

Evaluation of the immunogenic properties of a chimeric protein against West Nile virus: in-silico analysis and assessment in mouse

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ABSTRACT

Background and Objectives: West Nile virus (WNV) is a mosquito-borne flavivirus that represents a significant public health concern, and the development of effective vaccine candidates remains a priority. This study aimed to design a chimeric multi-epitope protein against WNV using immunoinformatics approaches and evaluate its immunogenicity in mice.

Materials and Methods: Selected regions of the WNV envelope (E) and non-structural 1 (NS1) proteins were analyzed to identify B- and T-cell epitopes using immunoinformatics tools. The selected epitopes were incorporated into a chimeric construct fused with cholera toxin B subunit, and its antigenicity, allergenicity, structural properties, and TLR3 interaction were evaluated in silico. The optimized construct was expressed in *Escherichia coli*, purified by Ni-NTA affinity chromatography, and confirmed by SDS-PAGE and western blotting. BALB/c mice were immunized with the recombinant protein plus adjuvant, and immune responses were assessed using ELISA, cytokine assays, and flow cytometry.

Results: Immunized mice developed high IgG antibody titers and showed detectable IFN- γ and IL-5 production, suggesting induction of Th1-associated responses with limited Th2-related cytokine production. The chimeric protein also stimulated CD4⁺ and CD8⁺ T-cell responses, indicating activation of both humoral and cellular immunity.

Conclusion: The designed chimeric protein showed immunogenic potential and induced humoral and cellular immune responses in mice, supporting further evaluation as a candidate for WNV vaccine development.

Keywords: West Nile virus; Immunogenicity; Epitopes; Recombinant fusion proteins

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne member of the genus *Flavivirus* within the family *Flaviviridae* (1, 2). The virus was first identified in 1937 in

the West Nile district of Uganda. Since its initial detection, WNV has expanded its geographic distribution and is now reported across Africa, Europe, the Middle East, North America, and parts of Asia (1).

WNV particles exhibit an icosahedral architecture

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and contain a positive sense, single stranded RNA genome that encodes a single polyprotein from one open reading frame (1, 2). Translation of this genome produces a polyprotein that undergoes co and post-translational processing to generate three structural proteins—capsid, pre-membrane/membrane (prM/M), and envelope (E)—as well as several non-structural proteins (NS1–NS5). Among these, the envelope (E) glycoprotein is the dominant surface antigen involved in receptor binding and membrane fusion and represents the primary target of neutralizing antibodies (2). Structurally, the E protein comprises three domains: domain I (central), domain II containing the conserved fusion loop, and domain III, which possesses an immunoglobulin-like fold that contributes to receptor interactions (3).

In nature, WNV transmission is maintained through a bird–mosquito enzootic cycle mainly involving *Culex* spp. mosquitoes and avian hosts, which serve as amplifying reservoirs. The spread of the WNV is influenced by bird migration, climate change, and ecological factors affecting mosquito populations (1). Humans and horses, although susceptible to infection, typically do not develop sufficient viremia to support further transmission and are therefore regarded as dead-end hosts. In humans, most infections remain asymptomatic; however, approximately 20% lead to febrile illness, and a small proportion develop severe neuroinvasive disease, particularly among elderly or immunocompromised individuals (4). To date, several vaccines have been licensed for use in horses; however, no licensed vaccines or specific antiviral therapies are available for humans. Consequently, the development of safe and effective vaccines for humans and horses, together with preventive measures such as mosquito population control, remains a critical public health priority (1). In this context, novel vaccine design approaches have gained considerable attention in modern vaccinology. In particular, epitope based vaccine platforms have emerged as promising strategies for the rational development of next generation vaccines. By incorporating selected cytotoxic T lymphocyte (CTL), helper T lymphocyte (HTL), and B-cell epitopes, multi-epitope vaccine approaches aim to induce targeted and broad antigen-specific immune responses against infectious diseases while minimizing the inclusion of unnecessary antigenic components (5).

In this study, we designed a chimeric multi-epitope

protein incorporating selected regions of the WNV E and NS1 proteins using in silico approaches, expressed the construct in *Escherichia coli* BL21(DE3), and evaluated its immunogenicity in mice.

MATERIALS AND METHODS

Selection of antigenic domain for multi-epitope protein. The amino acid sequences of the WNV polyprotein were retrieved from the NCBI database and aligned using ClustalW (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html) (6) to generate a consensus sequence. The E (residues 291–791) and NS1 (residues 792–1143) protein regions were then selected for further analysis.

B and T cell epitopes prediction. B cell epitopes were predicted using SVMTrip (7), ABCpred (8), Bcpreds (8), and BepiPred (9) servers. MHC class I and II binding epitopes were identified using the IEDB analysis resource with the NetMHCpan BA 4.1 method (10). Only epitopes with high binding affinity ($IC_{50} < 500$ nM) were selected for further analysis.

Design and validation of a chimeric multi-epitope subunit immunogen for WNV. The sequence of the *Vibrio cholerae* toxin B subunit (CTB; UniProtKB: P01556) was fused to the selected peptide regions using A(EAAAK)_nA linkers to construct multi epitope protein. Antigenicity, allergenicity, and physicochemical properties of the designed construct were evaluated using VaxiJen (11), ANTIGENpro (12), AllerTOP (13), AlgPred (14), and ProtParam (15) servers. TMHMM (16) and SignalP v5.0 (17) were used to confirm the absence of transmembrane helices and signal peptides.

Secondary and tertiary structures were predicted using the GOR4 (18) and RaptorX (19) servers. The predicted 3D model was further refined and validated using GalaxyRefine (20), ProSA web (21), PROCHECK (22), and ERRAT (22).

Codon optimization was performed according to the *Escherichia coli* codon usage table (Kazusa database) using the Optimizer tool (<http://genomes.urv.es/OPTIMIZER>) (23). *EcoRI* and *HindIII* restriction sites were introduced at the 5' and 3' termini of the synthetic gene, and a 6X His-tag was added at the C-terminus to facilitate purification. RNA

secondary structure and minimum free energy were predicted using RNAfold (24). Unless otherwise stated, default parameters were used for all prediction servers.

Molecular docking of the chimeric protein against WNV. The TLR3 structure (PDB ID: 1ZIW) was obtained from the RCSB PDB database, and both the refined chimeric protein structure and TLR3 were submitted to ClusPro (<https://cluspro.bu.edu/login.php>) (25) for docking. Binding affinity was assessed using the PRODIGY (26) server, and residue level interactions were analyzed via PDBsum (27).

Bacterial strains and media for the expression of the WNV chimeric protein. The WNV chimeric protein was cloned in *E. coli* DH5 α and expressed in *E. coli* BL21(DE3). Bacterial cultures were grown in nutrient broth supplemented with 50 μ g/ml kanamycin (Sigma Aldrich, St. Louis, MO, USA).

Expression and purification of the WNV chimeric protein. The synthetic gene encoding the chimeric protein was subcloned into pET28a and transformed into *E. coli* BL21 (DE3). Cultures were grown at 37°C in LB medium with kanamycin. Protein expression was induced by 1 mM IPTG for 6-8 hours. Cells were harvested by centrifugation at 6000 $\times$ g for 10 min at 25°C, and pellets resuspended in lysis buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 8.0). Lysates were sonicated (3 $\times$ 20 s, high power) and centrifuged at 10,000 $\times$ g for 15 min at 4°C. Recombinant protein was purified by Ni-NTA affinity chromatography (Qiagen) using a pH gradient from 8 to 4, analyzed by 12% SDS-PAGE, dialyzed against PBS, and quantified by Bradford assay.

Western blotting for confirmation of the WNV recombinant protein. The purified WNV chimeric protein was analyzed by 12% SDS PAGE and electrotransferred onto a nitrocellulose membrane overnight at 15 V using transfer buffer containing 39 mM glycine, 48 mM Tris base, 0.037% SDS, and 20% methanol. The membrane was blocked with 5% non fat skim milk in TBST (50 mM Tris Cl, 150 mM NaCl, pH 7.5, 0.05% Tween 20) for 2 h and incubated with HRP conjugated mouse anti-His tag antibody (Southern Biotech, USA; 1: 10,000 in PBS containing 0.05% Tween 20). Signal development was performed using 3,3 diaminobenzidine (DAB; Sigma Aldrich, St. Louis, MO,

USA) and stopped by immersion in distilled water.

Animal immunization. Six week old female BAL-B/c mice (Pasteur Institute of Iran, Tehran) were divided into two groups (n=5 per group). The experimental group received the chimeric protein formulated with adjuvant, whereas the control group received 1X PBS containing the adjuvant.

Mice were immunized weekly for four consecutive weeks. The initial dose included 20 μ g of antigen emulsified in complete Freund's adjuvant (CFA), followed by booster doses of 15 μ g and 10 μ g of antigen formulated in incomplete Freund's adjuvant (IFA), and a final intraperitoneal injection of 2 μ g purified protein (Table 1). Antigens were soluble in 1X PBS and mixed with equal volumes of CFA (primary dose) or IFA (boosters). Control mice received PBS formulated with CFA for the initial injection and IFA for boosters. For immunological analyses, sera were collected one day before each injection beginning after the second dose and stored at -20°C until assessment for WNV specific antibodies.

Ethics statement. All animal procedures were approved by the Ethics Committee of the National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran (IR.NIGEB.EC.1403.4.16.F), and were conducted in accordance with institutional guidelines for the care and use of laboratory animals.

Determination of IgG response in serum. IgG antibody levels were determined by ELISA, and the optical density (OD) of diluted sera from mice immunized with the WNV chimeric protein was used to estimate the IgG response. ELISA plates (Nunc, Roskilde, Denmark) were coated overnight with 1 μ g/well of purified chimeric protein in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) and washed three times with 1X PBS containing 2.5% Tween 20 (PBST). Plates were blocked with 5% skim milk, and serially diluted sera from immunized mice (primary antibody) were added and incubated for 1 h at 37°C. Goat anti mouse IgG-peroxidase conjugate was used as the secondary antibody, and color development was performed using 3,3' diaminobenzidine (DAB). The reaction was stopped with 1 N H₂SO₄, and absorbance was measured at 492 nm. Antibody titers were defined as the highest serum dilution yielding an OD₄₉₂ exceeding twice the mean OD₄₉₂ of control samples.

Table 1. Antigen administration schedule and dosing regimen in BALB/c mice

Dose	Day	Recombinant Protein (µg per mouse)	Freund's Adjuvant	Injection Route
First Injection	0	20	Complete	Subcutaneous
Second Injection	7	15	Incomplete	Subcutaneous
Third Injection	15	10	Incomplete	Subcutaneous
Fourth Injection	23	2	-	Intraperitoneal

The reactivity of mouse sera to the recombinant WNV protein was also assessed using the Euroimmun WNV IgG ELISA kit in a semi quantitative format. Sera were diluted 1:100 in PBS and added to antigen coated wells. Detection was performed using an HRP conjugated goat anti mouse IgG secondary antibody (Sigma-Aldrich, USA).

Cytokine analysis. Splenocytes were isolated from immunized BALB/c mice (n=5 per group) and single cell suspensions were prepared under sterile conditions. Cells (3×10^6 cells/mL; 3×10^5 cells/well; 100 µL) were seeded in triplicate in 96 well flat bottom plates (Orange Scientific, Belgium) and cultured in complete RPMI 1640 (Gibco, Invitrogen, Scotland, UK) supplemented with 5% FCS (Sigma, USA), 2.3×10^{-2} mM 2-mercaptoethanol, penicillin streptomycin (100 U–100 µg/mL), and 10 mM HEPES. Concanavalin A (5 µg/mL) and medium alone served as positive and negative controls, respectively, and recombinant antigen (4 µg/mL) was added to test wells. Cultures were maintained at 37°C in 5% CO₂. Supernatants were collected at 72 h (IL 5), and 120 h (IFN γ), and cytokine levels were measured using commercial ELISA kits (R&D Systems, Minneapolis, USA). Cytokine concentrations in negative controls were subtracted from antigen stimulated values.

CD4+, CD8+ T cells, and cell cycle analysis. To quantify CD4+ and CD8+ T cells and assess cell cycle distribution by flow cytometry, splenocytes from each group (n = 5) were cultured two weeks after the final immunization as previously described. Antigen stimulated splenocytes were collected after 72 h, washed with PBS, and stained with anti CD4 FITC and anti CD8 APC monoclonal antibodies (R&D Systems, USA) to determine CD4+ and CD8+ T cell populations. For cell cycle analysis, cells were stained with propidium iodide (EMD Millipore, USA). Quantitative analysis included CD4+ and CD8+ T cell frequencies, G0/G1, S, and G2/M phases, and calculation

of the proliferation index using Formula 1.

$$(1) \quad PI = \frac{S+G2M}{G0G1+S+G2M} \times 100$$

Statistical analysis. To compare differences between the sample and control groups, the non parametric Mann–Whitney U test was applied. All statistical analyses were performed using GraphPad Prism 9.

RESULTS

Immunogens selection and prediction of B and T cell epitopes. Linear B cell epitopes from the E (291–794) and NS1 (795–1146) proteins were predicted, and epitopes with scores >0.7 and antigenicity >0.4 were selected. For T cell analysis, 9 mer mouse MHC I (CTL) and 15 mer mouse MHC II (HTL) epitopes were predicted, and antigenic epitopes with IC50 <500 were selected.

Predicted epitopes were mapped onto the amino acid sequences of the E and NS1 proteins, and regions with higher epitope density were identified. These regions were analyzed using the IEDB Epitope Conservancy tool across *Flaviviridae*, and segments with less than 60% sequence conservation were selected. Consequently, one region from domain I, the entire domain III of the E protein, and a region of NS1 were chosen for chimeric construct design.

The design of chimeric protein. The selected regions were joined using A(EAAAK)_nA linkers to maintain structural separation between domains (Figs. 1A and B). A C-terminus 6X His-tag was incorporated for downstream purification.

Physicochemical properties. The construct showed high predicted antigenicity (0.91 in VaxiJen; 0.50 in ANTIGENpro) and was classified as non-allergenic.

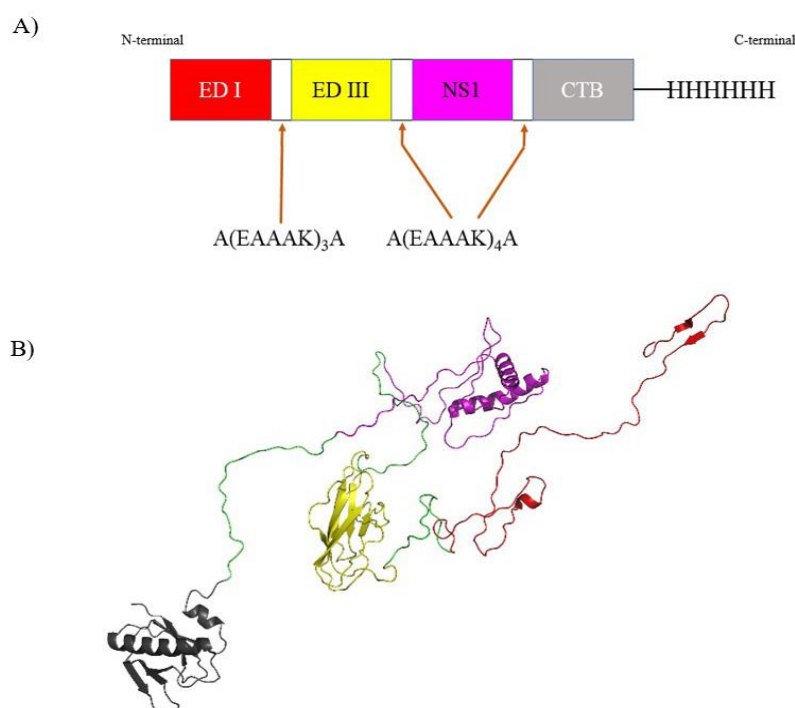


Fig. 1. A) The schematic structure of the candidate vaccine construct against West Nile Virus. B) The predicted tertiary structure of the designed protein for West Nile Virus. The selected regions of domain I and domain III of the E protein, NS1 (Non-Structural Protein 1), and CTB as a bio-adjuvant are highlighted in red, yellow, pink, and gray, respectively. A(EAAAK)_nA was used as a linker and depicted in white color. To facilitate purification, a 6x His-tag was incorporated at the C-terminal end of the construct.

ProtParam estimated a molecular weight of ~51 kDa and a pI of 6.79, and a half life of >10 h in *E. coli*. The instability index (30.66), aliphatic index (77.40), and GRAVY value (-0.296) indicated a stable, thermally resistant, and soluble protein. No transmembrane regions or signal peptides were detected.

Prediction of secondary and tertiary structures. Secondary structure prediction indicated that the chimeric protein consists predominantly of α helices (47.87%), followed by random coils (36.6%) and β strands (15.53%). The predicted 3D structure showed spatial separation of the epitope regions by the A(EAAAK)_nA linker. After refinement, the first model yielded the following metrics: GDT HA (0.94), RMSD (0.46 Å), MolProbity score (1.47), clash score (7.2), and (0.3%) poor rotamers. Structural validation produced a ProSA z score of -4.83, an ERRAT score of 91.07, and Ramachandran analysis indicated that 93.6% of residues were located in favored regions.

Protein-protein docking. The chimeric protein was docked with human TLR3, and model 0 was se-

lected based on the lowest energy score (-1301.4 kJ/mol). The interaction map showed 10 salt bridges, 41 hydrogen bonds, and 400 non-bonded contacts with TLR3.

Codon optimization and the stability of mRNA.

Codon optimization for expression in *E. coli* yielded a codon adaptation index of 0.97 and a GC content of 56.5%. RNAfold predicted a stable mRNA secondary structure with a minimum free energy of -499.8 kcal/mol.

Chimeric protein expression and purification.

The gene was subcloned under a T7 promoter and expressed in *E. coli* BL21(DE3). The protein appeared on a 12% SDS-PAGE (Fig. 2A), was purified by Ni-NTA under denaturing conditions (Fig. 2B), and verified by Western blotting using an anti-His tag antibody (Fig. 2C).

Antibody titer analysis. IgG antibody response in mice serum against purified chimeric protein was evaluated by ELISA (Fig. 3). Antibody levels

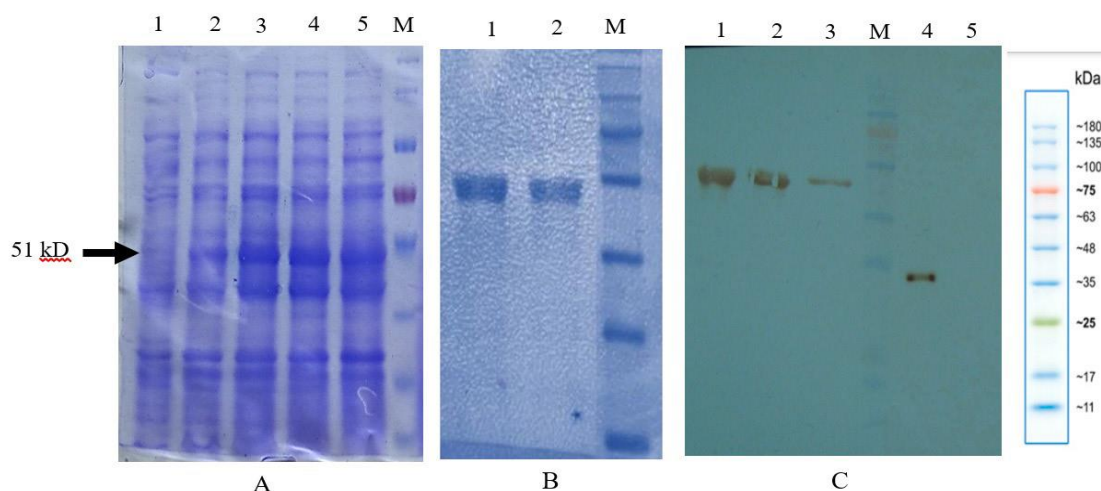


Fig. 2. Expression, purification, and Western blot confirmation of the WNV chimeric protein. (A) SDS-PAGE analysis of chimeric protein expression in *E. coli* BL21(DE3) following IPTG induction. Lanes 1–5 represent protein expression at 0, 2, 4, 6, and 8 h after induction, respectively. Lane M, protein molecular weight marker. (B) SDS-PAGE analysis of the purified chimeric protein obtained by Ni-NTA affinity chromatography. Lanes 1 and 2 represent purified protein fractions. (C) Western blot analysis using an anti-His-tag antibody. Lanes 1–3, chimeric protein; lane 4, purified recombinant His-tagged protein as positive control; lane 5, negative control. Lane M, protein molecular weight marker.

increased after each immunization, with mice receiving three or four doses plus Freund's adjuvant showing higher OD values at all serum dilutions than those immunized twice. Moreover, mice given four immunizations showed a slower decline in serum titer, indicating a stronger immune response. The control group (PBS 1× plus Freund's adjuvant) displayed minimal OD values throughout. No significant IgA antibody titers were detected in serum or feces in either group (data not shown).

Assessment of serum reactivity using the WNV IgG ELISA kit showed that mice immunized with the chimeric protein plus adjuvant developed detectable IgG responses, while control sera displayed minimal reactivity ($p < 0.01$).

Analysis of cellular immune responses in immunized mice. The immune response induced by the chimeric protein was evaluated via cytokine secretion, flow cytometry, and cell cycle analysis of splenocytes. Antigen-treated mice exhibited low IL-5 levels (52.67 pg/ml), indicating limited Th2 activation, while IFN- γ levels were significantly elevated (862.88 pg/ml, $p < 0.05$) compared to controls (Fig. 4). Flow cytometry showed a significant increase ($p < 0.05$) in CD4⁺, CD8⁺ T cells, and the CD4⁺/CD8⁺ ratio in the treated group (Fig. 5). Cell cycle analysis revealed that splenocytes from immunized mice

had significantly higher percentages in S (22.17%) and G2/M (4.79%) phases versus negative (10.89% S, 0.07% G2/M) and positive (12.64% S, 2.28% G2/M) controls ($p < 0.0001$) (Fig. 6). The proliferation index was also markedly increased in the antigen group, confirming enhanced splenocyte proliferation.

DISCUSSION

Over the past two decades, WNV has emerged as a major zoonotic pathogen worldwide. Although vaccination represents a rational control strategy, no licensed vaccine is yet available for humans (1). Multi-epitope vaccine design enables genome-wide identification of conserved and immunogenic epitopes while minimizing pathogenic components. Accordingly, we developed a chimeric protein incorporating immunologically relevant epitopes of WNV for evaluation in mice, which remains a widely used system for assessing vaccine efficacy.

The E protein is a major determinant of humoral immunity in flaviviruses and contains several well-characterized protective epitopes (28, 29). Although NS1 is a non-structural protein, its surface expression and secretion allow immune recognition, and previous studies have shown that NS1-specific immunity can contribute to protection against flavi-

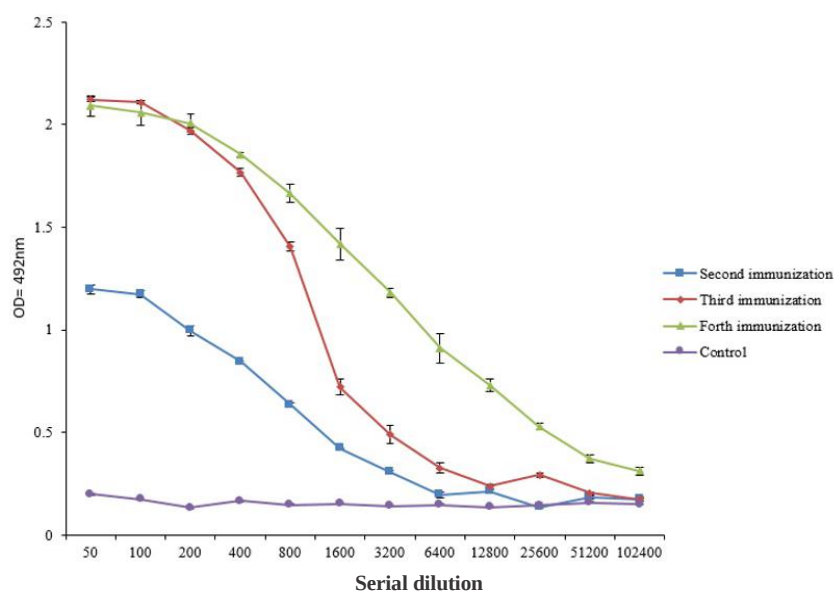


Fig. 3. The IgG antibody response induced by the chimeric protein designed against West Nile Virus in combination with CFA and IFA was measured after the second, third, and fourth immunizations. Optical density values for serial dilutions of serum samples collected from mice following injection with the chimeric protein were used to estimate the IgG antibody response. The control group was administered PBS 1X- Freund's adjuvant. The antibody titer was determined as the reciprocal of the highest serum dilution yielding an OD value exceeding the cut-off threshold. The cut-off threshold was defined as twice the mean OD at 492 nm in control mice.

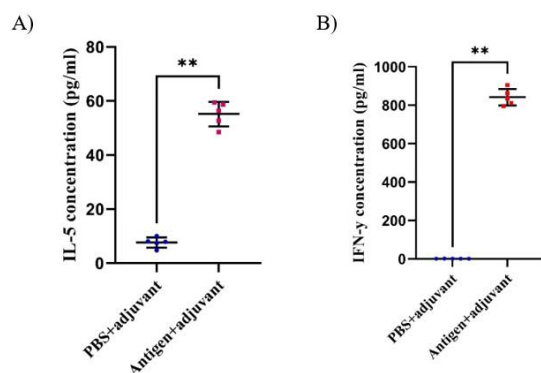


Fig. 4. Analysis of IL-5 (A) and IFN- γ (B) concentrations in the culture supernatant of splenocytes derived from mice immunized with the chimeric protein formulated with Freund's adjuvant against West Nile Virus. The control group received PBS 1X combined with Freund's adjuvant. Data are displayed as means $\pm$ SD (n=5). **p < 0.01.

virus infection (30).

Based on these considerations, E and NS1 were selected for construct design, and consensus sequences were generated by aligning amino acid sequences from multiple viral strains. CD4⁺ T cells and B cells play key roles in protection against WNV (31); con-

sequently, regions enriched with high-scoring B- and T-cell epitopes were identified. Epitope mapping facilitated the inclusion of regions with high determinant density, which may improve the preservation of conformational epitopes. Considering the potential risk of cross-reactivity among flaviviruses and the theoretical risk of antibody-dependent enhancement (ADE), regions with less than 60% sequence conservation were considered during construct design (31). Although epitope selection was optimized for the BALB/c model, future studies evaluating human HLA diversity and population coverage will be important for assessing its potential for human vaccine development.

In the designed construct, selected regions were linked to CTB using repeats of the A(EAAAK)_nA linker (2 < n < 5) to facilitate structural separation between the selected domains. Based on these observations, our structural predictions similarly indicated that A(EAAAK)₃A and A(EAAAK)₄A segments preserved spatial separation between domains (Fig. 1). To facilitate purification by immobilized metal affinity chromatography, a 6X His-tag sequence was added to the C-terminus of the chimeric pro-

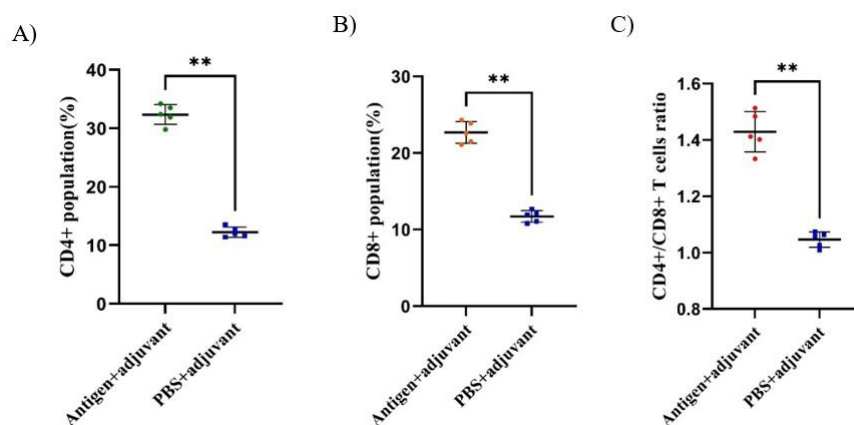


Fig. 5. Quantitative analysis of CD4+ (A) and CD8+ (B) T cells, as well as the CD4+/CD8+ ratio (C), in the spleens of mouse groups immunized with the chimeric protein formulated with Freund's adjuvant against West Nile Virus. The control group received PBS 1X combined with Freund's adjuvant. The data are depicted as means with $\pm$ SD (n=5). **p < 0.01.

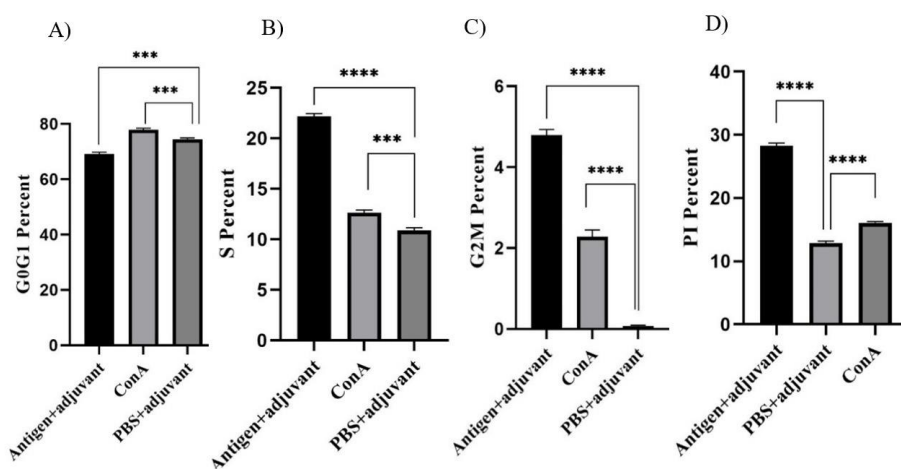


Fig. 6. Cell cycle distribution of splenocytes isolated from immunized BALB/c mice after stimulation with the chimeric antigen formulated with Freund's adjuvant, Concanavalin A (ConA, positive control), or PBS + adjuvant (negative control). The percentages of cells in (A) G0/G1, (B) S, (C) G2/M phases, and (D) proliferative index (PI) are shown. Data are presented as mean $\pm$ SEM (n = 5). Statistical significance: ***p < 0.001; ****p < 0.0001.

tein. Physicochemical analyses predicted a molecular weight of approximately 51 kDa, an extended half-life of >10 h, and a negative GRAVY value, consistent with the solubility of the construct in *E. coli*. Secondary and tertiary structure analyses further indicated appropriate domain separation, and structural validation using ProSA-web, ERRAT, and PROCHECK confirmed acceptable model stereochemistry. TLR3 recognizes double-stranded RNA (dsRNA) intermediates generated during the replication cycle of flaviviruses, including WNV, and activates innate immune pathways leading to type I interferon production and antiviral responses (32). Therefore,

considering its important role in antiviral immunity, docking studies with TLR3 were performed. The predicted interaction with TLR3 suggests a potential immunostimulatory capacity of the construct; however, further validation, including molecular dynamics simulations and experimental approaches, is required to confirm whether this interaction contributes to innate immune activation.

Evaluation of immunogenicity in mice demonstrated that the chimeric protein induced measurable antibody responses, with higher IgG titers observed in immunized groups (Fig. 3). To evaluate whether antibodies present in the sera of mice treated with the

chimeric protein could recognize the recombinant WNV antigen included in a commercially available human ELISA kit, a semi-quantitative approach was employed. Since the kit's calibrator and controls were based on human antibodies and the samples originated from mice, this method was considered appropriate. Although the data are relative and semi-quantitative and do not reflect absolute IgG concentrations in mouse sera, the results indicate that the chimeric protein was able to induce antigen-specific humoral responses in mice.

Immunized mice exhibited a robust increase in IFN- γ , a cytokine critically involved in antiviral defense and central nervous system protection during WNV infection (33, 34), together with detectable IL 5 secretion. The observed cytokine profile indicates that the immune response was predominantly skewed toward a Th1 phenotype, with limited Th2 involvement. This finding is consistent with the significant increase in IFN- γ levels and the detectable production of IL-5 (Fig. 4). The induction of CD4 $^{+}$ and CD8 $^{+}$ T cells is particularly relevant, as these cells contribute to viral clearance and protection in the central nervous system (35). Consistent with this, flow cytometry showed increased CD4 $^{+}$ and CD8 $^{+}$ populations in the treated group (Fig. 5), and the higher CD4 $^{+}$ /CD8 $^{+}$ ratio suggests a relative predominance of CD4 $^{+}$ T-cell responses within the activated T-cell population. Additionally, a greater proportion of splenocytes in S and G2/M phases, together with a higher proliferation index, indicated robust cellular activation following antigen exposure (Fig. 6).

Recombinant E-based vaccine approaches have demonstrated the ability to stimulate antigen-specific antibody production, IFN- γ secretion, and T-cell activation (28, 36). For example, De Filette et al. reported enhanced antibody responses and increased IFN- γ production by CD8 $^{+}$ T cells following an E protein-based DNA prime-protein boost strategy (28). Similarly, NS1-containing vaccines and single-cycle WNV vaccine candidates have highlighted the contribution of NS1-specific immunity and CD4 $^{+}$ /CD8 $^{+}$ T-cell responses to vaccine-induced protection (37). Consistent with previous findings, the E/NS1 chimeric construct evaluated in the present study induced coordinated humoral and cellular immune responses, including increased IFN- γ production and activation of CD4 $^{+}$ and CD8 $^{+}$ T cells when administered with the selected adjuvant formulation. The simultane-

ous induction of humoral responses and both CD4 $^{+}$ and CD8 $^{+}$ T-cell activation suggests that the E/NS1-based vaccine formulation, together with the selected adjuvant, may elicit a broader immune profile compared with conventional approaches primarily focused on a single antigenic component. Although direct comparisons with previous vaccine candidates are limited due to differences in vaccine platforms, adjuvant formulations, and experimental models, the present findings support the potential of E/NS1-based vaccine formulations as a promising approach for WNV vaccine development.

Further studies evaluating the protective efficacy of this construct through viral challenge and neutralization assays are warranted to confirm its ability to induce functional antiviral immunity. In addition, optimization of vaccine formulations, including evaluation of alternative adjuvants, assessment in larger animal cohorts, and consideration of human HLA diversity, will be important steps toward further evaluating the translational potential of this vaccine candidate.

CONCLUSION

We developed a multi-epitope chimeric protein against WNV with promising immunogenicity. Using epitopes from NS1 and E proteins identified by computational and experimental methods, we observed immune responses in mice, including CD4 $^{+}$ /CD8 $^{+}$ T-cell activation, IFN- γ and IL-5 production, and spleen cell proliferation, indicating effective humoral and cellular immunity. Further studies with pathogen challenge and additional animal models are needed to confirm protective efficacy.

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