Replication-Defective West Nile Virus with NS1 Deletion as a New Vaccine Platform for Flavivirus

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ABSTRACT We previously produced a replication-defective West Nile virus (WNV) lacking NS1 (WNV-ΔNS1) that could propagate at low levels (10⁵ infectious units [IU]/ml) in a 293T cell line expressing wild-type (WT) NS1. This finding indicates the potential of developing WNV-ΔNS1 as a noninfectious vaccine. To explore this idea, we developed an NS1-expressing Vero cell line (Vero_{NS1}) that significantly improved the yield of WNV-ΔNS1 (10⁸ IU/ml). We evaluated the safety and efficacy of WNV-ΔNS1 in mice. WNV-ΔNS1 appeared to be safe, as no replicative virus was found in naive Vero cells after continuous culturing of WNV-ΔNS1 in Vero_{NS1} cells for 15 rounds. WNV-ΔNS1 was noninfectious in mice, even when IFNAR^{-/-} mice were administered a high dose of WNV-ΔNS1. Vaccination with a single dose of WNV-ΔNS1 protected mice from a highly lethal challenge with WT WNV. The antibody response against WNV correlated well with the protection of vaccinated mice. Our study demonstrates the potential of the NS1 *trans* complementation system as a new platform for flavivirus vaccine development.

IMPORTANCE Many flaviviruses are significant human pathogens that frequently cause outbreaks and epidemics around the world. Development of novel vaccine platforms against these pathogens is a public health priority. Using WNV as a model, we developed a new vaccine platform for flaviviruses. WNV containing a NS1 deletion (WNV-ΔNS1) could be efficiently *trans* complemented in Vero cells that constitutively expressed WT NS1 protein. A single-dose immunization with WNV-ΔNS1 elicited robust immune responses in mice. The immunized animals were fully protected against pathogenic WNV infection. No adverse effects related to the WNV-ΔNS1 vaccination were observed. The results have demonstrated the potential of the NS1 complementation system as an alternative platform for flavivirus vaccine development, especially for highly pathogenic flaviviruses.

KEYWORDS flavivirus, NS1, West Nile virus, vaccine

West Nile virus (WNV) is an important mosquito-transmitted human pathogen. WNV causes no illness or a mild, self-limiting, febrile illness in most cases, but it causes more severe disease in elderly and immunocompromised individuals. Since its emergence in New York in 1999, WNV has remained an important public health threat in the United States (1). The virus has now been reported in many other regions, including Africa, Europe, and West Asia (2, 3), indicating that WNV may be a global public health threat.

WNV belongs to the genus *Flavivirus* in the family *Flaviviridae* (4). The *Flavivirus* genus also includes many other important human pathogens, such as dengue virus, yellow fever virus, Zika virus, and tick-borne encephalitis virus. The flavivirus genome is a single-stranded, positive-sense RNA with approximately 11,000 nucleotides, comprising a 5' untranslated region (UTR), a single open reading frame (ORF), and a 3' UTR. The

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single ORF encodes three structural proteins (capsid, membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (5–7). The structural proteins play essential roles in virus entry, fusion, and assembly, while the nonstructural proteins are important for viral replication, virion assembly, and evasion of the innate immune response.

NS1 is a highly conserved glycoprotein, with a molecular mass of about 46 to 50 kDa, that plays important roles in viral replication and immune responses (8–11). The nascent NS1 is hydrophilic and water soluble. Following its cleavage from a polyprotein in the endoplasmic reticulum (ER), NS1 undergoes glycosylation and homodimerization. After dimerization, NS1 acquires partial hydrophobicity, traffics to the replication complex, and plays an essential role in the early stage of viral RNA replication (12, 13). The protein functions in viral RNA synthesis by modulating different viral and host factors. For example, NS1 was found to interact genetically or physically with viral transmembrane proteins NS4A and NS4B, which are responsible for replication complex formation (8); such interactions allow the lumen-resident NS1 to regulate viral replication that occurs on the cytoplasmic side of the ER (12, 14). Mutations of NS1 decrease viral replication, and the replication defect can be restored in *trans* by ectopic expression of wild-type (WT) NS1 (11, 15–18).

Since there are no licensed antivirals against WNV infection, vaccination is a good means to prevent infections and disease. It has been well demonstrated that humoral immunity is sufficient to control WNV infection (19, 20), although WNV-specific T cells also contribute to protection and the clearance of WNV (20). The envelope of WNV is the main viral antigen. Mapping of human and murine antibody repertoires against the envelope has revealed important insights into the protective immune response against the virus (19). A number of vaccine strategies have been explored to combat WNV infection (21, 22), including inactivated vaccine, subunit vaccine (23), live-attenuated or chimeric vaccines (24–27), DNA-based vaccine (28–32), and virus-like particle vaccine (33). Among these different types of vaccines, live-attenuated vaccines are superior to other vaccines as they can elicit robust humoral and cellular adaptive immune responses. However, the safety of live-attenuated vaccine is a major concern, particularly for highly pathogenic pathogens.

In this study, higher titers of replication-defective WNV with a NS1 deletion (WNV- Δ NS1) were produced in a selected Vero cell line that expresses WT NS1 protein (Vero_{NS1}). WNV- Δ NS1 was evaluated as a vaccine candidate in mice. WNV- Δ NS1 was generated with a WNV infectious clone by deleting most coding sequences of NS1. WNV- Δ NS1 was not able to replicate in naive Vero cells unless NS1 protein was provided in *trans* (11). In animals, we demonstrated the safety of WNV- Δ NS1 in IFNAR^{-/-} mice, a susceptible animal model deficient in type I interferon (IFN) receptor (IFNAR). A single dose of WNV- Δ NS1 immunization protected C57BL/6 mice from a lethal challenge with WT WNV. The antibody response against WNV correlated well with the protection efficacy of WNV- Δ NS1. Our study demonstrates the potential of the NS1 complementation system as an alternative platform for flavivirus vaccine development.

RESULTS

High-titer production of WNV- Δ NS1 in Vero_{NS1} cell line. The goal of this study was to examine whether a replication-defective WNV- Δ NS1 could elicit protective immunity against WNV infection and serve as a safe vaccine platform for highly pathogenic flaviviruses. Although WNV- Δ NS1 was genetically stable after serial passaging in 293T_{NS1} cells without recombination (11), the viral titer of WNV- Δ NS1 was low (around 10⁵ infectious units [IU]/ml), which was not ideal for vaccine production. In the present study, we selected a new NS1 helper cell line, using Vero cells (Vero_{NS1}) to produce WNV- Δ NS1 (Fig. 1A). In Vero_{NS1} cells, WNV- Δ NS1 replicated efficiently with a mean peak viral titer of 1 × 10⁸ IU/ml (Fig. 1B) at 72 h postinfection (hpi), about 1,000-fold higher than that observed in the previously reported 293T_{NS1} cells (11). In addition, WNV- Δ NS1 could propagate efficiently in serum-free medium in the Vero_{NS1}

FIG 1 High-titer production of WNV-ΔNS1 in Vero_{NS1} cell line. (A) Method for production of WNV-ΔNS1. WNV-ΔNS1 was generated by transfection of WNV-ΔNS1 RNA (containing a deletion of residues 4 to 298 in the NS1 coding sequence) into the puromycin-resistant Vero/293T cell line stably expressing WNV-NS1 protein. (B) Comparison of growth kinetics of WNV-ΔNS1 in Vero_{NS1} and 293T_{NS1} cells. Vero_{NS1} and 293T_{NS1} cells were infected with WNV-ΔNS1 virus at an MOI of 0.1. The supernatants were harvested at the indicated time points, and viral titers were determined by IFA in Vero_{NS1} cells, as described in Materials and Methods. Two independent experiments were performed in triplicate. (C) Comparison of growth kinetics of WNV-ΔNS1 in serum-free medium and serum-containing medium. Vero_{NS1} cells were infected with WNV-ΔNS1 virus at an MOI of 0.1 and incubated in virus production serum-free medium (VP-SFM) or serum-containing medium. The supernatants were harvested at the indicated time points, and viral titers were determined as described above. Data represent the mean $\pm$ standard deviation of triplicate measurements in a representative experiment. The asterisks denote statistical differences between the indicated groups. *, $P < 0.05$.

cell line, although the viral titer was about 5- to 10-fold lower than that in serum-containing medium (Fig. 1C).

Characterization of WNV-ΔNS1. To characterize WNV-ΔNS1, the viral growth and plaque morphology were compared with those of WT WNV (Fig. 2). Similar viral growth curves were observed for these two viruses in the Vero_{NS1} cell line at a multiplicity of infection (MOI) of 0.1 (Fig. 2A), with the highest titer being about 1×10^8 IU/ml at 72 hpi. The replication foci of WNV-ΔNS1 and WT viruses were also similar when detected using an immunostaining-based focus-forming assay (Fig. 2B). After concentration and purification through polyethylene glycol 8000 (PEG8000) precipitation and ultracentrifugation, both viruses were analyzed by SDS-PAGE with Coomassie brilliant blue staining. Two bands, at about 55 and 15 kDa, corresponding to viral envelope (E) and capsid (C) proteins, respectively, were observed in both WNV-ΔNS1 and WT samples (Fig. 2C). The identities of the E and C bands were further confirmed by Western blotting with specific antibodies (Fig. 2D).

To examine the antigenicity of WNV-ΔNS1, we performed neutralization assays in Vero_{NS1} cells using two different monoclonal neutralization antibodies against WNV envelope. Dose-dependent decreases in viral infection were observed for WT WNV and WNV-ΔNS1 (Fig. 3A and B). In addition, the antisera against WNV could recognize WNV-ΔNS1 coated on enzyme-linked immunosorbent assay (ELISA) plates, suggesting that the WNV-ΔNS1 virion remains antigenically intact (Fig. 3C). These results indicate that the WNV-ΔNS1 virion has antigenic properties similar to those of WT WNV.

Stability and safety of WNV-ΔNS1. For stability testing, WNV-ΔNS1 was passaged serially on Vero_{NS1} cell lines for 15 rounds (passages 0 to 15). The resulting passage 0 to passage 15 WNV-ΔNS1 viruses were used to infect Vero_{NS1} and naive Vero cells. All passaged WNV-ΔNS1 viruses produced robust indirect immunofluorescence assay (IFA)-positive signals in Vero_{NS1} cells, whereas they did not generate any IFA-positive signals

FIG 2 Characterization of WNV-ΔNS1. (A) Comparison of growth kinetics between WT WNV and WNV-ΔNS1 virus in Vero_{NS1} cells. Vero_{NS1} cells were infected with either WT WNV or WNV-ΔNS1 virus at an MOI of 0.1. The supernatants were harvested at the indicated time points, and viral titers were determined as described above. Data represent the mean $\pm$ standard deviation of triplicate measurements in a representative experiment. n.s., no statistical difference. (B) Comparison of immunostained foci between WT WNV and WNV-ΔNS1 virus in Vero_{NS1} cells. The plaque morphology of WNV-ΔNS1 was analyzed by a standard immunostaining-based focus-forming assay and compared with that of WT virus. (C) Coomassie brilliant blue staining analysis of purified WT WNV and WNV-ΔNS1 virions. Following concentration and purification through PEG8000 precipitation and ultracentrifugation, equal amounts of purified WT and ΔNS1 virions were loaded onto a SDS-polyacrylamide gel, and protein bands were visualized by Coomassie brilliant blue staining. The bands corresponding to E and capsid proteins are indicated. (D) Western blotting analysis of purified WT WNV and WNV-ΔNS1 virions. The virus antigens described above were analyzed by Western blotting with an anti-E monoclonal antibody and anti-C polyclonal antibody.

in naive Vero cells (Fig. 4A), suggesting that WNV-ΔNS1 was genetically stable and did not recombine with the WT NS1 sequence in the Vero_{NS1} cells. To further confirm the replication defect of WNV-ΔNS1, we infected both naive Vero and Vero_{NS1} cells with the *Gaussia* luciferase (Gluc) reporter WNV-ΔNS1-Gluc at an MOI of 100. As shown in Fig. 4B, viral translations were observed in both cell lines at 1 to 6 hpi with similar luciferase activities, whereas only Vero_{NS1} cell lines produced significantly increased luciferase activities after 8 hpi (representing viral replication). The results demonstrated that WNV-ΔNS1-Gluc could replicate only in Vero_{NS1} cells and not in naive Vero cells. To further evaluate the biosafety of WNV-ΔNS1, IFNAR^{-/-} mice were inoculated intraperitoneally (i.p.) with 2×10^8 IU of WNV-ΔNS1 or 10^2 IU of WT WNV (Fig. 4C). The mice infected with WNV-ΔNS1 showed no signs of illness, with steadily increasing body weight (Fig. 4C), and survived during the entire observation period (Fig. 4D). In contrast, WT WNV caused disease in mice, such as ruffled fur and weight loss from day 2 postinfection (Fig. 4C), and all mice died by day 5 (Fig. 4D). These data demonstrate that WNV-ΔNS1 is not pathogenic in IFNAR^{-/-} mice. Overall, the results showed that WNV-ΔNS1 is genetically stable in Vero_{NS1} cells and safe in IFNAR^{-/-} mice.

Protection of C57BL/6 mice against WNV challenge. To assess the potential of WNV-ΔNS1 as a vaccine, we examined the antibody response induced by WNV-ΔNS1 infection in immunocompetent C57BL/6 mice. Four-week-old C57BL/6 mice ($n = 5$ in each group) were immunized with 4.8×10^7 IU of WNV-ΔNS1, with a booster with the same dose 28 days after the first immunization (Fig. 5A). As shown in Fig. 5B, WNV-ΔNS1 immunization induced robust ELISA antibody titers on day 14 (1:3,200) and day 28 (1:9,600); the booster on day 28 resulted in an additional increase in antibody titers to 1:204,800 on day 42 and afterward (Fig. 5B). In agreement with the ELISA titers, neutralizing antibody titers reached 1:33, 1:66, 1:2,560, and 1:1,706 on days 14, 28, 42,

FIG 3 Immunogenicity of WNV-ΔNS1. (A and B) PRNT₅₀ assay. PRNT₅₀ testing against both WT WNV and WNV-ΔNS1 was performed with two different neutralizing antibodies against WNV envelope, i.e., FD011-1 (A) and FD011-2 (B). Serial 2-fold dilutions of neutralizing antibodies were incubated with WT WNV or WNV-ΔNS1 viruses. The inhibition rates were calculated by defining no-antibody treatment of WT WNV (0 μg/ml) as 0%. (C) ELISA of the antisera against WNV and WNV-ΔNS1. Two independent experiments were performed in triplicate. Data represent the mean $\pm$ standard deviation of triplicate measurements in a representative experiment. The asterisks denote statistical differences between the indicated groups. *, $P < 0.05$; **, $P < 0.01$; n.s., no statistical difference.

and 56, respectively (Fig. 5C). The results demonstrated that WNV-ΔNS1 could induce a robust antibody response.

To determine the protective efficacy of WNV-ΔNS1 virus, both phosphate-buffered saline (PBS)- and WNV-ΔNS1-immunized mice were i.p. challenged with 3×10^7 PFU of WT WNV at 30 days after booster immunization (Fig. 5A). As expected, all PBS-immunized mice started to lose weight on day 6 postchallenge (Fig. 5D), displayed signs of disease (such as ruffled fur and lameness), and succumbed to infection by 15 days postchallenge (Fig. 5E). In contrast, the WNV-ΔNS1-vaccinated mice survived (Fig. 5E) without any signs of sickness or body weight loss (Fig. 5D) in 21 days. The viremia levels were measured on day 2 postchallenge (Fig. 5F). All PBS-immunized mice developed a high level of viremia (viral RNA copy numbers of $>10^7$ copies/μl), compared to no detectable viral RNA from the WNV-ΔNS1-vaccinated group (Fig. 5F). IgG antibody titers against WNV increased 1.6-fold and 4-fold at day 14 and day 28 postchallenge, respectively (Fig. 5G). Taken together, these results demonstrated that WNV-ΔNS1 virus could protect mice from a highly lethal challenge with WT WNV.

FIG 4 Stability and safety of WNV-ΔNS1. (A) IFA detection of WNV-ΔNS1 virus at different passages using the 4G2 monoclonal antibody. WNV-ΔNS1 viruses were blind passaged for 15 rounds in VerON51 cells. The viruses at passages 0, 5, 10, and 15 were used to infect naive VerON51 cells and Vero cells. (B) Detection of the Gluc activities in cell lysates at different time points after WNV-ΔNS1-Gluc infection. Naive VerON51 cells and Vero cells were infected with WNV-ΔNS1-Gluc reporter virus at an MOI of 100, and the cell lysates collected at the indicated time points were subjected to the luciferase assay. Data represent the mean $\pm$ standard deviation of triplicate measurements in a representative experiment. (C and D) Morbidity (C) and survival (D) analyses of IFNAR^{-/-} mice after inoculation with WT and WNV-ΔNS1 viruses. Six-week-old IFNAR^{-/-} female mice were inoculated i.p. with 10^2 IU of WT WNV or 2×10^8 IU of WNV-ΔNS1. All mice ($n = 5$ for each group) were monitored for morbidity and death for 28 days. Two experiments were performed independently. The asterisks denote statistical differences between the indicated groups. ***, $P < 0.001$.

Protective efficacy of single immunization with WNV-ΔNS1 in C57BL/6 mice. To further determine whether one-shot vaccination with WNV-ΔNS1 was sufficient to elicit protective immunity against WNV, C57BL/6 mice were immunized with WNV-ΔNS1 only once before challenge. Three dosages (3×10^6 IU, 1.2×10^7 IU, and 4.8×10^7 IU) of WNV-ΔNS1 were evaluated. Sera collected on days 14 and 28 were subjected to ELISA (Fig. 6A). The three dosages induced antibody responses in a dose- and time-dependent manner (Fig. 6B and C). On day 14, the average IgG titers were 1:800, 1:3,200, and 1:3,200 for the groups treated with 3×10^6 IU, 1.2×10^7 IU, and 4.8×10^7 IU, respectively; on day 28, the antibody titers rose to 1:3,200, 1:9,600, and 1:9,600, respectively. Corroborating the ELISA IgG titers, neutralizing antibody titers reached 1:20, 1:26, and 1:33 on day 14 and 1:26, 1:80, and 1:66 on day 28 for the lowest to the highest dosages, respectively (Fig. 6C). At 30 days postimmunization, all mice, including PBS-vaccinated ones, were i.p. challenged with 3×10^7 PFU of WT WNV. Mice were then monitored daily for weight loss and visible signs of disease. All mice vaccinated with WNV-ΔNS1, even at the lowest vaccine dosage, were fully protected and survived without any significant weight loss or detectable viremia, whereas the mice immunized with PBS exhibited severe disease or death, weight loss, and high levels of viremia (Fig. 6D to F). Additionally, the IgG antibody titers were measured at 0, 14, and 28 days postchallenge. The titers increased significantly in mice immunized with different dosages of WNV-ΔNS1, relative to their counterparts prior to challenge (Fig. 6B and G). Overall, our results illustrated that WNV-ΔNS1 could protect mice from a highly lethal challenge with only one dose of immunization.

CD8⁺ T cell response in vaccinated mice. To assess T cell responses in immunized mice, the mice were immunized with 2×10^8 IU WNV-ΔNS1, with a booster on day 28 after primary immunization. Splenocytes were isolated from mice on day 7 and day 14 after booster immunization, and the levels of CD8⁺ T cell activation were analyzed by

FIG 5 Antibody responses of mice immunized with WNV-ΔNS1 virus. (A) Animal experimental schedule. Four-week-old C57BL/6 female mice ($n = 5$ for each group) were i.p. immunized with 4.8×10^7 IU of WNV-ΔNS1, followed by the same dose as a booster immunization 28 days later. (B and C) ELISA and PRNT₅₀ assay. Sera were harvested from mice at the indicated time points to determine the IgG antibody and neutralizing antibody titers against WNV, using the ELISA (B) and PRNT₅₀ assay (C), respectively. ND, not detected. (D and E) Morbidity and survival analyses. All mice were i.p. injected with 3×10^7 PFU of WT WNV 30 days after the booster immunization, and weight loss (D) and survival (E) were monitored for 21 days after the challenge. (F) Viremia levels. Serum viremia levels were measured by real-time RT-PCR on day 2 postchallenge. Mice without any treatment (mock) were used as a negative control. (G) Postchallenge IgG antibody titers. On day 14 and day 28 after challenge, IgG antibody titers in sera were quantified using ELISA. The dashed lines in panels B, C, F, and G represent the limits of detection. Data represent the mean $\pm$ standard deviation of 5 mice at each time point in each group. The asterisks denote statistical differences between day 28 and day 42, which indicated that the booster dose resulted in enhanced antibody titers. *, $P < 0.05$; ****, $P < 0.0001$; n.s., no statistical difference. Two independent experiments were performed, and data from one experiment are presented.

flow cytometry after stimulation with the peptide of CD8⁺ T cell epitopes corresponding to the WNV envelope. Nonspecific stimulation with phorbol myristate acetate (PMA) and ionomycin (IONO) was used as a positive control. Time-dependent enhancement of IFN- γ production by CD8⁺ T cells (IFN- γ ⁺ CD8⁺ cells) was observed in the WNV-ΔNS1-vaccinated mice in response to either specific (peptide E) or nonspecific (PMA plus

FIG 6 Protective efficacy of one-shot immunization with WNV-ΔNS1 in C57BL/6 mice. (A) Animal experimental schedule. (B and C) Antibody titer measurements. Four-week-old C57BL/6 female mice ($n = 5$ for each group) were i.p. immunized once with different dosages of WNV-ΔNS1 (3×10^6 IU, 1.2×10^7 IU, or 4.8×10^7 IU), and sera were collected on day 14 and day 28 for IgG antibody (B) and neutralizing antibody (C) titer measurements. ND, not detected. (D to G) Analyses of morbidity, survival, and virus and antibody levels. All mice were i.p. injected with 3×10^7 PFU of WT WNV at 30 days postimmunization and monitored for weight loss (D), survival (E), viremia (F), and postchallenge IgG antibody titers (G). The dashed lines in panels B, C, and F represent the limit of detection. Data represent the mean $\pm$ standard deviation of 5 mice at each time point in each group. The asterisks denote statistical differences between the indicated groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

IONO) stimulation. In contrast, antigen-specific stimulation did not cause any increase in the percentage of IFN- γ^+ CD8 $^+$ cells in the mock-vaccinated group. There were significant differences in the percentages of antigen-specific IFN- γ^+ CD8 $^+$ cells between the mock- and WNV-ΔNS1-vaccinated mice (0.17% versus 0.3% on day 7 [$P < 0.05$] and 0.17% versus 0.93% on day 14 [$P < 0.0001$]) (Fig. 7A). Taken together, these data suggested that WNV-ΔNS1 could induce T cell responses in mice.

FIG 7 CD8⁺ T cell responses in WNV-ΔNS1-vaccinated C57BL/6 mice. (A) Summary of the percentages of IFN-γ⁺ CD8⁺ T cells in the total number of CD8⁺ T cells without stimulation (non-stimulation) or with stimulation with WNV peptide (peptide E) or PMA and IONO at 7 and 14 days after booster immunization. Mock-vaccinated mice were used as a negative control. Data represent the mean $\pm$ standard deviation of 5 mice at each time point in each group. The asterisks denote statistical differences between the indicated groups. *, $P < 0.05$; ****, $P < 0.0001$. (B) Representative flow cytometry contour plots of IFN-γ⁺ CD8⁺ T cells on day 14 after booster immunization. The CD8⁺ T cells were gated, and the percentages of IFN-γ⁺ CD8⁺ cells are indicated in the top right corners.

DISCUSSION

In this study, we assessed the potential of the replication-defective virus WNV-ΔNS1 as a vaccine candidate. WNV-ΔNS1 provides complete protection against WT WNV infection in mice, with strong neutralizing antibody responses. Viremia was not observed in the vaccinated mice, compared with the mock-vaccinated mice. No specific safety-associated adverse effects related to the vaccine were observed. Although effective CD8⁺ T cell responses were also observed in mice receiving the high dose (2×10^8 IU), the role of the cellular response in preventing WNV infection with WNV-ΔNS1 needs further study. CD8⁺ T cell depletion experiments following vaccination and prior to challenge (20, 34) will help elucidate how cellular responses contribute to survival and viral clearance.

WNV is a biosafety level 3 (BSL3) pathogen. We tried to obtain a balance between safety and immunogenicity by using WNV-ΔNS1 as a vaccine through *trans* complementation of NS1. WNV-ΔNS1 was safe in IFNAR^{-/-} mice, without causing any symptoms of disease (Fig. 4C). The safety profile of WNV-ΔNS1 can be explained by the fact that WNV-ΔNS1 undergoes only viral entry and translation, without viral replication, in naive cells (Fig. 4B). This safety property is stably maintained after serial passaging in the Vero_{NS1} cell line (Fig. 4A). The replication-defective nature of WNV-ΔNS1 in normal cells, despite its ability to replicate to exceptionally high titers in Vero_{NS1} cells, highlights the safety advantage of WNV-ΔNS1 as a vaccine.

Owing to the defective replication of WNV-ΔNS1 in normal cells, a chemical or physical inactivation step is not required for the production of this vaccine candidate. Thus, native and functional antigens could be maximally preserved to provide an antigen presentation capacity as native virus particles (35). Without the need for inactivation, WNV-ΔNS1 combines the ease of production of live-attenuated vaccines and the safety of inactivated vaccines with immunogenicity and can be manufactured at relatively low cost. It should be noted that the viral titers were much higher in the complementary Vero_{NS1} cells than in the previously reported 293T_{NS1} cells (Fig. 1B). This may result from the inability of Vero cells to generate type I IFN (36). Additionally, different cell lines for selection may have different NS1 expression levels, which may lead to different complementing efficiencies.

In summary, our results showed that WNV-ΔNS1 produced from an NS1 complementary cell line provides a new flavivirus vaccine platform. Due to its nonreplicative nature, this vaccine platform is safe and immunogenic. We think that the NS1 complementation approach could be applied to vaccine development for other highly pathogenic flaviviruses, for which safety is one of the major concerns.

MATERIALS AND METHODS

Cells, viruses, and antibodies. The Vero_{NS1} cell line was established following the same method as described previously for the generation of 293T_{NS1} (11). Vero_{NS1} and 293T_{NS1} cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 5 μg/ml or 2 μg/ml of puromycin (Invitrogen), respectively, in 5% CO₂ at 37°C. BHK-21 and Vero cells were grown in DMEM as described above without puromycin. Recombinant WT WNV was generated by electroporation of BHK-21 cells with *in vitro*-transcribed viral genomic RNA from the linearized infectious cDNA clone of strain 3356 from New York City (37, 38). The backbone of the WT WNV cDNA clone was used to construct WNV-ΔNS1 and WNV-ΔNS1-Gluc vectors, which were originally produced in the 293T_{NS1} cell line from a previous study (11), and the resultant replication-deficient WNV-ΔNS1 and WNV-ΔNS1-Gluc viruses were amplified in the Vero_{NS1} cell line in DMEM as described above but supplemented with 2% FBS. The supernatants of transfected cells were harvested at 72 h posttransfection and frozen at -80°C.

Monoclonal antibody 4G2 against the flavivirus envelope protein was kindly provided by Cheng-Feng Qin (Beijing Institute of Microbiology and Epidemiology, Beijing, China). Monoclonal neutralization antibodies FD011-1 and FD011-2 against WNV envelope were from the State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology (38). Anti-WNV capsid polyclonal antibody was reclaimed from the sera of BALB/c mice immunized with purified capsid proteins. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse/rabbit IgG and horseradish peroxidase (HRP)-conjugated goat anti-mouse/rabbit IgG secondary antibodies were purchased from Proteintech (China).

Mice. Four-week-old C57BL/6 mice were used for virus immunization and challenge studies, and 6-week-old type I IFN receptor-deficient IFNAR^{-/-} mice, which are sensitive to WNV infection, were used to test the biosafety of WNV-ΔNS1. All mouse infection experiments were performed in an animal BSL3

facility at Wuhan Institute of Virology under a protocol approved by the Laboratory Animal Ethics Committee of Wuhan Institute of Virology (permit WIVA26201801).

Viral titer quantification with immunofluorescence assay. Vero_{NS1} cells seeded on coverslips in 12-well plates (2×10^5 Vero_{NS1} cells per well) were infected with 10-fold dilutions of WNV or WNV-ΔNS1 for 1 h at 37°C and then overlaid with 2% methylcellulose. At 36 hpi, the infected cells were washed three times with PBS and then fixed with cold 5% acetone in methanol for 15 min at room temperature. The fixed cells were stained with 4G2 antibody for 1 h at room temperature. After three washes with PBS, the cells were incubated with FITC-conjugated goat anti-mouse IgG polyclonal secondary antibody. The coverslips were mounted on glass slides with 90% glycerol after three washes with PBS. Images were captured under a fluorescence microscope (Nikon Eclipse TE2000). The infectious titer of viruses was calculated as IU per milliliter.

Immunostaining-based focus-forming assay. To compare the plaque sizes of WT WNV and WNV-ΔNS1, an immunostaining-based focus-forming assay was performed as described previously (39), in 12-well plates seeded with 2×10^5 Vero_{NS1} cells per well. The 4G2 antibody and goat anti-mouse IgG conjugated with HRP were used as primary and secondary antibodies, respectively. An enhanced HRP-3,3'-diaminobenzidine (DAB) chromogenic substrate kit (Tiangen) was used for color development.

Virus growth kinetics. The virus growth kinetics of both WNV and WNV-ΔNS1 were assessed in Vero_{NS1} cells. The cells (in 12-well plates, at 2×10^5 cells per well) were infected with viruses at an MOI of 0.1. At the indicated times postinfection, the culture medium was collected and subjected to viral titer quantification as described above.

Concentration and purification of viral particles. To obtain highly purified and concentrated viral stocks, four 175-cm² flasks of Vero_{NS1} cells were infected with WT or WNV-ΔNS1 at an MOI of 0.1. The supernatants were reclaimed at 72 hpi and subjected to sequential centrifugation at 4°C for 10 min at $400 \times g$ and for 20 min at 5,000 rpm, to remove cells and cell debris, respectively. The clarified supernatants were then incubated overnight at 4°C with PBS containing 8% (wt/vol) PEG8000 (Sigma), followed by centrifugation at 4°C for 50 min at $10,500 \times g$. The pellets were then gently resuspended in PBS and subjected to ultracentrifugation at 4°C for 1.5 h at $105,000 \times g$ with a 24% sucrose cushion, using a SW41 rotor in an Optima MAX-XP ultracentrifuge (Beckman). The pellets were finally resuspended in 50 μl of PBS.

Gel electrophoresis. Purified virions were denatured by heating at 95°C for 10 min in SDS sample buffer containing β-mercaptoethanol and were resolved on a 15% SDS-PAGE gel. The gel was then stained with Coomassie brilliant blue or subjected to Western blotting analysis, as described previously (11).

Luciferase assay. The infected cells were lysed with 200 μl of lysis buffer (Thermo Scientific) and subjected to the luciferase assay according to the manufacturer's instructions. Briefly, 20 μl of cell lysate was mixed with 50 μl of substrate (Thermo Scientific), followed by immediate measurement on a multimode microplate reader (Varioskan Flash; Thermo Fisher, Finland). The relative light units (RLUs) were recorded for each sample. All luciferase assays were performed in triplicate.

Animal safety test. For safety evaluations, 6-week-old female IFNAR^{-/-} mice were infected i.p. with 10^2 IU of WT WNV or 2×10^8 IU of WNV-ΔNS1. The animals were monitored for survival and body weight loss for 28 days.

Immunization and challenge studies. Four-week-old female C57BL/6 mice were i.p. immunized with 4.8×10^7 IU of WNV-ΔNS1, followed by the same dose of booster 28 days later; unvaccinated mice were immunized with PBS as a negative control. At 30 days after booster vaccination, all mice were challenged i.p. with 3×10^7 PFU of WT WNV and monitored for survival and weight loss for 21 days. For one-shot immunization, mice were i.p. immunized once with 3×10^6 , 1.2×10^7 , or 4.8×10^7 IU of WNV-ΔNS1, challenged i.p. with 3×10^7 PFU of WT WNV at 30 days postimmunization, and monitored for weight loss and survival for 15 days. Viremia was measured, with a real-time reverse transcription (RT)-PCR assay, in the serum of challenged and unchallenged (mock-treated) mice at 2 days after challenge, as described previously (39); the limit of detection was $10^{3.3}$ viral RNA copies/μl. Total RNA was extracted from 100 μl blood of mice using TRIzol reagent (TaKaRa, China), according to the manufacturer's protocols. Postchallenge IgG antibody titers were also quantified on day 14 and day 28 after challenge.

Antibody titers. WNV-specific IgG antibody levels of vaccinated mice were examined with an ELISA. Briefly, 96-well plates were coated with WNV-ΔNS1 and blocked with PBS containing 5% skim milk. The coated wells were incubated with 4-fold serial dilutions of heat-inactivated (56°C for 30 min) sera from vaccinated mice, starting with a 1:50 dilution, and the HRP-conjugated goat anti-mouse IgG secondary antibody. A two-component 3,3',5,5'-tetramethylbenzidine (TMB) color development kit (Beyotime Biotechnology) was used for color development for detection of bound antibodies. The optical density at 450 nm was measured after the addition of 1 M H₂SO₄ stop solution, using a multimode microplate reader (Varioskan Flash; Thermo Fisher), according to the manufacturer's instructions. The IgG antibody titers were defined as the highest dilution of serum giving an optical density twice that of the nonimmune serum (20, 26, 40, 41).

The neutralizing antibody titers were determined with the standard 50% plaque reduction/neutralization titer (PRNT₅₀) assay, as described previously (38, 39). Briefly, serial 2-fold dilutions of serum samples were preincubated with WT WNV for 1 h at 37°C and added to a monolayer of BHK-21 cells seeded into 12-well plates. The mixture was then replaced with a layer of 2% methylcellulose. After further incubation at 37°C in 5% CO₂ for 3 days, the overlay was removed. The cells were then fixed with 3.7% formaldehyde containing 1% crystal violet for plaque formation, and the plaques were counted. The

neutralizing antibody titers were calculated as the highest dilution of serum yielding a 50% reduction in virus control plaque numbers (42, 43).

Intracellular staining and flow cytometry. C57BL/6 mice were immunized with 2×10^8 IU of WNV-ΔNS1, with a booster 28 days after the first immunization. On day 7 and day 14 after booster immunization, splenocytes were isolated from the spleens of immunized mice for cytokine analysis. Briefly, the isolated splenocytes were first stimulated with 1 μg/ml of WNV E peptide (IALFLAV) (no. 3, K^b-restricted) (26, 34, 44, 45) or 50 ng/ml of PMA plus 1 μg/ml of IONO at 37°C for 5 h in the presence of GolgiPlug (1 μl/ml; BD Biosciences). The treated cells were then washed with staining buffer (2% FBS in PBS) and stained at 4°C for 30 min with anti-CD8 antibody conjugated with allophycocyanin (APC)-Cy7 (BD Pharmingen). After an additional wash, the cells were fixed and permeabilized with BD Fix/Perm buffer at 4°C for 20 min and stained with anti-IFN-γ antibody conjugated with phycoerythrin (PE)-Cy7 or an isotype control (BD Pharmingen). Finally, the cells were washed and analyzed by flow cytometry. The percentage of IFN-γ-positive CD8⁺ T cells in the total number of CD8⁺ T cells was calculated.

Statistical analysis. The unpaired *t* test was used to determine whether there were significant (*P* < 0.05) differences for all experiments. Kaplan-Meier survival curves were analyzed by the log-rank test. The statistical analyses were performed using the nonparametric test in GraphPad Prism 5.0.

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