Biosafety and Health 5 (2023) 308–319

Contents lists available at ScienceDirect

Biosafety and Health

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

Original Research

Incorporation of *Escherichia coli* heat-labile enterotoxin B subunit into rabies virus particles enhances its immunogenicity in mice and dogs

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ARTICLE INFO

Article history:
Received 13 February 2023
Revised 4 May 2023
Accepted 5 May 2023
Available online 09 May 2023

Keywords:
RABV
Inactivated vaccine
Incorporation
LTB
Dendritic cells
Activation

ABSTRACT

Although inactivated vaccines against rabies have the advantage of high safety, effective protection against rabies virus (RABV) infection often requires multiple, high-dose immunization. Incorporating a molecular adjuvant into the viral particles has been found to be a useful strategy to promote the immune effectiveness of inactivated vaccines. In this study, we constructed a recombinant virus, rCVS11-LTB, which chimerically expresses a molecular adjuvant heat-labile enterotoxin B subunit (LTB) protein on the surface of the RABV particles. Immunogenicity *in vivo* was found to be promoted by rCVS11-LTB through the activation of dendritic cells (DCs). Our results demonstrated that inactivated rCVS11-LTB was able to induce higher levels of virus-neutralizing antibodies (VNAs) in both mice and dogs than the parent virus rCVS11, to enhance the cellular immune response and T cell immune memory in mice, and was also able to provide 100% protection in mice from lethal doses of rabies virus, indicating its potential as a safe and effective inactivated rabies vaccine candidate.

1. Introduction

Rabies is a zoonotic disease with high fatality and is still prevalent in many developing countries. It is caused by the rabies virus (RABV), which belongs to the family *Rhabdoviridae*, genus *Lyssavirus* [1,2]. Almost all warm-blooded animals are susceptible to this pathogen, and 99% of cases of human rabies are transmitted from RABV-infected dogs in developing countries [3]. Although cases of rabies in humans and dogs are extremely rare in developed countries, new threats from RABV-infected wild animals occur continuously [4,5]. Due to the unique neurotropism and immune privileges in the central nervous system (CNS) of RABV, there are no effective drugs to treat rabies, and vaccination is the most effective way to prevent and control this disease worldwide. The World Health Organization (WHO) has reported that more than 75% immunization coverage is necessary for effectively controlling the rabies epidemic in humans [3,6,7]. Therefore, there is an urgent need to develop efficient and inexpensive rabies vaccines for animals to help achieve the goal of eliminating dog–human rabies transmissions by 2030 [8].

Many kinds of rabies vaccines have been developed. These vaccines include a G protein subunit vaccine, a polypeptide vaccine, a gene-deleted recombinant virus vaccine, an attenuated vaccine, an inactivated vaccine and others. Of these, the inactivated vaccine is the most widely used.

Since vaccination plays an irreplaceable role in rabies prevention, many attempts have been made to improve the immunogenicity of developed rabies vaccines. Initially, attempts were made to construct replication-deficient RABV vaccines which can express high levels of glycoprotein (G), which is the major antigenic determinant for RABV [9,10], resulting in the elicitation of stronger innate and acquired immune responses while remaining nonpathogenic in hosts. Many studies have demonstrated that cytokines can be used as molecular adjuvants to improve the vaccine immunogenicity [11,12], and recombinant rabies viruses expressing cytokines such as GM-CSF [13] or

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[1] Given his role as an Editorial Board Member, Hualei Wang had no involvement in the peer-review of this article and had no access to the information regarding its peer review. Full responsibility for the editorial process for this article was delegated to the Editor William J. Liu.

https://doi.org/10.1016/j.bsheal.2023.05.005
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HIGHLIGHTS

Scientific question

To reduce and eliminate the rabies epidemic, it is essential to improve the effectiveness of the rabies vaccine.

Evidence before this study

Many studies have demonstrated that adjuvants, particularly molecular adjuvants targeting the immune cells, play an important role in improving the immune efficacy of inactivated vaccines. *Escherichia coli* heat-labile enterotoxin B subunit (LTB) shows advantages in promoting the immune efficacy of vaccines by activating the dendritic cells.

New findings

A recombinant virus rCVS11-LTB was constructed in this study, which could display the LTB protein on the surface of viral particles. Inactivated rCVS11-LTB was able to induce the higher levels of virus-neutralizing antibodies (VNAs) in both mice and dogs, and to induce faster and stronger cellular immune responses and the production of $CD4^+$ CTM in mice than rCVS11 post immunization. In addition, two doses of inactivated rCVS11-LTB could completely protect the mice from the challenge with a lethal dose of rabies virus.

Significance of the study

The recombinant virus rCVS11-LTB chimeric-expressing the molecular adjuvant LTB can induce high levels of humoral and cellular immune responses *in vivo* and provide 100% protection against RABV challenge in mice, demonstrating its potential as an effective inactivated rabies vaccine candidate.

HMGB1 [14] have been constructed. Immunizing animals with these recombinant viruses have been found to induce high levels of humoral immunity and the activation of dendritic cells (DCs) and B cells in the early stages of immunity. However, cytokines can only be expressed during the replication of the recombinant viruses, restricting their use in rabies vaccines. Further studies incorporated molecular adjuvants into RABV particles, and these recombinant viruses showed better immunogenicity than the parent virus when used as inactivated vaccines [15,16].

Escherichia coli heat-labile enterotoxin B subunit (LTB) is known as a typical mucosal adjuvant and has been used for developing many kinds of vaccines for large number of diseases [17–19]. Recently, it has been demonstrated that LTB can promote the activation of DCs and the uptake of antigens by binding to the GM1 ganglioside [20,21]. Given that the activation of DCs plays an important role in improving the immune effect of rabies vaccines, we constructed a recombinant RABV, rCVS11-LTB, which chimerically expresses LTB on the surface of the viral particles to promote its immunogenicity when used as an inactivated vaccine. Animal studies demonstrated that inactivated rCVS11-LTB induced stronger humoral and cellular immune responses in mice and dogs than the parent virus CVS11.

2. Materials and methods

2.1. Antibodies, plasmids, and cells

The mouse anti-RABV G mAb (MAB8727) and FITC-anti-rabbit antibody were purchased from Millipore (Billerica, USA). A fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) against the RABV N protein (800–092) was purchased from Fujirebio (Melvin, USA). Rabbit anti-LTB polyclonal antibody was prepared by our laboratory. The TRITC-conjugated goat anti-mouse IgG (T5393) was purchased from Sigma (St. Louis, USA). Flow cytometry antibodies, including PE-Cy7 labeled CD11c antibody and APC labeled CD80 antibody, were purchased from BD Pharmingen Inc. The CVS11 full-length plasmid pCDNA3.0-CVS11 and the helper plasmids pD-N, pD-P, pD-L, and pD-G [22] required to rescue the virus were constructed and stored in our laboratory. Mouse neuroblastoma N2a (NA) cells, hamster kidney (BHK21) cells, and BSR cells (derived from BHK-21 cells expressing T7 RNA polymerase) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA).

2.2. Construction of the recombinant virus rCVS11-LTB

The full length of the LTB gene was amplified with PCR, and the signal peptide and the transmembrane and cytoplasmic domains (TMCD) of the G protein of RABV CVS11 strain were ligated to the 3′ and 5′ ends of the product, respectively. The fragment was inserted into the genome of CVS11 between the G and L genes to construct the full-length plasmid pCDNA3.0-CVS11-LTB. Full-length plasmids pCDNA3.0-CVS11-LTB (2.5 μg) and pCDNA3.0-CVS11 (2.5 μg) were co-transfected into BSR cells with helper plasmids pD-N (0.625 μg), pD-P (0.3125 μg), pD-L (0.125 μg), and pD-G (0.1875 μg) [22,23], respectively, to rescue the rCVS11-LTB and rCVS11 recombinant viruses. After 72 h, the supernatants were collected for direct immunofluorescence assay (DFA).

2.3. Growth curve

NA cells and BSR cells were infected with the recombinant virus rCVS11-LTB or rCVS11 at MOI = 1 or 0.1, respectively. Virus titer was determined every 24 h and the growth curve was drawn.

2.4. Confocal microscopy

NA cells were seeded into a 24-well plate (2.5×10^4/mL) and infected with either rCVS11-LTB or rCVS11 at an MOI of 0.4. After being cultured for 48 h, the samples were fixed with 4% paraformaldehyde at 4 ˚C and blocked with 1% BSA at room temperature (RT) for 30 min. Rabbit anti-LTB polyclonal antibody (1:200) and mouse anti-RABV G mAb (1:500) were used as primary antibodies. FITC-labeled anti-rabbit antibody (1:300) and TRITC-labeled anti-mouse antibody (1:500) were used as secondary antibodies. After incubation with the secondary antibodies for 1 h, the samples were stained with DAPI and observed using laser confocal microscopy.

2.5. Inactivation and purification of recombinant viruses

The viruses were inactivated by mixing with β-propiolactone (1:2,000) and incubated at 4 ˚C for 24 h. The inactivated viruses were centrifuged at 4 ˚C, 3,000 rpm for 30 min. The supernatants were mixed with 1 M zinc acetate (2%, v/v) and incubated at 4 ˚C for 30 min. The samples were centrifuged at 12,000 rpm for 30 min and the precipitate was then resuspended in an appropriate amount of saturated EDTA. The mixture then was then purified by sucrose gradient centrifugation (20%, 30%, 40%, 55% sucrose) and the samples retained in 30–40% sucrose were collected and resuspended in STE buffer (pH = 7.4, 0.15 M NaCl, 0.001 M EDTA and 0.01 M Tris). The purified viruses were quantified by the BCA method.

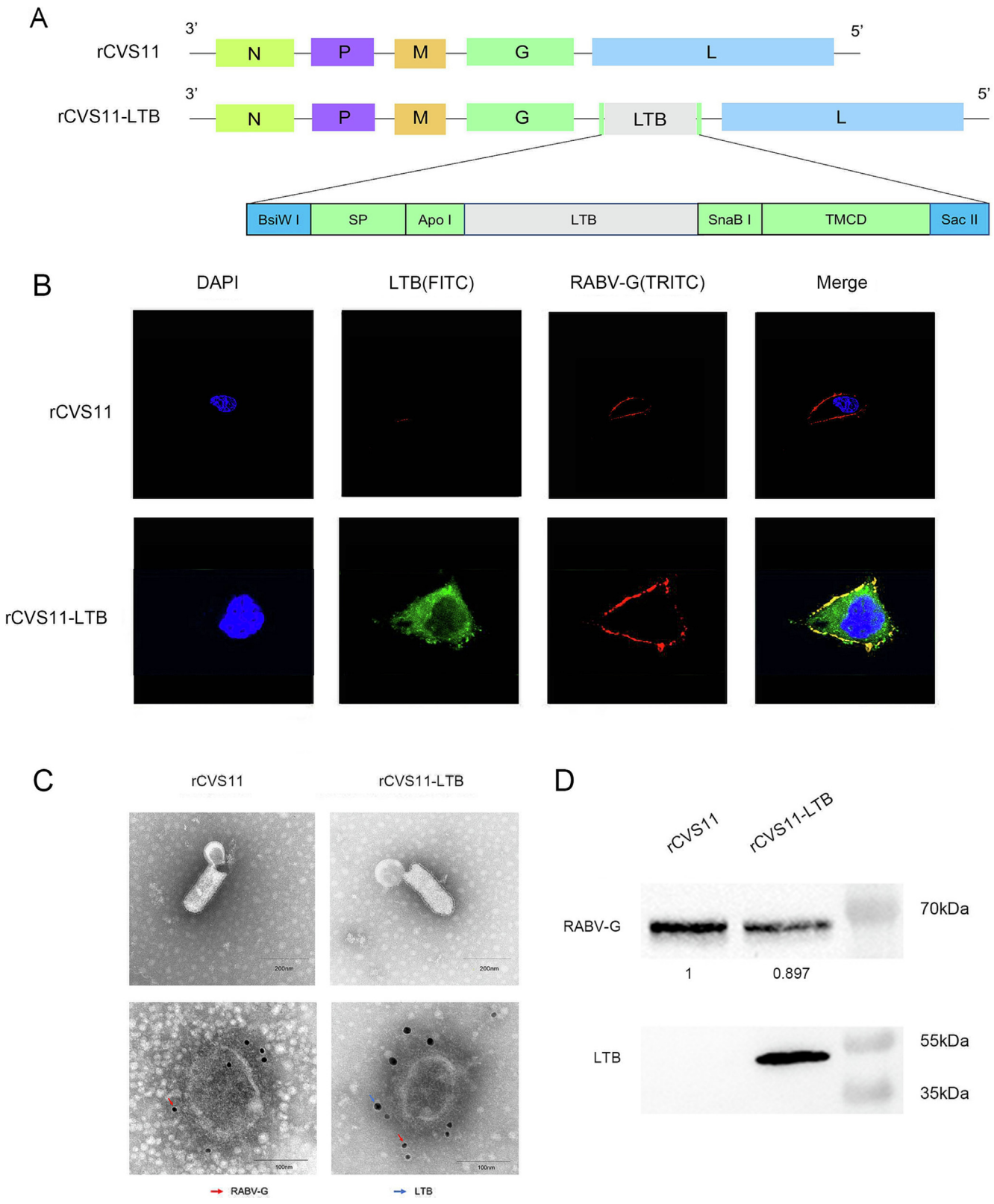


Fig. 1. Construction and characterization of rCVS11-LTB. A) Construction diagram of the recombinant plasmid pCDNA3.0-CVS11-LTB. B) NA cells were infected with either the recombinant virus rCVS11-LTB or rCVS11 at 0.1 MOI. After 48 h, the cells were incubated with rabbit polyclonal LTB antibody (1:500) and anti-RABV G antibody as the primary antibodies, with FITC-labeled goat anti-rabbit antibody as the secondary antibody (1:500). C) The purified recombinant viruses rCVS11-LTB and rCVS11 were analyzed using electron microscopy (EM) at a magnification of 40,000×. D) The purified virus was analyzed with WB. Mouse anti-RABV G monoclonal antibody (1:500) and rabbit polyclonal LTB antibody (1:200) were used as primary antibodies, and enzyme-labeled goat anti-mouse IgG (1:1,000) and enzyme-labeled goat anti-rabbit IgG (1:2,000) were used as secondary antibodies. Abbreviations: LTB, heat-labile enterotoxin B subunit; RABV, rabies virus; FITC, fluorescein isothiocyanate; WB, Western blotting.

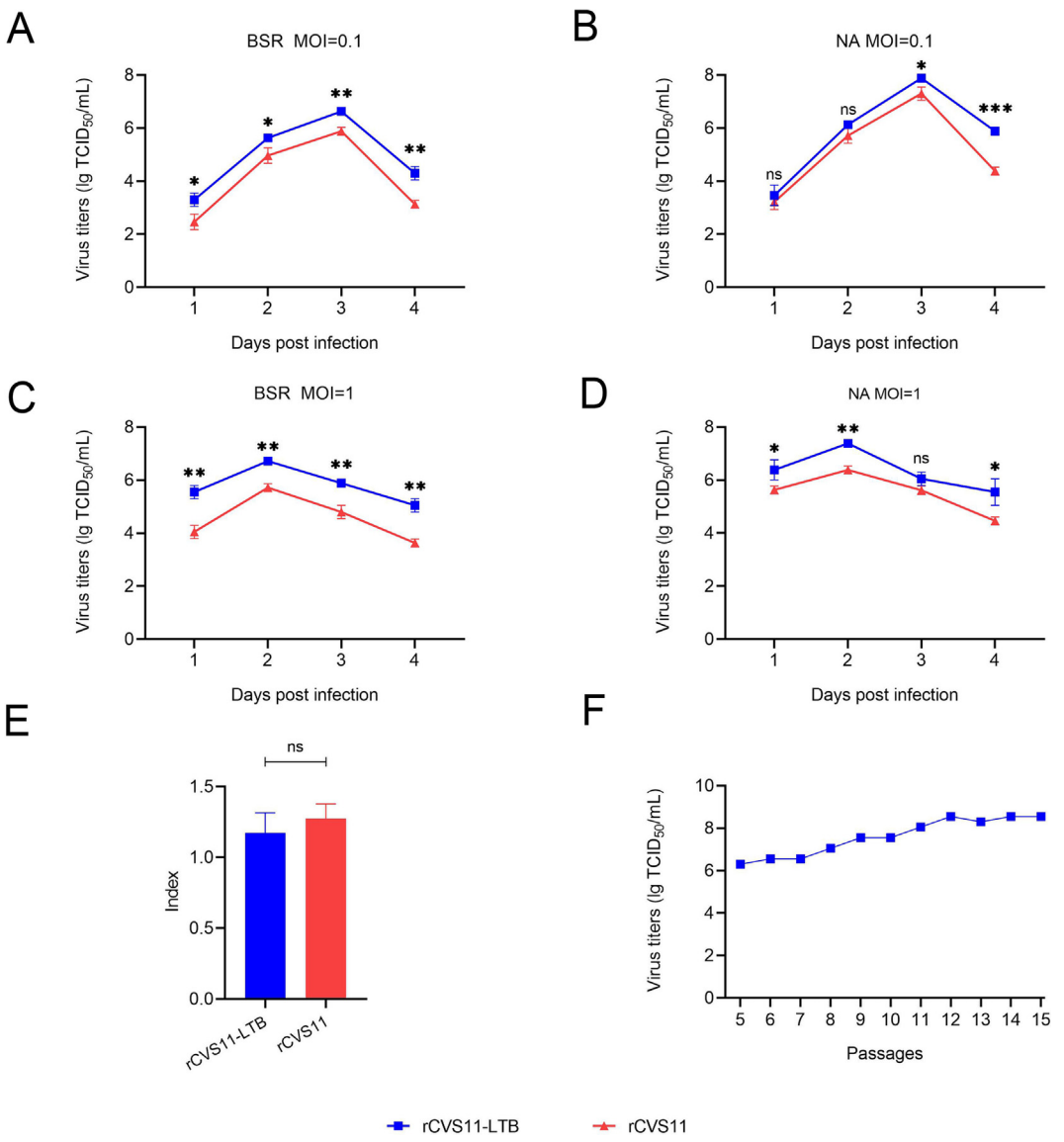


Fig. 2. Growth characteristics of rCVS11-LTB. A and B) Multi-step growth curves of the recombinant viruses. The recombinant rCVS11-LTB and rCVS11 viruses were separately inoculated into either NA or BSR cells at MOI = 0.1. The cell cultures were collected on the 1st, 2nd, 3rd, and 4th days post-infection, and the viral titer were determined using the fluorescent antibody virus neutralization test (FAVN) method. C and D) One-step growth curves of the recombinant viruses. The recombinant rCVS11-LTB and rCVS11 viruses were separately inoculated into either NA or BSR cells at MOI = 1. The cell cultures were collected on the 1st, 2nd, 3rd, and 4th days post-infection, and the viral titers were determined by the FAVN method. E) Neurotropism analysis of the recombinant virus rCVS11-LTB. The viral titers of the recombinant viruses rCVS11-LTB or rCVS11 in NA cells were compared with those in BSR cells. All of the experiments in A-E were repeated independently three times and the data are presented as the mean ± SD for each group. F) The recombinant virus was serially passaged, and the viral titer of each passage after the fifth generation was determined by the FAVN method. Abbreviations: LTB, heat-labile enterotoxin B subunit; NA, neuronal.

2.6. Immunoelectromicroscopy

A total of 20 μL of recombinant virus CVS11-LTB or rCVS11 was incubated on copper mesh at RT for 20 min, and were fixed with 4 % paraformaldehyde. The samples were then blocked with 1 % skim milk in PBS at RT for 1 h. Mouse anti-RABV G antibody and rabbit anti-LTB polyclonal antibody were used as primary antibodies. Anti-mouse gold-labeled antibody (10 nm) and anti-rabbit gold-labeled antibody (18 nm) were used as secondary antibodies.

2.7. Western blotting (WB)

A total of 50 μg of rCVS11-LTB and rCVS11 recombinant viruses were analyzed by SDS-PAGE. The gels were then transferred onto nitrocellulose (NC) membranes and blocked with 5% skim milk in PBST at RT for 2 h. Mouse anti-RABV G mAb (1:5,000) or rabbit anti-LTB polyclonal antibody (1:200) was used as primary antibodies and incubated at 4 ˚C overnight. HRP-conjugated goat anti-mouse IgG antibody (1:10,000) or HRP-conjugated goat anti-rabbit IgG antibody (1:20,000) was used as secondary antibodies and incubated at RT for 1 h. Electrochemiluminescence (ECL) Western Blotting Substrate (Pierce, USA) was then added, and the membranes were analyzed using a Tanon-5200 Chemiluminescent imaging system (Tanon, China).

2.8. Immunization in mice and dogs

The immunogen was prepared by mixing inactivated recombinant virus rCVS11-LTB, rCVS11 or Gel02 adjuvant in a 5:1 ratio. 6 ~ 8 weeks old BALB/c mice were randomly divided into 3 groups

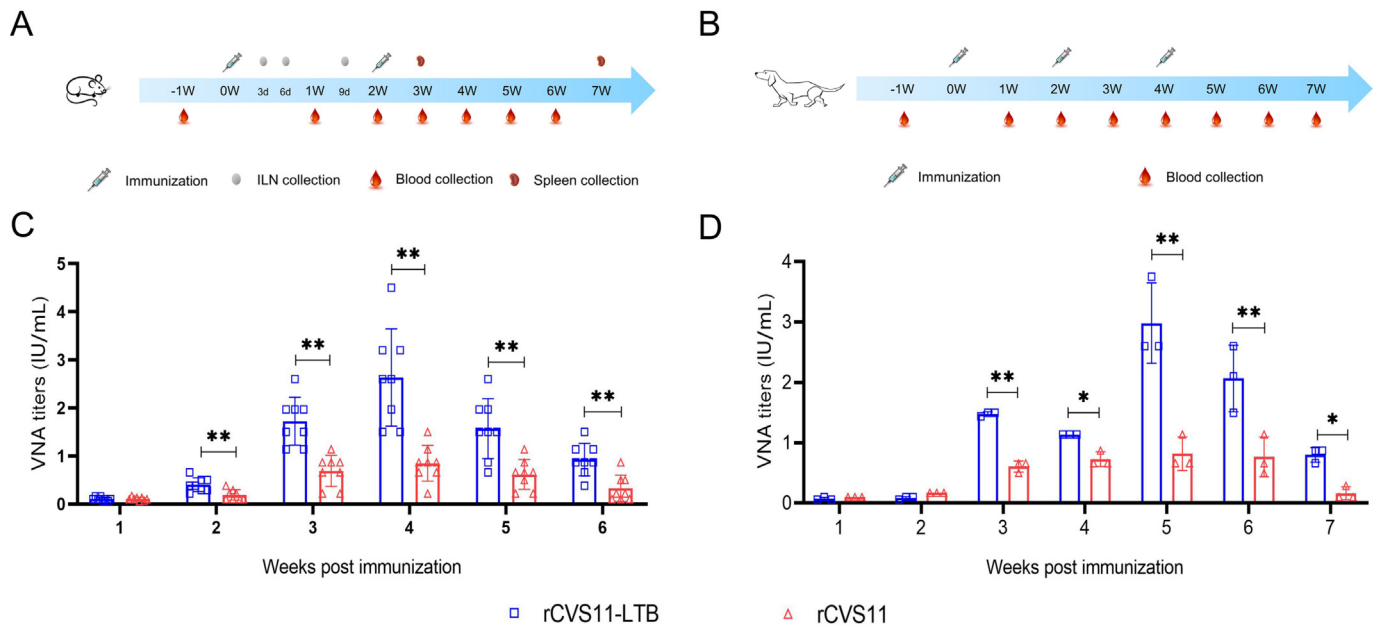


Fig. 3. rCVS11-LTB induced higher VNA in mice and dogs. A) Immunization scheme in mice. 6- to 8-week-old BALB/c mice were randomly divided into three groups (n = 23/group). The immunogen was prepared by mixing inactivated recombinant virus with Gel02 adjuvant (5:1). Mice were immunized intramuscularly with 100 μL of immunogen, with a total of two doses, two weeks apart. The mouse image was from OPENCLIPART. B) Immunization scheme in dogs. Beagle dogs (17- to 21-month-old) were randomly divided into two groups (n = 3/group). The dogs were immunized intramuscularly with 1 mL of immunogen every two weeks for a total of three immunizations. The image of dog was obtained from OPENCLIPART. C) RABV-specific VNAs in mouse sera at weeks 1, 2, 3, 4, 5, and 6 post-immunization were measured using a FAVN test. D) RABV-specific VNAs in dog sera at weeks 1, 2, 3, 4, 5, 6, and 7 post-immunizations were measured using a FAVN test. Data are presented as the mean ± SD for each group. *, $P < 0.05$; **, $P < 0.01$. Abbreviation: LTB, heat-labile enterotoxin B subunit.

(n = 23/group). Mice were immunized intramuscularly with 100 μL immunogen (10^7TCID$_{50}$), and booster immunization was performed 2 weeks after the first immunization. Mouse blood was collected at the 1st, 2nd, 3rd, 4th, 5th, and 6th week after first immunization. The mouse inguinal lymph nodes (ILNs) (n = 3/group/day) were collected at 3rd, 6th, and 9th day post-immunization (dpi). Mouse spleens (n = 3/group/day) were collected at 3rd and 7th weeks after the first immunization. The 17- to 21- month-old beagle dogs were randomly divided into two groups (n = 3/group), and immunized intramuscularly with 1 mL of immunogen (10^8TCID$_{50}$) for three times every two weeks. Blood samples were collected from the dogs at the 1st, 2nd, 3rd, 4th, 5th, 6th and 7th week after the first immunization.

2.9. Flow cytometry

The collected mouse ILNs and spleens were processed to prepare single-cell suspensions [23]. The single-cell suspensions of ILNs were seeded into a 96-well plate with 5×10^5 cells/well and were detected by antibodies (CD11c, CD80, MHC-II and MHC-I are the markers for DC cells, CD4, CD8, CD69 are the markers for T cells, CD40, CD19 are the markers for B cells). The single-cell suspensions of spleen were subjected to the detection of TCM cells (CD4, CD8, CD44, CD62L), T cells (CD4, CD8, CD69), B cells (CD40, CD69, CD19). Finally, the assay was performed using a FACS calibur flow cytometer (BD Biosciences, USA).

2.10. Elispot

A total of 5×10^5 splenocyte cells, prepared as described above, were seeded onto the ELISpot plates and were stimulated with the purified rCVS11 (10 μg/well). After incubation at 37 °C for 24 h, the plates were washed five times with PBST, and then incubated with biotin-labeled interferon (IFN)-γ or interleukin (IL)-4 antibodies (1:1,000) (provided with the ELISpot kits, 3321-4HPW-2) for 2 h at RT. The plates were subsequently washed again and incubated with streptavidin-conjugated HRP (1:1,000) for 1 h at RT. The TMB substrate was then added, and the spot-forming cells (SFCs) in the plates were counted using an ELISpot reader.

2.11. Challenge experiment in mice

The 6 to 8-week-old BALB/c mice were challenged with 10^5 TCID$_{50}$ of RABV standard attack strain CVS11 by i.m. injection 4 weeks after the first immunization, and were observed daily for 21 days. During the observation period, any mouse that developed clinical signs of rabies was humanely euthanized by cervical dislocation under isoflurane anesthesia.

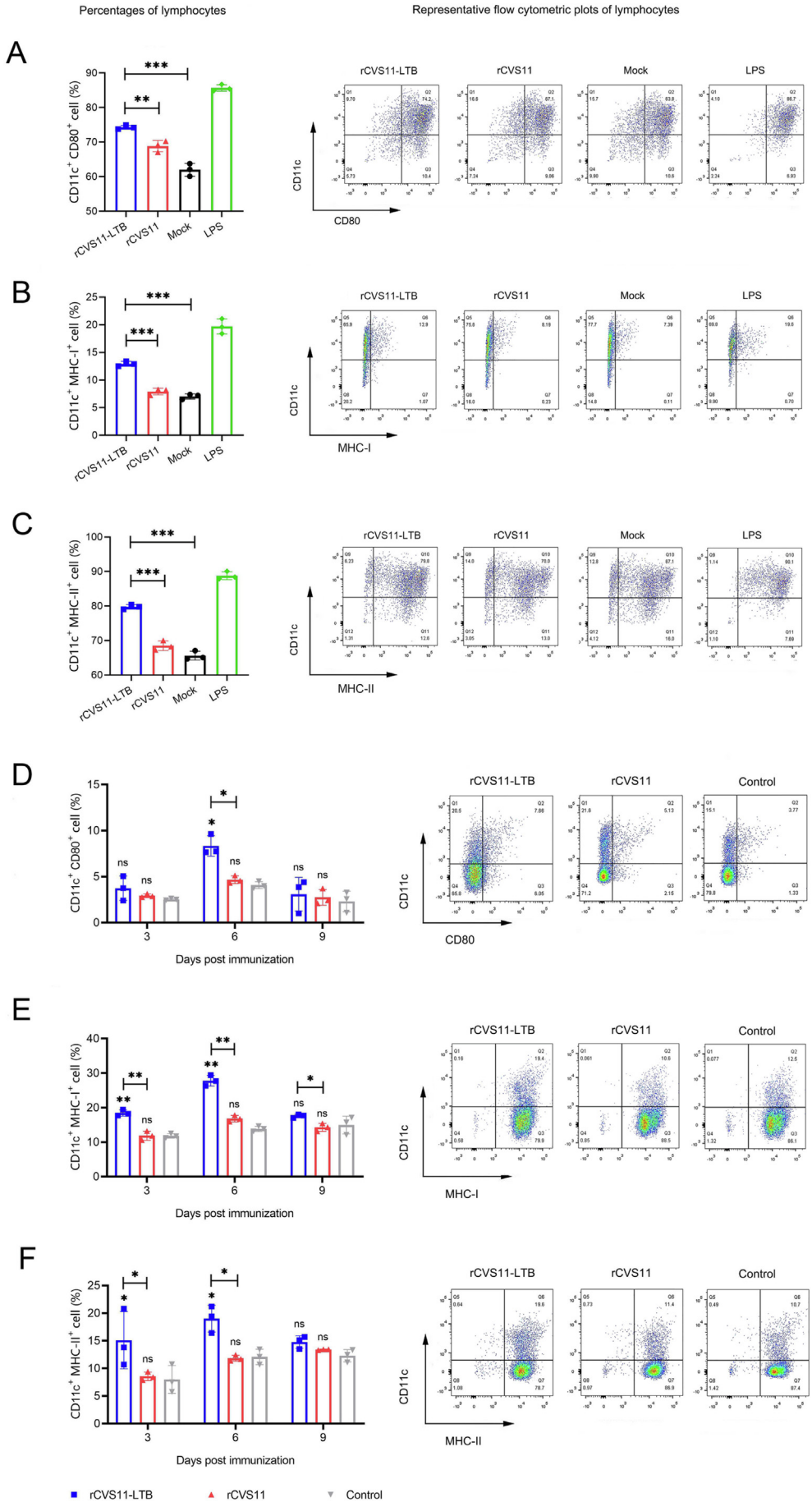
2.12. Statistical analysis

Statistical analysis of the data was performed using a one-way ANOVA in the GraphPad Prism software. All the experiments were performed independently at least three times. Error bars represent the standard deviation (SD) in each group, as indicated in the figure legends (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3. Results

3.1. Construction and characterization of rCVS11-LTB

The 3′ and 5′ ends of the target fragment LTB were added with the signal peptide and the TMCD structural domain of the RABV CVS11 strain. The target fragment was inserted between G-L of the full-length sequence of CVS11 to construct the full-length plasmid pCDNA3.0-CVS11-LTB (Fig. 1A). The full-length plasmid and helper plasmid were co-transfected into BSR cells to rescue the recombinant virus. To verify the expression of LTB protein by rCVS11-LTB, NA cells were infected with the recombinant viruses rCVS11-LTB or rCVS11.


Percentages of lymphocytes
Representative flow cytometric plots of lymphocytes
A
B
C
D
E
F
rCVS11-LTB
rCVS11
Mock
LPS
Control
CD11c+ CD80+ cell (%)
CD11c+ MHC-I+ cell (%)
CD11c+ MHC-II+ cell (%)
CD11c
CD80
MHC-I
MHC-II
Days post immunization
ns

The confocal results showed that in rCVS11-LTB-infected NA cells but not in rCVS11-infected cells, LTB was expressed both in the cytoplasm and on the cell membrane, and co-localized with the RABV G protein (Fig. 1B). The recombinant viruses were purified and analyzed by electron microscopy and WB. Electron microscopy results showed that the recombinant virus rCVS11-LTB had the same size and structure as the parental virus rCVS11 (Fig. 1C). WB results showed that the expression of RABV-G protein in purified recombinant virus rCVS11-LTB was similar to that of rCVS11, indicating that the insertion of LTB did not affect the expression of RABV-G. In addition, LTB could be detected with rabbit anti-LTB polyclonal antibody, which resulted in a 60 kDa band (a pentamer of LTB) in purified recombinant virus rCVS11-LTB, but this band was not detected in purified rCVS11 (Fig. 1D). These results all indicated that the LTB protein was successfully chimerically expressed on the surface of the viral particles by the recombinant rCVS11-LTB virus.

3.2. Growth characteristics of rCVS11-LTB

In order to investigate whether the insertion of the LTB gene into the RABV genome affected the replication of the recombinant rCVS11-LTB virus, we measured the growth curves of recombinant viruses in NA (neuronal) cells and BSR (somatic) cells. The results showed that rCVS11-LTB had similar multi-step (Fig. 2A and B) and one-cycle (Fig. 2C and D) growth curves to rCVS11, with higher viral titers. Neutrophilic analysis showed that rCVS11-LTB had similar neutrophilic properties to rCVS11 (Fig. 2E). The titer of recombinant virus rCVS11-LTB was stable in continuous passages, with the highest titer being $10^{8.55}TCID_{50}$/mL (Fig. 2F). Meanwhile, the LTB protein was stably expressed in different passages of the recombinant virus rCVS11-LTB (Figure S1). The pathogenicity of the recombinant virus was evaluated, and the results showed that the rCVS11-LTB group had milder clinical symptoms (Figure S2A), lower weight changes (Figure S2B) and higher survival rates (Figure S2C) than the rCVS11 group.

3.3. rCVS11-LTB induced higher VNA than rCSV11 in mice and dogs

The inactivated recombinant viruses rCVS11-LTB and rCVS11 were mixed with the Gel02 adjuvant to prepare the immunogen. Mouse blood, ILNs and spleen samples (Fig. 3A) and dog blood samples (Fig. 3B) were collected at the indicated time points for further analysis. The results showed that the inactivated recombinant virus rCVS11-LTB could induce higher level of virus-neutralizing antibodies (VNAs) in both mice and dogs than the parent virus rCVS11 (Fig. 3C and D).

3.4. Activation of DCs by rCVS11-LTB in vitro and vivo.

We next evaluated the recruitment and/or activation of the inactivated recombinant virus rCVS11-LTB on DC cells *in vitro* and *in vivo*. The mouse BMDCs [24,25] were prepared and incubated with inactivated rCVS11-LTB or rCVS11. LPS was used as a positive control and RPMI 1640 (mock) as a negative control. The results showed that the number of $CD11c^+CD80^+$ (Fig. 4A), $CD11c^+MHC\text{-}I^+$ (Fig. 4B), and $CD11c^+MHC\text{-}II^+$ (Fig. 4C) double positive cells in the rCVS11-LTB group was significantly higher than that in the rCVS11 or mock groups. Meanwhile, the results of the MFI analysis showed that the MFI of CD80 (Figure S3A), MHC-I (Figure S3B), MHC-II (Figure S3C) in the rCVS11-LTB group were higher than those in the rCVS11 or mock groups, indicating that rCVS11-LTB could activate BMDC in vitro.

The activation of DCs by inactivated rCVS11-LTB was evaluated in vivo. ILNs were collected at 3rd, 6th and 9th dpi and the numbers of $CD11c^+$ and $CD80^+$, $MHC\text{-}I^+$, $MHC\text{-}II^+$ double positive cells were examined by flow cytometry. The results showed that the numbers of $CD11c^+$ $CD80^+$(Fig. 4D), $CD11c^+$ $MHC\text{-}I^+$(Fig. 4E), and $CD11c^+$ $MHC\text{-}II^+$(Fig. 4F) double positive cells increased significantly after rCVS11-LTB immunization, while there was no increase in the rCVS11 group, suggesting that the inactivated recombinant virus rCVS11-LTB can activate more DCs than can rCVS11 *in vivo*.

3.5. Recruitment and/or expansion of T and B cells by inactivated rCVS11-LTB

The activation of DCs enhances their ability to present antigens and promotes the recruitment and/or expansion of B cells and T cells. ILNs were collected at 3rd, 6th and 9th dpi and the numbers of $CD4^+$, $CD8^+$, $CD40^+$, $CD19^+$, $CD69^+$ cells were examined using flow cytometry. Compared with the rCVS11 group, the rCVS11-LTB group significantly promoted the recruitment of $CD4^+$ T cells at the 3rd and 6th dpi (Fig. 5A), and significantly promoted the recruitment of B cells at the 3rd dpi (Fig. 5B). These results indicate that the inactivated recombinant virus rCVS11-LTB is capable of enhancing the recruitment and/or expansion of T cells and B cells in mice.

3.6. Production of T helper 1 (Th1) and T helper 2 (Th2) cytokines by rCVS11-LTB

To evaluate the effect of rCVS11-LTB on the Th1 and Th2 immune responses, the secretion of Th1 and Th2 cytokines by the spleens of mice immunized with recombinant viruses was analyzed at the 3rd and 7th week post-immunization. As expected, compared with the rCVS11 group, more IFN-γ (Fig. 6A) and IL-4 (Fig. 6B) SFCs were detected in the spleens of mice inoculated with rCVS11-LTB, indicating that the recombinant virus rCVS11-LTB could simultaneously enhance the production of Th1 and Th2 cytokines after immunization.

3.7. rCVS11-LTB induced good immune memory after immunization

To assess the immunologic memory induced by the recombinant viruses in mice, splenic lymphocytes were isolated from the immunized mice at the 3rd and 7th weeks after the first immunization. The splenic lymphocytes were stimulated with the purified rCVS11-LTB and analyzed using flow cytometry. The gate control strategy is shown in Figure S4. The results showed that the rCVS11-LTB group recruited more $CD4^+$ TCM ($CD4^+$, $CD44^+$, and $CD62L^+$) at the 3rd and 7th weeks after the first immunization compared to the control

Fig. 4. Activation of DCs by rCVS11-LTB *in vitro* and *vivo*. Mouth BMDCs were isolated and cultured, and stimulated with inactivated recombinant virus rCVS11-LTB, rCVS11, LPS, and RPMI 1640, respectively. The single cell suspensions were prepared and stained with antibodies against DC activation markers for flow cytometric analysis. A) The representative flow cytometric plots and percentages of $CD11c^+$ and $CD80^+$ activated DCs in BMDC. B) The representative flow cytometric plots and percentages of $CD11c^+$ and $MHC\text{-}I^+$ activated DCs in BMDC. C) The representative flow cytometric plots and percentages of $CD11c^+$ and $MHC\text{-}II^+$ activated DCs in BMDC. D) The representative flow cytometric plots (6th dpi) and percentages of $CD11c^+$ and $CD80^+$ recruited DCs in the ILNs of immunized mice. E) The representative flow cytometric plots (3rd dpi) and percentages of $CD11c^+$ and $MHC\text{-}I^+$ recruited DCs in the ILNs of immunized mice. F) The representative flow cytometric plots (6th dpi) and percentages of $CD11c^+$ and $MHC\text{-}II^+$ recruited DCs in the ILNs of immunized mice. Data are presented as the mean ± SD for each group. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Abbreviations: BMDC, bone marrow-derived dendritic cells; LTB, heat-labile enterotoxin B subunit.

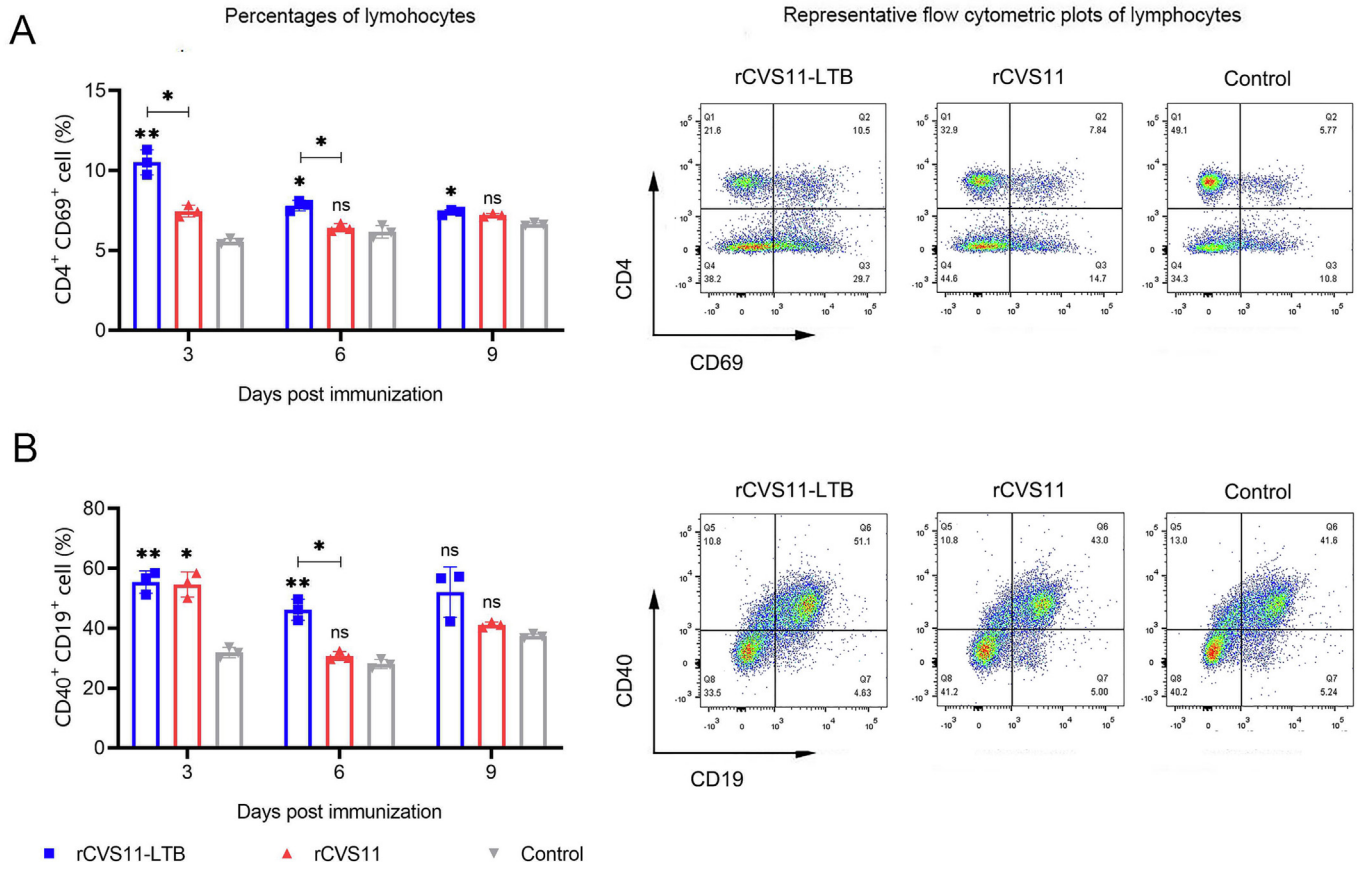


Fig. 5. rCVS11-LTB vaccination leads to rapid activation of T cells and B cells in ILNs. At 3rd, 6th and 9th dpi after the first vaccination, mouse ILNs were collected, and single cell suspensions were prepared and stained with antibodies against T-cell and B-cell activation markers for flow cytometric analysis. A) The representative flow cytometric plots (3th dpi) and percentages of $CD4^+$ and $CD69^+$ T cells in the ILNs of immunized mice. B) The representative flow cytometric plots (6th dpi) and percentages of $CD40^+$ and $CD19^+$ B cells in the ILNs of immunized mice. Data are presented as the mean ± SD for each group. ns, not significant; *, $P < 0.05$; **, $P < 0.01$. Abbreviations: LTB, heat-labile enterotoxin B subunit; ILNs, inguinal lymph nodes.

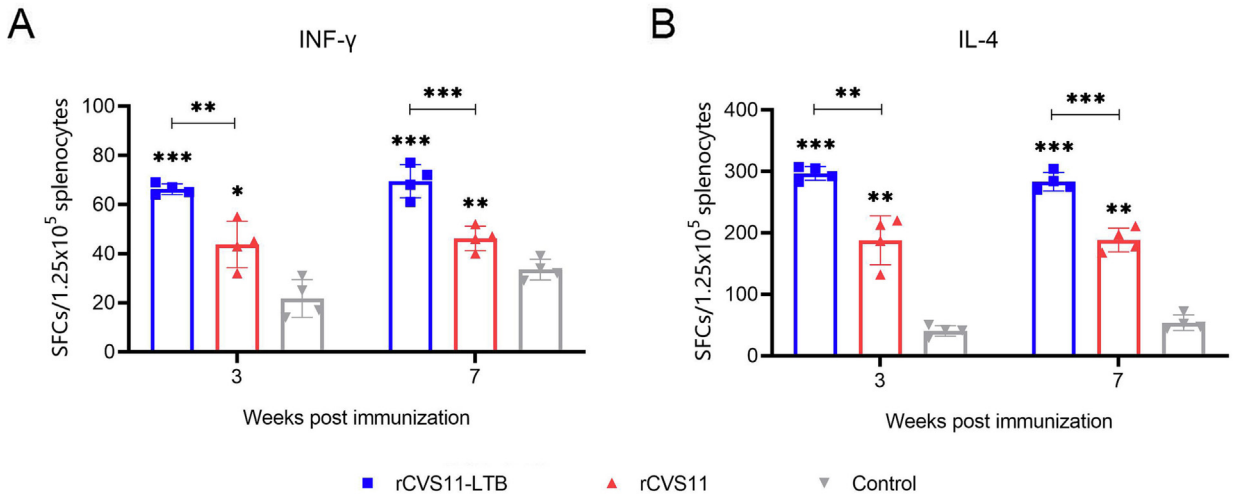
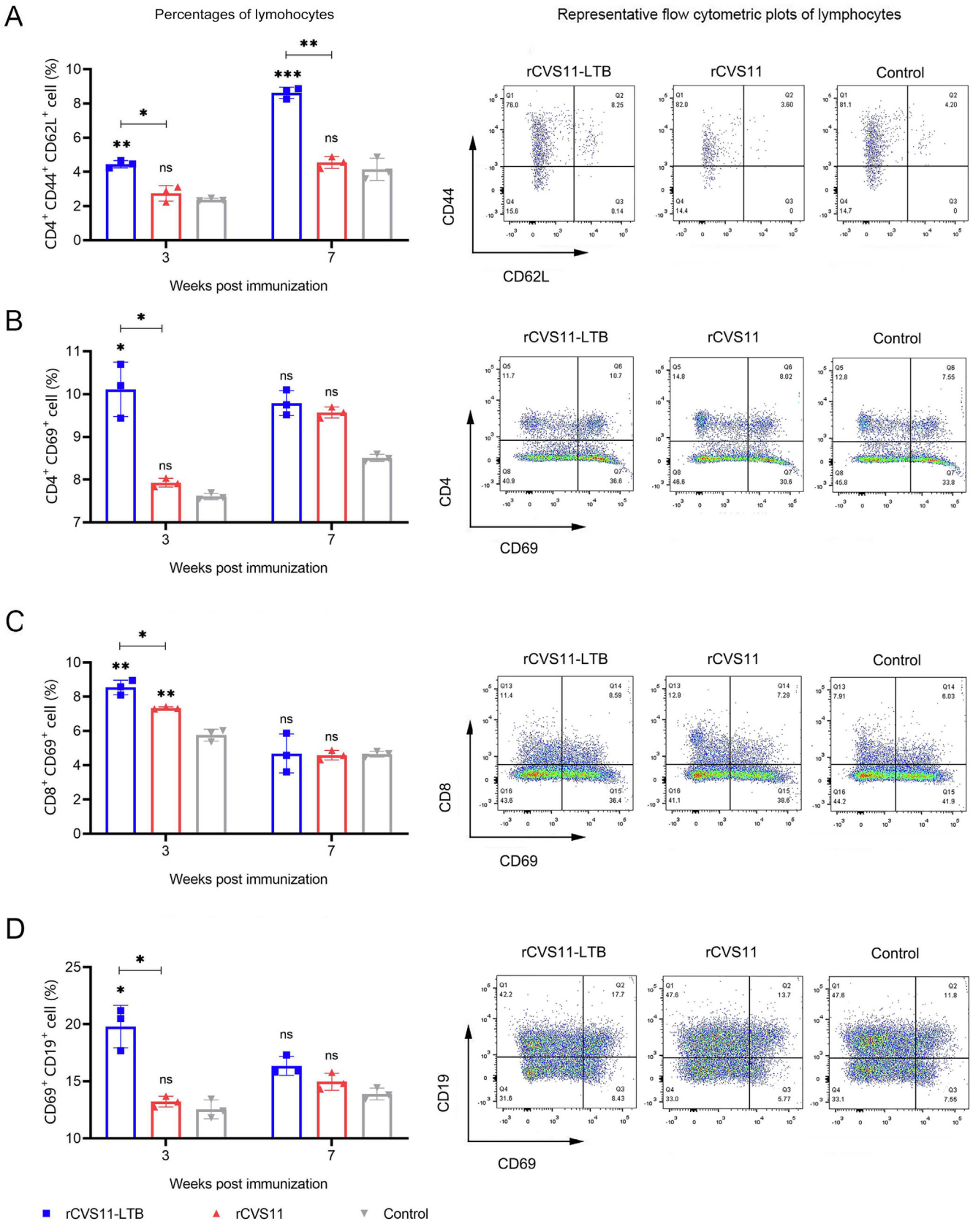


Fig. 6. Induction of Th1 and Th2 cytokine production by rCVS11-LTB. Mouse spleens were collected at the 3rd and 7th week following the first immunization, and were prepared as splenocyte suspensions. IFN-γ (A) or IL-4 (B) SFCs were quantified using ELISpot assays. Data are presented as the mean ± SD for each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Abbreviations: LTB, heat-labile enterotoxin B subunit; IFN, interferon; IL, interleukin; SFC, spot-forming cell.

group (Fig. 7A). Meanwhile, the rCVS11-LTB group elicited more $CD4^+$ T cells (Fig. 7B), $CD8^+$ T cells (Fig. 7C) and B cells at the 3rd week after the first immunization. In summary, mice immunized with the recombinant virus rCVS11-LTB developed a higher level of immune memory than those vaccinated with rCVS11. This means that those immunized with rCVS11-LTB may be able to produce the VNAs necessary to resist reinfection with RABV more rapidly than those vaccinated with rCVS11.


A
Percentages of lymohocytes
Representative flow cytometric plots of lymphocytes
CD4+ CD44+ CD62L+ cell (%)
Weeks post immunization
rCVS11-LTB
rCVS11
Control
CD44
CD62L
B
CD4+ CD69+ cell (%)
CD4
CD69
C
CD8+ CD69+ cell (%)
CD8
D
CD69+ CD19+ cell (%)
CD19
rCVS11-LTB
rCVS11
Control

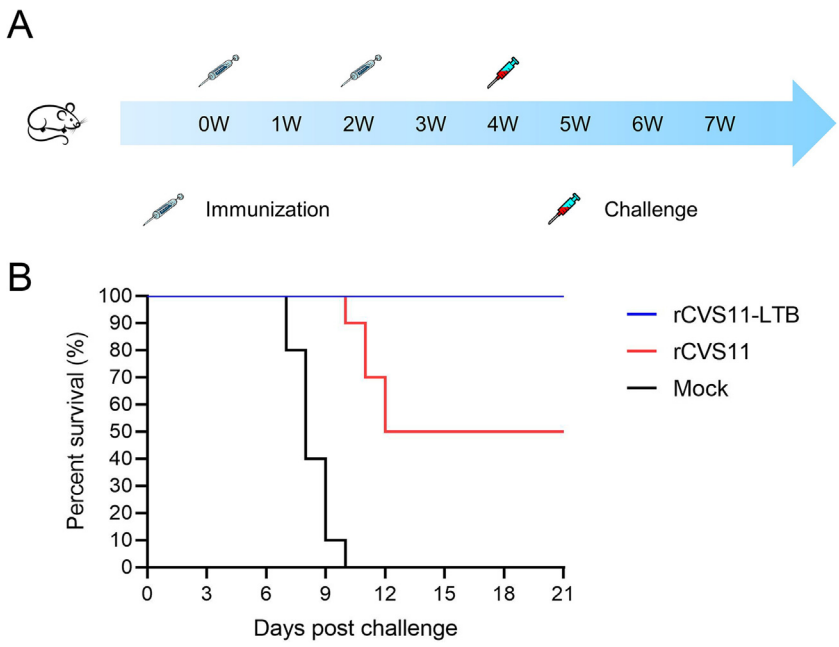


Fig. 8. Immunization with inactivated recombinant virus rCVS11-LTB protected mice from lethal challenge with rabies virus (RABV). A) Challenge test program. Six- to 8-week-old BALB/c mice were randomly divided into three groups (n = 10/group). The immunogen was prepared by mixing inactivated recombinant virus with Gel02 adjuvant (5:1). Mice were immunized intramuscularly twice with 100 μL of immunogen, with a two-week interval between immunizations. All mice in the three groups were inoculated with 10^5 $TCID_{50}$ of CVS11via the i.m. route and were monitored daily for 21 days. B) The percentages of surviving mice in the different groups at different time points after challenge were recorded and analyzed.

3.8. Immunization with rCVS11-LTB protects mice from the lethal challenge of rabies

To determine whether the immune responses induced by rCVS11-LTB could protect mice against challenge with RABV, the mice in the three groups were vaccinated with different inactivated recombinant viruses or with PBS, and then inoculated with 10^5 $TCID_{50}$ of CVS11 by the i.m. route and monitored daily for 21 days (Fig. 8A). All of mice vaccinated with inactivated rCVS11-LTB survived the lethal RABV challenge and showed no symptoms of rabies during the 21-day observation period. However, half of the mice in the rCVS11 group developed clinical signs of rabies and died within 12 days, resulting in a 50% survival rate in this group. All of the mice in the PBS group died (Fig. 8B). These results suggested that the recombinant virus rCVS11-LTB could completely protect mice from lethal attack by RABV.

4. Discussion

An advantage of inactivated rabies vaccines is their high level of safety, but they often require multiple, high-dose immunizations to stimulate the host body to produce effective protection against RABV infection. Incorporating a molecular adjuvant into viral particles has been found to be a useful strategy to enhance the immune efficacy of vaccines, particularly in the development of inactivated vaccines. Previous studies have reported that recombinant RABVs chimerically expressing the DC-binding peptide or B cell activating factor boosted both the speed and magnitude of vaccine-induced antibody responses, and provided better protection against lethal virus challenge than the parent RABV [15,16]. Here, we constructed a recombinant virus rCVS11-LTB chimerically expressing LTB protein, a molecular adjuvant capable of activating DCs, on the surface of the RABV particles (Fig. 1), to improve the immune efficacy of this recombinant virus as an inactivated vaccine.

LTB is the non-toxic pentamer subunit of the *Escherichia coli* heat-labile toxin (LT). Numerous studies have shown that LTB is a potent mucosal immune adjuvant, stimulating antigen-specific B cells and lymphocytes and modulating immune activity, including the production of cytokines, lymphocyte apoptosis, and the expression of B-cell-activating molecules [26]. A fusion protein consisting of RABV glycoprotein and LTB was developed and delivered into attenuated *Salmonella* strain LH430. Oral immunization of mice with this fusion protein can induce the production of virus neutralizing antibodies (VNA) [27]. Another study reported that LTB chimerically expressed on the surface of RABV virus-like particles could help to induce higher VNA production in mice and could protect mice from lethal RABV attack [20]. It has also been reported that LTB can enhance the immune effect of vaccines by activating DCs [28–30].

In our study, the recombinant virus could induce significant levels of VNAs in mice and dogs, which were much higher and were main-

Fig. 7. rCVS11-LTB induced good immune memory after immunization. At 3rd and 7th weeks after primary immunization, mouse spleens were collected and single cell suspensions were prepared and stained with antibodies against TCM, T-cell and B-cell activation markers for flow cytometry analysis. A) The representative flow cytometric plots (7th week post immunization) and percentages of $CD4^+$, $CD44^+$ and $CD62L^+$ in recruited $CD4^+$ T cells in the spleens of immunized mice. B) The representative flow cytometric plots (3rd week post immunization) and percentages of $CD4^+$ and $CD69^+$ recruited $CD4^+$ T cells in the spleens of immunized mice. C) The representative flow cytometric plots (3rd week post immunization) and percentages of $CD8^+$ and $CD69^+$ recruited $CD8^+$ T cells in the spleens of immunized mice. D) The representative flow cytometric plots (3rd week post immunization) and percentages of $CD69^+$ and $CD19^+$ B cells in the spleens of immunized mice. Data are presented as the mean ± SD for each group. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Abbreviation: LTB, heat-labile enterotoxin B subunit.

tained longer than those in the rCVS11 group (Fig. 3). We also demonstrated that inactivated rCV11-LTB-based vaccination led to the sustained accumulation of activated DCs in draining lymph nodes (Fig. 4), enhanced the antigen presentation and uptake ability by the DCs, and then promoted the proliferation of CD4 + T cells, thus triggering stronger T-cell and B-cell responses in mice (Fig. 5). This recombinant virus was also able to promote the induction of Th1 and Th2 responses by increasing the secretion of the cytokines IFN-γ and IL-4 (Fig. 6), stimulating the host body to produce better and longer lasting immune memory than the parent virus rCVS11 (Fig. 7). When the rCVS11-LTB immunized mice were challenged with the standard virus CVS11, 100 % survived (Fig. 8), while the mice immunized with rCVS11 showed only a 50% survival rate, indicating that the molecular adjuvant LTB chimerically expressed on the viral particles plays an important role in enhancing the vaccine-induced host immune response.

It is well known that molecular adjuvants are important for vaccine efficacy [31–33], but there are often problems with the preparation of molecular adjuvants, such as short half-life, maintenance of conformation, and preparation cost. In this study, the LTB gene was inserted into the genome of RABV. This allows the LTB protein to be stably and persistently expressed throughout viral passage (Fig. 2). Chimeric expression of LTB on the surface of intact virions allows LTB to maintain a stable conformation, prolong its half-life, and exert its adjuvant properties even when the recombinant virus has been inactivated. Meanwhile, compared to rCVS11, the recombinant virus rCVS11-LTB had higher viral titers in both NA and BSR cells (Fig. 2) and a lower pathogenicity in mice (Figure S2). These characteristics make the recombinant virus rCVS11-LTB highly suitable for vaccine production, with high titers and excellent safety.

In summary, the recombinant rabies virus rCVS11-LTB can induce a more efficient humoral and cellular immune response in both mice and dogs by activating DCs, and provided 100% protection for mice against lethal doses of the rabies virus, indicating that it has the potential to be used as a safe and efficient inactivated rabies vaccine candidate. The mucosal immune properties of LTB also provide new insights into the development of rabies vaccines [34,35]. In the future, we will construct a recombinant gene deletion vaccine with chimeric expression of LTB based on RABV reverse genetic system, conduct oral or nasal feeding vaccine research, and apply the newly developed vaccine to the immunization of wild animals.

Ethics statement

The study of all mice and dogs used in this study complied with the Chinese ethical guidelines for the welfare of laboratory animals (GB 14925-2010). The study was approved by the Animal Welfare and Ethics Committee of Jilin University (Laboratory Animal Care and Use Committee Authorization permit number SY202207010). All the mice used in this study were female and the dogs were male.

Acknowledgements

This study is supported by the National Key Research and Development Program of China (No. 2022YFD1800100).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Hualei Wang: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Zhiyuan Gong:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Validation. **Hongli Jin:** Methodology, Validation, Investigation, Data curation, Validation. **Yujie Bai:** Methodology, Validation, Investigation, Validation. **Hailun Li:** Methodology, Validation, Investigation, Validation. **Meichen Qian:** Methodology, Validation, Investigation, Validation. **Mengyao Zhang:** Validation, Investigation. **Jingxuan Sun:** Validation, Investigation. **Cuicui Jiao:** Validation, Investigation. **Pei Huang:** Formal analysis, Investigation, Data curation. **Yuanyuan Li:** Formal analysis, Investigation, Supervision. **Haili Zhang:** Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bsheal.2023.05.005.

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