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

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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-NS1Δ63) with a truncated NS1 protein (NS1Δ63) 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-NS1Δ63 was assessed by determining its toxicity in vitro and in vivo. Protective capability of LTB-NS1Δ63 and its-induced antiserum was evaluated in the mice challenged with JEV by analyzing mortality and morbidity.

Findings: LTB-NS1Δ63 induced immune responses to a similar level as LTB-NS1, but more robust than NS1Δ63 alone, particularly in the context of oral immunization of mice. Oral vaccination of LTB-NS1Δ63 led to a higher survival rate than that of NS1Δ63 or live-attenuated JEV vaccine SA14 14–2 in the mice receiving lethal JEV challenge. LTB-NS1Δ63 protein also significantly decreases the morbidity of JEV-infected mice. In addition, passive transfer of LTB-NS1Δ63-induced antiserum provides a protection against JEV infection in mice.

Interpretation: NS1Δ63 bears JEV NS1 antigenicity. Besides, LTB-NS1Δ63 could serve as a novel protein-based mucosa vaccine targeting JEV and other flaviviruses.

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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...
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. Moreover, the fusion of LTB with NS1 protein bears NS1 antigenicity and is highly safe both in vitro and in vivo. 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-NS1Δ63) by fusing the truncated NS1 protein, lacking the hydrophobic C-terminal 63 amino acids (NS1Δ63), with LTB, a potent mucosal adjuvant. LTB-NS1Δ63 protein was highly expressed in E. coli and induced immune responses to a similar level as LTB-NS1, but more robust than NS1Δ63 protein, particularly by oral vaccine delivery. LTB-NS1Δ63 protein protected about 90% of mice from the lethal challenge of JEV, which is either comparable (subcutaneous vaccination) or better (oral vaccination) than NS1Δ63 protein or live-attenuated vaccine SA14-14-2. LTB-NS1Δ63 protein also significantly decreased the morbidity of mice upon JEV challenge. Additionally, LTB-NS1Δ63-induced antisera provided similar protection against JEV infection to SA14-14-2-induced antisera.

Implications of all the available evidence

E.coli-expressed NS1Δ63 bears JEV NS1 antigenicity. LTB-NS1Δ63 could serve as a novel protein-based mucosa vaccine strategy targeting JEV as well as other flaviviruses.

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-NS1Δ63) by fusing the truncated JEV NS1 (NS1Δ63), lacking the hydrophobic C-terminal 63 amino acids, with LTB. LTB-NS1Δ63 protein bears NS1 antigenicity and is highly safe both in vitro and in vivo. Moreover, the fusion of LTB with NS1Δ63 enhances mucosa immunity of NS1Δ63 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 (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 (RID: AB_11,232,599), actin (RID: AB_2,687,938), HRP-labeled goat anti-mouse (RID: 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 (RID: AB_775,815) was purchased from Abcam (Wuhan, China). Alexa Fluor 488-conjugated anti-mouse IgG (RID: AB_2,556,548) and Trizol RNA extraction kit (Cat:15,596,018) were purchased from Invitrogen (California, USA). Lactate dehydrogenase kit (Cat: ml003416), ELISA Kit of Swine JEV antibody (Cat: m1611403), and anti-mouse IFN-γ/IL-1β antibodies (Cat:ml059749/Cat:ml063132) were purchased from Shanghai Enzyme-linked Biotechnology (Shanghai, China). Anti-mouse Japanese encephalitis antibody (IgG) ELISA kit (Cat: CBEB-10752220MO) was purchased from Wuhan Huamei Biological Engineering Co., Ltd (Wuhan, China). Mouse-1L-4 pre-coated ELISA kit (Cat:1,210,402) was purchased from Dakewe Biotech Co., Ltd (Shenzhen, China). Mouse IFN-γ ELISpot kit (Cat: ALP) (Cat: 3321-APW-2) was purchased from Mabtech (Stockholm, Sweden). Ni-plates (Cat:163,038,916) were purchased from QIAGEN (Germany). Guinea pig complement (Cat: GC999-0005) 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.iedb.org/main/). NS1 hydrophobicity was analyzed using the software Protscale (https://www.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 (NS1Δ63) 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-NS1Δ63, and pET-LTB. Overlapping PCR was used to amplify the fused segments LTB-NS1 and LTB-NS1Δ63, which were further cloned into the pET-28a vector to get pET-LTB-NS1 and pET-LTB-NS1Δ63, respectively. All the cloned gene fragments were confirmed by sequencing.

2.5. Expression and purification of recombinant proteins

Recombinant plasmids pET-NS1, pET-NS1Δ63, pET-LTB-NS1Δ63, pET-LTB-NS1, and pET-LTB were transformed into E.coli BL21 (DE3) strain
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 antisera 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 Na$_2$CO$_3$, 0.034 M NaHCO$_3$, 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$_{63}$ 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 cytolysis 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 cytolyis 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 × (experimental LDH release - spontaneous LDH release)/ (maximum LDH release - spontaneous LDH release).

2.10. Cytokine profiling in serum

Sera were collected from protein-immunized mice. Cytokines including IL-1β, 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 μL of biotinylated antibody was added to each well and incubated for 90 min at 37 °C. After 4 times of wash, 100 μL 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 μg of each protein and 10$^5$ splenocytes were washed with PBS and added to 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 μL/well MTT (5 mg/mL stock in PBS) was added and further incubated for 4 h. After media removal, 150 μL 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$_{63}$ protein at a concentration ranging from 100 μg to 400 μg/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$_{63}$ 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$_{63}$ on vascular leakage, each BALB/c mouse was injected intraperitoneally with 250 μL of 400 μg of recombinant protein, 0.1% (w/v) histamine phosphate (positive control) [17], or PBS (negative control). Subsequently, 100 μL 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.
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 NS1Δ63, 120 μg of LTB-NS1Δ63, 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 intraperitoneally injected intraperitoneally with 1 × 10^5 or 1 × 10^6 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-NS1Δ63–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 °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-NS1Δ63−5 group), or day −1 (LTB-NS1Δ63−1 group) or day 1 (LTB-NS1Δ63+1 group). Viral load in sera at day 1, 4, 7 (LTB-NS1Δ63−1 group) or day 2, 5, 8 (control group and LTB-NS1Δ63+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 Protoscale 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 Protoscale 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 (NS1Δ63) 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 NS1Δ63 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 NS1Δ63 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-NS1Δ63, 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 NS1Δ63, LTB-NS1Δ63, LTB-NS1, and LTB above 90%...
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) NS1Δ63 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-NS1Δ63 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

| Projects | LTB | NS1Δ63 | NS1 | LTB-NS1Δ63 | 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 NS1Δ63, LTB-NS1Δ63, NS1, LTB-NS1Δ63, and LTB proteins in E.coli.

3.3. Antigenicity of recombinant NS1Δ63 and LTB-NS1Δ63 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 sup-porting 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 NS1Δ63 and LTB-NS1Δ63 proteins with the sera from JEV-infected mice. The data from both immunoblotting and ELISA assays unveiled that NS1Δ63 and LTB-NS1Δ63 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. NS1Δ63 and LTB-NS1Δ63 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 NS1Δ63 and LTB-NS1Δ63 proteins (Fig. 3e). Among 40 serum samples derived from either healthy or JEV-infected pigs, 97.5% of agreement ratio between NS1Δ63-based and NS1-based ELISA highlighted the comparable antigenicity between NS1Δ63 and NS1 proteins (Fig. 3f, Table 2). Altogether, these data demonstrated E.coli-expressed recombinant proteins NS1Δ63 and LTB-NS1Δ63 retained NS1 antigenicity.

3.4. NS1Δ63 and LTB-NS1Δ63 proteins elicit JEV NS1-specific antibody in mice

Data that E.coli-expressed NS1Δ63 and LTB-NS1Δ63 Proteins recognize JEV-positive sera (Fig. 3c–f) prompts us to address whether NS1Δ63- and LTB-NS1Δ63-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 NS1Δ63-induced antisera or commercial anti-NS1 antibody were used as detecting reagents, implying that NS1Δ63-induced mouse antiserum could react with JEV NS1 protein (Fig. 4c). Furthermore, immunoblotting assays showed that the antisera from NS1Δ63- and LTB-NS1Δ63-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-NS1Δ63-immunized mice (Fig. 4d–f). As negative controls, cell lysate from mock-infected BHK-21 cells failed to interact with the antisera from NS1Δ63-, LTB-NS1Δ63- or LTB-NS1-immunized mice (Fig. 4d–f).

Taken together, these results demonstrate that NS1Δ63 and LTB-NS1Δ63 proteins elicit JEV NS1-specific antibodies in mice.

3.5. LTB-NS1Δ63 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-NS1Δ63 induced more robust antibody titers than those of NS1Δ63 during the whole examined period, especially on week 2 (1:8200 for LTB-NS1Δ63 and 1:6600 for NS1Δ63), week 4 (1:13,700 for LTB-NS1Δ63 and 1:10,100 for NS1Δ63), and week 8 (1:17,900 for LTB-NS1Δ63 and 1:15,100 for NS1Δ63) (Fig. 5b, Table 3).

Interestingly, a similar pattern was detected in the oral vaccine regimen, especially on week 6 (1:16,200 for LTB-NS1Δ63 and 1:4600 for NS1Δ63), week 7 (1:16,600 for LTB-NS1Δ63 and 1:4800 for NS1Δ63), and week 8 (1:17,800 for LTB-NS1Δ63 and 1:4800 for NS1Δ63) (Fig. 5c, Table 4). Notably, in the oral context, boosters of LTB-NS1Δ63 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-NS1Δ63-immunized mice lysed 81.04 ± 9.19% of JEV-infected cells, which were comparable with those from LTB-NS1-immunized mice (81.78 ± 6.54%), but higher than those from NS1Δ63-immunized mice (67.07 ± 8.23%). As controls, the antisera from mice pre-treated with SA14–14–2 exhibited high cytolytic (90.36 ± 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 cytosis might serve as a mechanism adopted by LTB-NS1Δ63 protein to induce protection against JEV infection.

3.6. LTB-NS1Δ63 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-1β) 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 NS1Δ63 protein and SA14–14–2 vaccine, but not LTB protein or PBS, triggered mice to secrete cytokines substantially (Fig. 6b). Moreover, all of the cytokines examined, LTB-NS1Δ63 induced higher secretions than NS1Δ63, while comparable to LTB-NS1 upon subcutaneous delivery (Fig. 6b). Oral delivery of LTB-NS1 or LTB-NS1Δ63 protein elicited mice to secrete cytokines at similar levels as the subcutaneous administration (Fig. 6b-c). Interestingly, oral immunization of NS1Δ63 protein and SA14–14–2 vaccine to mice triggered much less secretions of cytokines (IL-1β, 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-NS1Δ63 group as compared with that of LTB-treated group (P = 0.0099, Student’s t-test), NS1Δ63 group (P = 0.0002, Student’s t-test), and SA14–14–2 group (P = 0.0003,
Fig. 3. Antigenicity of recombinant NS1Δ63 and LTB-NS1Δ63 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 NS1Δ63-coated ELISA with commercial NS1-coated ELISA. JEV-positive and -negative swine serum samples (n = 40) were tested by NS1Δ63-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 NS1Δ63 and commercial NS1 proteins is highlighted by the arrow.
Student's t-test (Fig. 6d). As expected, the spot numbers of LTB-NS1Δ63 group and LTB-NS1 group were comparable (P = 0.6442, Student's t-test) (Fig. 6d). All the results above collectively demonstrate that LTB-NS1Δ63 protein is capable to initiate robust cellular immunity.

### 3.7. LTB-NS1Δ63 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 NS1Δ63 or LTB-NS1Δ63 protein followed by MTT assay. The data showed that up to 500 µg/ml (11.6 µM) of NS1Δ63 or 500 µg/ml (8.3 µM) of LTB-NS1Δ63 had no adverse effect on cell viability (Fig. 7a). The safety of LTB-NS1Δ63 was next examined with mice. BALB/c mice receiving LTB-NS1Δ63 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-NS1Δ63. We found that all mice displayed comparable retention times on the rotarod before and after protein inoculation (Fig. 7c), suggesting LTB-NS1Δ63 protein did not affect mouse vitality.

### Table 2
Comparison of NS1Δ63-coated ELISA with commercial NS1-coated ELISA.

| Serum   | ELISA (NS1Δ63-coated) | Commercial kit (NS1-coated) |
|---------|------------------------|-----------------------------|
| 39      | Same                   | Same                        |
| 1       | Negative               | Positive                    |
| Coincidence rate | 97.5%                  |

Fig. 4. NS1Δ63 and LTB-NS1Δ63 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 cytopathology was observed by microscopy. (c) JEV-infected cells were subjected to immunofluorescence using NS1Δ63-induced antiserum, commercial JEV NS1 antibody, or control (negative serum); (d–f) Immunoblotting assays using NS1Δ63 (d), LTBS-NS1Δ63 (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$_{D63}$, LTB-NS1$_{D63}$, and LTB-NS1. (b,c) BALB/c mice (4-week old) were administrated subcutaneously (b) or orally (c) with 2 nmol of NS1$_{D63}$, LTB-NS1$_{D63}$ or LTB-NS1 protein, or 10$^4$ 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 strain 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\_D63 | LTB-NS1\_D63 | 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) | $P=0.0066$ | $P=0.0247$ | $P=0.0138$ |

All P values are calculated using Student’s t-test.

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

| Comparison between weeks | NS1\_D63 | LTB-NS1\_D63 | 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) | $P=0.0004$ | $P<0.0001$ | $P=0.0003$ |

All 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\_D63, LTB-NS1\_D63, and LTB-NS1. (b,c) BALB/c mice (4-week old) were administrated subcutaneously (b) or orally (c) with 2 nmol of NS1\_D63, LTB-NS1\_D63, or LTB-NS1 protein, or 10^6 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 $\mu$g of each protein at 10^5 cells/well. For PBS group, no protein was used while for SA14\_14\_2 group, NS1\_D63 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. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$. 
Motor coordination and balance. Moreover, BALB/c mice receiving LTB-NS1\textsubscript{D63} 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\textsubscript{D63} can induce vascular leakage. Treatment of 400 \textmu g LTB-NS1\textsubscript{D63} 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\textsubscript{D63} protein demonstrated favorable safety profile for mouse immunization.

3.8. LTB-NS1\textsubscript{D63} protein vaccination confers protection against JEV challenge

We next focused on LTB-NS1\textsubscript{D63} 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...
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_{D63} provided similar protection against JEV infection as those of NS1_{D63} and SA14–14–2 (Fig. 8b, left panel), whereas oral delivery of LTB-NS1_{D63} protein protected 90% of mice from death, much higher than that of NS1_{D63} 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_{D63} 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_{D63}-, LTB-NS1_{D63}-immunized groups. Both subcutaneous and oral immunization of LTB-NS1_{D63} 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_{D63} or LTB-NS1_{D63}, 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
immunization of LTB-NS1\textsubscript{D63} 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\textsubscript{D63}, suggesting that LTB enhances the protective immunity of NS1\textsubscript{D63} 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\textsubscript{D63}-immunized and nonimmunized control group. Oral immunization of LTB-NS1\textsubscript{D63} 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\textsubscript{D63} immunization can reduce histopathology caused by JEV infection.

3.9. LTB-NS1\textsubscript{D63}-induced antiserum provides protection by inhibiting JEV replication

Passive transfer of NS1 antiserum has been reported to induce protection against lethal challenge\cite{24}. We wonder whether LTB-NS1\textsubscript{D63}-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\textsubscript{D63}-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\textsubscript{D63} 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\textsubscript{D63}-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\textsubscript{D63}-induced antiserum conferred protection against JEV in a dose-dependent manner (Fig. 9e,f). Collectively, these findings demonstrate that LTB-NS1\textsubscript{D63}-induced antiserum is protective against lethal challenge by limiting JEV replication.
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 safety, rapid development, and low-cost. Viral structural proteins are particularly mucosal immunity (Fig. 5b,c, Fig. 6b–d), further supporting that NS1Δ63 elicits higher levels of antibodies and cytokines than NS1Δ63 protein, particularly in the context of oral immunization of mice (Fig. 5b,c, Fig. 6b–d). Notably, the presence of LTB augments NS1Δ63-induced production of IFN-γ (Fig. 6b,c), which is a type II IFN that possesses broad-spectrum anti-viral activity, thus providing cross-protection of infections from other viruses including flaviviruses. On the ground that NS1Δ63 has much higher expression than NS1 in E.coli, and both LTB-NS1Δ63 and LTBNS1Δ63 can be recognized by JEV-positive sera from mice, pigs, and humans (Fig. 3b–e). In addition, mouse antisera elicited by both NS1Δ63 and LTB-NS1Δ63 also recognize viral NS1 protein from JEV-infected BHK-21 cells (Fig. 4c–f). These findings suggest that E.coli-expressed NS1Δ63 alone or fused with LTB keeps native NS1 antigenic epitopes. LTB conjugated proteins LTB-NS1Δ63 and LTB-NS1 elicit similar levels of antibodies and cytokines including TNF-α, IFN-γ, IL-1β, and IL-4 in mice (Fig. 5b,c, Fig. 6b–d), further supporting that NS1Δ63 maintains NS1 immunogenicity. LTB-NS1Δ63 elicits higher levels of antibodies and cytokines than NS1Δ63 protein, particularly in the context of oral immunization of mice (Fig. 5b,c, Fig. 6b–d). In addition, mouse antisera elicited by both NS1Δ63 and LTB-NS1Δ63 also recognize viral NS1 protein from JEV-infected BHK-21 cells (Fig. 4c–f). These findings suggest that E.coli-expressed NS1Δ63 alone or fused with LTB keeps native NS1 antigenic epitopes. LTB conjugated proteins LTB-NS1Δ63 and LTB-NS1 elicit similar levels of antibodies and cytokines including TNF-α, IFN-γ, IL-1β, and IL-4 in mice (Fig. 5b,c, Fig. 6b–d), further supporting that NS1Δ63 maintains NS1 immunogenicity. LTB-NS1Δ63 elicits higher levels of antibodies and cytokines than NS1Δ63 protein, particularly in the context of oral immunization of mice (Fig. 5b,c, Fig. 6b–d), suggesting that LTB as an adjuvant enhances NS1Δ63-induced immune responses, particularly mucosal immunity (Fig. 5b,c, Fig. 6b–d). Notably, the presence of LTB augments NS1Δ63-induced production of IFN-γ (Fig. 6b,c), which is a type II IFN that possesses broad-spectrum anti-viral activity, thus providing cross-protection of infections from other viruses including flaviviruses. On the ground that NS1Δ63 has much higher expression than NS1 in E.coli, and both LTB-NS1Δ63 and LTB-NS1 exhibit similar ability to induce immune responses in examined mice (Figs. 5,6), we decided to use NS1Δ63 and LTB-NS1Δ63 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 reverting 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 virus, instead, they enhance the uptake of the virion-antibody complex by Fcγ-receptor (FcγR)-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 Fcγ-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 elevating 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 neuro-invasion [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 NS1Δ63 or LTB-NS1Δ63 followed by lethal viral infection (Fig. 8b). Moreover, both NS1Δ63 and LTB-NS1Δ63 immunization dramatically reduced JEV-induced morbidity (Fig. 8c,d). In agreement with previous finding that LTB augments oral NS1Δ63-induced immune responses (Figs. 5,6), oral immunization with LTB-NS1Δ63 confers better protection than oral immunization with NS1Δ63 in JEV infection setting (Fig. 8b–d), suggesting that fused LTB as an adjuvant enhances protective immunity of NS1Δ63 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-NS1Δ63-immunized mouse sera. Protective immunity is observed in the mice receiving LTB-NS1Δ63-immunized antisera followed by JEV infection (Fig. 9b,c,e,f). Taken together, these data demonstrate that LTB-NS1Δ63 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-NS1Δ63 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 NS1Δ63 and LTB-NS1Δ63 proteins are nontoxic to BHK-21 cells, even when the dosage reaches 500 μg/ml (Fig. 7a). The safety test with animals demonstrates that LTB-NS1Δ63 protein had no obvious adverse influence on mouse vitality, body weight and temperature, with a dosage reaching 400 μg per mouse (Fig. 7b,e). These findings imply that E.coli-expressed LTB-NS1Δ63 is safe as a vaccine candidate.

In conclusion, we describe a novel JEV NS1-based vaccine, LTB-NS1Δ63, which induces robust humoral and cellular immune responses, confers protection against lethal JEV challenge in subcutaneous and oral immunization. Passive transfer of LTBS-NS1Δ63-induced antisera also provides protective immunity against JEV infection (Fig. 10). LTB-NS1Δ63 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-NS1Δ63 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-NS1Δ63 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.

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Supplementary materials

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

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