



Research paper

Novel Japanese encephalitis virus NS1-based vaccine: Truncated NS1 fused with *E. coli* heat labile enterotoxin B subunit

Jiawu Wan^{a,1}, Ting Wang^{a,1}, Jing Xu^a, Tao Ouyang^a, Qianruo Wang^a, Yanni Zhang^b, Shiqi Weng^a, Yihan Li^a, Yu Wang^a, Xiu Xin^a, Xiaoling Wang^a, Sha Li^{a,*}, Lingbao Kong^{a,*}

^a Institute of Pathogenic Microorganism and College of Bioscience and Engineering, Jiangxi Agricultural University, Nanchang, Jiangxi, China

^b Jiangxi Province Center for Disease Control and Prevention, Nanchang, Jiangxi, China



ARTICLE INFO

Article History:

Received 19 November 2020

Revised 6 March 2021

Accepted 8 April 2021

Available online xxx

Keywords:

Flavivirus

JEV

Truncated NS1

LTB

Mucosa vaccine

ABSTRACT

Background: Current vaccines against Japanese encephalitis virus (JEV) of flaviviruses have some disadvantages, such as the risk of virulent reversion. Non-structural protein NS1 is conserved among flaviviruses and confers immune protection without the risk of antibody-dependent enhancement (ADE). Therefore, NS1 has become a promising vaccine candidate against flaviviruses.

Methods: A NS1-based vaccine (LTB-NS1_{Δ63}) with a truncated NS1 protein (NS1_{Δ63}) fused to *E. coli* heat-labile enterotoxin B subunit (LTB) was expressed in *E. coli* and explored for its ability to induce immune responses. Safety of LTB-NS1_{Δ63} was assessed by determining its toxicity *in vitro* and *in vivo*. Protective capability of LTB-NS1_{Δ63} and its-induced antisera was evaluated in the mice challenged with JEV by analyzing mortality and morbidity.

Findings: LTB-NS1_{Δ63} induced immune responses to a similar level as LTB-NS1, but more robust than NS1_{Δ63} alone, particularly in the context of oral immunization of mice. Oral vaccination of LTB-NS1_{Δ63} led to a higher survival rate than that of NS1_{Δ63} or live-attenuated JEV vaccine SA14-14-2 in the mice receiving lethal JEV challenge. LTB-NS1_{Δ63} protein also significantly decreases the morbidity of JEV-infected mice. In addition, passive transfer of LTB-NS1_{Δ63}-induced antisera provides a protection against JEV infection in mice.

Interpretation: NS1_{Δ63} bears JEV NS1 antigenicity. Besides, LTB-NS1_{Δ63} could serve as a novel protein-based mucosa vaccine targeting JEV and other flaviviruses.

Funding: This work was supported by the National Natural Science Foundation, Jiangxi Province Science and Technology Committee, Education Department of Jiangxi Province.

© 2021 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

1. Introduction

Japanese encephalitis virus (JEV) is the leading cause of viral encephalitis. JEV-caused encephalitis (JE) results in 25–30% of mortality. Up to 50% of surviving patients suffer neurologic or psychiatric sequelae [1,2]. JEV transmission is a complex cycle including birds, mosquitoes, pigs, horses, and humans. JEV transmits mainly through mosquito bite. JE occurs predominantly in the countries of Asia-Pacific region. Recently, there are shreds of evidences that birds and pigs were infected with JEV in Europe which were long considered free of JEV infection [3,4].

JEV belongs to the *Flavivirus* genus, which also includes Zika virus (ZIKV), dengue virus (DENV), and yellow fever virus (YFV), resulting

in outbreaks of Zika fever, dengue, and yellow fever, respectively [3]. Flaviviruses are endemic in many regions of the world and responsible for the illness ranging from mild flu-like symptoms to severe hemorrhagic, neurologic, and cognitive manifestations leading to death [5]. Distribution and outbreak of flaviviruses correlate with the geographic location of their vectors such as mosquitoes and ticks, and reservoirs such as birds and pigs [5]. Flaviviruses are enveloped, positive-sense, single-stranded RNA viruses. RNA genome of flaviviruses encodes one polyprotein, which undergoes proteolytic cleavages and produces three structural proteins including precursor membrane protein (prM), envelope protein (E), and capsid protein (C), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Among nonstructural proteins, NS1 is highly conserved and exists in diverse forms including monomer, dimer (membrane-bound protein), and hexamer (secreted protein).

Accumulating evidence shows that flavivirus NS1 plays important roles in viral replication, immune protection, and pathogenesis [5]. The economic and social burden of flavivirus-associated disease

* Corresponding authors.

E-mail addresses: lisha@jxau.edu.cn (S. Li), lingbaok@mail.jxau.edu.cn (L. Kong).

¹ These authors contributed equally.

Research in context

Evidence before this study

Live-attenuated SA14–14–2 strain is the most widely used vaccine against Japanese encephalitis virus (JEV), a member of flaviviruses. However, the possibility that a live-attenuated vaccine might reverse to a high virulence strain can never be excluded with certainty. In addition, antibody-dependent enhancement (ADE) is a general concern for the development of vaccines against flavivirus since the antibodies targeting flaviviral structural proteins have been found to amplify the infection. However, the flavivirus vaccines targeting viral non-structural proteins can avoid the risk of ADE. Among non-structural proteins of flaviviruses, NS1 is a highly conserved viral protein and protective against flavivirus infection. *E. coli* heat-labile enterotoxin B subunit (LTB) could function as a potent mucosal immunogen and adjuvant. However, whether LTB as an adjuvant can enhance JEV NS1-induced protective immune responses remains unknown.

Added value of this study

We developed a novel subunit vaccine (LTB-NS1_{Δ63}) by fusing the truncated NS1 protein, lacking the hydrophobic C-terminal 63 amino acids (NS1_{Δ63}), with LTB, a potent mucosal adjuvant. LTB-NS1_{Δ63} protein was highly expressed in *E. coli* and induced immune responses to a similar level as LTB-NS1, but more robust than NS1_{Δ63} protein, particularly by oral vaccine delivery. LTB-NS1_{Δ63} protein protected about 90% of mice from the lethal challenge of JEV, which is either comparable (subcutaneous vaccination) or better (oral vaccination) than NS1_{Δ63} protein or live-attenuated vaccine SA14-14-2. LTB-NS1_{Δ63} protein also significantly decreased the morbidity of mice upon JEV challenge. Additionally, LTB-NS1_{Δ63}-induced antisera provided similar protection against JEV infection to SA14-14-2-induced antisera.

Implications of all the available evidence

E. coli-expressed NS1_{Δ63} bears JEV NS1 antigenicity. LTB-NS1_{Δ63} could serve as a novel protein-based mucosa vaccine strategy targeting JEV as well as other flaviviruses.

(Shanghai, China) and maintained in Dulbecco's Modified Eagle's medium (Merck, Cat: D5546) supplemented with 10% fetal bovine serum (Gibco, Cat:10,100,147). JEV vaccine strain SA14-14–2 and JEV virulent strain P3 were maintained in our lab as previously described [2–10]. BALB/c female mice (three-week old) were purchased from Hunan SJA laboratory animal Co., Ltd (Changsha, China). Antibody against His-tag (RIID: AB_11,232,599), actin (RIID: AB_2,687,938), HRP-labeled goat anti-mouse (RIID: AB_2,722,565), anti-human (Cat: SA00001-17), or anti-swine IgG (Cat:SA00001-5), and anti-mouse TNF- α ELISA Kit (Cat: KE10002) were purchased from Proteintech (Chicago, USA). Anti-JEV NS1 antibody (RIID: AB_775,815) was purchased from Abcam (Wuhan, China). Alexa Fluor 488-conjugated anti-mouse IgG (RIID: AB_2,556,548) and Trizol RNA extraction kit (Cat:15,596,018) were purchased from Invitrogen (California, USA). Lactate dehydrogenase kit (Cat: m1003416), ELISA Kit of Swine JEV antibody (Cat: m1611403), and anti-mouse IFN- γ /IL-1 β antibodies (Cat:m1059749/Cat:m1063132) were purchased from Shanghai Enzyme-linked Biotechnology (Shanghai, China). Anti-mouse Japanese encephalitis antibody (IgG) ELISA kit (Cat: CSB-EQ027220MO) was purchased from Wuhan Huamei Biological Engineering Co., Ltd (Wuhan, China). Anti-mouse IL-4 pre-coated ELISA kit (Cat:1,210,402) was purchased from Dakewe Biotech Co.,Ltd (Shenzhen, China). Mouse IFN- γ ELISpot^{PLUS} kit (ALP) (Cat: 3321-4APW-2) was purchased from Mabtech (Stockholm, Sweden). Ni pillars (Cat:163,038,916) were purchased from QIAGEN (Frankfort, Germany). Guinea pig complement (Cat: GPCL99-0005) was purchased from Equitech-Bio (Beijing, China). Porcine sera were collected from pig farms in Jiangxi province of China. Human sera were obtained from Jiangxi Children's Hospital.

2.2. Ethics

Infection assays were carried out in a biosafety level 2 (BSL-2) facility. Mouse experiments were approved by Animal Care and User Committee and Laboratory Animal Ethics Committee at Jiangxi Agricultural University (reference number: JXAC20180046) and performed under the approved guidelines.

2.3. Prediction of antigenic epitopes and hydrophobicity of NS1 protein

Prediction of NS1 immune epitopes were performed using the predictor of Bepipred on the IEDB website (<http://tools.immuneeptope.org/main/>). NS1 hydrophobicity was analyzed using the software ProtScale (<https://web.expasy.org/protscale/>).

2.4. Plasmid construction

JEV SA14–14–2 strain (GenBank accession number AF315119) was used to design the NS1 gene, which was synthesized by overlapping PCR and codon-optimized for enhanced bacterial expression by DNAWorks (<http://helixweb.nih.gov/dnaworks/>). Synthesized full-length NS1 gene was subsequently used as the template to amplify truncated NS1 gene (NS1_{Δ63}) lacking the sequence encoding C-terminal 63 amino acids. LTB was generated similarly based on the gene information from LtpB-5 (GenBank accession number AAL55672.1). These genes were separately cloned into the pET-28a vector to generate plasmid pET-NS1, pET-NS1_{Δ63}, and pET-LTB. Overlapping PCR was used to amplify the fused segments LTB-NS1 and LTB-NS1_{Δ63}, which were further cloned into the pET-28a vector to get pET-LTB-NS1 and pET-LTB-NS1_{Δ63}, respectively. All the cloned gene fragments were confirmed by sequencing.

2.5. Expression and purification of recombinant proteins

Recombinant plasmids pET-NS1, pET-NS1_{Δ63}, pET-LTB-NS1_{Δ63}, pET-LTB-NS1, and pET-LTB were transformed into *E. coli* BL21 (DE3) strain

outbreak around the world have made flavivirus NS1 protein a focus in terms of its role in diagnosis and therapeutics. Secreted NS1 is an ideal diagnostic marker for flavivirus infection since it is found in the blood at the early stages and with a relatively high concentration. NS1-based ELISA has been used as a diagnostic tool for JEV, West Nile virus (WNV), and DENV infections. Immunization of NS1 or passive transfer of NS1-specific antibody confers protection against flavivirus challenge [5,6]. LTB is a potent mucosal immunogen and adjuvant [7–9]. We here develop a novel *E. coli*-expressed subunit vaccine (LTB-NS1_{Δ63}) by fusing the truncated JEV NS1 (NS1_{Δ63}), lacking the hydrophobic C-terminal 63 amino acids, with LTB. LTB-NS1_{Δ63} protein bears NS1 antigenicity and is highly safe both *in vitro* and *in vivo*. Moreover, the fusion of LTB with NS1_{Δ63} enhances mucosa immunity of NS1_{Δ63} in mice.

2. Methods

2.1. Materials

Baby hamster kidney cell line BHK-21 [C-13] (Cat: GNHa10) was purchased from the National Collection of Authenticated Cell Cultures

separately. The expression of these proteins were induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG). After induction, the expression levels in the whole cell lysate, the supernatant and the sediment were examined by SDS-PAGE gel. Purification of recombinant proteins was performed as described previously [11]. Briefly, cells were pelleted and suspended in 6 M urea buffer, followed by centrifugation at 12,000 g, 4 °C for 30 min. The resultant supernatant was applied to Ni-NTA affinity column. Purified proteins were eluted with buffers containing different concentrations of imidazole. Samples were stored at -80 °C for further use.

2.6. Immunoblotting

Immunoblotting was performed as described previously [12,13]. Briefly, protein samples were separated by SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After protein transfer, the membranes were blocked for 1 h with 10% non-fat dry milk. The blots were then incubated with a primary antibody at 4 °C overnight. The primary antibody was the antiserum from mouse immunized with the recombinant protein or SA14-14-2, antiserum from healthy or JEV-infected swine; antiserum from SA14-14-2 immunized or non-immunized human individuals; or commercial antibody against NS1, His-tag, or actin. The membrane was then incubated with HRP-conjugated secondary antibody: goat anti-mouse, anti-human, or anti-swine antibody. Finally, the proteins were visualized with Clarity ECL Immunoblotting substrate (Bio-Rad). Antisera were used in a blinded manner.

2.7. Recombinant protein-coated ELISA

ELISA was performed as described previously [11]. ELISA plates were coated overnight at 4 °C with 50 ng/well of each purified recombinant protein or cell lysate dissolved in coating buffer (0.016 M Na₂CO₃, 0.034 M NaHCO₃, pH 9.6) followed by blocking with 4% non-fat milk for one hour at room temperature. After extensive washes with PBS, mouse, human or pig serum samples were added to wells and incubated at 37 °C for 1 hour. After extensive wash, HRP-conjugated goat anti-mouse, anti-human, or anti-swine antibody was added to the well for one hour-incubation at room temperature. Finally, substrates were added to the plate. The absorbance of each well was measured with a Bio-Rad microplate reader at a wavelength of 450 nm.

2.8. Immunofluorescence assay (IFA)

IFA was performed as described previously [2–14]. In brief, JEV- or mock-infected cells were fixed with acetone-methanol (1:1) for 10 min at -20 °C. After blocking with 5% BSA solution, the cells were incubated with either commercial anti-NS1 antibody or lab-made mouse anti-NS1 Δ ₆₃ serum for one hour at room temperature. The cells were then stained with Alexa Fluor 488-conjugated anti-mouse antibody. Images were taken with an Olympus IX73 inverted microscope.

2.9. Antibody-dependent complement-mediated cytolytic assay (ADCC assay)

Anti-sera from the mice on week 4 post-immunization were pre-heated at 56 °C for 30 min to inactivate endogenous complement. BHK-21 cells grown in 96-well plates were infected with JEV virulent strain P3 at the multiplicity of infection (MOI) of 5 overnight. Infected BHK-21 cells were incubated with mouse anti-sera (1:20 dilution) and guinea pig complement (1:20 dilution) at 37 °C for 2–3 h. The levels of cytolysis were measured by the release of lactate dehydrogenase with a commercial kit. The maximum LDH release was determined from the wells containing the target cells lysed with 1% Triton

X-100. Spontaneous LDH release was determined from the wells containing the target cells and medium only. The percent specific lysis was calculated as follows: $100 \times (\text{experimental LDH release} - \text{spontaneous LDH release}) / (\text{maximum LDH release} - \text{spontaneous LDH release})$.

2.10. Cytokine profiling in serum

Sera were collected from protein-immunized mice. Cytokines including IL-1 β , IL-4, TNF- α , and IFN- γ in serum samples were measured by ELISA as described previously [15]. Briefly, the harvested sera were added to the pre-coated ELISA plate. After incubation for one hour at room temperature, 50 μ l of biotinylated antibody was added to each well and incubated for 90 min at 37 °C. After 4 times of wash, 100 μ l of streptavidin-HRP was added to each well. The plates were sealed and incubated at 37 °C for 30 min. After adding substrate for development, the absorbance was recorded at a wavelength of 450 nm.

2.11. IFN- γ ELISpot assay

Mouse IFN- γ ELISpot assay was performed according to the manufacturer's instruction with minor modifications. ELISpot plates pre-coated with anti-mouse IFN- γ antibody were washed with PBS 4 times. After blocking with cell culture medium containing 10% fetal bovine serum for 30 min at room temperature, 0.4 μ g of each protein and 10⁵ splenocytes /well from immunized and PBS-treated mice were added. The plates were cultured for 40 h at 37 °C. Cells were then removed and plates were developed with biotinylated anti-mouse IFN- γ antibody and then streptavidin-alkaline phosphatase. Spots corresponding to cytokine-secreting cells were enumerated by using Bioreader 2000 (BioSys, Frankfurt, Germany).

2.12. Safety evaluation of recombinant proteins

Safety of recombinant proteins was measured by cytotoxicity, rotarod and vascular leakage assays. The cytotoxicity of the recombinant proteins in BHK-21 cells was tested using MTT assay as described previously [16]. Briefly, the cells were treated with each protein at various concentrations for 36 h. 20 μ L/well MTT (5 mg/mL stock in PBS) was added and further incubated for 4 h. After media removal, 150 μ L of DMSO was added to each well for cell lysis, and the absorbance was read at 490 nm with a microplate reader. Cell viability of the control group was set to 100%.

The effect of the recombinant proteins on mouse vitality was evaluated by behavior observation and rotarod assay. To this end, mice were injected subcutaneously with LTB-NS1 Δ ₆₃ protein at a concentration ranging from 100 μ g to 400 μ g/mouse. Mouse alert, weight, and body temperature were monitored at indicated time-points. For rotarod assay, all mice received a training prior to rotarod trial to make them accustomed to the fixed rod. A standard 5-min rotarod assay was adopted to evaluate the biosafety of LTB-NS1 Δ ₆₃ protein. Briefly, protein-immunized or non-immunized mice were placed on a rotarod. For every 5-minute trial, the rod accelerated from 4 to 40 rpm in the first 3 min, and then continued rotating at 40 rpm for the remaining 2 min. The latency to fall was recorded for each mouse. 300 s were recorded if a mouse succeeded staying on the rotating tripod for more than 5 min. Mice performed three trials with a 30-min interval break.

To assess the impact of recombinant protein LTB-NS1 Δ ₆₃ on vascular leakage, each BALB/c mouse was injected intraperitoneally with 250 μ L of 400 μ g of recombinant protein, 0.1% (w/v) histamine phosphate (positive control) [17], or PBS (negative control). Subsequently, 100 μ L of 1% (w/v) of Evans blue dye was injected by tail vein. After one hour, dye leakage in mouse brain and abdomen was observed.

Evans blue content in tissue was analyzed at a wavelength of 610 nm as described previously [18].

2.13. Mouse immunization, challenge, and protection

BALB/c mice were randomly assigned to the following groups and immunized with 2 nmol of the recombinant proteins (corresponding to 86 μg of NS1 $_{\Delta 63}$, 120 μg of LTB-NS1 $_{\Delta 63}$, and 126 μg of LTB-NS1) in the absence of adjuvant (oral) or the presence of complete Freund's adjuvant (CFA) (subcutaneous). The mice were subsequently boosted by the same method twice at 2-week intervals. In the subcutaneous boosts, incomplete Freund's adjuvant was used to substitute CFA. Blood was harvested at different time-points. Mice receiving PBS and SA14-14-2 served as negative and positive controls. For the challenge assay, two weeks after the final boost, each mouse was injected intraperitoneally with 1×10^3 or 1×10^5 PFU of JEV P3 strain followed by a sham intracerebral injection. Mouse mortality and morbidity were monitored daily as indicated. Hematoxylin and eosin (H&E) staining assays were performed on brain samples at indicated time-points as described previously [2]. Clinical scores for individuals were recorded for 7 days post-infection in a blinded manner.

2.14. Passive protection assay

Mice received one dose (100 μL) or two doses (200 μL) of immune sera collected from LTB-NS1 $_{\Delta 63}$ -immunized mice. Antiserum collected from mice vaccinated with SA14-14-2 was used as a positive control while that from mice injected with PBS serves as a negative control. All sera were pre-incubated at 56 $^{\circ}\text{C}$ for 30 min. The individual mouse was intraperitoneally challenged with 10^3 or 10^4 PFU of JEV P3 strain at day 0 and received antiserum either at day -5 (LTB-NS1 $_{\Delta 63}^{-5}$ group), or day -1 (LTB-NS1 $_{\Delta 63}^{-1}$ group) or day 1 (LTB-NS1 $_{\Delta 63}^1$ group). Viral load in sera at day 1,4,7 (LTB-NS1 $_{\Delta 63}^{-5}$ group and LTB-NS1 $_{\Delta 63}^{-1}$ group) or day 2,5,8 (control group and LTB-NS1 $_{\Delta 63}^1$ group) post-infection were determined by plaque assay using BHK-21 cells as described previously [2]. The survival of mice was monitored for 21 days after JEV infection.

2.15. Statistical analysis

Data analysis was performed using GraphPad Prism 8.0 software to determine statistical significance. Survival statistical analysis was performed using a log-rank test. Statistical differences between two groups were tested with Student's *t*-test. $P < 0.05$ was regarded as the statistical significance.

2.16. Role of funders

The funders of this study do not affect the experimental design, sample collection, data analysis, result interpretation, manuscript preparation, or decision to submit for publication.

3. Results

3.1. Prediction of potential antigenic epitopes and hydrophobic regions within JEV NS1 protein

JEV NS1 protein sequence was subjected to software of Bepipred and ProtScale to forecast its linear B cell epitopes and hydrophobic features, respectively. Using a threshold value ≥ 0.35 (corresponding to a specificity cutoff of 75%) for Bepipred, 16 potential B cell epitopes were predicted, some of which were previously identified as B-cell antigenic epitopes [19]. Interestingly, the predicted immunogenic epitopes for B cells dispersed along with NS1 protein from amino acid 1 to 360, with a stretch of around 70 amino acids at the C-terminus hardly bearing any epitope (Fig. 1a). Thus, deleting the C-terminal fragment of ~ 70 amino acids may have little effect on NS1-induced humoral response. Consistently, two evident hydrophobic regions (aa 360–375 and aa 380–400) revealed by ProtScale analysis were located in the C-terminus without apparent B cell antigenic epitopes (Fig. 1b). Since the hydrophobic region is not on the protein surface, which is unlikely to serve as antigenic stimuli in physiological condition, these results collectively indicate that JEV NS1 protein with a truncation of 63 amino acids at the C-terminus (NS1 $_{\Delta 63}$) may harbor a similar potential in inducing host immune responses as the full-length one.

3.2. Expression and purification of recombinant JEV NS1 protein and its variants

To compare the features of JEV NS1 and NS1 $_{\Delta 63}$ proteins, we constructed the full-length and truncated forms of JEV NS1 gene with a His-tag at each terminus (Fig. 2a). The expression of recombinant NS1 protein was negligible regardless of the presence of IPTG (Suppl. Fig. 1a). In contrast, a decent amount of NS1 $_{\Delta 63}$ protein was detected in whole cell lysates upon IPTG induction, which were highly enriched in the sediment of the cells, up to 31.3% of total proteins (Table 1, Suppl. Fig. 1b). Similarly, the expressions of LTB-NS1 $_{\Delta 63}$, LTB-NS1, and LTB, predominated in the inclusion bodies of *E.coli* (Suppl. Fig. 1c-e). Interestingly, the fusion of LTB with NS1 dramatically enhanced the expression of NS1 (Table 1, Suppl. Fig. 1a,1d).

Purification by Ni-column made the purities of all recombinant proteins including NS1 $_{\Delta 63}$, LTB-NS1 $_{\Delta 63}$, LTB-NS1, and LTB above 90%

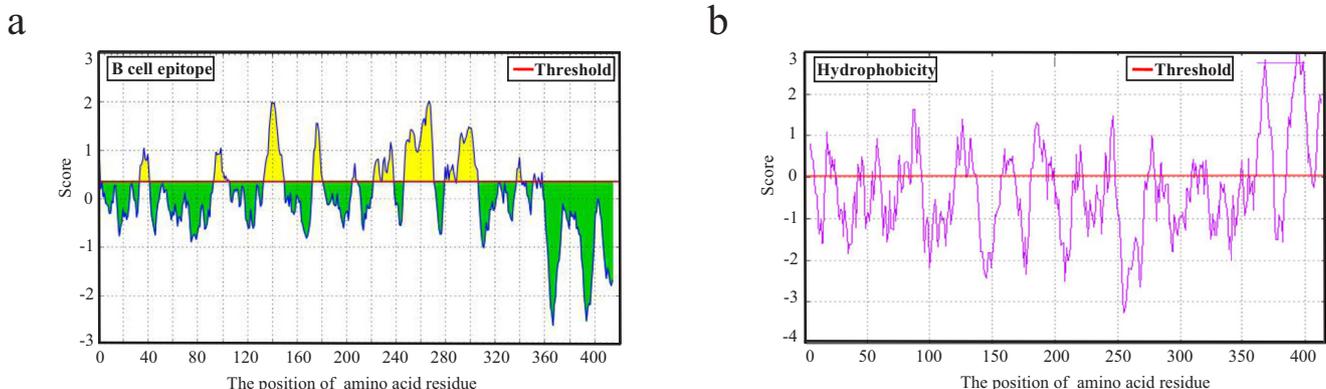


Fig. 1. Predictions of B-cell antigenic epitopes and hydrophobic regions of the NS1 protein. (a) Prediction of B-cell potential antigenic epitopes using BepiPred with the threshold value ≥ 0.35 . (b) Prediction of hydrophobic regions using ProtScale.

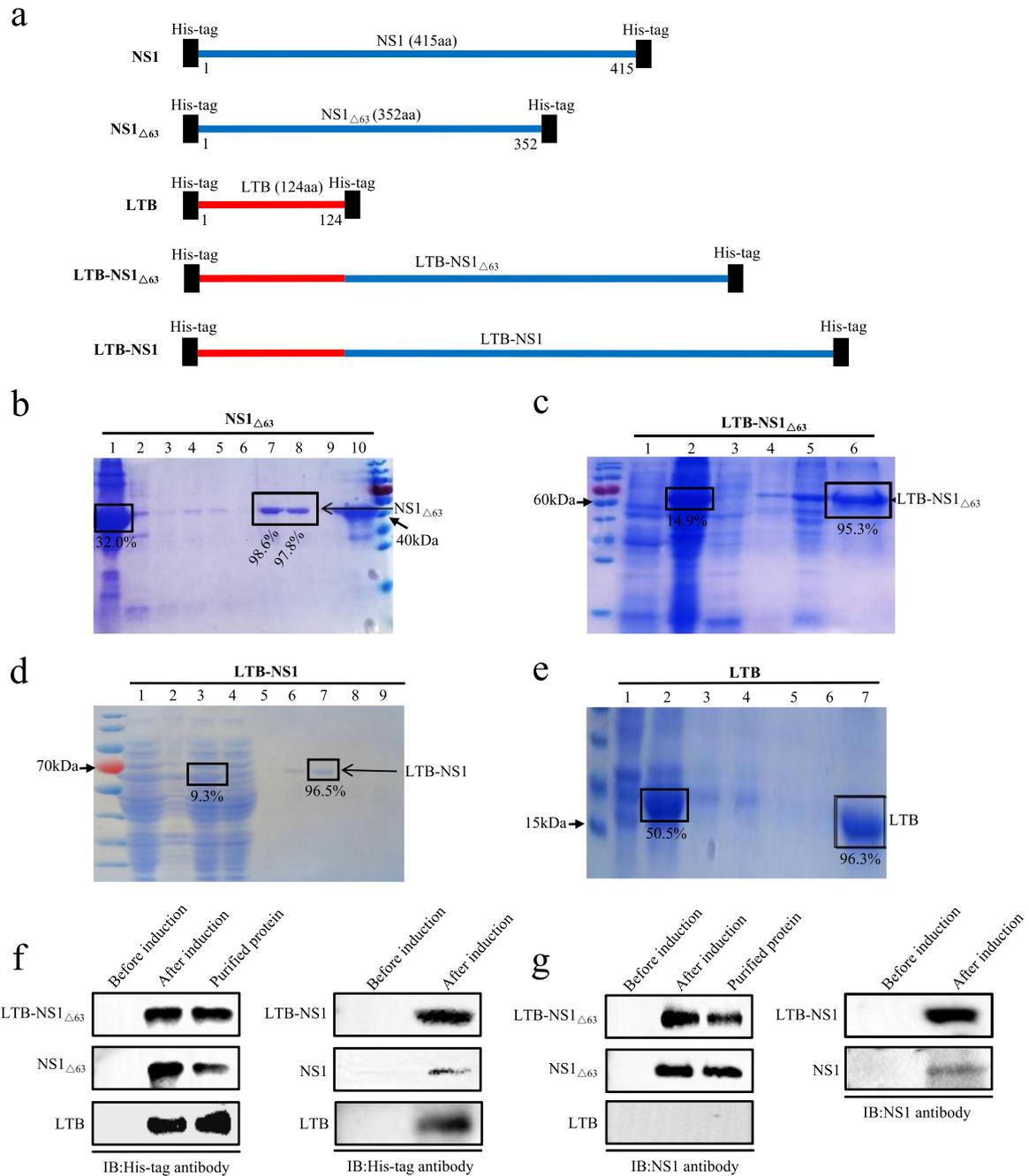


Fig. 2. Expression and purification of recombinant proteins. (a) Diagram of recombinant constructs. (b,e) Purification of recombinant proteins. Recombinant proteins were purified by Ni-NTA affinity column and eluted with elution buffers. (b) NS1 Δ_{63} protein. Lane 1: IPTG-induced cell lysate; Lane 2: Elution from Ni-NTA column; Lane 3–9: Elution fractions with imidazole of 5, 10, 20, 40, 60, 80, and 100 mM; Lane 10: cellular inclusion bodies. (c) LTB-NS1 Δ_{63} protein. Lane 1: Un-induced cell lysate; Lane 2: Induced cell lysate; Lane 3–6: Elution fractions with imidazole of 5, 10, 20, and 80 mM. (d) LTB-NS1 protein. Lane 1–3: whole cell lysate, supernatant, and sediment; Lane 4: Elution from Ni-NTA column; Lane 5–9: Elution fractions with imidazole of 20, 40, 80, 120, and 160 mM; (e) LTB protein. Lane 1: Un-induced cell lysates; Lane 2: Induced cell lysates; Lane 3–7: Elution fractions with imidazole of 10, 20, 40, 60, and 80 mM. The percentages shown in the images indicated ratios of recombinant protein to total proteins. (f,g) Identification of recombinant proteins by immunoblotting. Recombinant proteins were subjected to immunoblotting analysis using commercial anti-His-tag antibody (f) or anti-NS1 antibody (g). Images were representative of three independent experiments.

Table 1
Production and purification characteristics of recombinant protein.

Projects	LTB	NS1 Δ_{63}	NS1	LTB-NS1 Δ_{63}	LTB-NS1
Bacterial wet weight yield (g/L)	29.1 ± 0.30	32.8 ± 0.57	30.0 ± 1.22	31.9 ± 0.79	32.0 ± 0.76
Proportion of recombinant protein to total protein (%)	11.8 ± 2.26	26.8 ± 6.08	—	17.7 ± 3.56	17.0 ± 3.83
Purity of protein (%)	95.4 ± 1.07	97.2 ± 1.72	—	96.5 ± 1.08	96.4 ± 2.20
Purification efficiency (%)	68.9 ± 6.04	67.2 ± 4.84	—	78.7 ± 3.15	62.6 ± 2.75
Purified protein concentration (mg/mL)	0.42 ± 0.05	0.98 ± 0.22	—	1.12 ± 0.24	0.75 ± 0.07

except NS1 protein that we could not achieve high yield after several attempts (Fig. 2b–e, Table 1). To confirm the identities of *E. coli*-derived recombinant proteins, we performed immunoblotting assays with an anti-His-tag antibody or anti-NS1 antibody. Expected bands were detected for all recombinant proteins in IPTG-induced bacterial lysate samples, but not un-induced ones, demonstrating the identities of *E. coli*-expressed proteins (Fig. 2f–g). As controls, LTB protein could be detected only by anti-His-tag antibody, but not anti-NS1 antibody (Fig. 2f–g). It should be noted that recombinant protein NS1 can be detected by immunoblotting assay, although SDS-PAGE images show that its expression seems invisible, suggesting that the expression of NS1 protein is weak in *E. coli* system (Fig. 2f–g, Suppl. Fig. 1a). Overall, we successfully expressed recombinant NS1_{Δ63}, LTB-NS1_{Δ63}, NS1, LTB-NS1, and LTB proteins in *E. coli*.

3.3. Antigenicity of recombinant NS1_{Δ63} and LTB-NS1_{Δ63} proteins

Sera were harvested from protein-immunized mice, JEV-infected mice or pig, JEV-immunized human to examine the immunogenicity of recombinant proteins (Fig. 3a). Immunoblotting results showed that recombinant protein-immunized murine sera specifically detected the corresponding proteins expressed in IPTG-induced transformed *E. coli*, but not in the un-induced samples (Fig. 3b, left panel). As a negative control, sera from mice treated with PBS did not recognize any recombinant proteins (Fig. 3b, left panel). Another supporting evidence came from ELISA assays with recombinant proteins as coating reagents and corresponding antisera as primary antibodies (Fig. 3b, right panel). We further evaluated NS1-specific immunogenicity of NS1_{Δ63} and LTB-NS1_{Δ63} proteins with the sera from JEV-infected mice. The data from both immunoblotting and ELISA assays unveiled that NS1_{Δ63} and LTB-NS1_{Δ63} recognized JEV-positive mouse sera but not JEV-free mouse sera (Fig. 3c). To confirm the data in mice, we further used the sera from other species including human and swine. NS1_{Δ63} and LTB-NS1_{Δ63} proteins successfully distinguished live-attenuated vaccine SA14–14–2-immunized human blood samples from unimmunized ones in both immunoblotting and ELISA assays (Fig. 3d). Similar results were obtained when sera from JEV-infected and JEV-free pigs were used to test NS1-specific immunogenicity of NS1_{Δ63} and LTB-NS1_{Δ63} proteins (Fig. 3e). Among 40 serum samples derived from either healthy or JEV-infected pigs, 97.5% of agreement ratio between NS1_{Δ63}-based and NS1-based ELISA highlighted the comparable antigenicity between NS1_{Δ63} and NS1 proteins (Fig. 3f, Table 2). Altogether, these data demonstrated *E. coli*-expressed recombinant proteins NS1_{Δ63} and LTB-NS1_{Δ63} retained NS1 antigenicity.

3.4. NS1_{Δ63} and LTB-NS1_{Δ63} proteins elicit JEV NS1-specific antibody in mice

Data that *E. coli*-expressed NS1_{Δ63} and LTB-NS1_{Δ63} proteins recognize JEV-positive sera (Fig. 3c–f) prompts us to address whether NS1_{Δ63}- and LTB-NS1_{Δ63}-induced antibodies can specifically interact with JEV NS1 protein. Antisera were collected from mice subcutaneously received each recombinant protein and used in immunofluorescence assays to examine BHK-21 cells untreated or pre-treated with JEV SA14–14–2 (Fig. 4a and 4c). Morphology data showed that JEV infected-cells exhibited an apparent cytopathy at day 4 post-infection, demonstrating successful viral infection (Fig. 4b). Comparable amounts of virus-infected cells were observed in the contexts when NS1_{Δ63}-induced antisera or commercial anti-NS1 antibody were used as detecting reagents, implying that NS1_{Δ63}-induced mouse antiserum could react with JEV NS1 protein (Fig. 4c). Furthermore, immunoblotting assays showed that the antisera from NS1_{Δ63}- and LTB-NS1_{Δ63}-immunized mice resulted in decent NS1 protein bands after incubation with cell lysate from JEV-infected BHK-21 cells, consistent with positive control data using the antisera from

LTB-NS1-immunized mice (Fig. 4d–f). As negative controls, cell lysate from mock-infected BHK-21 cells failed to interact with the antisera from NS1_{Δ63}-, LTB-NS1_{Δ63}- or LTB-NS1-immunized mice (Fig. 4d–f). Taken together, these results demonstrate that NS1_{Δ63} and LTB-NS1_{Δ63} proteins elicit JEV NS1-specific antibodies in mice.

3.5. LTB-NS1_{Δ63} protein induces robust humoral responses in mice

Given that distinct immunization regimens induce various levels of humoral responses, we compared the end-point antibody titers in the context of subcutaneous and oral immunization (Fig. 5a). Subcutaneously administration of LTB-NS1_{Δ63} induced more robust antibody titers than those of NS1_{Δ63} during the whole examined period, especially on week 2 (1:8200 for LTB-NS1_{Δ63} and 1:6600 for NS1_{Δ63}), week 4 (1:13,700 for LTB-NS1_{Δ63} and 1:10,100 for NS1_{Δ63}), and week 8 (1:17,900 for LTB-NS1_{Δ63} and 1:15,100 for NS1_{Δ63}) (Fig. 5b, Table 3). Interestingly, a similar pattern was detected in the oral vaccine regimen, especially on week 6 (1:16,200 for LTB-NS1_{Δ63} and 1:4600 for NS1_{Δ63}), week 7 (1:16,600 for LTB-NS1_{Δ63} and 1:4800 for NS1_{Δ63}), and week 8 (1:17,800 for LTB-NS1_{Δ63} and 1:4800 for NS1_{Δ63}) (Fig. 5c, Table 4). Notably, in the oral context, boosters of LTB-NS1_{Δ63} further elevated the antibody titers to 1:17,000 on week 6 and afterwards, remarkably higher than those of JEV vaccine SA14–14–2 (antibody titers are around 1:6500 on week 6–8) (Fig. 5c, Table 4).

NS1-specific antibodies have been reported to exhibit cytolytic activity against JEV-infected cells in a complement-dependent manner [20]. To address whether the antisera specific to the recombinant proteins were able to lyse JEV-infected cells in the presence of complement, we performed an ADCC assay with the antisera from mice at week 4 post-immunization. As shown in Fig. 5d, the antisera from LTB-NS1_{Δ63}-immunized mice lysed $81.04 \pm 9.19\%$ of JEV-infected cells, which were comparable with those from LTB-NS1-immunized mice ($81.78 \pm 6.54\%$), but higher than those from NS1_{Δ63}-immunized mice ($67.07 \pm 8.23\%$). As controls, the antisera from mice pre-treated with SA14–14–2 exhibited high cytolysis ($90.36 \pm 5.52\%$) when complement was included, whereas the sera from PBS or LTB-immunized mice showed no specific activity to JEV-infected targets. These results indicated that antibody-dependent complement-mediated cytolysis might serve as a mechanism adopted by LTB-NS1_{Δ63} protein to induce protection against JEV infection.

3.6. LTB-NS1_{Δ63} protein induces robust cellular responses in mice

Cellular immune responses play an important role in the defense against viruses [21]. Recent studies show that NS1 of flaviviruses such as ZIKV and JEV confers protection against virus infection through NS1-specific cellular immune responses [21,22]. Therefore, we performed ELISA assays to examine levels of Th1 (TNF- α , IFN- γ , and IL-1 β) and Th2 (IL-4) cytokines in mouse sera upon immunization with different proteins through oral or subcutaneous immune routes (Fig. 6a). It is no surprise that the subcutaneous delivery of NS1_{Δ63} protein and SA14–14–2 vaccine, but not LTB protein or PBS, triggered mice to secrete cytokines substantially (Fig. 6b). Moreover, of all the cytokines examined, LTB-NS1_{Δ63} induced higher secretions than NS1_{Δ63}, while comparable to LTB-NS1 upon subcutaneous delivery (Fig. 6b). Oral delivery of LTB-NS1 or LTB-NS1_{Δ63} protein elicited mice to secrete cytokines at similar levels as the subcutaneous administration (Fig. 6b–c). Interestingly, oral immunization of NS1_{Δ63} protein and SA14–14–2 vaccine to mice triggered much less secretions of cytokines (IL-1 β , IFN- γ , and IL-4), compared with the subcutaneous delivery (Fig. 6b,c). To further evaluate cellular immunity at a single-cell level, an IFN- γ ELISpot assay was performed using the splenocytes from orally immunized mice. The significantly higher spot number was evidenced in LTB-NS1_{Δ63} group as compared with that of LTB-treated group ($P < 0.0001$, Student's *t*-test), NS1_{Δ63} group ($P = 0.0002$, Student's *t*-test), and SA14–14–2 group ($P = 0.0003$,

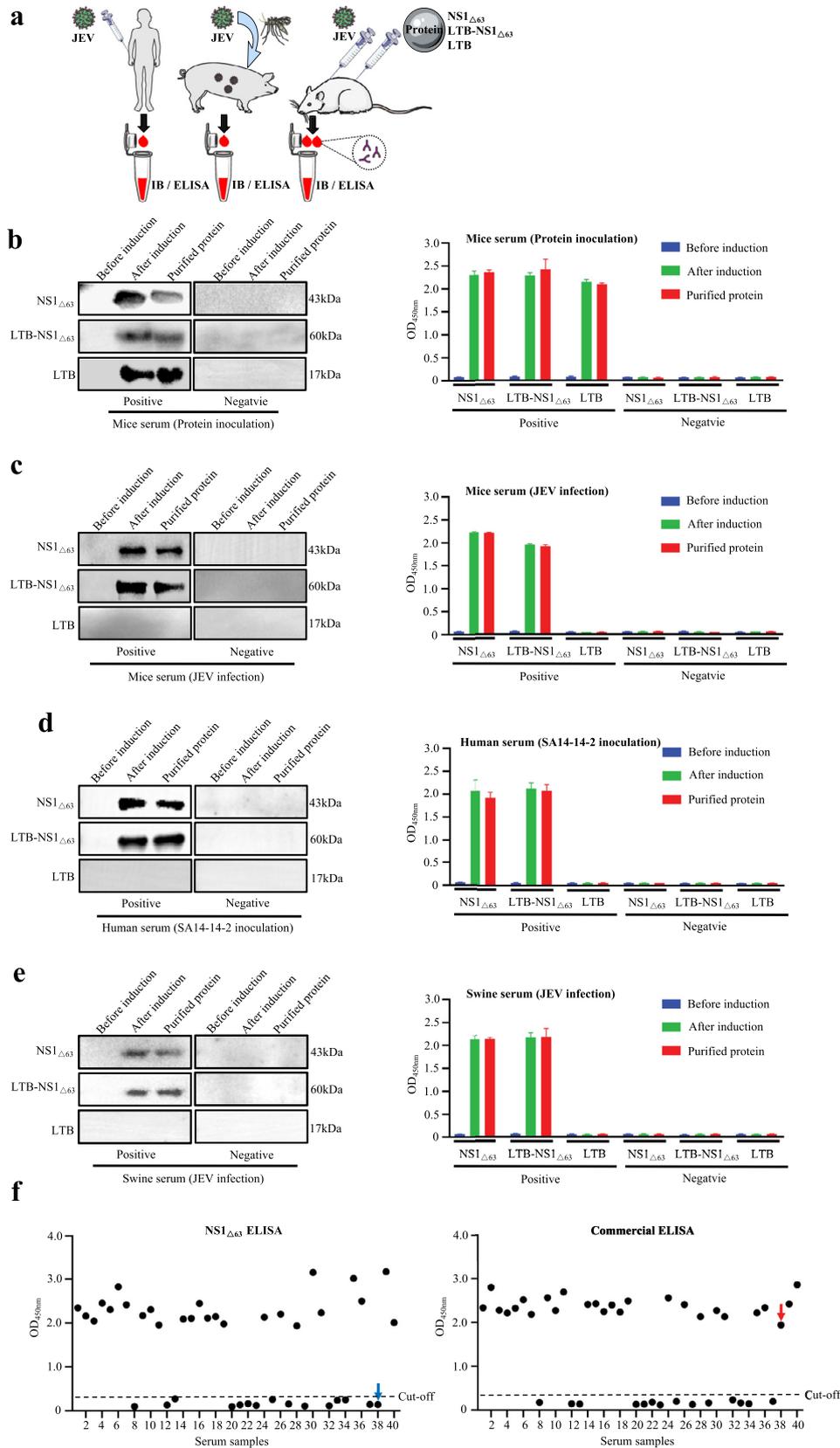


Fig. 3. Antigenicity of recombinant NS1 Δ 63 and LTB-NS1 Δ 63 proteins. (a) Scheme of JEV infection and protein immunization. IB, immunoblotting. (b–f) Reactivity of recombinant proteins with sera from mice (b,c), humans (d), and pigs (e,f). (b–e) The lysates from un-induced or IPTG-induced *E.coli* cells, and purified protein were used to react with corresponding protein-immunized mouse sera (b), JEV- Infected mouse sera (c), vaccine SA-14–14–2–immunized human sera(d), JEV-positive pig sera (e) and control sera (b–e) in the immunoblotting (b–e, left panels) and ELISA (b–e, right panels) assays. For immunoblotting, images were representative of three independent experiments. For ELISA, error bars indicate standard deviations (SD) of the means ($n = 3$). (f) Comparison of NS1 Δ 63-coated ELISA with commercial NS1-coated ELISA. JEV-positive and -negative swine serum samples ($n = 40$) were tested by NS1 Δ 63-coated ELISA (left panel) and commercial NS1-coated ELISA (right panel). Each dot represents a sample, and the dashed line indicates cutoff value. The one with a discrepancy in reactivity to NS1 Δ 63 and commercial NS1 proteins is highlighted by the arrow.

Table 2
Comparison of NS1 $_{\Delta 63}$ -coated ELISA with commercial NS1-coated ELISA.

Serum	ELISA (NS1 $_{\Delta 63}$ -coated)	Commercial kit (NS1-coated)
39	Same	Same
1	Negative	Positive
Coincidence rate	97.5%	

Student's *t*-test (Fig. 6d). As expected, the spot numbers of LTB-NS1 $_{\Delta 63}$ group and LTB-NS1 group were comparable ($P=0.6442$, Student's *t*-test) (Fig. 6d). All the results above collectively demonstrate that LTB-NS1 $_{\Delta 63}$ protein is capable to initiate robust cellular immunity.

3.7. LTB-NS1 $_{\Delta 63}$ protein is well-tolerable in vitro and in vivo

To assess the safety of the truncated form of NS1 protein as a vaccine candidate, we incubated BHK-21 cells with different concentrations of NS1 $_{\Delta 63}$ or LTB-NS1 $_{\Delta 63}$ protein followed by MTT assay. The data showed that up to 500 $\mu\text{g/ml}$ (11.6 μM) of NS1 $_{\Delta 63}$ or 500 $\mu\text{g/ml}$ (8.3 μM) of LTB-NS1 $_{\Delta 63}$ had no adverse effect on cell viability (Fig. 7a). The safety of LTB-NS1 $_{\Delta 63}$ was next examined with mice. BALB/c mice receiving LTB-NS1 $_{\Delta 63}$ protein with the dose ranging from 100 μg to 400 μg remained alert, active, and well-groomed (Fig. 7b). We further adopt a rotarod assay to assess the vitality of mice treated with LTB-NS1 $_{\Delta 63}$. We found that all mice displayed comparable retention times on the rotarod before and after protein inoculation (Fig. 7c), suggesting LTB-NS1 $_{\Delta 63}$ protein did not affect mouse

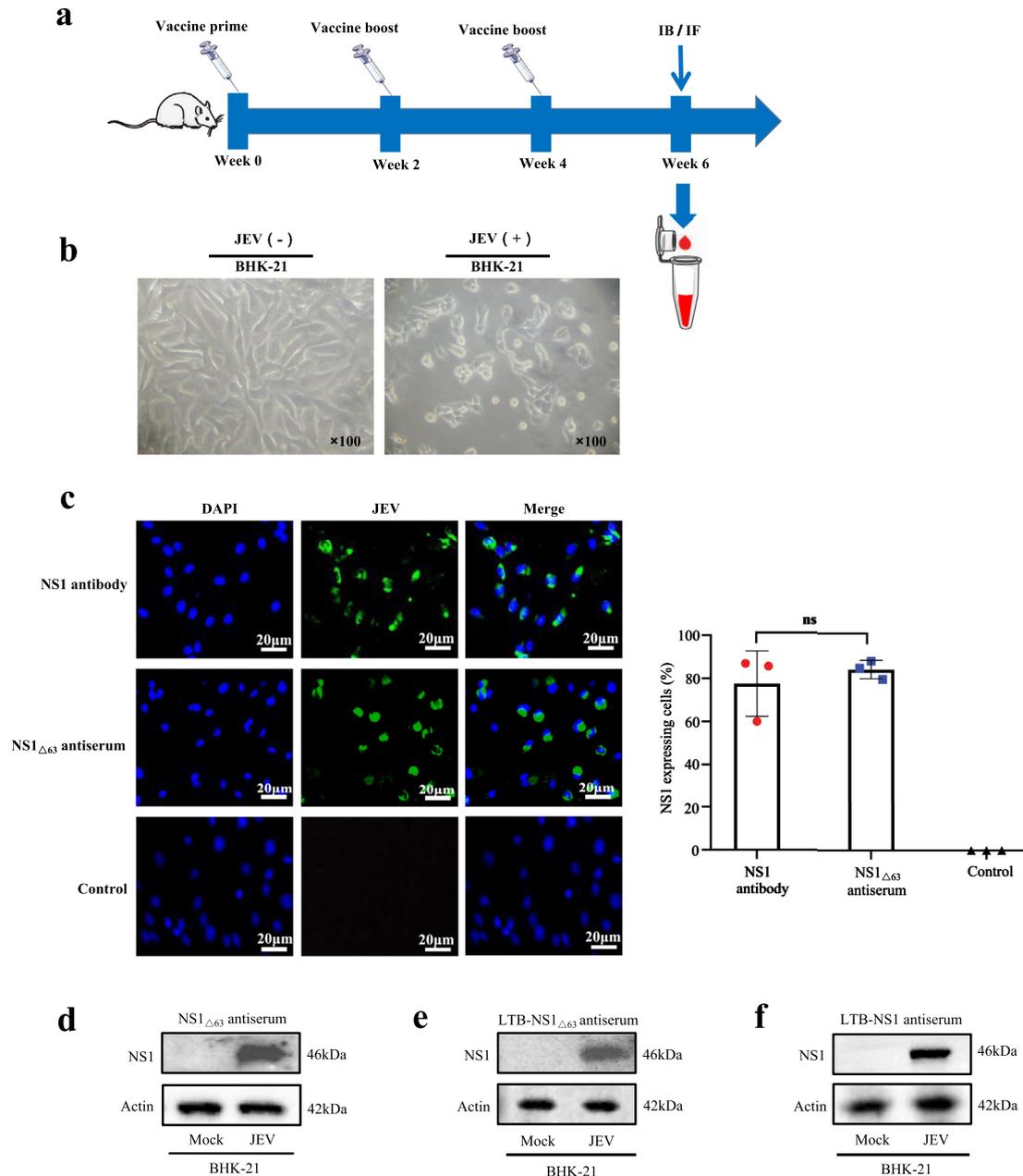


Fig. 4. NS1 $_{\Delta 63}$ and LTB-NS1 $_{\Delta 63}$ proteins elicit JEV NS1-specific antibody in mice. (a) Scheme of protein immunization and antiserum analysis. IB, immunoblotting; IF, Immunofluorescence. (b–f) BHK-21 cells were infected with JEV SA14–14–2 strain at MOI of 5. Mock-infected cells were used as negative control. (b) Four days post JEV infection, the cytopathy was observed by microscopy. (c) JEV-infected cells were subjected to immunofluorescence using NS1 $_{\Delta 63}$ -induced antiserum, commercial JEV NS1 antibody, or control (negative serum). (d–f) Immunoblotting assays using NS1 $_{\Delta 63}$ - (d), LTB-NS1 $_{\Delta 63}$ - (e), and LTB-NS1-induced antiserum (f). Student's *t*-test was used to evaluate the intergroup difference. Images were representative of three independent assays. Error bars indicate standard deviations (SD) of the means ($n=3$). ***, $P<0.001$; ****, $P<0.0001$.

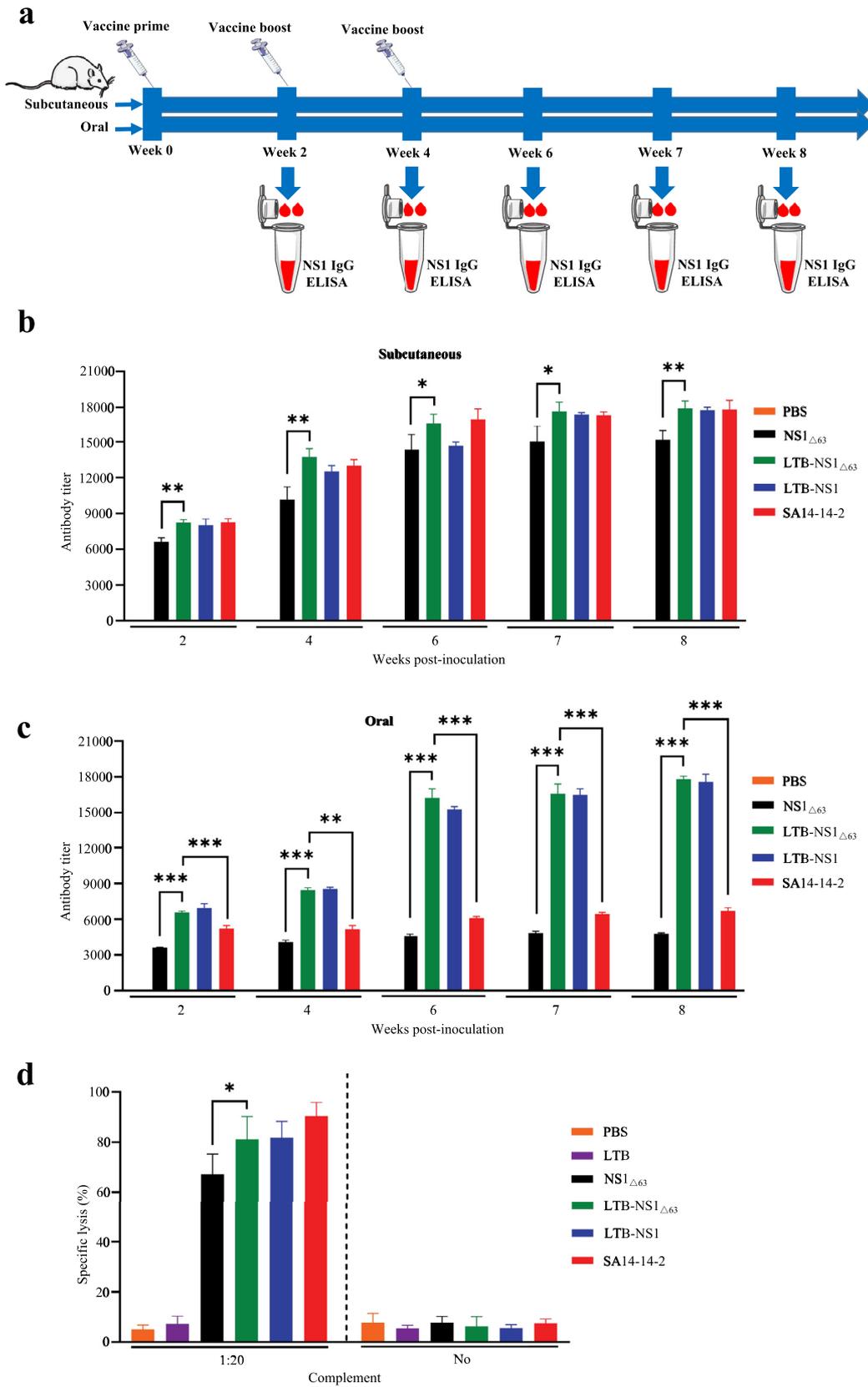


Fig. 5. Humoral responses induced by subcutaneous or oral immunization of recombinant proteins or SA14-14-2. (a) Scheme of immunization strategy and antibody analysis. Boost was performed for recombinant proteins NS1 Δ_{63} , LTB-NS1 Δ_{63} , and LTB-NS1. (b,c) BALB/c mice (4-week old) were administrated subcutaneously (b) or orally (c) with 2 nmol of NS1 Δ_{63} , LTB-NS1 Δ_{63} , or LTB-NS1 protein, or 10⁴ PFU JEV vaccine SA14-14-2. Antisera were collected and subjected to ELISA for detecting JEV NS1 antibody. (d) ADCC assay. BHK-21 cells were infected with JEV P3 stain at MOI of 5 overnight, then incubated with pre-heated mouse anti-sera and guinea pig complement at 37 °C for 2–3 h. The release of lactate dehydrogenase was used as the index of cytolysis. Student's *t*-test was adopted to evaluate the intergroup difference. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001. Error bars indicate SD of the means (*n* = 3).

Table 3
The impact of subcutaneous boost immunization on antibody production.

Comparison between weeks	NS1 $_{\Delta 63}$	LTB-NS1 $_{\Delta 63}$	LTB-NS1
Week 4 vs Week 2 (First boost)	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Week 6 vs Week 4 (Second boost)	$P = 0.0066$	$P = 0.0247$	$P = 0.0138$

^QAll P values are calculated using Student's *t*-test.

Table 4
The impact of oral boost immunization on antibody production.

Comparison between weeks	NS1 $_{\Delta 63}$	LTB-NS1 $_{\Delta 63}$	LTB-NS1
Week 4 vs Week 2 (First boost)	$P < 0.0001$	$P = 0.0036$	$P = 0.0009$
Week 6 vs Week 4 (Second boost)	$P = 0.0004$	$P < 0.0001$	$P = 0.0003$

^QAll P values are calculated using Student's *t*-test.

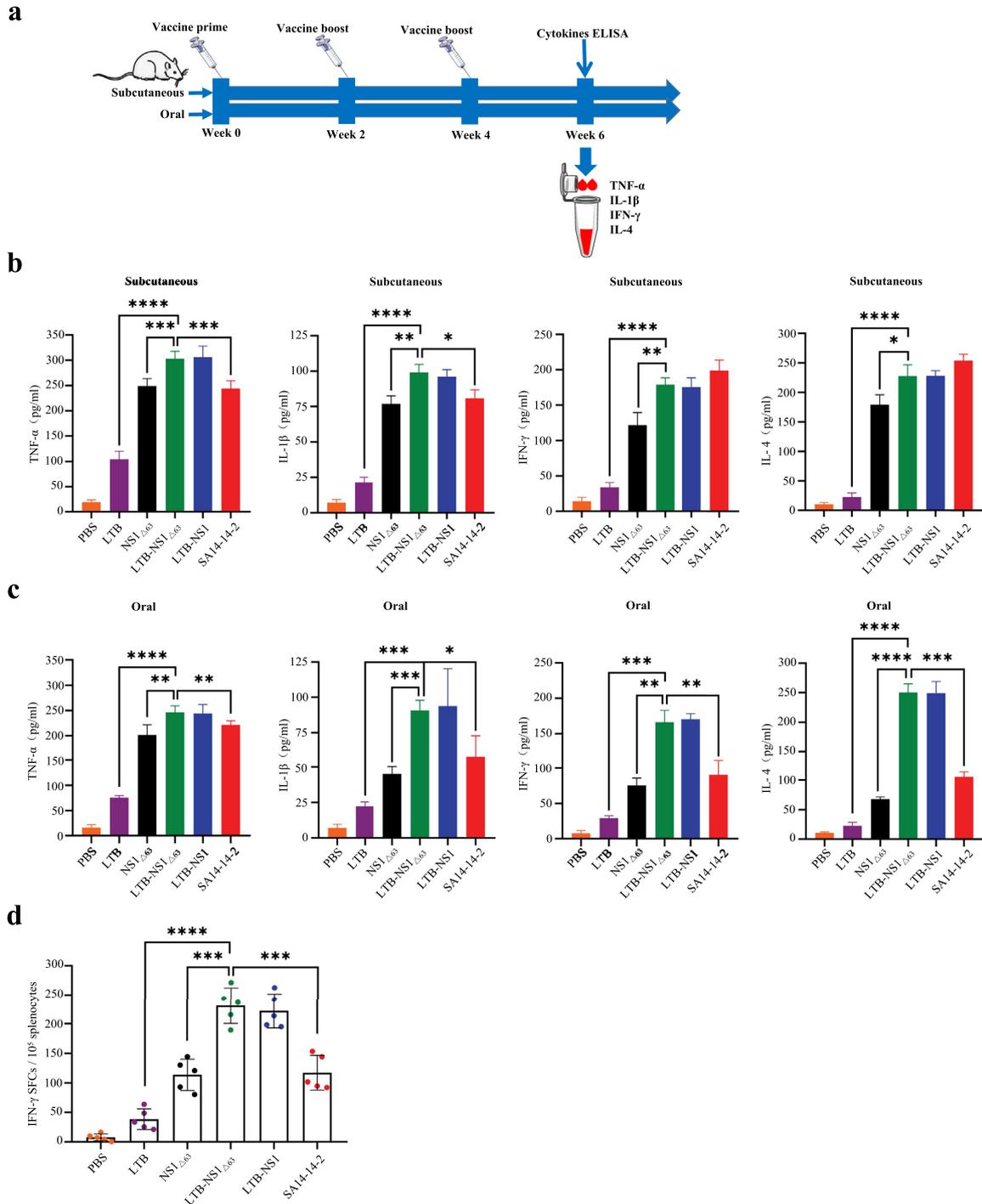


Fig. 6. Cytokines induced by subcutaneous or oral immunization of recombinant proteins. (a) Scheme of vaccine immunization and cytokine analysis. Boost was performed for recombinant proteins NS1 $_{\Delta 63}$, LTB-NS1 $_{\Delta 63}$, and LTB-NS1. (b,c) BALB/c mice (4-week old) were administrated subcutaneously (b) or orally (c) with 2 nmol of NS1 $_{\Delta 63}$, LTB-NS1 $_{\Delta 63}$, or LTB-NS1 protein, or 10^4 PFU JEV vaccine SA14–14–2. Antisera were collected and subjected to ELISA for detecting cytokines. Error bars indicate SD of the means ($n = 3$). (d) ELISpot assay. Splenocytes were harvested from orally immunized mice and incubated with $0.4 \mu\text{g}$ of each protein at 10^5 cells/well. For PBS group, no protein was used while for SA14–14–2 group, NS1 $_{\Delta 63}$ protein was added. After incubation for 40 h, the plates were developed. Spots corresponding to cytokine-secreting cells were enumerated and compared. Error bars indicate SD of the means ($n = 5$). Student's *t*-test was used to evaluate the intergroup difference. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

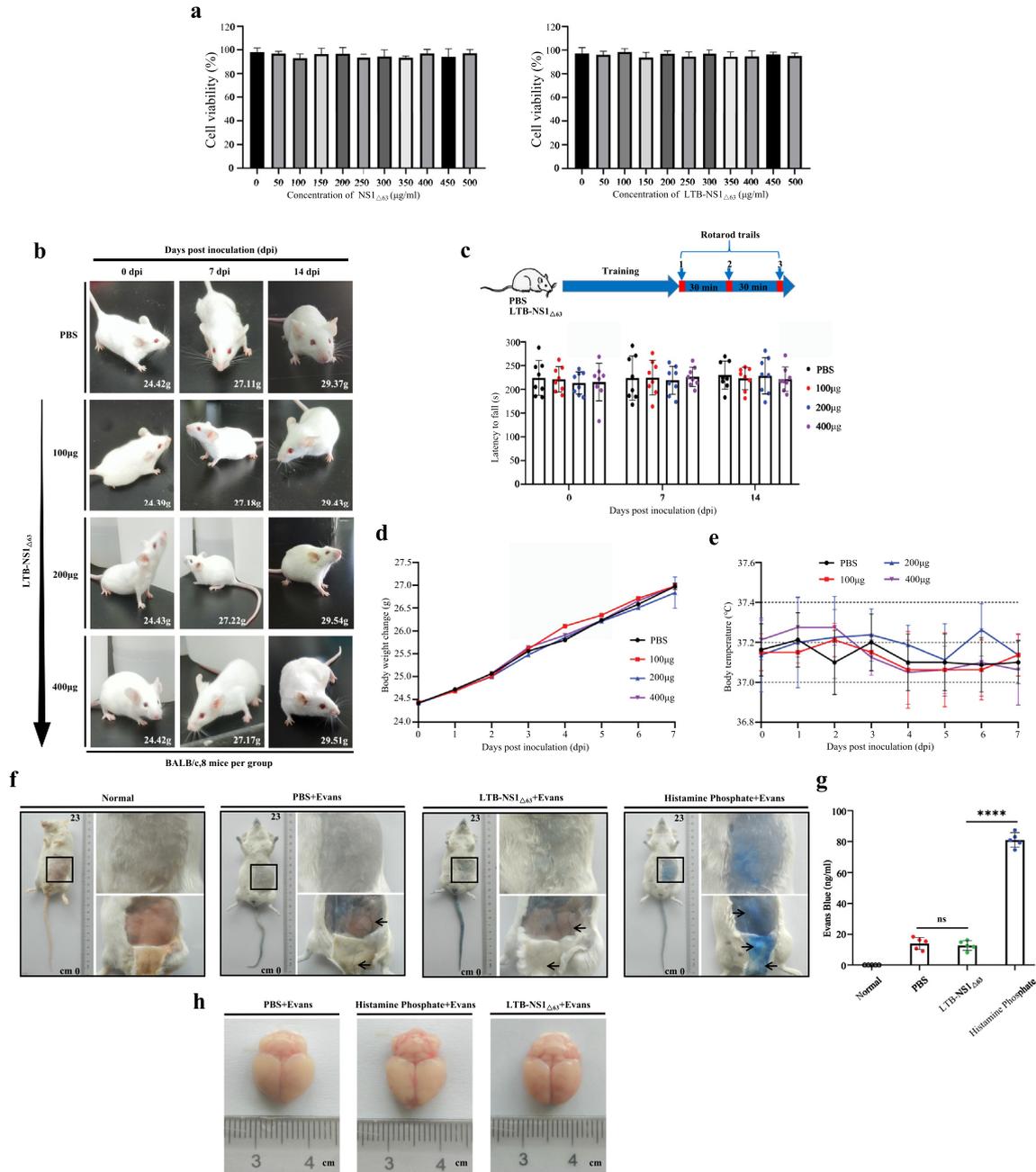


Fig. 7. Safety evaluation of recombinant proteins. (a) Cytotoxicity analysis of NS1 Δ_{63} (left panel) and LTB-NS1 Δ_{63} (right panel) in BHK-21 cells by MTT assays. Error bars indicate SD of the means ($n = 3$). (b–e) Effect of LTB-NS1 Δ_{63} protein on mouse physiological parameters. An individual mouse was subcutaneously immunized with LTB-NS1 Δ_{63} protein with a concentration ranging from 100 μ g to 400 μ g. (b,c) Mouse vitality was assessed by behavior observation (b) and rotarod assay (c). (d–e) Changes in body weight (d) and temperature (e) were examined at indicated time-points. For behavior observation, images were representative of mice from each group ($n = 8$). Error bars indicate standard deviations (SD) of the means ($n = 8$). (f–h) Impact of LTB-NS1 Δ_{63} protein on vascular leakage. Each mouse was intraperitoneally injected with 250 μ L of PBS, 250 μ L of 0.1% (w/v) histamine phosphate, 400 μ g of LTB-NS1 Δ_{63} protein or untreated, followed by the injection of 100 μ L of 1% (w/v) of Evans blue dye by tail vein. One hour later, the extent of dye leakage in mouse abdomen (f,g) and brain (h) was examined. Representative images from each group ($n = 5$) were shown (f–h). The boxed areas (f) show enlargements in the corresponding panels in the right (upper, epilation; lower, exuviation). Evans blue dye in abdomen (f) was quantified (g). Error bars indicate the SD of the mean ($n = 5$). Student's *t*-test was used to evaluate the intergroup difference. ****, $P < 0.0001$.

motor coordination and balance. Moreover, BALB/c mice receiving LTB-NS1 Δ_{63} protein gained daily body weight regularly (Fig. 7d), and maintained a healthy rectal temperature (Fig. 7e). Since flavivirus NS1 has been reported to trigger endothelial barrier dysfunction [23], we also examined whether LTB-NS1 Δ_{63} can induce vascular leakage. Treatment of 400 μ g LTB-NS1 Δ_{63} did not cause any vascular leakage in mouse brain and abdomen, in consistent to the negative control (PBS treatment). As a positive control, histamine phosphate treatment induced vascular leakage in the mouse abdomen (Fig. 7f–g). We did not observe vascular leakage in the brain, which may be due

to short treatment time of histamine phosphate or its inability to penetrate blood-brain barrier (Fig. 7h). Overall, LTB-NS1 Δ_{63} protein demonstrated favorable safety profile for mouse immunization.

3.8. LTB-NS1 Δ_{63} protein vaccination confers protection against JEV challenge

We next focused on LTB-NS1 Δ_{63} protein's function in anti-JEV infection. To this end, mice were immunized with either different recombinant proteins or vaccine JEV SA14–14–2, followed by

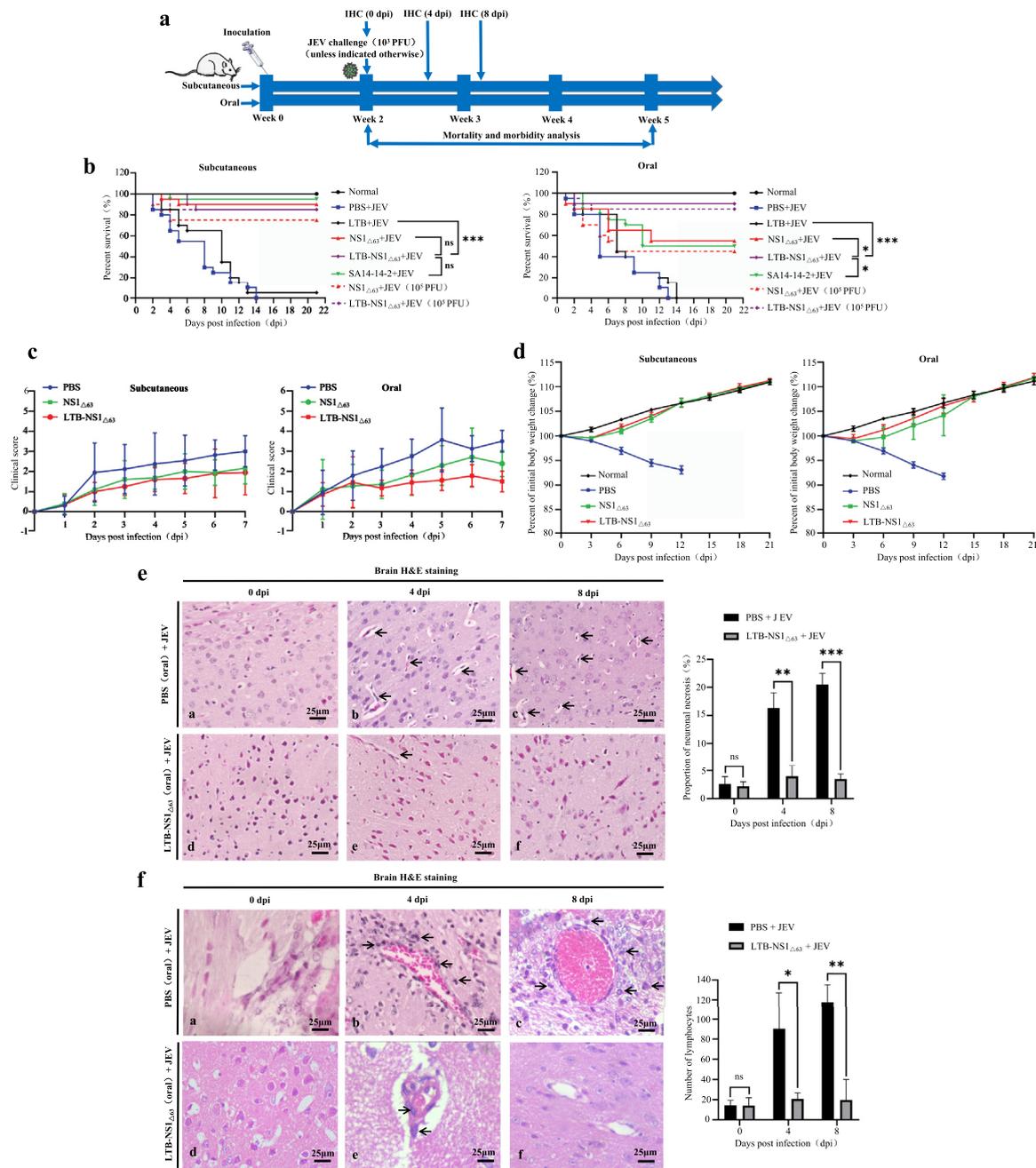


Fig. 8. LTB-NS1 $_{\Delta 63}$ protein confer protection against JEV challenge. (a) Scheme of protein immunization and JEV challenge. (b–f) Each mouse was administrated either subcutaneously or orally with 2 nmol of each recombinant protein followed by intraperitoneal injection of 10^3 or 10^5 PFU JEV P3 strain. Mice receiving 10^4 JEV vaccine SA14–14–2 were used as the positive controls. The survival rate (b, 20 mice/group), clinical score (c, 20 mice/group), body weight (d, 8 mice/group), and encephalitic pathogenic manifestation (e, f, 3 mice/group) were examined. (c) clinical score: 0=healthy; 1=anorexia, deprementia; 2=anorexia, deprementia, ruffled fur, hunched; 3=hindlimb weakness, drowsiness; 4=tetany, disorder of consciousness; 5=death. (e, f) Left panels show representative images; right panels show quantitation data. Error bars indicate SD of the mean ($n = 3–8$ or 20). A log-rank test was used to evaluate the inter-group difference in survival rate, and Student's *t*-test was used elsewhere. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

challenge with virulent JEV P3 (Fig. 8a). The survival data showed that all mice immunized with LTB or PBS died within 14 days regardless of the immunization regimens, indicating that 10^3 PFU of JEV P3 strain was lethal dose (Fig. 8b,c). Additionally, subcutaneous immunization with LTB-NS1 $_{\Delta 63}$ provided similar protection against JEV infection as those of NS1 $_{\Delta 63}$ and SA14–14–2 (Fig. 8b, left panel), whereas oral delivery of LTB-NS1 $_{\Delta 63}$ protein protected 90% of mice from death, much higher than that of NS1 $_{\Delta 63}$ protein (55% of survival rate) and SA14–14–2 (50% of survival rate) (Fig. 8b, right panel). One thing that needs to emphasize is the administration of LTB-NS1 $_{\Delta 63}$ protein still conferred 85% of protection even upon the challenge with 10^5 PFU of JEV P3 strain regardless protein administration routes

(subcutaneous or oral) (Fig. 8b,c). We also compared clinical scores of mice among PBS-, NS1 $_{\Delta 63}$ -, LTB-NS1 $_{\Delta 63}$ -immunized groups. Both subcutaneous and oral immunization of LTB-NS1 $_{\Delta 63}$ protein apparently lowered the clinical scores from day 2 (subcutaneous) or day 3 (oral) as compared to PBS-treated group (Fig. 8c). A decrease in body weight was observed in all treated mice regardless of the immunized route during the first 3 days post-infection (Fig. 8d). Later on, a gradual recovery in body weight occurred in mice immunized with NS1 $_{\Delta 63}$ or LTB-NS1 $_{\Delta 63}$, which caught up with that of normal mice at day 12 (subcutaneous) or day 15 (oral) after JEV infection (Fig. 8d). In the negative control group, a durative decrease in body weight was observed (Fig. 8d). One tendency that needs to note is oral

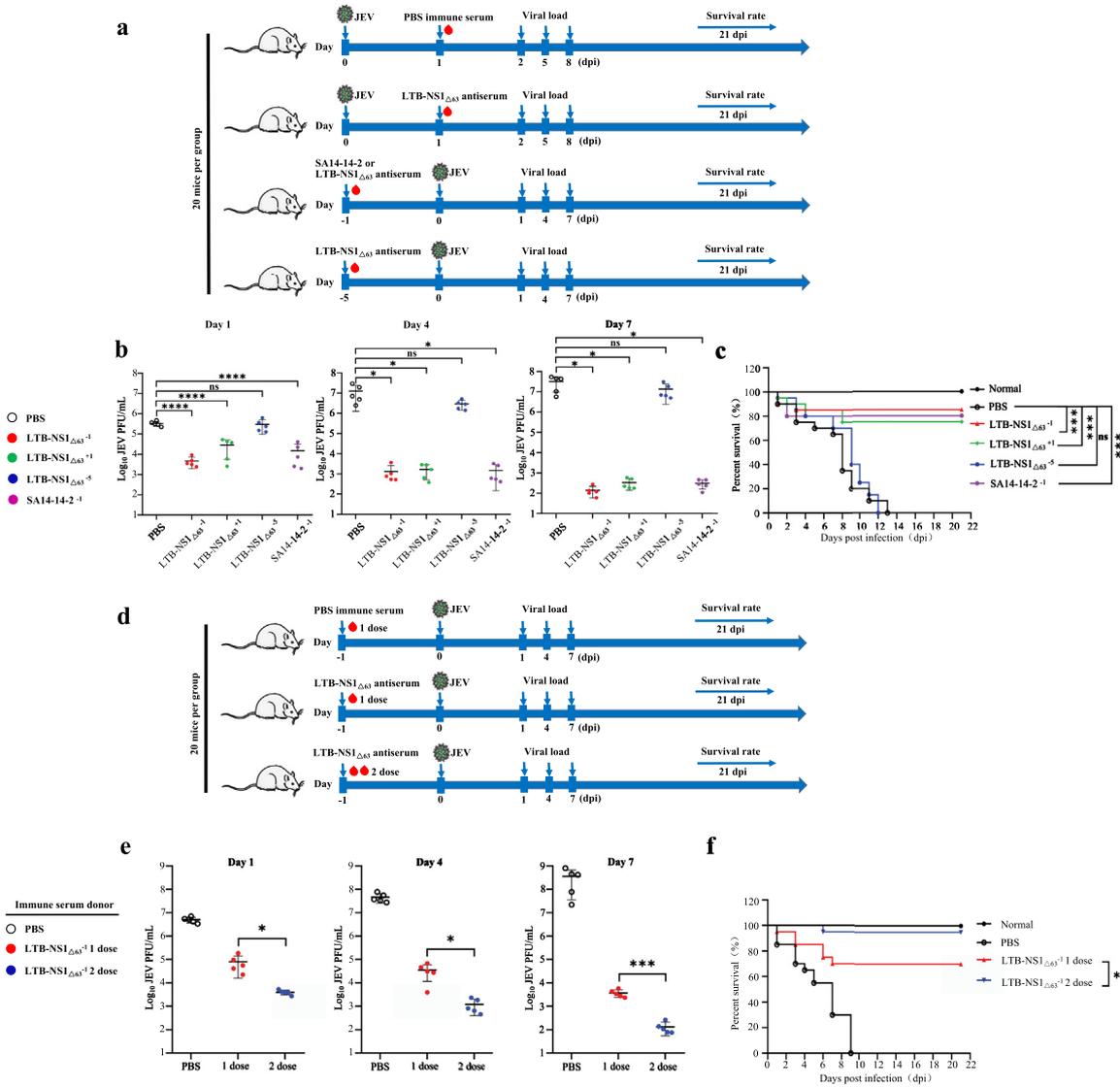


Fig. 9. Passive transfer of LTB-NS1 Δ 63-induced antisera rendered protection by inhibiting JEV replication. (a–d) Schemes of antiserum inoculation into mice in the context of JEV infection. 10^3 (a) or 10^4 (d) PFU of JEV P3 strain was used to infect mice. (b,c) Effect of different inoculation time-points of antiserum on the protection consequence. (e,f) Impact of the antiserum dose on its protective effect. (b,c, e,f) Viral load in mouse serum was determined by plaque assay with 5 mice per group. The survival rate of mice (20/group) was monitored for 21 days post-infection. A log-rank test was used to evaluate the inter-group difference, and Student’s *t*-test was used elsewhere. Error bars indicate SD of the mean (*n* = 5). *, *P* < 0.05; ***, *P* < 0.001; ****, *P* < 0.0001.

immunization of LTB-NS1 Δ 63 has lower clinical scores from 4 days post-infection and a faster recovery in body weight from 6 days post-infection than that of NS1 Δ 63, suggesting that LTB enhances the protective immunity of NS1 Δ 63 in the oral route (Fig. 8c-d). Neuro-pathogenesis is a crucial parameter in evaluating JEV infection. Therefore, we monitored histopathological changes in mouse brains following JEV infection between LTB-NS1 Δ 63-immunized and nonimmunized control group. Oral immunization of LTB-NS1 Δ 63 protein dramatically reduced the level of neuro-pathogenesis and the number of infiltrated lymphocytes in the brain at day 4 and 8 after JEV infection, compared to PBS-injection (Fig. 8e,f), suggesting that LTB-NS1 Δ 63 immunization can reduce histopathology caused by JEV infection.

3.9. LTB-NS1 Δ 63-induced antiserum provides protection by inhibiting JEV replication

Passive transfer of NS1 antiserum has been reported to induce protection against lethal challenge [24]. We wonder whether LTB-NS1 Δ 63-induced antiserum can inhibit JEV infection, thus protecting

against lethal viral challenge. BALB/c mice were treated according to the scheme showed in Fig. 9a. Passive transfer of LTB-NS1 Δ 63-induced antiserum at day 1 before or after challenge inhibited JEV replication and increased the survival rate of mice (Fig. 9b-c). A similar protection pattern was also observed in the group receiving SA14–14–2-immunized anti-serum at day 1 before the challenge (Fig. 9b,c). However, passive transfer of antiserum induced by LTB-NS1 Δ 63 at day 5 before challenge failed to restrict mouse JEV replication and increase the survival rate, possibly because of antibody degradation (Fig. 9b,c). Next, we examined the effect of antiserum dose on JEV replication and mouse survival ratio (Fig. 9d). As expected, the administration of two doses of LTB-NS1 Δ 63-induced antiserum caused a lower viral load and an increase in mouse survival rate than those of one dose of the same antiserum at day 4 and 7 after the virus challenge, suggesting that LTB-NS1 Δ 63-induced antiserum conferred protection against JEV in a dose-dependent manner (Fig. 9e,f). Collectively, these findings demonstrate that LTB-NS1 Δ 63-induced antiserum is protective against lethal challenge by limiting JEV replication.

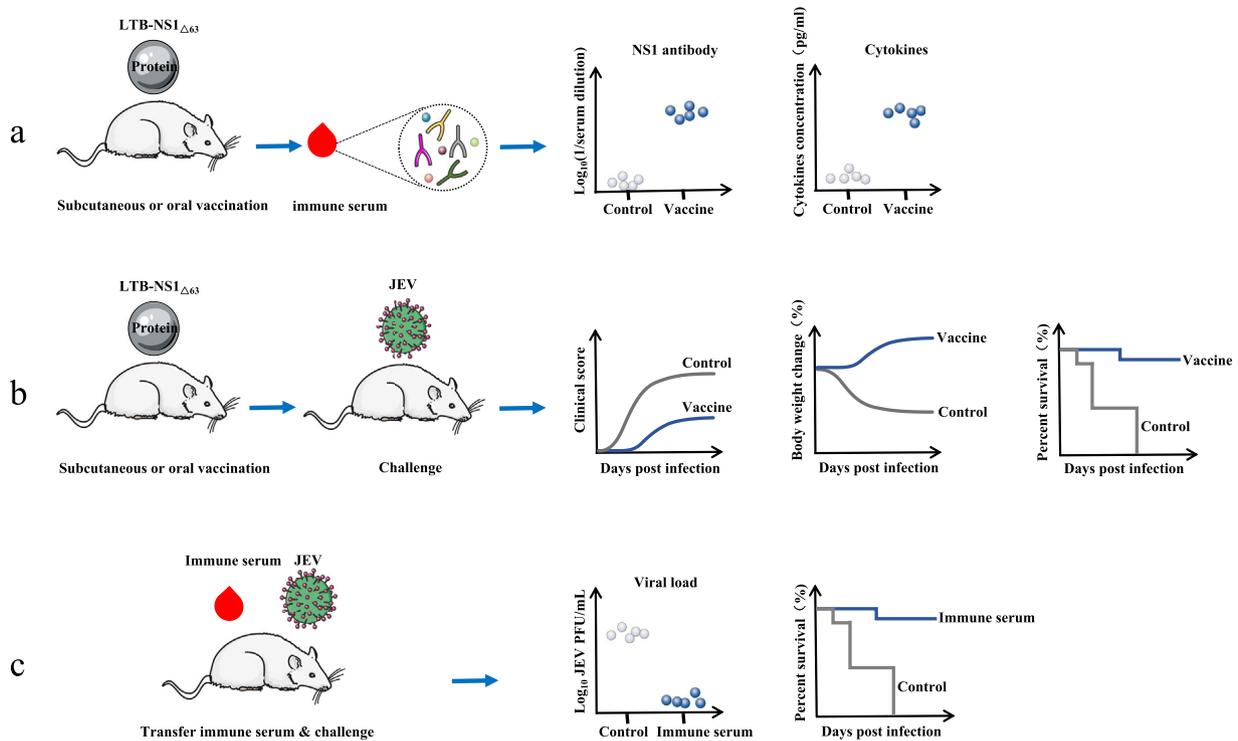


Fig. 10. Schematic diagram of LTB-NS1 Δ ₆₃-induced protective immunity. (a,b) Subcutaneous or oral administration of LTB-NS1 Δ ₆₃ induces humoral and cellular immune responses (a) and protection against JEV challenge (b). (c) LTB-NS1 Δ ₆₃-induced antisera confer mice resistant to JEV infection.

4. Discussion

Efforts have been made to express JEV NS1 protein using mammalian cells, insect cells, yeast, and bacterial cells [3,7,9,19,24–31]. *E. coli* is a lucrative expression system for recombinant proteins due to its rapid growth, easy manipulation, low cost, and high yield. The expression of full-length NS1 amplified from SA14–14–2 strain in *E. coli* was low (Suppl. Fig. 1a). Protscale hydrophilicity analysis reveals that C-terminus of NS1 contains two strong hydrophobic regions (aa 360–375 and 380–400), which could contribute to low production of NS1 protein (Fig. 1b). Therefore, we constructed the NS1 Δ ₆₃ lacking 63 amino acids at the C terminus of NS1. As expected, NS1 Δ ₆₃ had a much higher expression in *E. coli* than full-length one in our experiment conditions (Fig. 2a, Suppl. Fig. 1b). LTB is a potent mucosal immunogen and is commonly used as an adjuvant to stimulate an immune response [8]. Compared with traditional adjuvants like Alum or Freund's adjuvant, genetically detoxified *E. coli* heat-labile enterotoxin (LT_{C33D}) as an adjuvant has been reported to yield better efficacy against DENV challenge following DENV NS1 vaccination [32]. To enhance the mucosal immunogenicity of NS1 Δ ₆₃, we constructed LTB-NS1 Δ ₆₃ by fusing NS1 Δ ₆₃ with LTB. Consistent with NS1 Δ ₆₃, LTB-NS1 Δ ₆₃ was highly expressed in *E. coli* in the same conditions (Fig. 2b, Suppl. Fig. 1b,c). Both NS1 Δ ₆₃ and LTB-NS1 Δ ₆₃ proteins were expressed predominantly in an insoluble form (Suppl. Fig. 1b,c), which might be due to a high level of expression in cells or lack of post-translational modifications. Similarly, earlier works on the expression of JEV NS1 protein have also reported its tendency to form the inclusion within *E. coli* host [3–25].

Accumulating evidence demonstrates that JEV NS1 protein expressed in *E. coli* can retain its immunogenicity and elicit protective immune responses [3–25–28–30–33,34]. Prediction of potential B-cell antigenic epitopes reveals that the stretch of around 70 amino acids at JEV NS1 C-terminus hardly bear apparent linear B cell epitopes (Fig. 1a), implying that NS1 Δ ₆₃ hardly loses native antigenicity of NS1 in terms of humoral immunity. Consistently, both NS1 Δ ₆₃ and

LTB-NS1 Δ ₆₃ can be recognized by JEV-positive sera from mice, pigs, and humans (Fig. 3b–e). In addition, mouse antisera elicited by both NS1 Δ ₆₃ and LTB-NS1 Δ ₆₃ also recognize viral NS1 protein from JEV-infected BHK-21 cells (Fig. 4c–f). These findings suggest that *E. coli*-expressed NS1 Δ ₆₃ alone or fused with LTB keeps native NS1 antigenic epitopes. LTB conjugated proteins LTB-NS1 Δ ₆₃ and LTB-NS1 elicit similar levels of antibodies and cytokines including TNF- α , IFN- γ , IL-1 β , and IL-4 in mice (Fig. 5b,c, Fig. 6b–d), further supporting that NS1 Δ ₆₃ maintains NS1 immunogenicity. LTB-NS1 Δ ₆₃ elicits higher levels of antibodies and cytokines than NS1 Δ ₆₃ protein, particularly in the context of oral immunization of mice (Fig. 5b,c, Fig. 6b–d), suggesting that LTB as an adjuvant enhances NS1 Δ ₆₃-induced immune responses, particularly mucosal immunity (Fig. 5b,c, Fig. 6b–d). Notably, the presence of LTB augments NS1 Δ ₆₃-induced production of IFN- γ (Fig. 6b,c), which is a type II IFN that possesses broad-spectrum antiviral activity, thus providing cross-protection of infections from other viruses including flaviviruses. On the ground that NS1 Δ ₆₃ has much higher expression than NS1 in *E. coli*, and both LTB-NS1 Δ ₆₃ and LTB-NS1 exhibit similar ability to induce immune responses in examined mice (Figs. 5,6), we decided to use NS1 Δ ₆₃ and LTB-NS1 Δ ₆₃ for subsequent protective immune study in the context of viral challenge.

Vaccines play a dominant role in dealing with viral diseases. The complex zoonotic cycle for JEV highlights extreme importance of vaccine in controlling JEV transmission and related diseases. Live-attenuated vaccine SA14–14–2 is the most widely used vaccine. However, there is always a risk of live-attenuated strain reversing to high virulence strain. In addition, inactivated JEV vaccine has disadvantages including incomplete inactivation, multiple immunizations, and high costs [3]. Therefore, a safer and economical JEV vaccine is urgently needed in JE endemic areas, particularly poverty regions. Until now, recombinant protein-based vaccines, plasmid DNA-based vaccines, and virus vector-based vaccines against JEV have been developed with variable efficacy in animal models [35]. Recombinant protein-based vaccines represent promising approaches because of their safety, rapid development, and low-cost. Viral structural proteins are

ordinarily used for recombinant subunit vaccines since these proteins can induce neutralizing antibodies and interfere in the first stage of viral infection into host cells, virus entry. However, one potential risk for viral structural components as vaccine candidates is the phenomenon of ADE. ADE occurs when preexisting antibodies are unable to fully neutralize the infecting virion, instead, they enhance the uptake of the virion-antibody complex by Fc γ -receptor (Fc γ R)-bearing cells, thus promoting viral entry and disease severity. One remarkable example is some flavivirus E proteins result in antibody cross-reactivity and subsequent ADE of infection [22]. Thus, ADE issue inspires researchers to explore viral nonstructural proteins as potential subunit vaccines. Encouragingly, some nonstructural proteins from viruses have been demonstrated to induce protective immunity against viral challenges albeit without the capacity to elicit neutralizing antibodies [36]. Since flavivirus NS1 lacks on the virion's surface, ADE is not a concern for NS1-based vaccines. Antibodies against NS1 protein were shown to be protective against a number of different flaviviruses by multiple mechanisms such as Fc γ receptor-mediated viral clearance, complement-mediated cytotoxicity and complement-independent phagocytosis [24–36,37]. Interestingly, recent evidence indicates that NS1 immunization reduces flaviviral diseases by alleviating the role of NS1 protein, suppressing the immune response in mosquito midgut and disrupting endothelial barriers [38,39]. Additionally, the protective activity provided by the antibody against JEV NS1 could result from its binding to NS1, an elongated form of JEV NS1, which is involved in viral replication and neuroinvasion [40]. The above findings, taken together, suggest that flavivirus NS1 has been an attractive target for vaccine development. Various attempts have been made for NS1-based vaccines against JEV, DENV, YFV, or ZIKV. Flavivirus NS1 either alone or in combination with other viral proteins confer either partial or complete protection of mice or monkeys from lethal viral challenge [5–22–36–41]. More than 80% protection rates were observed in the mice immunized subcutaneously with NS1 $_{\Delta 63}$ or LTB-NS1 $_{\Delta 63}$ followed by lethal viral infection (Fig. 8b). Moreover, both NS1 $_{\Delta 63}$ and LTB-NS1 $_{\Delta 63}$ immunization dramatically reduced JEV-induced morbidity (Fig. 8c,d). In agreement with previous finding that LTB augments oral NS1 $_{\Delta 63}$ -induced immune responses (Figs. 5,6), oral immunization with LTB-NS1 $_{\Delta 63}$ confers better protection than oral immunization with NS1 $_{\Delta 63}$ in JEV infection setting (Fig. 8b–d), suggesting that fused LTB as an adjuvant enhances protective immunity of NS1 $_{\Delta 63}$ in the context of oral immunization. Previous studies have shown that passive immunization with the antibodies against NS1 of DENV, WNV, or ZIKV confers complete or partial protection upon a lethal viral challenge in animal models [24–36–42–43]. We also evaluate protection efficiency of LTB-NS1 $_{\Delta 63}$ -immunized mouse sera. Protective immunity is observed in the mice receiving LTB-NS1 $_{\Delta 63}$ -immunized antiserum followed by JEV infection (Fig. 9b,c, 9e,f). Taken together, these data demonstrate that LTB-NS1 $_{\Delta 63}$ could serve as a potent oral JEV vaccine. JEV has been reported to transmit between pigs by contact or oronasal inoculation [44]. Thus, the activation of mucosal immunity plays an important role against JEV transmission. Accumulating data has emerged to develop oral vaccines against JEV and demonstrate the effectiveness of mucosal vaccination approach for JEV vaccine, particularly in the presence of the adjuvant [28,45–52]. Most of the flaviviruses are arboviruses that transmit mainly through ticks or mosquito bites. Oral vaccines against other arboviruses such as DENV and ZIKV have also been developed [53,54]. All of these evidence highlights the value of oral vaccines against arboviruses including JEV.

In addition to eliciting protective immune responses, flavivirus NS1 has been implicated in the pathogenesis in the infected host as mentioned above [38,39–41]. Flavivirus NS1 triggers tissue-specific vascular endothelial dysfunction, reflecting disease tropism. NS1 from DENV that causes systemic disease induces permeability in various endothelial cells *in vitro*, whereas NS1 from JEV that causes encephalitis induces permeability only in brain endothelial cells *in*

vitro. DENV NS1 *in vivo* has been shown to induce vascular leakage and high level of this protein is associated with severe disease. However, whether JEV NS1 induces vascular leakage *in vivo* and whether there is a correlation between vascular leakage and JEV-related diseases remain unknown [23]. It should be noticed that flavivirus NS1-induced endothelial permeability correlates with the level and category of NS1 protein [39]. Similar to flavivirus NS1 protein, NS1 antibodies have also been reported to be involved in the pathogenesis of flavivirus. Compelling evidence obtained *in vitro* and from animal models indicates that DENV NS1 antibody might cross-react with surface components on human platelets and endothelial cells. Such interactions may result in vascular leakage and other dengue-related symptoms [37]. On the other hand, DENV NS1 immunization was reported to reduce dengue-related diseases by inhibiting the disruption of NS1 protein on endothelial barriers possibly via the engagement of NS1-induced antibody with tNS1 protein [38,39]. Therefore, DENV NS1 antibody can either be protective or deleterious to the host, possibly depending on its level and category [37]. The C-terminal region of DENV NS1 protein contains cross-reactive epitopes, which results in cross-reactivity of DENV NS1 antibody with human platelets and endothelial cells and subsequent functional disturbances. Chimera DENV NS1 protein replacing C-terminal region with the corresponding JEV NS1 fragment reduces DENV-induced prolonged bleeding time, local skin hemorrhage, and viral load compared to original one [42]. In contrast, JEV NS1 antibody does not cause cell damage probably because of the structural differences in the β -ladder domain of C-termini between DENV and JEV [37]. Similarly, our data show that immunization of LTB-NS1 $_{\Delta 63}$ had no induction on vascular leakage (Fig. 7f–h), which is consistent with the fact that JEV infection does not cause hemorrhagic manifestations. In addition, cytotoxicity experiments show that both NS1 $_{\Delta 63}$ and LTB-NS1 $_{\Delta 63}$ proteins are nontoxic to BHK-21 cells, even when the dosage reaches 500 μ g/ml (Fig. 7a). The safety test with animals demonstrates that LTB-NS1 $_{\Delta 63}$ protein had no obvious adverse influence on mouse vitality, body weight and temperature, with a dosage reaching 400 μ g per mouse (Fig. 7b,e). These findings imply that *E.coli*-expressed LTB-NS1 $_{\Delta 63}$ is safe as a vaccine candidate.

In conclusion, we describe a novel JEV NS1-based vaccine, LTB-NS1 $_{\Delta 63}$, which induces robust humoral and cellular immune responses, confers protection against lethal JEV challenge in subcutaneous and oral immunization. Passive transfer of LTB-NS1 $_{\Delta 63}$ -induced antiserum also provides protective immunity against JEV infection (Fig. 10). LTB-NS1 $_{\Delta 63}$ has no risk inducing ADE in individuals living in areas endemic for flaviviruses since this protein is absent from the virion's surface. Moreover, our laboratory work shows that LTB-NS1 $_{\Delta 63}$ is highly safe *in vitro* and *in vivo* without obvious side effects. Based on its protective efficiency, high safety, low-cost, convenient immune approach, and high conservation of flavivirus NS1, LTB-NS1 $_{\Delta 63}$ could be a promising oral vaccine candidate against JEV as well as other flaviviruses. Our research also offers a promising strategy to develop other oral flavivirus vaccines.

5. Contributors

All authors have read and approved the final version of the manuscript, have had access to the raw data, and ensure it is the case. Jiawu Wan and Ting Wang performed the majority of experiments, interpreted the data, and wrote the manuscript. Jing Xu performed antibody titration and cytokine measurement. Tao Ouyang collected swine samples and performed ELISA assays; Shiqi Weng obtained human sera and carried out the immune blotting assays. Qianruo Wang and Yanni Zhang performed protein expression and purification. Yihan Li and Xiaoling Wang performed writing editing and review; Yu Wang and Xiu Xin constructed plasmids and performed data analysis. Jiawu Wan, Ting Wang, Jing Xu, Tao Ouyang, Shiqi Weng, Sha Li, and Lingbao Kong verified the accuracy of the

underlying data. Sha Li and Lingbao Kong developed the research plan and experimental strategy, analyzed data, and wrote the paper.

6. Data sharing statement

The main data supporting the findings of this study are available within the paper and its supplementary materials. The data not shown can be available from the corresponding author SL and LK.

Declaration of Competing Interest

The authors have declared no conflicts of interest.

Acknowledgements

This study was funded by research grants from National Natural Science Foundation of China (grants 31460667, 31860038, and 31960699) and Jiangxi Province (grants 20161BBF60084, GJJ180239, and GJJ200405).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103353.

References

- Guo F, Yu X, Xu A, Xu J, Wang Q, Guo Y, et al. Japanese encephalitis virus induces apoptosis by inhibiting Foxo signaling pathway. *Vet Microbiol* 2018;220:73–82. doi: 10.1016/j.vetmic.2018.05.008.
- Wang Q, Xin X, Wang T, Wan J, Ou Y, Yang Z, et al. Japanese encephalitis virus induces apoptosis and encephalitis by activating the PERK pathway. *J Virol* 2019;93(17). doi: 10.1128/JVI.00887-19.
- Choi JW, Eom HJ, Kim HY. Non-structural protein 1 from Japanese encephalitis virus expressed in *E. coli* retains its molecular weight and immunogenicity. *Protein Expr Purif* 2020;169:105548. doi: 10.1016/j.pep.2019.105548.
- Chakraborty S, Barman A, Deb B. Japanese encephalitis virus: a multi-epitope loaded peptide vaccine formulation using reverse vaccinology approach. *Infect Genet Evol* 2020;78:104106. doi: 10.1016/j.meegid.2019.104106.
- Rastogi M, Sharma N, Singh SK. Flavivirus NS1: a multifaceted enigmatic viral protein. *Virology* 2016;13:131. doi: 10.1186/s12985-016-0590-7.
- Poonsiri T, Wright G, Diamond MS, Turtle L, Solomon T, Antonyuk SV. Structural study of the C-terminal domain of nonstructural protein 1 from Japanese encephalitis virus. *J Virol* 2018;92(7). doi: 10.1128/JVI.01868-17.
- Konishi E, Shoda M, Ajiro N, Kondo T. Development and evaluation of an enzyme-linked immunosorbent assay for quantifying antibodies to Japanese encephalitis virus nonstructural 1 protein to detect subclinical infections in vaccinated horses. *J Clin Microbiol* 2004;42(11):5087–93. doi: 10.1128/JCM.42.11.5087-5093.2004.
- Tiwari S, Verma PC, Singh PK, Tuli R. Plants as bioreactors for the production of vaccine antigens. *Biotechnol Adv* 2009;27(4):449–67. doi: 10.1016/j.biotechadv.2009.03.006.
- Flamand M, Deubel V, Girard M. Expression and secretion of Japanese encephalitis virus nonstructural protein NS1 by insect cells using a recombinant baculovirus. *Virology* 1992;191(2):826–36. doi: 10.1016/0042-6822(92)90258-q.
- Huang M, Xu A, Wu X, Zhang Y, Guo Y, Guo F, et al. Japanese encephalitis virus induces apoptosis by the IRE1/JNK pathway of ER stress response in BHK-21 cells. *Arch Virol* 2016;161(3):699–703. doi: 10.1007/s00705-015-2715-5.
- Xiang ZH, Cai WJ, Zhao P, Kong LB, Ye LB, Wu ZH. Purification and application of bacterially expressed chimeric protein E1E2 of hepatitis C virus. *Protein Expr Purif* 2006;49(1):95–101. doi: 10.1016/j.pep.2006.02.013.
- Wang T, Fang L, Zhao F, Wang D, Xiao S. Exosomes mediate intercellular transmission of porcine reproductive and respiratory syndrome virus. *J Virol* 2018;92(4). doi: 10.1128/JVI.01734-17.
- Kong L, Fujimoto A, Nakamura M, Aoyagi H, Matsuda M, Watashi K, et al. Prolactin regulatory element binding protein is involved in hepatitis C virus replication by interaction with NS4B. *J Virol* 2016;90(6):3093–111. doi: 10.1128/JVI.01540-15.
- Kong L, Aoyagi H, Yang Z, Ouyang T, Matsuda M, Fujimoto A, et al. Surfite 4 contributes to the replication of hepatitis C virus using double-membrane vesicles. *J Virol* 2020;94(2). doi: 10.1128/JVI.00858-19.
- Li S, Choi HJ, Felio K, Wang CR. Autoreactive CD1b-restricted T cells: a new innate-like T-cell population that contributes to immunity against infection. *Blood* 2011;118(14):3870–8. doi: 10.1182/blood-2011-03-341941.
- Kong L, Li S, Liao Q, Zhang Y, Sun R, Zhu X, et al. Oleoic acid and ursolic acid: novel hepatitis C virus antivirals that inhibit NS5B activity. *Antivir Res* 2013;98(1):44–53. doi: 10.1016/j.antiviral.2013.02.003.
- Simons FE, Silver NA, Gu X, Simons KJ. Skin concentrations of H1-receptor antagonists. *J Allergy Clin Immunol* 2001;107(3):526–30. doi: 10.1067/mai.2001.113080.
- Ahishali B, Kaya M. Evaluation of blood-brain barrier integrity using vascular permeability markers: Evans blue, sodium fluorescein, albumin-alexa fluor conjugates, and horseradish peroxidase. *Methods Mol Biol* 2020. doi: 10.1007/978-1-4939-9316-1_16.
- Wang B, Hua RH, Tian ZJ, Chen NS, Zhao FR, Liu TQ, et al. Identification of a virus-specific and conserved B-cell epitope on NS1 protein of Japanese encephalitis virus. *Virus Res* 2009;141(1):90–5. doi: 10.1016/j.virusres.2009.01.004.
- Lin YL, Chen LK, Liao CL, Yeh CT, Ma SH, Chen JL, et al. DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. *J Virol* 1998;72(1):191–200.
- Jain N, Oswal N, Chawla AS, Agrawal T, Biswas M, Vrati S, et al. CD8 T cells protect adult naive mice from JEV-induced morbidity via lytic function. *PLOS Negl Trop Dis* 2017;11(2):e0005329. doi: 10.1371/journal.pntd.0005329.
- Grubor-Bauk B, Wijesundara DK, Masavuli M, Abbink P, Peterson RL, Prow NA, et al. NS1 DNA vaccination protects against Zika infection through T cell-mediated immunity in immunocompetent mice. *Sci Adv* 2019;5(12):eaax2388. doi: 10.1126/sciadv.aax2388.
- Puerta-Guardo H, Glasner DR, Espinosa DA, Biering SB, Patana M, Ratnasiri K, et al. Flavivirus NS1 triggers tissue-specific vascular endothelial dysfunction reflecting disease tropism. *Cell Rep* 2019;26(6):1598–1613.e8. doi: 10.1016/j.celrep.2019.01.036.
- Li Y, Counor D, Lu P, Duong V, Yu Y, Deubel V. Protective immunity to Japanese encephalitis virus associated with anti-NS1 antibodies in a mouse model. *Virology* 2012;9:135. doi: 10.1186/1743-422X-9-135.
- Dhanze H, Bhilegaonkar KN, Rawat S, Chethan KH, Kumar A, Gulati BR, et al. Development of recombinant nonstructural 1 protein based indirect enzyme linked immunosorbent assay for sero-surveillance of Japanese encephalitis in swine. *J Virol Methods* 2019;272:113705. doi: 10.1016/j.jviromet.2019.113705.
- Hua RH, Liu LK, Chen ZS, Li YN, Bu ZG. Comprehensive mapping antigenic epitopes of NS1 protein of Japanese encephalitis virus with monoclonal antibodies. *PLOS One* 2013;8(6):e67553. doi: 10.1371/journal.pone.0067553.
- Peck KA, Lomax DP, Olson OP, Sol SY, Swanson P, Johnson LL. Development of an enzyme-linked immunosorbent assay for quantifying vitellogenin in Pacific salmon and assessment of field exposure to environmental estrogens. *Environ Toxicol Chem* 2011;30(2):477–86. doi: 10.1002/etc.390.
- Lin TS, Chuang CC, Hsu HL, Liu YT, Lin WP, Liang CC, et al. Role of amphotericin B upon enhancement of protective immunity elicited by oral administration with liposome-encapsulated-Japanese encephalitis virus nonstructural protein 1 (NS1) in mice. *Microb Pathog* 2010;49(3):67–74. doi: 10.1016/j.micpath.2010.04.002.
- Krishna VD, Rangappa M, Satchidanandam V. Virus-specific cytolytic antibodies to nonstructural protein 1 of Japanese encephalitis virus effect reduction of virus output from infected cells. *J Virol* 2009;83(10):4766–77. doi: 10.1128/JVI.01850-08.
- Lin CW, Liu KT, Huang HD, Chen WJ. Protective immunity of *E. coli*-synthesized NS1 protein of Japanese encephalitis virus. *Biotechnol Lett* 2008;30(2):205–14. doi: 10.1007/s10529-007-9529-9.
- Xu G, Xu X, Li Z, He Q, Wu B, Sun S, et al. Construction of recombinant pseudorabies virus expressing NS1 protein of Japanese encephalitis (SA14-14-2) virus and its safety and immunogenicity. *Vaccine* 2004;22(15–16):1846–53. doi: 10.1016/j.vaccine.2003.09.015.
- Amorim JH, Diniz MO, Cariri FA, Rodrigues JF, Bizerra RS, Goncalves AJ, et al. Protective immunity to DENV2 after immunization with a recombinant NS1 protein using a genetically detoxified heat-labile toxin as an adjuvant. *Vaccine* 2012;30(5):837–45. doi: 10.1016/j.vaccine.2011.12.034.
- Tafuku S, Miyata T, Tadano M, Mitsumata R, Kawakami H, Harakuni T, et al. Japanese encephalitis virus structural and nonstructural proteins expressed in *Escherichia coli* induce protective immunity in mice. *Microbes Infect* 2012;14(2):169–76. doi: 10.1016/j.micinf.2011.09.004.
- Tripathi NK, Kumar JS, Biswal KC, Rao PV. Production of recombinant nonstructural 1 protein in *Escherichia coli* for early detection of Japanese encephalitis virus infection. *Microb Biotechnol* 2012;5(5):599–606. doi: 10.1111/j.1751-7915.2012.00344.x.
- Nath B, Vandna Saini HM, Prasad M, Kumar S. Evaluation of Japanese encephalitis virus E and NS1 proteins immunogenicity using a recombinant Newcastle disease virus in mice. *Vaccine* 2020;38(7):1860–8. doi: 10.1016/j.vaccine.2019.11.088.
- Bailey MJ, Duehr J, Dulin H, Broecker F, Brown JA, Arumemi FO, et al. Human antibodies targeting Zika virus NS1 provide protection against disease in a mouse model. *Nat Commun* 2018;9(1):4560. doi: 10.1038/s41467-018-07008-0.
- Reyes-Sandoval A, Ludert JE. The dual role of the antibody response against the flavivirus non-structural protein 1 (NS1) in protection and Immuno-Pathogenesis. *Front Immunol* 2019;10:1651. doi: 10.3389/fimmu.2019.01651.
- Liu J, Liu Y, Nie K, Du S, Qiu J, Pang X, et al. Flavivirus NS1 protein in infected host sera enhances viral acquisition by mosquitoes. *Nat Microbiol* 2016;1(9):16087. doi: 10.1038/nmicrobiol.2016.87.
- Beatty PR, Puerta-Guardo H, Killingbeck SS, Glasner DR, Hopkins K, Harris E. Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. *Sci Transl Med* 2015;7(304):304ra141. doi: 10.1126/scitranslmed.aaa3787.
- Zhou D, Li Q, Jia F, Zhang L, Wan S, Li Y, et al. The Japanese encephalitis virus NS1 protein inhibits type I IFN production by targeting MAVS. *J Immunol* 2020;204(5):1287–98. doi: 10.4049/jimmunol.1900946.
- Muller DA, Young PR. The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. *Antivir Res* 2013;98(2):192–208. doi: 10.1016/j.antiviral.2013.03.008.
- Wan SW, Lu YT, Huang CH, Lin CF, Anderson R, Liu HS, et al. Protection against dengue virus infection in mice by administration of antibodies against modified

- nonstructural protein 1. PLOS One 2014;9(3):e92495. doi: [10.1371/journal.pone.0092495](https://doi.org/10.1371/journal.pone.0092495).
- [43] Chung KM, Nybakken GE, Thompson BS, Engle MJ, Marri A, Fremont DH, et al. Antibodies against West Nile Virus nonstructural protein NS1 prevent lethal infection through Fc gamma receptor-dependent and -independent mechanisms. J Virol 2006;80(3):1340–51. doi: [10.1128/JVI.80.3.1340-1351.2006](https://doi.org/10.1128/JVI.80.3.1340-1351.2006).
- [44] Ricklin ME, Garcia-Nicolas O, Brechbuhl D, Python S, Zumkehr B, Nougairede A, et al. Vector-free transmission and persistence of Japanese encephalitis virus in pigs. Nat Commun 2016;7:10832. doi: [10.1038/ncomms10832](https://doi.org/10.1038/ncomms10832).
- [45] Appaiahgari MB, Saini M, Rauthan M, Jyoti Vrati S. Immunization with recombinant adenovirus synthesizing the secretory form of Japanese encephalitis virus envelope protein protects adenovirus-exposed mice against lethal encephalitis. Microbes Infect 2006;8(1):92–104. doi: [10.1016/j.micinf.2005.05.023](https://doi.org/10.1016/j.micinf.2005.05.023).
- [46] Harakuni T, Kohama H, Tadano M, Uechi G, Tsuji N, Matsumoto Y, et al. Mucosal vaccination approach against mosquito-borne Japanese encephalitis virus. Jpn J Infect Dis 2009;62(1):37–45.
- [47] Khang G, Cho JC, Lee JW, Rhee JM, Lee HB. Preparation and characterization of Japanese encephalitis virus vaccine loaded poly(L-lactide-co-glycolide) microspheres for oral immunization. Biomed Mater Eng 1999;9(1):49–59.
- [48] Li P, Zheng QS, Wang Q, Li Y, Wang EX, Liu JJ, et al. Immune responses of recombinant adenoviruses expressing immunodominant epitopes against Japanese encephalitis virus. Vaccine 2008;26(46):5802–7. doi: [10.1016/j.vaccine.2008.08.035](https://doi.org/10.1016/j.vaccine.2008.08.035).
- [49] Ramakrishna C, Desai A, Shankar SK, Chandramuki A, Ravi V. Oral immunisation of mice with live Japanese encephalitis virus induces a protective immune response. Vaccine 1999;17(23–24):3102–8. doi: [10.1016/S0264-410X\(99\)00137-1](https://doi.org/10.1016/S0264-410X(99)00137-1).
- [50] Ramakrishna C, Ravi V, Desai A, Subbakrishna DK, Shankar SK, Chandramuki A. T helper responses to Japanese encephalitis virus infection are dependent on the route of inoculation and the strain of mouse used. J Gen Virol 2003;84:1559–67 (Pt 6). doi: [10.1099/vir.0.18676-0](https://doi.org/10.1099/vir.0.18676-0).
- [51] Rauthan M, Kaur R, Appaiahgari MB, Vrati S. Oral immunization of mice with Japanese encephalitis virus envelope protein synthesized in Escherichia coli induces anti-viral antibodies. Microbes Infect 2004;6(14):1305–11. doi: [10.1016/j.micinf.2004.08.010](https://doi.org/10.1016/j.micinf.2004.08.010).
- [52] Wang Y, Deng H, Zhang X, Xiao H, Jiang Y, Song Y, et al. Generation and immunogenicity of Japanese encephalitis virus envelope protein expressed in transgenic rice. Biochem Biophys Res Commun 2009;380(2):292–7. doi: [10.1016/j.bbrc.2009.01.061](https://doi.org/10.1016/j.bbrc.2009.01.061).
- [53] Marquez-Escobar VA, Banuelos-Hernandez B, Rosales-Mendoza S. Expression of a Zika virus antigen in microalgae: towards mucosal vaccine development. J Biotechnol 2018;282:86–91. doi: [10.1016/j.jbiotec.2018.07.025](https://doi.org/10.1016/j.jbiotec.2018.07.025).
- [54] van Eerde A, Gottschamel J, Bock R, Hansen K, Munang'Andu HM, Daniell H, et al. Production of tetravalent dengue virus envelope protein domain III based antigens in lettuce chloroplasts and immunologic analysis for future oral vaccine development. Plant Biotechnol J 2019;17(7):1408–17. doi: [10.1111/pbi.13065](https://doi.org/10.1111/pbi.13065).