgA and IgG in vaginal wash.

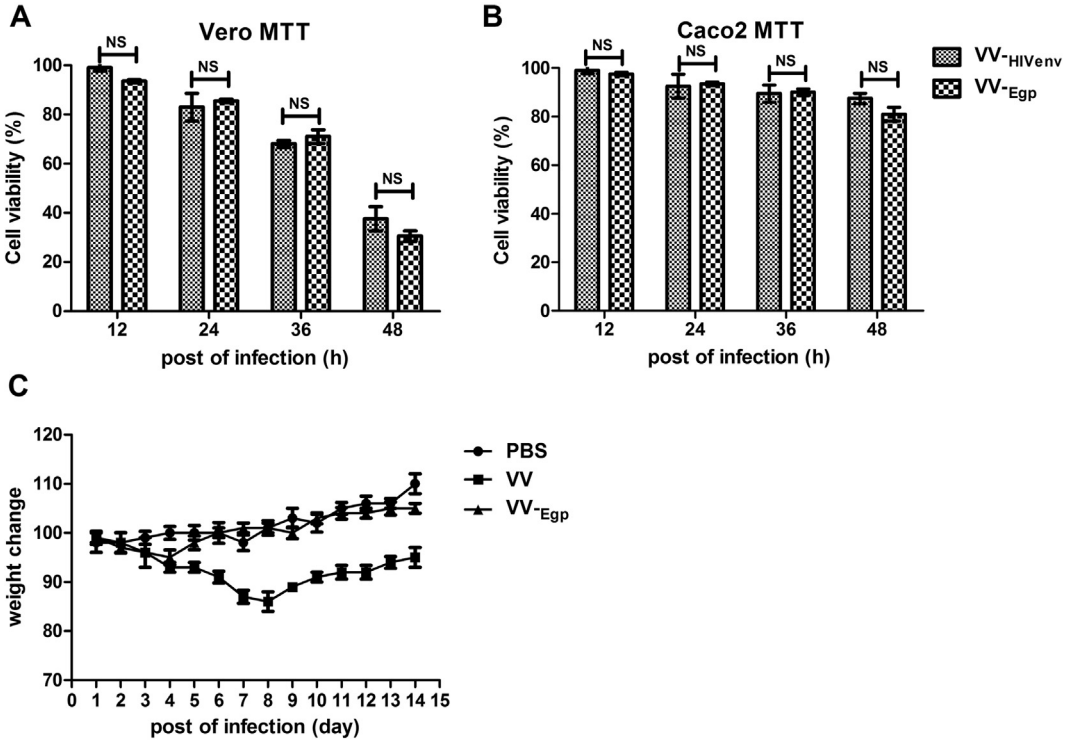

Fig. 5. Side-effects. (A). The virus-induced cytotoxicity on Vero cells and Caco2 cells were determined by MTT assay. Cell viability of Vero and Caco2 infected with the indicated viruses at the MOI of 0.05 were measured every 12 h over a 48 h period. (B). The side-effect on mice was determined by measuring the weight of mice after inoculation. Six-week-old BALB/c mice were intranasally inoculated with 5×10^6 pfu of the indicated viruses, and weight changes were monitored daily for 14 days after infection.

reproductive tract were also indispensable for clearing virus [26,27]. Therefore, recombinant VV-based attenuated virus provided us a versatile platform for the development of novel vaccine inducing mucosal immune responses. Despite the distinct immunogens used, protective mucosal immune responses were all successfully elicited [12,25], which was consistent with our current results. Intranasal vaccine candidates such as Adenovirus-based EBOV GP and truncated prokaryotic GP (258-601) induced local protective immune responses against intraperitoneal viral challenge of EBOV [12,25]. It was worth noting that the intranasal challenge model was more suitable for evaluating the efficacy of vaccine immunization compared with the currently applied intraperitoneal challenge approach.

Due to biosafety concerns, manipulation of infectious EBOV is not allowed even in the P3 laboratory. Therefore, the EBOV luciferase report pseudovirus system instead of infectious EBOV was used to evaluate the efficacy of vaccine candidates. We generated the EBOV luciferase report pseudovirus by cotransfecting pNL4-3.Luc. R^-E^- and pEAK13-GP to 293 T. The 2G4, a well known EBOV-neutralizing mAb [22], was found to significantly inhibit pseudovirus attachment, indicating that the pseudovirus report system in this study was reliable.

Needle-free-mediated mucosal immunization is safer than intramuscular vaccination when non-specialized personnel are performing the vaccination. More importantly, mucosal immunization of VV-$_{Egp}$ can stimulate protective mucosal and systemic immune responses. Therefore we here provided a novel promising vaccine candidate VV-$_{Egp}$ that could prevent not only viral systemic infection but also possible mucosal virus acquirement.

Acknowledgements

We sincerely thank Dr. Cao at the Huazhong Agricultural University for editorial assistance. This work was supported by grants from Hubei Provincial Natural Science Foundation of China 2018CFB449, High-level Scientific Research Foundation for the introduction of talent of Wuhan University of Bioengineering (2017KQ01), and National Natural Science Foundation of China (81202381).

Conflict of interest

The authors declared that they had no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.04.070.

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