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Original Research

The recombinant truncated envelope protein of West Nile virus adjuvanted with Alum/CpG induces potent humoral and T cell immunity in mice

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ABSTRACT

West Nile virus (WNV) is a mosquito-transmitted flavivirus distributed globally for decades and can cause disease in humans and animals. So far, no WNV vaccine has been licensed for human use. Therefore, the development of novel candidate vaccines and the improvement of vaccination strategies is imperative. As the WNV envelope (E) glycoprotein plays an important role in mediating viral binding to cellular receptors and virus-cell membrane fusion, it is a critical target for the host humoral response. Here, we prepared a recombinant truncated envelope protein of WNV (rWNV-80E) and developed a WNV subunit vaccine formulation with a combination of aluminum hydroxide (alum) and a synthetic oligonucleotide CpG as adjuvants. C57BL/6 mice were immunized twice intramuscularly at 28-day intervals with 5 μg purified rWNV-80E adjuvanted with Alum/CpG. WNV E-specific IgG was detected by enzyme-linked immunosorbent assay and neutralizing antibodies (nAbs) was detected using single-round infectious particles of WNV. Furthermore, T cell immunity was detected by enzyme-linked immunospot assay and intracellular cytokine staining assay. Notably, rWNV-80E was highly immunogenic and elicited potent humoral and cell immunity, as evidenced by significant levels of IFN-γ and TNF-α secretion in the T cells of mice. In summary, the Alum/CpG-adjuvanted rWNV-80E subunit vaccine elicited potent and balanced B- and T-cell immunity in mice, and therefore it is a promising candidate vaccine that warrants further investigation for use in human or veterinary applications.

1. Introduction

West Nile virus (WNV) was first isolated from the blood of a febrile woman in Uganda in 1937. It is mainly transmitted between birds via *Culex pipiens* mosquitoes and it also can infect humans and other vertebrates as final hosts [1]. The geographic distribution of WNV is expanding and outbreaks in humans and animals are increasing [2], making WNV became an important public health concern. WNV is a positive-sense, single-stranded RNA virus that is divided into nine lineages. Lineages 1 and 2 have the largest geographic range and have been associated with human outbreaks [3,4]. The West Nile virus genome is composed of a 5′ untranslated region (UTR), a long open reading frame (ORF), and a 3′ UTR. The ORF-encoded polyprotein precursors are hydrolyzed by viral and host proteases into three structural proteins, capsid protein (C), anterior membrane or membrane protein (PrM/M), and envelope protein (E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [5,6]. The E protein on the surface of WNV is crucial for virus attachment and entry into host cells, it is also the major antigen eliciting neutralizing antibody responses. Crystallographic studies on the ectodomain of E protein have revealed three distinct domains of DI, DII, and DIII, among which domain DIII has an immunoglobulin-like structure containing multiple epitopes recognized by neutralizing antibodies (nAbs) [7–9]. An epitope of a humanized monoclonal antibody (mAb) localized to the DIII of the WNV E protein protected over 90% of the mice against WNV virus challenges, providing evidence that DIII of the E protein would be a feasible region for generating subunit vaccines [10–13]. Therefore, WNV E is an important target for vaccine development.

Several WNV vaccines have been developed, which have significantly reduced the incidence rates in horses [14]. WNV vaccines for human use are currently in clinical trials and mainly include inactivated [15,16], live attenuated [17,18], DNA [19,20], and subunit vac-

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HIGHLIGHTS

Scientific question

Although several novel West Nile virus (WNV) vaccines have been developed, none of the vaccines has been licensed for human use. Novel WNV candidate vaccines induced both humoral and cellular immunity is imperative.

Evidence before this study

The envelope protein of WNV is an important vaccine target. The protection conferred by WNV E subunit vaccines in mice has been described in previous reports. The appropriate adjuvant is an effective means to improve the WNV E subunit vaccine immunity.

New findings

We prepared a soluble recombinant truncated envelope protein of WNV based on the XJ11129 strain in a eukaryotic expression platform and developed a candidate vaccine formulation with Alum/CpG as adjuvants. The immunization results showed that Alum/CpG adjuvanted rWNV-80E vaccine not only elicited high and sustained titers of serum IgG and nAb but also significant levels of IFN-γ and TNF-α secretion of $CD8^+$ T cells in mice.

Significance of the study

The recombinant truncated envelope protein of WNV adjuvanted with Alum/CpG induces potent humoral and T cell immunity in mice, providing a promising vaccine candidate for further investigation for human or veterinary applications.

cines [21,22]. Recombinant E proteins derived from the flavivirus family, such as dengue fever serum type 1–4, Japanese encephalitis virus, hepatitis C, and WNV have been successfully produced based on a proprietary expression platform [21,23–26]. Recombinant truncated E proteins include the extracellular E protein domains I, II, and III and were used as WNV vaccine candidates, have several advantages over existing WNV immunization strategies. These include the ability to produce large quantities of a soluble protein, the production of neutralizing Abs and protective immunity, and better safety due to the exclusion of the risk of the virus undergoing recombination and changes in tropism. These proteins are truncated at the C-terminus, deleting the membrane-anchored part of the protein and leaving 80% of the natural envelope protein (80E) that is secreted into the extracellular medium. The expressed protein is glycosylated correctly and maintains its natural conformation, as determined by reactivity with conformation-sensitive monoclonal antibodies and X-ray crystallographic structure [24,25].

Humoral immunity is an important part of the immune response against WNV infection and nAbs are the main measure of vaccine efficacy against WNV and other flaviviruses. Experimental studies on passive immunity have shown that the transfer of nAbs to naive animals is sufficient to protect them from fatal WNV infections [10,27,28]. In addition, several studies have shown that WNV-specific T cells are an important part of protective immunity in mice and humans, demonstrating that clearing WNV infection in mice requires $CD8^+$ T cells. Increased WNV replication was observed in the CNS of mice lacking $CD8^+$ T cells [29,30]. The flavivirus vaccine predominantly induces protective T cell responses to avoid antibody-dependent enhancement. WNV vaccine approaches based on T cell epitopes have been described [31,32], but protection in animal models has only reached 75%. Therefore, it is necessary to use these vaccines in combination with neutralizing antibody-inducing antigens. Based on this evidence, it is believed that specific nAbs against WNV are responsible for terminating viremia, and $CD8^+$ T cells are important for virus clearance and lasting immune protection in the body.

The most representative WNV strain in clade 1a is NY99, which is highly pathogenic to humans. The WNV epidemic is also a threat in China. In 2011, the WNV strain of XJ11129 was isolated from *Culex pipiens* samples in the Xinjiang Uygur Autonomous Region of China. Sequence analysis showed that Xinjiang isolates belong to lineage 1a of the WNV, which is closely related to the internationally dominant neuroinvasive strain, exhibited the highest nucleotide identity (97.1%) and amino acid identity (99.4%) with NY99 [33]. We previously demonstrated that the combination of CpG and alum adjuvants in subunit vaccine formulations increased Th1 and Th2 immunity in mice. In this study, a novel recombinant 80E protein with amino acid 1–401 of E protein derived from the WNV strain of XJ11129 was prepared and purified in a eukaryotic expression platform and named rWNV-80E. C57BL/6 mice were immunized with combined adjuvant aluminum hydroxide (Alum)/CpG and evaluated for E-specific humoral immunity and $CD8^+$ T cell response. Our results demonstrated that this novel WNV subunit vaccine is strongly immunogenic in mice and warrants further evaluation.

2. Materials and methods

2.1. Plasmids and cells

pCMV-d1-nLuc-Rep-fs is a subgenomic replicon plasmid wherein the structural genes have been replaced with luciferase reporter (nLuc) [34]. pCag-d1c is a capsid expression plasmid of the WNV-NY99 strain. Both plasmids were kindly provided by Professor Ryosuke Suzuki (National Institute of Infectious Diseases of Japan). The pcWNV-prME plasmid encoding the structural gene prM-E of the WNV NY99 strain was provided by Professor Atsushi Yamanaka (Osaka University, Japan). To create the recombinant plasmid of pcWNV-80E, the gene encoding the truncated E protein (E protein 1–401 amino acid sequence) of WNV-XJ11129-3 (GenBank: JX442279) was synthesized for codon optimization and inserted into the pcDNA3.1 expression vector with 6xHis-tag at the C-terminus (Fig. 1A). Human embryonic kidney cells (HEK-293 T) and African green monkey kidney cells (Vero) were obtained from ATCC (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium containing 25 mM HEPES (Invitrogen, Waltham, MA, USA) and supplemented with 10% fetal bovine serum (Gibco, NY, USA) and 100 U/mL penicillin–streptomycin (Gibco, NY, USA). HEK-293 T and Vero cells were maintained at 37 °C in the presence of 5% CO_2.

2.2. Immunofluorescence assay and western blot analysis

Immunofluorescence assays were performed as previously described [26]. Briefly, HEK-293 T cells were transiently transfected with pcWNV-80E or empty vector plasmid pcDNA3.1 as a control, using the jetPRIME® kit (Polyplus, Illkirch-Graffenstaden, France). After 24 h, the cells were fixed with paraformaldehyde, and incubated with anti-WNV-E mouse mAb (Sino Biological Inc., Beijing, China) as the primary antibody. After washing with PBS, the cells were stained with FITC-labeled goat anti-mouse IgG as secondary antibodies at 37 °C for 1 h and stained with 0.1% DAPI. Finally, a laser-scanning confocal microscope was used for signal detection.

Western blot analysis was performed as previously described [26–27]. Briefly, cell lysates were collected at 48 h post-transfection, and 1% (100 mM) phenylmethylsulfonyl fluoride (PMSF) was added to the native lysis buffer (Solarbio Life Sciences, Beijing, China). The denatured samples were separated by SDS-PAGE (12% resolving gel) and transferred to a nitrocellulose membrane. Anti-WNV E mouse

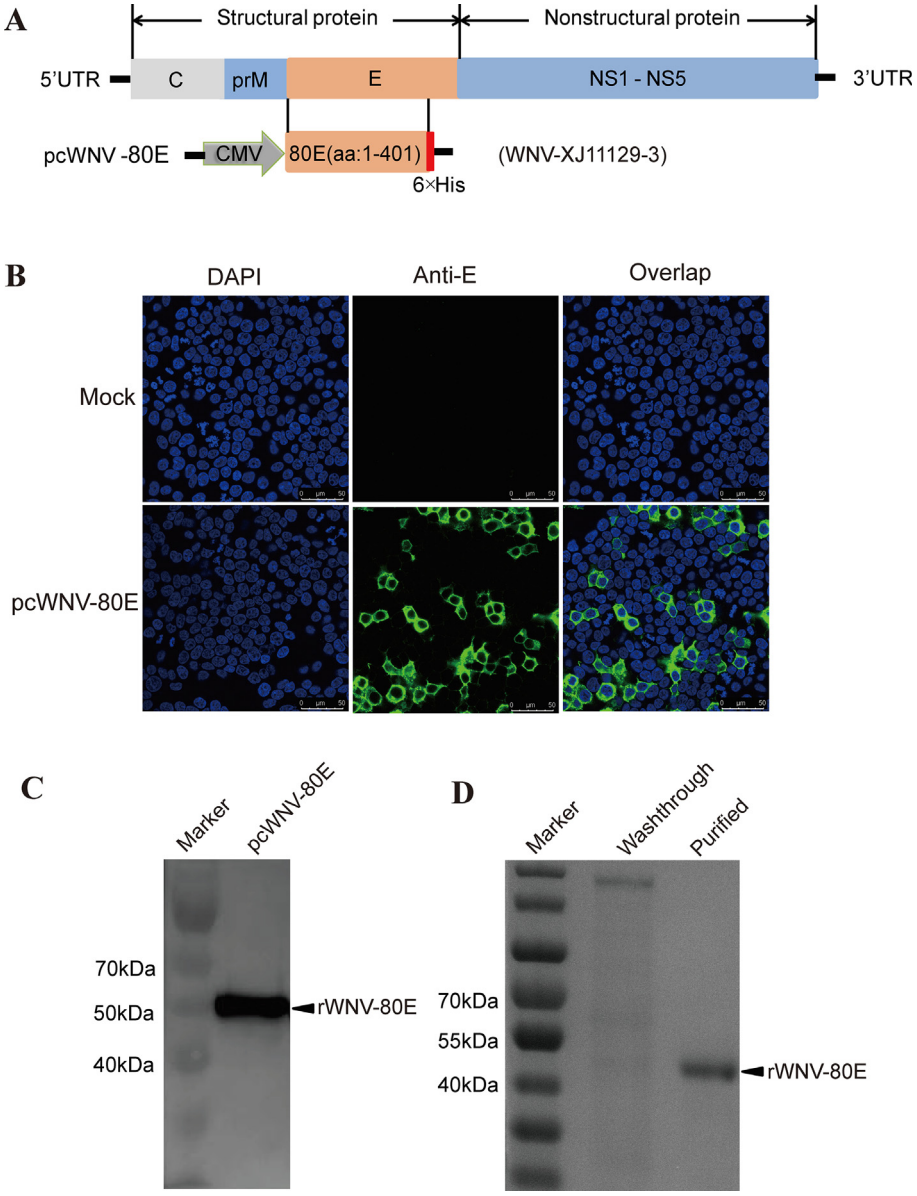

Fig. 1. Identification of pcWNV-80E expression and purified recombinant truncated WNV E (rWNV-80E). A) Schematic diagram of pcWNV-80E construct. B) Identification of pcWNV-80E expression using immunofluorescence assay. C) Identification of pcWNV-80E expression by performing western blot analysis, Molecular weight standards (in kDa) are indicated at the left. D) Identification of purified rWNV-80E by SDS-PAGE after second step purification of rWNV-80E protein by immunoaffinity chromatography. Molecular weight standards (in kDa) are indicated at the left.

mAb (Sino Biological) was used as the primary antibody, and horseradish peroxidase-conjugated (HRP) goat anti-mouse IgG was used as the secondary antibody. Protein bands were developed with a chemiluminescent substrate and analyzed using a chemiluminescent imager (KeChuangRuiXin, Beijing, China).

2.3. Purification and characterization of recombinant truncated envelope protein rWNV-80E

For the preparation of the recombinant truncated envelope protein rWNV-80E (rWNV-E), the pcWNV-80E plasmid was transfected into HEK-293 T cells with the jetPRIME® kit (Polyplus, Illkirch-Graffenstaden, France). The supernatant was harvested after 48 h and the soluble rWNV-80E proteins were purified by affinity chromatography. The supernatant from several large flask cultures of HEK-293 T cells transfected with pcWNV-80E was collected, and then concentrated and purified using Ni-Sepharose (GE Healthcare, Chicago, IL, USA), followed by immunoaffinity chromatography. The purified protein was diafiltered using a storage buffer, underwent filter sterilization, and stored at −70 °C. The purity and specificity of the recombinant rWNV-80E proteins were identified by SDS-PAGE and western blotting, and the concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Endotoxins mainly liberated by gram-negative bacteria, are potential contaminants of the protein solutions originating from biological products. It is possible that the remnant endotoxin in the final product has some adjuvant effect. In this study, we determined the endotoxin levels using the Limulus Amebocyte Lysate assay for endotoxin (Zhanjiang A & C Biological Ltd, China). The content of endotoxin in WNV-80E protein was < 0.25EU/ml, lower than the detection limit.

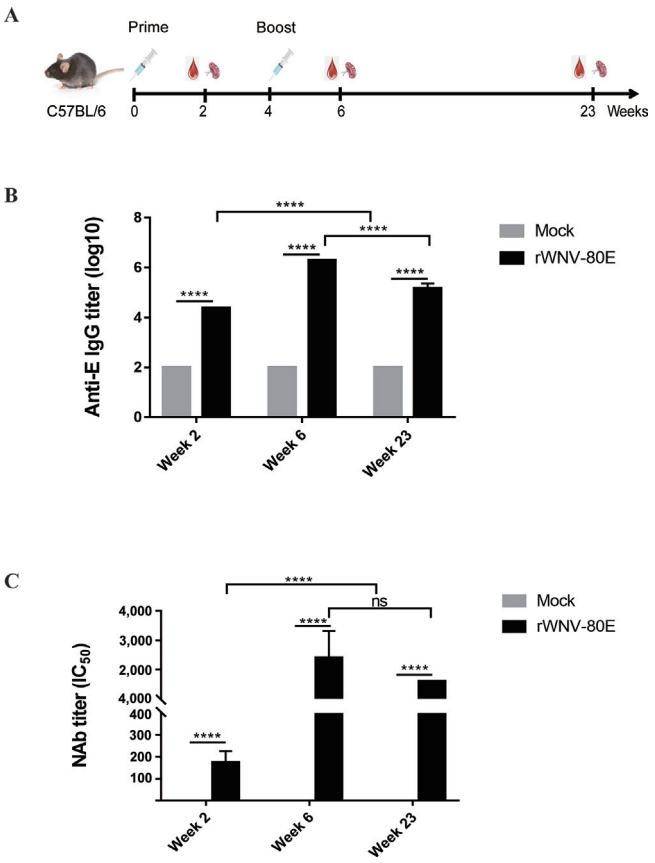

Fig. 2. Detection of humoral immune response in mice vaccinated with rWNV-80E subunit vaccine using ELISA and WNV-SRIP neutralization. A) Experiment schedule. C57BL/6 mice were immunized twice intramuscularly at Day 0 and Day 28. On Day 14, 42, and 161, blood sample was collected to perform serological assays, and animals in each group were sacrificed (N = 4 or 5) to conduct ELISPOT or ICS assay. B) WNV E specific binding antibody (IgG) in mouse serum detected by ELISA with WNV-E-DIII protein as the antigen. C)The neutralizing titers (IC_{50}) in mouse serum were detected by WNV-SRIP neutralization. ****, $P < 0.0001$.

2.4. Mice immunization

Six to eight weeks-old Female C57BL/6 mice were obtained from the Vital River Laboratory Animal Technology Co., Ltd., (Beijing, China). All mouse experiments in this study followed the Regulations for Administration of Laboratory Animals of the National Institute for Viral Disease Control and Prevention, China CDC. Two groups of 12 mice were immunized intramuscularly with 5 μg purified rWNV-80E antigen and adjuvant combinations in a total volume of 100 μL. The mice immunization schedule was shown in Fig. 2A. The CpG oligodeoxynucleotide (ODN) motif containing unmethylated cytosine preceding guanosine (5′-TCCATGACGTTCCTGACGTT-3′) with a phosphorothioate backbone was custom-synthesized (TAKARA BIO. Inc., Beijing, China). The amount of CpG ODN used per injection was 10 μg and the final concentration of Alum was 1 mg/mL. The vaccine formulation was administered twice by intramuscular injection at an interval of four weeks. Blood and spleen cells were collected two weeks after each immunization to detect and analyze humoral and cellular immune responses.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Antigen-specific IgG antibody responses were determined by ELISA, as described previously. Briefly, purified WNV-E-DIII protein expressed in a yeast system (Sino Biological) was used (50 ng per well) to coat the ELISA plate wells. Serum samples, diluted in 1% bovine serum albumin phosphate-buffered saline, were incubated for 2 h at 37 ℃, washed three times, and incubated with goat anti-mouse IgG HRP-conjugated secondary antibody, and 3,3′,5′,’-tetramethylbenzidine (TMB) substrate was used as a chromogen. For IgG subtyping, ELISA plates were incubated with biotinylated anti-mouse IgG1 and IgG2c (Abcam, Cambridge, UK) conjugated to streptavidin-horseradish peroxidase (BD Biosciences, Beijing, China). The optical density at 450 nm was measured using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). Absorbance values greater than or equal to 2.1-fold of that obtained using blank control were considered positive, and the highest dilution ratio determined to be positive was considered the endpoint.

2.6. Preparation of single-round infectious particles (SRIPs) of WNV and detection of neutralizing antibody

Infectious WNV-SRIPs were produced by transfecting 293 T cells with pCMV-D1-nluc-rep, pCAG-D1C, and prME-expression plasmids, as previously described [34]. The neutralization assays were performed as previously described [34]. Briefly, serial 2-fold dilutions of serum samples were mixed with SRIPs at a 1:1 ratio and incubated at 37 ˚C for 1 h before each mixture was added to 96-well tissue culture plates containing a Vero cell monolayer. After 24 h incubation at 37 ˚C, the medium was replaced with 2% fetal bovine serum in Dulbecco's Modified Eagle Medium for further incubation at 37 ˚C. The luciferase activity of cells was subsequently determined three days post-infection, using the Nano-Glo Luciferase Assay System (Promega, Madison, WI, USA). The neutralization titer was defined as the serum dilution that inhibited more than 50% of the SRIP inoculum without serum (IC_{50}).

2.7. ELISPOT assay

To determine the frequency of IFN-γ-producing cells, we used antigen-specific IFN-γ ELISPOT kits (BD, Franklin Lakes, NJ, USA), according to the manufacturer's instructions [26]. Six WNV E-specific peptides (FNCLGMSNRDFLEGV, ANLAEVRSYCYLATV, VRSYCYLATVSDLST, SSAGSTVWRNRETLM, TVWRNRETLMEEP, and IALTFLAVGGVLLFL) were used to stimulate mouse splenocytes [30]. The nonspecific stimulants phorbol ester and ionomycin (Dako, Carpinteria, CA, USA) were used as positive controls. The plates were imaged using an ImmunoSpot reader (Cellular Technologies Ltd., Shaker Heights, OH, USA), and the number of spots per well was counted using Biospot 5.0.

2.8. Intracellular cytokine staining (ICS)

ICS was performed to measure antigen-specific functional T cell response, as previously described [26]. Briefly, splenocytes were stimulated with mixed peptides as described above. Next, these cells were stained for surface markers CD3 and CD8, processed, and stained intracellularly with fluorochrome-conjugated antibodies specific for IFN, IL-2, TNF, and IL-4, followed by flow cytometry (BD Biosciences) evaluation and analysis using GraphPad Prism 6 software.

2.9. Data analysis

Data was analyzed and graphically represented as mean ± SEM by GraphPad Prism 6 software. Statistical significance was determined by two-way analysis of variance, and results with $P < 0.05$ were considered statistically significant. All preliminary data were tabulated and basic calculations were performed using Microsoft Excel.

3. Results

3.1. Identification and purification of rWNV-80E

To prepare the soluble rWNV-80E protein, a codon-optimized truncated E protein gene was constructed into pcDNA3.1 plasmid (Fig. 1A), and the 293 T cells were transiently transfected with pcWNV-80E. The cells were fixed for immunofluorescence assay, the supernatants were collected for western blotting assay, and the purity of soluble rWNV-80E protein was identified by SDS-PAGE assay. Immunofluorescence analysis demonstrated that rWNV-80E protein reacted with mAb specific to WNV E protein and it could be detected in the cytoplasm (Fig. 1B). Western blotting showed that rWNV-80E protein were detected in supernatant with a band of approximately 50 kDa as expected (Fig. 1C). Densitometry analysis of Coomassie blue-stained SDS-PAGE gels was used to estimate the purity of the rWNV-80E protein. SDS-PAGE of the wash-through and the purified rWNV-80E protein with grey scans analysis showed the purity of rWNV-80E was greater than 95% (Fig. 1D).

3.2. rWNV-80E-based subunit vaccine induced robust and lasting IgG and neutralizing antibody response

To evaluate the immunogenicity of the rWNV-80E-based subunit vaccine candidates, C57BL/6 mice were immunized twice and serum samples were collected (Fig. 2A). WNV E-specific IgG and IgG subtype were detected by ELISA with WNV-E-DIII protein as coated antigen (Fig. 2B). DIII-specific IgG titers were detected after a single administration of rWNV-80E-based subunit vaccine, with high titers of IgG antibody more than 1:10,000. The IgG antibody levels increased significantly two weeks post-boost immunization, with an average titer of approximately 1:1,900,000. Furthermore, the E-specific antibody response persisted in mice, with an average IgG titer above 1:100,000 at 19 weeks post-boost immunization. In addition, WNV E-specific IgG subtypes of IgG1 and IgG2c induced by rWNV-80E subunit vaccine were detected from the mice two weeks post-boost immunization, IgG subtypes level results showed an average titer of 1:107,635 for IgG1 and 1:128,000 for IgG2c, the ratio of IgG2c:IgG1 was close to 1, which suggested that rWNV-80E subunit vaccine formulation induced a balanced Th1 and Th2 immune response in mice.

The nAb titer of IC_{50} above 160 against infectious WNV-SRIPs was also detected in mice serum two weeks after a single immunization (Fig. 2C), and significantly increased at two weeks post-boost immunization, with an average NT_{50} titer of 2,600. Notably, this nAb activity persisted in mice, with an average NT_{50} titer above 1,200 at 19 weeks post-boost immunization. Collectively, the rWNV-80E-based subunit vaccine induced robust and lasting IgG and nAbs in mice.

3.3. rWNV-80E-based subunit vaccine shown a potential to induce antigen-specific T cell immune response

To evaluate antigen-specific T cell responses, ELISPOT and ICS were performed after in vitro re-stimulation of splenocytes obtained from mice immunized with peptide pools derived from WNV E protein. Although no E-specific T cell immunity was detected at two weeks post-prime immunization, ELISPOT results showed significant levels of IFN-γ, with average spot forming units (SFU) greater than 200, in mouse splenocytes at two weeks post-boost immunization (Fig. 3A). However, the significant level of IFN-γ secretion was not detected in splenocytes at 19 weeks post-boost immunization, suggesting that the T cell response did not last as long as the humoral immune response in mice for the rWNV-80E based subunit vaccine.

To further evaluate the functionality of T cells in spleens of mice challenged with rWNV-80E-based subunit vaccine, ICS was performed concurrently with ELISPOT two weeks post-boost immunization (Fig. 3B and 3C). The total number of both IFN-γ and TNF-α $CD8^+$ T cells in the spleen increased when mice were stimulated with WNV-E-specific peptide, while no significant increase was observed for IL-2 and IL-4 $CD8^+$ T cells. Taken together, these data indicated that the rWNV-80E-based subunit vaccine induced specific T cell immunity in mice.

4. Discussion

This study focused on developing a recombinant subunit WNV vaccine candidate using the soluble truncated viral E protein (rWNV-80E) produced from 293 T cell as the target antigen. Several WNV candidate vaccines have been evaluated in animal models and approved for use in horses. The safety of WNV vaccine is imperative because the target groups for vaccination include the elderly, individuals with low immunity, and those with chronic diseases. Compared to inactivated, live attenuated, or chimeric vaccines, one obvious advantage of the recombinant subunit WNV vaccine is its safety and scalability. In this study, we showed for the first time that immunization with recombinant soluble truncated rWNV-80E prepared from combined with adjuvant Alum/CpG was strongly immunogenic in mice, as evidenced by high level and sustained titers of serum IgG and protective nAb, as well as significant levels of IFN-γ and TNF-α secretion T cell immune response in C57BL/6 mice. The balanced Th1 / Th2 immune response was observed by detecting anti-E protein DIII-specific IgG2c and IgG1. These data are encouraging and require further research to evaluate their application in clinical settings.

WNV E protein is involved in receptor binding, virus entry, and membrane fusion, and is the most immunogenic glycoprotein [35,36]. For this reason, E-proteins have been extensively evaluated as candidate vaccines in both preclinical animal models and clinical settings. Recombinant truncated E protein (80E, derived from WNV-NY99) produced in the Drosophila cell expression system was explored as a WNV subunit vaccine previously and elicited high-titer nAbs and protected immunized mice, hamsters, and rhesus monkeys against WNV challenge[21,22,37]. The nAbs are critical for protective immunity against flavivirus infection. The World Health Organization considers nAb titer as a relevant factor for protection against the Japanese encephalitis virus [38,39]. The WNV strain of XJ11129 isolated in China is classified in clade 1a of lineage 1 and showed high sequence identity with the pathogenic strain NY99 (GenBank: DQ211652), six amino acid differences were located in the E protein: Y90F, T126I, T159V, F167L, S277N, and S467A. Whether the truncated E protein of XJ11129 exhibits similar to that of NY99 needs experimental verification. In this study, we prepared rWNV-80E based on the coding sequence of WNV strain XJ11129-3 (Xinjiang strain) and expressed the protein in a eukaryotic expression system (293 T cell). Although five amino acid sequence variations were found in the truncated 80 E protein between WNV-NY99 and WNV-XJ11129-3 strains, a robust nAb response against WNV-The NY99 strain in response for the rWNV-80E immunization mice, which maintained a high level of cross-nAb for at least 19 weeks after booster vaccination.

The protection conferred by subunit vaccines in immunocompromised animals and young and old animals has been described in previous reports [40]. Several subunit vaccines based on E proteins have been developed. Of these, the WN-80E protein is clinically the most advanced candidate vaccine (Clinical Trial#: NCT00707642) and was found to be safe when adjuvanted with Alhydrogel. However, the immunogenicity needs to be enhanced further because the overall titer of virus-specific nAb induced by this vaccine after three doses was still low relative to live-attenuated vaccines. Currently, the results available from clinical trials suggest that the Alum/CpG adjuvant is safe and useful for boosting the immunogenicity of co-administered vaccines. The suitability of using alum and/or CpG DNA as adjuvants

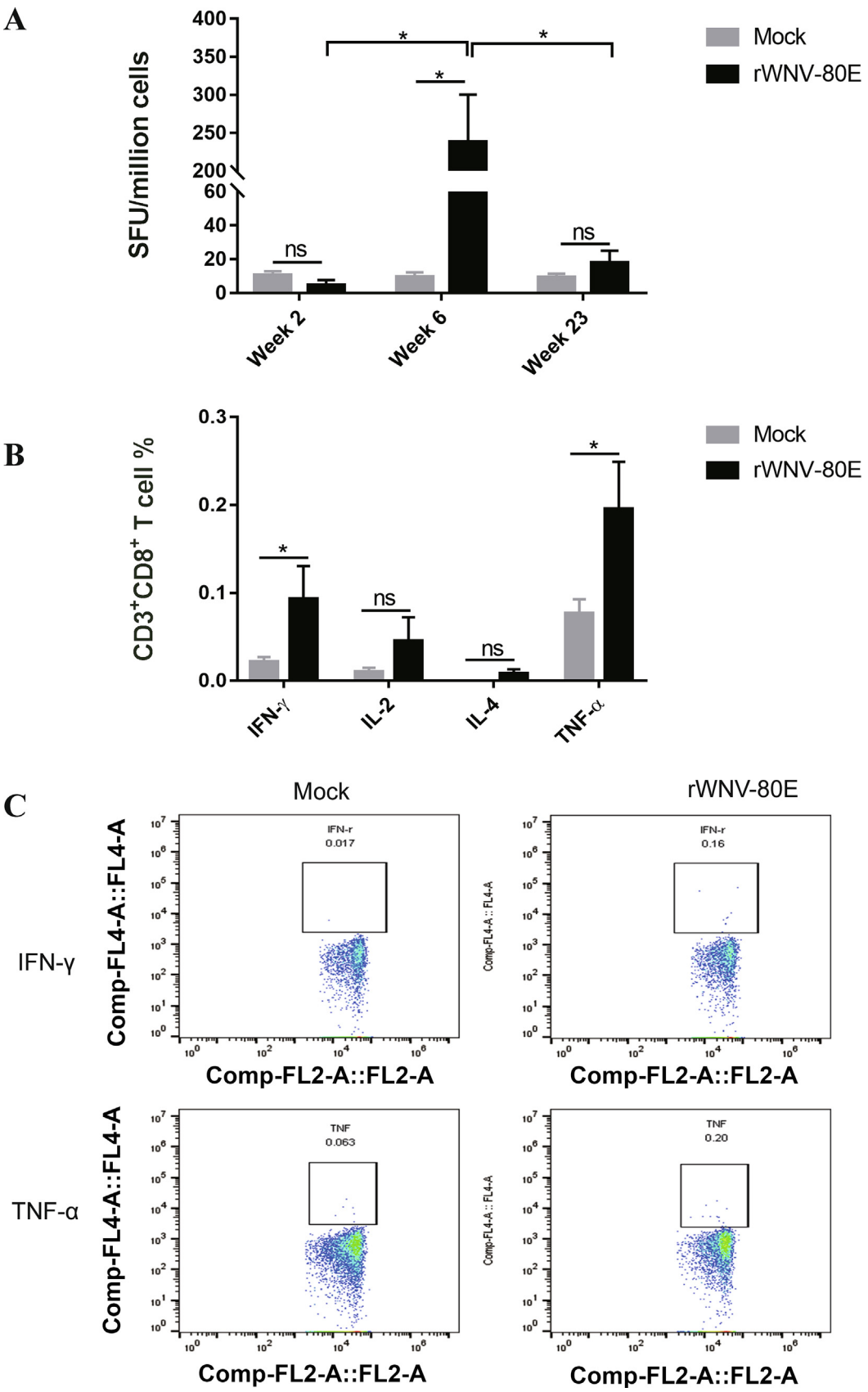

Fig. 3. Detection of antigen-specific T cell immunity in mice challenged with rWNV-80E subunit vaccine using ELISPOT and ICS. A) ELISPOT assay. Specific T-cell responses to WNV-80E antigens in mice splenocytes were analyzed by performing ELISPOT assay at two weeks post prime immunization (Prime), two weeks post-boost immunization (Boost), and 19 weeks post-boost. The amount of antigen-specific IFN-γ is shown as SFU per million splenocytes. B) ICS analyses of the splenocytes at two weeks post-boost immunization. The splenocytes were stimulated with WNV-E peptides, and cytokine production was measured using specific antibody staining and flow cytometric analysis. The mean (±SEM) percentage of IFN-γ, IL-2, TNF-α, and IL-4 producing $CD3^+$ $CD8^+$ T cells was obtained from three mice per group following stimulation with WNV-E peptides. Cytokine levels that differed significantly between the groups are indicated by asterisks *. ns, not significant, $P > 0.05$; *, $P < 0.05$. C) Representative dot plots show the population of $CD3^+$ $CD8^+$ T cells secreting IFN- γ or TNF- α in response to WNV-E peptides stimulation.

to enhance the protective effectiveness of the recombinant WNV subunit vaccine has also been evaluated [41]. Previous studies have shown that co-administration of CpG adjuvant with WNV E DIII protein significantly boosted Ab production and increased the production of Th1 cytokines (IFN-γ and IL-2) compared to mice immunized with WNV E DIII protein alone [41]. Recent data suggests that this E DIII antigen does not induce ADE for dengue virus and Zika virus [42]. Adjuvants are critical for the effective development of protective responses of subunit vaccines. For recombinant proteins with low immunogenicity, pairing them with appropriate adjuvants is an effective means to improve their vaccine immunity. As a very preliminary study, we mainly focus on the immunogenicity of rWNV-80E adjuvant with the combination of Alum and CpG in C57BL/6 mice. We choose these adjuvants for two reasons. Firstly, alum was used as the adjuvant because it has been successfully employed in licensed human vaccines against other flaviviruses and WNV-80E subunit vaccines [41,43,44,45]. Secondly, CpG are immunomodulatory synthetic oligonucleotides designed to specifically agonize Toll-like receptor 9 (TLR9), promoted Th1 immune responses, and enhance the immunogenicity of many vaccines with high safety in some clinical research [41,44,45]. Adjuvant formulations of aluminum and TLRs have been shown to enhance vaccine immune responses compared to aluminum or TLRs alone. CpG could boost the immunogenicity of alum-based vaccines against infectious diseases including hepatitis B, anthrax and influenza [45]. To overcome the shortcomings of aluminum-hydroxide-based adjuvants and to evoke more potent immune responses, we focused on the adjuvant of Alum and CpG.

In this study, rWNV-80E combined with adjuvant Alum/CpG not only elicited high and sustained titers of serum IgG and nAb but also significant levels of IFN-γ and TNF-α secretion of $CD8^+$ T cells in mice, which is critical for the development of Ag-specific CTL immune response that protect vaccinated individuals from WNV infection. Of note, CpG is classified into 4 major classes, with distinct activation profiles of human cells, and different DNA motifs are required for optimal stimulation of mouse and human immune cells. The human clinical adjuvant activity trials of TLR9 ligands have focused on B-type CpG, which could stimulate strong B cell, NK cell, and pDCs activation. The CpG sequence selected in this study is a well-known B-type CpG 1826, containing unmethylated cytosine preceding guanosine (5-TCCATGACGTTCCTGACGTT-3) with a phosphorothioate backbone. Whether the CpG sequence selected in this study still has the same effect on other animals remains to be verified, more animal models such as hamsters, geese, and nonhuman primate animals should be investigated in future study. Vaccines containing adjuvants must be tested for safety and effectiveness in preclinical and clinical trials before they are licensed for use. Further toxicological evaluation and mechanism studies should be conducted to enrich the data related to cellular and humoral immune responses. Indeed, preclinical safety evaluations are one of the key phases in the development of new vaccines, to expand the safety analysis of the rWNV-80E subunit vaccine formulation with Alum/CpG, safety evaluations should be conducted on rats or rhesus macaques for clinical and histopathological exploration of changes in different tissues in detail. In addition, Further mechanism studies should be conducted to enrich the data related to cellular and humoral immune responses, and further explain the immune-enhancing effect of the vaccine at the molecular level by means of single-cell sequencing and so on.

There are several limitations to our study. Firstly, as the WNV is a highly pathogenic virus that needs to be handled at biosafety level 3 laboratory. Our limited infrastructure and facilities did not allow us to use live viruses for neutralizing antibody detection, nor we could perform animal challenge experiments to further optimize immunization procedures and analyze mechanisms underlying immune protection. Secondly, ELISPOT and ICS assay results proved that our rWNV-80E subunit vaccine induced significant T cell immunity at two weeks post-boost immunization, but it lasted < 19 weeks. A new immunization scheme needs to be devised for eliciting long-term cellular immunity in response to subunit vaccines. Thus, further studies are warranted to evaluate this rWNV-80E vaccine in other preclinical models before it can be used as a candidate vaccine in humans. In addition, as a preliminary study, we mainly focus on the immunogenicity of rWNV-80E adjuvant with Alum/CpG in C57BL/6 mice, more animal models such as hamsters, geese, and nonhuman primate animals should be investigated in future. Finally, no safety evaluation nor mechanism study was performed in this study, a detailed safety evaluation experiment would be helpful to evaluate the application of WNV subunit vaccine in clinical.

5. Conclusions

In summary, we prepared a soluble recombinant truncated envelope protein of West Nile virus and developed a WNV subunit vaccine formulation with Alum/CpG as adjuvants. Immunization results showed that the Alum/CpG adjuvant rWNV-80E triggered effective and lasting humoral immunity as well as T-cell immunity in mice. Our results provide a foundation for further research on developing vaccines for the WNV.

Ethics statement

All animal experiments were conducted strictly according to the instructions of SPF containments in the Chinese Center for Disease Control and Prevention. The protocol and animal ethics were approved by the National Institute for Viral Disease Control and Prevention Laboratory Animal Welfare and Ethics Committee (Approval number: 20200205008).

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Yongping Du: Data curation, Formal analysis, Methodology, Funding acquisition, Writing - original draft. **Yao Deng:** Data curation, Formal analysis, Methodology, Funding acquisition, Writing - original draft. **Ying Zhan:** Formal analysis, Funding acquisition, Methodology. **Ren Yang:** Formal analysis, Funding acquisition, Methodology. **Wen Wang:** Formal analysis, Funding acquisition, Methodology. **Baoying Huang:** Formal analysis, Writing - review & editing. **Wenjie Tan:** Conceptualization, Methodology, Project administration, Writing - review & editing.

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