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Combination of Modified NS1 and NS3 as a Novel Vaccine Strategy against Dengue Virus Infection

Yu-San Kao,* Chia-Yi Yu,† Hong-Jyun Huang,* Sen-Mao Tien,* Wan-Yu Wang,* Martyr Yang,* Robert Anderson,‡,§ Trai-Ming Yeh,¶,‖ Yee-Shin Lin,*,‖ and Shu-Wen Wan‖,#

Dengue virus (DENV) causes a range of illness, including dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. DENV nonstructural protein (NS) 1 has been considered to be a desirable vaccine candidate for its ability to induce Ab and complement-dependent cytolysis of DENV-infected cells as well as to block the pathogenic effects of NS1. However a potential drawback of NS1 as a vaccine is that anti-DENV NS1 Abs can lead to endothelial cell damage and platelet dysfunction by antigenic cross-reactivity. Therefore, we modified the DENV NS1 by replacing the C-terminal cross-reactive epitopes with the corresponding region of Japanese encephalitis virus NS1 to generate a chimeric DJ NS1 protein. Active immunization with DJ NS1 induced a strong Ab response. To enhance cellular immunity, we further combined DJ NS1 with DENV NS3 to immunize mice and showed activation of Ag-specific $CD4^+$ and $CD8^+$ T cells in addition to Ab responses. We further detected NS3-specific CTL activities as well as CD107a expression of effector cells. Importantly, the protective effects attributed by DJ NS1 and NS3 immunization were demonstrated in a DENV-infected mouse model by reduced viral titers, soluble NS1 levels, mouse tail bleeding time, and vascular leakage at skin injection sites. Collectively, the results from this study reveal the humoral and cellular immune responses and the protective effects conferred by DJ NS1 and NS3 immunization in the mouse model of DENV infection and provide a potential strategy for dengue vaccine design. ***The Journal of Immunology*****, 2019, 203: 000–000.**

With increasing air travel and climate change, dengue is a spreading mosquito-borne disease in many tropical and subtropical parts of the world. It is estimated that there are annually ∼390 million dengue virus (DENV)–infected individuals, of which 96 million are symptomatic (1). Of this latter number, there are ∼500,000 individuals who develop severe disease, including hemorrhage, plasma leakage, and shock, and 20,000 deaths per year (2). Currently, there is only one licensed dengue vaccine for dengue-endemic countries; however, the safety in seronegative vaccine recipients remains a concern (3–5). The development of improved vaccines and/or antiviral therapies against DENV represents a global public health priority (6).

DENV is a positive-sense ssRNA virus belonging to the genus *Flavivirus* within the family *Flaviviridae*. There are four serotypes of DENV (DENV1-4), which are transmitted by *Aedes* mosquitoes (7). The DENV nonstructural protein (NS) 1 is of particular interest to DENV pathogenesis as well as to potential vaccine strategies. NS1 is expressed on the cell plasma membrane and is also secreted into the circulation. NS1 mediates pathogenesis through multiple pathways in the host (8, 9). The soluble NS1 (sNS1) can bind to prothrombin to interrupt the coagulation cascade and can direct complement against endothelial cells and induce endothelial cell apoptosis (10, 11). Recent studies showed that sNS1 can bind to TLR4, triggering cell release of proinflammatory cytokines, which contribute to vascular leakage (12, 13). NS1 has also been shown to activate TLR2 and TLR6, resulting in proinflammatory cytokine production (14). NS1 triggers endothelial permeability and vascular leakage that is prevented by NS1 immunization in mice (8, 15, 16). Furthermore, NS1 can directly disrupt the endothelial glycocalyx, leading to hyperpermeability (17, 18). Anti-NS1 Abs provide protection against DENV pathogenesis by reducing proinflammatory cytokine production, maintaining endothelial integrity, and inhibiting vascular leakage (19). Moreover, anti-NS1 Abs can fix complement and participate in Ab-dependent cell-mediated cytotoxicity to clear DENV-infected cells (8).

In dengue patients, levels of DENV-specific $CD8^+$ T cells are higher than DENV-specific $CD4^+$ T cells. The $CD8^+$ T cell activities mainly target NS3 and NS5, rather than NS1. In contrast, $CD4^+$ T cell responses are directed mainly against viral proteins that are also targeted by B cells (such as envelope, capsid, and NS1) (20). Analysis of the function of $CD4^+$ and $CD8^+$ T cells from DENV-exposed blood donors suggested an HLA-linked protective role for T cells against DENV infection in humans

*Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan; †National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Tainan 701, Taiwan; ‡Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada; §Canadian Center for Vaccinology, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada; ¶Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan; ‖Center of Infectious Disease and Signaling Research, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan; and #School of Medicine, College of Medicine, I-Shou University, Kaohsiung 840, Taiwan

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Address correspondence and reprint requests to Dr. Yee-Shin Lin or Dr. Shu-Wen Wan, Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, No. 1 University Road, Tainan 701, Taiwan (Y.-S.L.) or School of Medicine, College of Medicine, I-Shou University, No. 8 Yida Road, Kaohsiung 824, Taiwan (S.-W.W.). E-mail addresses: yslin1@mail.ncku.edu.tw (Y.-S.L.) or swwan@isu.edu.tw (S.-W.W.).

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Abbreviations used in this article: ADE, Ab-dependent enhancement; BHK, baby hamster kidney; DC, dendritic cell; DENV, dengue virus; LN, lymph node; LNDC, lymph node dendritic cell; MHC I, MHC class I; MHC II, MHC class II; NCKU, National Cheng Kung University; NS, nonstructural protein; sNS1, soluble NS1; ZIKV, Zika virus.

(21, 22). The predominance of CTL epitopes on the NS3 protein suggests a protective role in limiting viral replication. NS3 is the main target for $CD8^+$ T cell responses during DENV infection, yet there are only few studies evaluating the capacity of NS3 as DENV vaccine. One DNA vaccine candidate based on the NS3 from DENV2 has been reported. Both full-length NS3 and the helicase domain provided protection from lethal DENV challenge in mice. However, some mice still presented certain clinical signs especially in animal groups immunized with the helicase domain (23). These results suggest that an NS3-based vaccine has limited ability to prevent all aspects of DENV pathogenesis. Studies with mice inoculated with NS3 mAbs showed an increase in survival time after virus challenge, although most animals eventually died (24). Recent studies demonstrated that the addition of NS3 to a purified inactivated DENV2 virus vaccine could induce T cell responses and markedly increase total DENV-specific IgG titer as well as neutralizing Ab titer (25). Hence, the combination of NS3 with other DENV components may be advantageous in new DENV vaccine formulations.

Our previous studies indicated that anti-NS1 Abs cross-react with platelets and endothelial cells, leading to thrombocytopenia and platelet/endothelial cell dysfunction (26–31). We modified the DENV NS1 by replacing the cross-reactive C-terminal region with the corresponding region of Japanese encephalitis virus NS1 to generate a chimeric DJ NS1 protein. In this study, we show that the combination of recombinant DJ NS1 with NS3 proteins induces higher immune responses and more effective protective effects in the DENV-infected mouse model.

Materials and Methods

Mice

C3H/HeN mice were obtained from the Laboratory Animal Center of National Cheng Kung University (NCKU) College of Medicine and maintained on standard laboratory food and water. Their male 3-wk-old progeny (for DENV infection model, $n = 4$ per group) and 6–8-wk-old progeny (for other experiments, $n = 4$–5 per group) were used for our studies. Housing, breeding, and experimental use of the animals were performed in strict accordance with the Experimental Animal Committee of NCKU. The experiments were approved by the Institutional Animal Care and Use Committee of NCKU.

Preparation of recombinant proteins

DJ NS1 (aa 1–270 of DENV NS1 from the New Guinea C strain fused to aa 271–352 of Japanese encephalitis virus NS1 from the NT109 strain) cDNA was cloned into the pET28a vector with His-tag (31). The plasmid was prepared by the Proteomic Research Core Facility, Academia Sinica, Taiwan. Following introduction of the plasmids into *Escherichia coli* BL21, the recombinant proteins were induced by 1 mM isopropyl B-D-1-thiogalaactopyranoside (IPTG) (Calbiochem, San Diego, CA), solubilized in urea buffer (8 M urea, 500 mM NaCl, and 20 mM Tris-HCl), and purified on a Ni^{2+}-NTA affinity column (GE Healthcare Life Science). After purification, proteins were examined by SDS-PAGE, followed by staining with Coomassie Brilliant Blue R-250. Purified proteins were dialyzed in refolding buffer (50 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, 250 mM L-arginine, 10 mM GSH [glutathione, reduced], and 1 mM GSSG [glutathione, oxidized], and 5% glycerol) and concentrated by Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA).

DENV NS3 cDNA from the DENV2 PL046 strain was cloned into the pET21b vector with His-tag (provided from Dr. C.-Y. Yu, National Health Research Institutes). The plasmids were used to transform *E. coli* BL21. Cells were grown overnight at 37°C in Luria-Bertania medium containing 100 μg/ml ampicillin. Overnight cultures were diluted 100-fold in the Luria-Bertania medium with 100 μg/ml ampicillin and further incubated at 37°C until the OD at 600 nm reached 0.5. DENV2 NS3 expression was then induced by the addition of 1 mM IPTG for 6 h at 30°C. To analyze the expression of NS3 protein, samples were centrifuged at 8000 × *g* for 30 min at 4°C. Cells were harvested by centrifugation, and the pellets were stored at −80°C until use. The bacterial pellets were collected and analyzed by electrophoresis. For protein purification, pellets were resuspended in PBS and were lysed by sonication on ice. The cellular suspension was centrifuged at 13,000 × *g* for 30 min. The pellet containing inclusion bodies was resuspended in binding buffer (8 M urea, 0.5 M NaCl, and 20 mM Tris-HCl, pH 6.95) and lysed by sonication on ice again and clarified by centrifugation at 13,000 × *g* for 45 min at 4°C. The supernatant containing soluble protein was loaded onto a Ni^{2+} column. The column was washed with washing buffer (8 M urea, 0.5 M NaCl, 20 mM Tris-HCl, and 120 mM imidazole, pH 6.95). His-tagged proteins were eluted with eluting buffer (8 M urea, 0.5 M NaCl, 20 mM Tris-HCl, and 300 mM imidazole, pH 6.95). After purification, proteins were examined by SDS-PAGE. Purified proteins were freed of urea by dialysis in refolding buffer (50 mM Tris-HCl, 250 mM NaCl, 1 mM EDTA, 250 mM L-arginine 10% glycerol, 10 mM GSH, and 1 mM GSSG, pH 6.95) and concentrated by Amicon Ultra for centrifugation (Millipore).

Imject Alum (Pierce Biotechnology, Rockford, IL) was added dropwise with constant mixing to the Ag solution so the final volume ratio of Imject Alum to Ag was 1:1 (100 μl of Imject Alum to 100 μl of Ag). Mixing was continued overnight at 4°C to allow complete Imject Alum-Ag adsorption.

Cell cultures

Baby hamster kidney (BHK)-21 cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing antibiotics and 5% FBS. C6/36 cells and L929 cells were cultured in DMEM containing antibiotics and 10% FBS. Lymphocytes were collected from mouse lymph nodes (LNs) and cultured in RPMI 1640 medium containing antibiotics, 10% FBS, 1% nonessential amino acids, 1% sodium pyruvate, and 50 μM 2-ME at 37°C in 5% CO_2. HMEC-1 cells were obtained from the Centers for Disease Control and Prevention (32) and were passaged in culture flasks with endothelial cell growth medium M200 (Invitrogen) supplemented with 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 μg/ml heparin, and antibiotics. Cells were detached using detaching buffer with 1000 U/ml trypsin and 0.5 mM EDTA.

Virus culture

DENV2 strain 454009A was originally isolated from a dengue patient in Taiwan and maintained in C6/36 cells. Briefly, monolayers of C6/36 cells were incubated with DENV at a multiplicity of infection of 0.01 and incubated at 28°C in 5% CO_2 for 5 d. The cultured medium was harvested, and cell debris was removed by centrifugation at 1000 × *g* for 10 min. The virus supernatant was collected and stored at −70°C until use. Virus titer was determined by plaque assay using BHK-21 cells. Briefly, BHK-21 cells were plated into 12-well plates (8×10^4 cells per well) and cultured in DMEM under CO_2-enriched condition. After adsorption with serially diluted virus for 1 h, the inoculum was replaced with fresh DMEM containing 2% FBS and 0.8% methyl cellulose. Five days postinfection, the medium was removed, and the cells were fixed and stained with crystal violet solution consisting of 1% crystal violet, 0.64% NaCl, and 2% formalin.

Ab titer determination

DJ NS1 or NS3 proteins were coated on 96-well plates at 0.2 μg/well in coating buffer (Na_2CO_3 1.59 g, $NaHCO_3$ 2.93 g, pH 9.6, in 1 l ddH_2O) at 4°C overnight. The plates were blocked with 5% BSA in PBS at 4°C overnight and then washed three times with 0.05% Tween 20 in PBS. Mouse sera were diluted serially, and the diluted mouse sera were added to protein-coated wells and incubated at 4°C overnight. After washing three times, HRP-conjugated anti-mouse IgG (Cell Signaling, Danvers, MA) or IgM (KPL, Gaithersburg, MD) was added into each well and incubated for 2 h at room temperature. After washing, ABTS was added into each well, and the absorbance was measured by microplate reader at 415 nm (EMax Microplate Reader; Molecular Devices, Sunnyvale, CA). The Ab titers were determined as the last serum dilution to give a signal above the blank.

Analysis for dendritic cell and $CD4^+$ and $CD8^+$ T cell activation

In the ex vivo studies, pooled LN cells were double-stained or triple-stained with PE-conjugated anti-CD11c (eBioscience, San Diego, CA), combined with allophycocyanin-conjugated anti-CD40 or -CD86 (BioLegend, San Diego, CA), PE-Cy7–conjugated anti-CD80 (BioLegend), and FITC-conjugated MHC class I (MHC I) or MHC class II (MHC II) Abs (BioLegend) to determine dendritic cell (DC) activation. Cells were analyzed by flow cytometry (CytoFLEXs; Beckman Coulter, Brea, CA).

Pooled LN cells (5×10^5 cells/ml) were cultured in 24-well plates and stimulated with DJ NS1 or NS3 (5 μg/ml) for 3 d. Cells were harvested and triple-stained with PE-conjugated anti-CD25 (eBioscience), FITC-conjugated anti-CD4, and PE-Cy7–conjugated anti-CD8 Abs (BD Biosciences, San Jose,

CA) to determine T cell activation. Cells were analyzed by flow cytometry (CytoFLEXs).

Analysis for surface CD107a of CD8$^+$ T cells

Pooled LN cells (5×10^5 cells/ml) were cultured in 24-well plates and stimulated with DJ NS1 or NS3 (5 μg/ml) for 3 d. At day 3, cells were further stimulated with anti–CD3/CD28 (0.5 μg/ml) (eBioscience), and PerCP/cy5.5–conjugated CD107a Abs (BioLegend) were added to the cells prior to stimulation. The cultures were incubated for 4 h at 37˚C in 5% CO_2 in the presence of the secretion inhibitor, Brefeldin (BioLegend), and monensin (BD Biosciences) to inhibit cytotoxic granule acidification and receptor-mediated endocytosis retaining CD107a on cell surface. Cells were harvested and triple-stained with PE-Cy7–conjugated anti-CD8 Abs (BD Biosciences) to determine T cell degranulation. Cells were analyzed by flow cytometry (CytoFLEXs).

Intracellular staining of NS3 expression in L929 cells

L929 cells stably expressing NS3 were established by lentiviral transduction using the vectors as previously described (33). The ViralDuctin kit (LTV-200; Cell Biolab) was used to increase the transduction rate, and the NS3-expressing L929 cells were selected by blasticidin (10 μg/ml). NS3-expressing L929 cells (5×10^5 cells/ml) were washed with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. After washing with PBS, cells were permeabilized with permeabilized buffer (0.5 g of saponin, 5 g of BSA, and 0.5 g of NaN_3 in 500 ml of PBS) and stained with anti-NS3 Ab overnight at 4˚C. After washing with permeabilized buffer, cells were incubated with Alexa-488–conjugated anti-mouse IgG (Invitrogen) for 1 h at room temperature. After a final two washes with PBS, cells were analyzed by flow cytometry (CytoFLEXs).

Active immunization and DENV infection mouse model

Three-week-old mice (n = 4 per group) were immunized s.c. with 25 μg/mouse of DJ NS1 protein with alum, 12.5 μg/mouse of NS3 protein with alum, 25 μg of DJ NS1 protein plus 12.5 μg of NS3 per mouse with alum, alum alone, and PBS control at days 0 and 14. Mouse sera and LNs were collected at day 21 for assays. For the DENV infection model, mice were challenged i.v. with DENV2 (1×10^8 PFU/mouse) at day 17 and sacrificed, and the plasma and tissues were collected at day 19.

Fluorescent focus assay

Mouse plasma was serially diluted and incubated with BHK-21 cells for 2 h at 37˚C. The monolayers were then overlaid with DMEM containing 2% FBS and 0.8% methyl cellulose and incubated at 37˚C for 2–3 d. Virus foci were stained with anti-NS1 Ab (mAb 33D2 obtained from Dr. T.-M. Yeh) followed by Alexa 488–conjugated goat anti-mouse IgG (Invitrogen) and visualized with a fluorescence microscope.

NS1 quantitative ELISA

To quantify the concentration of NS1 in the blood of mice, homemade NS1 sandwich ELISA was performed. In brief, 5 μg/ml anti-NS1 mAb 33D2 was coated onto 96-well plates at 4˚C overnight. After blocking for 1 h with 1% BSA in PBS, mouse plasma (1:5 dilution) was coincubated with 1 μg/ml biotin-conjugated anti-NS1 mAb 31B2 (obtained from Dr. T.-M. Yeh) on wells at 37˚C for 1–2 h. HRP-labeled streptavidin solution (1:40) (R&D Systems, Minneapolis, MN) was added into wells at 37˚C for 20 min. After washing with PBST (PBS contained 0.01% Tween 20) three times, color development and visualization with tetramethylbenzidine (TMB) was performed. The absorbance was read following the addition of stop solution (2 N H_2SO_4) by microplate reader at OD 450 nm (EMax microplate reader; Molecular Devices).

Mouse tail bleeding time

Bleeding time was performed with a 3-mm tail-tip transection. Blood droplets were collected on filter paper every 30 s. Bleeding time was recorded when the blood spot was smaller than 0.1 mm in diameter (31).

Hemorrhage quantification

The degree of murine peritoneal hemorrhage was image-processed by Photoshop and digitally quantified by ImageJ software according to a published quantitative method (34). The samples of mouse peritoneum were taken for photography and adjusted to the same image sizes. Hemorrhagic areas were isolated and created as new images in Photoshop 6.0. To quantify the hemorrhagic portion, the processed images were loaded into ImageJ software and converted into 16-bit images. After setting image type to black and white, the total hemorrhage area was calculated and analyzed by Prism software.

Vascular permeability assay

To evaluate the effect of immune serum to inhibit NS1-induced vascular permeability increase, HMEC-1 cells were seeded to form monolayers on a 24-well Transwell polycarbonate membrane system (Transwell permeable support, 0.4 mM, 6.5-mm insert; Corning) and incubated with NS1 (10 μg/ml) to disrupt endothelial integrity. Transwell inserts containing untreated HMEC-1 cells were used as a negative control. After 24-h treatment with mouse sera, upper and lower chamber media were replaced with fresh endothelial cell medium. Endothelial permeability was evaluated by measuring HRP activity in the lower chamber 15 min after the addition of HRP-labeled streptavidin solution (R&D Systems) to the upper chamber. Color development and visualization with TMB was performed. The absorbance was read following the addition of stop solution (2 N H_2SO_4) by microplate reader at OD 450 nm (EMax microplate reader; Molecular Devices).

Statistical analysis

Data were expressed as the means ± SD. Comparisons between various treatments were performed by ANOVA and Kruskal-Wallis test with GraphPad Prism version 6.0. Statistical significance was set at $p < 0.05$.

Results

Specific Ab responses induced by active immunization with various doses of DJ NS1 and NS3

In our previous studies, we demonstrated that the DENV NS1 epitopes that are cross-reactive with self-Ags could be modified to avoid autoimmunity (28, 30). Thus modified DJ NS1 was used as vaccine candidate (31). Based on preliminary studies, including DC activation, Ab responses, and T cell activation (data not shown), we used a ratio of DJ NS1 and NS3 as 2:1 to provide optimized humoral and cell-mediated immune responses. To determine the Ab responses induced by active immunization with DJ NS1 and NS3, C3H/HeN mice were immunized s.c. with Ags twice at days 0 and 14. DJ NS1- or NS3-specific Ab titers were determined in the mouse sera at days 7 and 21. There was no significant increase in the serum levels of blood urea nitrogen, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) of DJ NS1-, NS3-, or DJ NS1- plus NS3-immunized mice (data not shown), indicating no toxicity of these proteins in vivo.

Results showed that the serum levels of IgG and IgM against DJ NS1 in 25 μg of DJ NS1 plus 12.5 μg of NS3-immunized mice were significantly higher than those in 25 μg of DJ NS1-immunized mice, especially on day 7 (Supplemental Fig. 1A–D). In addition, the serum levels of IgG and IgM against NS3 in 25 μg of DJ NS1 plus 12.5 μg of NS3-immunized mice were significantly higher than those in 12.5 μg of NS3-immunized mice on day 7 (Supplemental Fig. 1E, 1G). The serum levels of IgM against NS3 in 12.5 μg of NS3-immunized mice were dramatically increased compared with those in 25 μg of DJ NS1 plus 12.5 μg of NS3-immunized mice (Supplemental Fig. 1F), whereas the serum levels of IgG against NS3 in 25 μg of DJ NS1 plus 12.5 μg of NS3-immunized mice were dramatically increased compared with those in 12.5 μg of NS3-immunized mice (Supplemental Fig. 1H) on day 21. Furthermore, mice immunized with DJ NS1 alone induced anti-DJ NS1 IgM and IgG titers of 8000 and 8000, respectively, when determined at day 21. Mice immunized with DJ NS1 plus NS3 induced anti-DJ NS1 IgM and IgG titers of 8000 and 8000, respectively, at day 21. Mice immunized with NS3 alone induced anti-NS3 IgM and IgG titers of 16,000 and 128,000, respectively, at day 21. Finally, mice immunized with DJ NS1 plus NS3 induced anti-NS3 IgM and IgG titers of 8000 and 256,000, respectively, at day 21 (Supplemental Fig. 1). We observed that NS3 provided a marginal synergistic effect to DJ NS1 IgG titers after the first immunization (i.e., DJ NS1 alone

induced a DJ NS1 IgG titer of 2000 IgG, whereas DJ NS1 plus NS3 induced a DJ NS1 IgG titer of 4000). These results indicate that DJ NS1 and NS3 can induce specific Ab responses, and the anti-DJ NS1 IgG titers are slightly increased with the DJ NS1 plus NS3 combination.

Immunization with DJ NS1 and NS3 induces DC and specific CD4$^+$ and CD8$^+$ T cell activation in response to DJ NS1 or NS3 Ag ex vivo

To evaluate the DC activation after immunization, we analyzed the expression of CD40, CD80, CD86, MHC I, and MHC II in CD11c$^+$ LNDCs. The results showed that most CD11c$^+$ LNDCs express MHC I (Supplemental Fig. 2A), and mice immunized with 25 μg of DJ NS1- and 12.5 μg of NS3-induced LNDCs exhibited significantly higher MHC I than other groups, including alum alone, DJ NS1 alone, and NS3 alone (Supplemental Fig. 2B). The MHC II expression of CD11c$^+$ LNDCs was increased in mice immunized with DJ NS1 alone or NS3 alone, and although not statistically significant, there was a trend of increase in mice immunized with combination of DJ NS1 plus NS3 (Supplemental Fig. 2C). Active immunization with DJ NS1 alone, NS3 alone, or DJ NS1 plus NS3 in mice induced a trend of increase of CD40 expression in CD11c$^+$ LNDCs although with no statistical significance (Supplemental Fig. 2D). Active immunization with DJ NS1 alone, NS3 alone, or combination of DJ NS1 plus NS3 all significantly increased CD80 expression on CD11c$^+$ LNDCs as compared with PBS control at day 21 (Supplemental Fig. 2E). Only the combination of DJ NS1 plus NS3 significantly increased CD86 expression on CD11c$^+$ LNDCs as compared with alum alone and the PBS control group (Supplemental Fig. 2F). Thus, these results indicate that active immunization with DJ NS1 plus NS3 can induce DC activation as measured by increased DCs that express costimulatory receptors.

To evaluate whether DJ NS1 and NS3 can induce Ag-specific T cell responses, LN cells were collected from immunized mice and restimulated with 5 μg/ml DJ NS1 or NS3 proteins for 3 d. Anti-CD3 and anti-CD28 Abs were administered to all groups to partially mimic TCR signals and amplify responses. Active immunization of mice with DJ NS1 alone or DJ NS1 plus NS3 significantly induced CD4$^+$ T cell activation in response to DJ NS1 Ag stimulation compared with alum alone or PBS control (Fig. 1A, Supplemental Fig. 3A). Only active immunization with DJ NS1 plus NS3 in mice significantly induced CD4$^+$ T cell activation in response to NS3 Ag stimulation compared with other groups (Fig. 1B, Supplemental Fig. 3B). Active immunization with DJ NS1 plus NS3 also significantly induced CD8$^+$ T cell activation in response to DJ NS1 Ag stimulation (Fig. 1C, Supplemental Fig. 3C) as well as to NS3 Ag stimulation (Fig. 1D, Supplemental Fig. 3D) compared with other groups. Therefore, active immunization with DJ NS1 plus NS3 can induce both CD4$^+$ and CD8$^+$ T cell activation in response to DJ NS1 or NS3 Ag stimulation.

Active immunization with DJ NS1 and NS3 induces specific CTL responses

Previous studies showed that CD8$^+$ T cells could directly contribute to protection against DENV (35, 36). In our study, active immunization with DJ NS1 plus NS3 induced CD8$^+$ T cell activation (Fig. 1C, 1D). To examine whether a CTL response can be induced, we isolated LN cells from immunized mice and restimulated with DJ NS1 or NS3 protein and then cocultured with

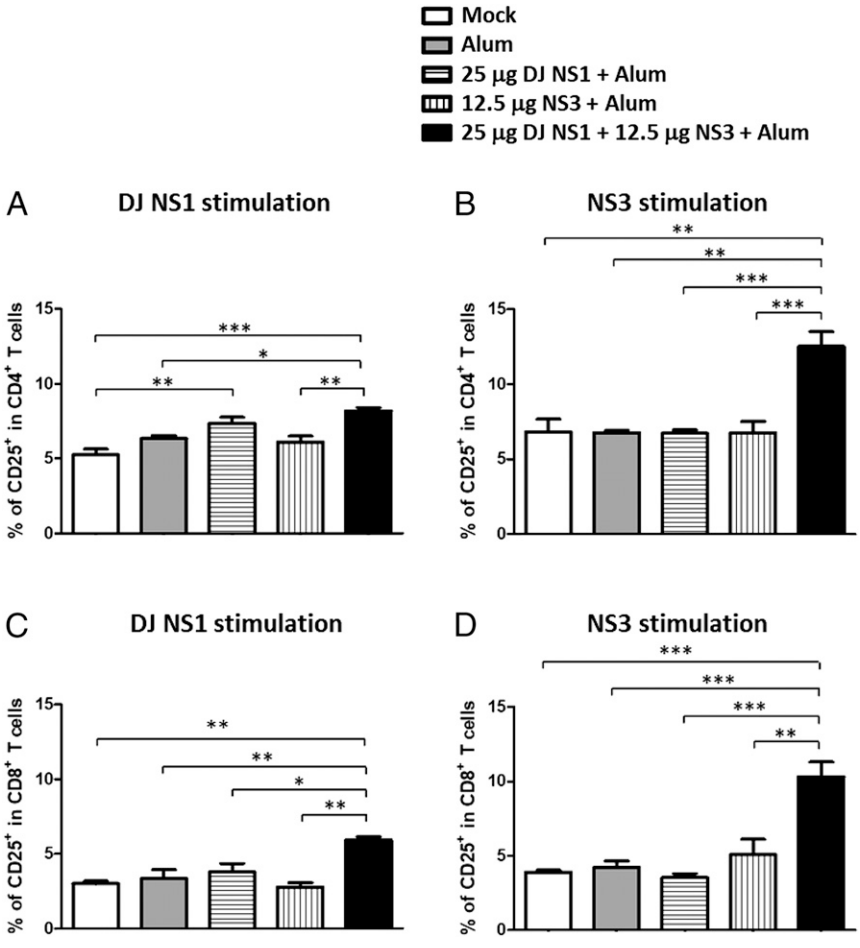

FIGURE 1. Active immunization with DJ NS1 and NS3 induces both CD4$^+$ and CD8$^+$ T cell activation in response to DJ NS1 or NS3 Ag stimulation ex vivo. C3H/HeN mice were immunized s.c. with different doses of immunogen (i.e., 25 μg of DJ NS1 plus 12.5 μg of NS3 per mouse, 25 μg of DJ NS1 per mouse, 12.5 μg of NS3 per mouse, alum alone, or PBS) at day 0 and boosted at day 14 ($n = 4$ per group; data are representative of two independent experiments). Mice were sacrificed at day 21, and LN cells were collected and restimulated with 5 μg/ml DJ NS1 or NS3 proteins for 3 d and incubated with anti-CD3/CD28 Abs for the final 4 h, then triple-stained with PE-labeled anti-CD25, FITC-labeled anti-CD4, and PE/Cy7–labeled anti-CD8 to analyze the CD25 expression of CD4$^+$ and CD8$^+$ T cells by flow cytometry. (**A**) Percentage of CD25$^+$ in CD4$^+$ T cells in response to DJ NS1 Ag stimulation. (**B**) Percentage of CD25$^+$ in CD4$^+$ T cells in response to NS3 Ag stimulation. (**C**) Percentage of CD25$^+$ in CD8$^+$ T cells in response to DJ NS1 Ag stimulation. (**D**) Percentage of CD25$^+$ in CD8$^+$ T cells in response to NS3 Ag stimulation. $n = 5$ for DJ NS1 plus NS3 group and $n = 4$ for other groups. The averages of each group ± SD are shown. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, as determined by one-way ANOVA with Tukey post hoc test.

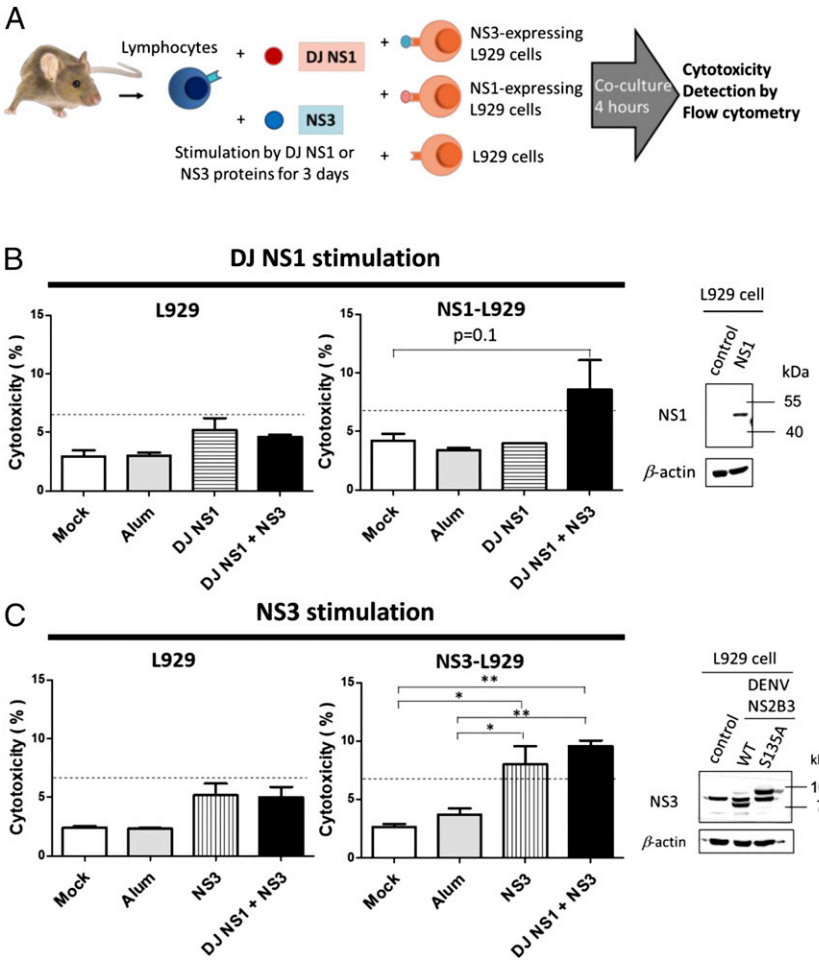

FIGURE 2. Active immunization with DJ NS1 and NS3 induces CTL responses against NS3-expressing L929 cells. C3H/HeN mice were immunized s.c. with different doses of immunogen (i.e., 25 μg of DJ NS1 plus 12.5 μg of NS3 per mouse, 25 μg of DJ NS1 per mouse, 12.5 μg of NS3 per mouse, alum alone, or PBS) at day 0 and boosted at day 14 ($n = 4$ per group; data are representative of two independent experiments). (**A**) Experimental design of CTL killing assay. LNs were collected and restimulated with 5 μg/ml DJ NS1 or NS3 proteins for 3 d as effector cells. (**B**) NS1-expressing L929 cells were cocultured with effector cells at E:T ratio of 20:1. After 4 h, target cells were collected and assayed for cell death by propidium iodide staining and flow cytometry. The NS1 expression levels were detected by Western blotting. (**C**) NS2B/3 with S135A was used for NS3-expressing cells, and the impaired protease activity of the mutated constructs was confirmed by analyzing the absence of NS2B3 self-processing by Western blotting. NS3-expressing L929 cells were cocultured with effector cells at E:T ratio of 20:1. After 4 h, target cells were collected and assayed for cell death by propidium iodide staining and flow cytometry. The averages of each group ± SD are shown. $*p < 0.05$, $**p < 0.01$, as determined by one-way ANOVA with Tukey post hoc test.

DJ NS1-expressing or NS3-expressing L929 cells as the target for 4 h (Fig. 2A). L929 cells were stably transduced with the lentiviral vector encoding wild type or proteolytically inactive (S135A) DENV protease NS2B3 as previously described (33) to generate NS3-expressing L929 cells. NS2B3-S135A–expressing L929 cells were then used as target cells because of better expression levels (data not shown). Results showed that only CTLs from DJ NS1- plus NS3-immunized mice lysed NS1-expressing L929 cells, however, with no statistical difference compared with other groups (Fig. 2B). In contrast, CTLs from NS3 alone or DJ NS1- plus NS3-immunized mice significantly lysed NS3-expressing L929 cells (Fig. 2C).

In addition to demonstrating CTL activity on target cells, we further determined the cytotoxic activity of effector cells directly. The lipid bilayer of cytotoxic granule in $CD8^+$ T cells contains lysosomal-associated membrane glycoproteins (LAMPs), including CD107a (LAMP-1); therefore, CTL degranulation leads to cumulative exposure of CD107a on the cell surface. Based on the fact that degranulation of activated $CD8^+$ T cells occurs rapidly after TCR stimulation, LN cells isolated from immunized mice were restimulated with DJ NS1 or NS3 protein for 3 d and incubated with anti-CD3 and anti-CD28 to partially mimic TCR signals during the last 4 h. Active immunization with DJ NS1 plus NS3 significantly increased CD107a expression on the surface of $CD8^+$ T cells in response to both DJ NS1 (Fig. 3A) and NS3 (Fig. 3B) stimulation compared with NS3 alone, DJ NS1 alone, alum alone, and PBS control. The CD107a expression on the surface of $CD8^+$ T cells from NS3-immunized mice was also significantly increased in response to NS3 stimulation compared with alum alone and PBS control but was less than that from DJ NS1- plus NS3-immunized mice (Fig. 3B). Results from Figs. 2, 3 indicate that active immunization with DJ NS1 plus NS3 can induce Ag-specific CTL responses.

Active immunization with DJ NS1 and NS3 provides protective effects against DENV infection

We next established a DENV infection model to evaluate the protection provided by active immunization with DJ NS1 plus NS3 as shown in Fig. 4A. We determined the viral titers and sNS1 in plasma at day 19. DJ NS1 protein can induce Ab responses that trigger Ab-dependent complement-mediated cytolysis of DENV-infected cells (37). Active immunization with DJ NS1 alone or NS3 alone presented a trend of reduced viral titers and sNS1 levels in the plasma compared with the DENV2 only and alum control but was not statistically significant (Fig. 4B). Active immunization with NS3 alone reduced viral titers and sNS1 levels in the plasma to a greater extent than with DJ NS1 alone. The NS3-specific T cell responses shown in the current study may thus contribute to viral clearance. The significant reductions of viral titers and sNS1 levels after active immunization with DJ NS1 plus NS3 were comparable to those after immunization with DENV2 only and alum control.

Our previous studies demonstrated that mice inoculated with high-titer DENV can produce pathogenic signs such as prolonged bleeding time and local hemorrhaging (31). Accordingly, we evaluated the protection provided by active immunization with DJ NS1 and NS3 by determining the DENV-induced prolonged bleeding time. The results showed that DENV infection caused

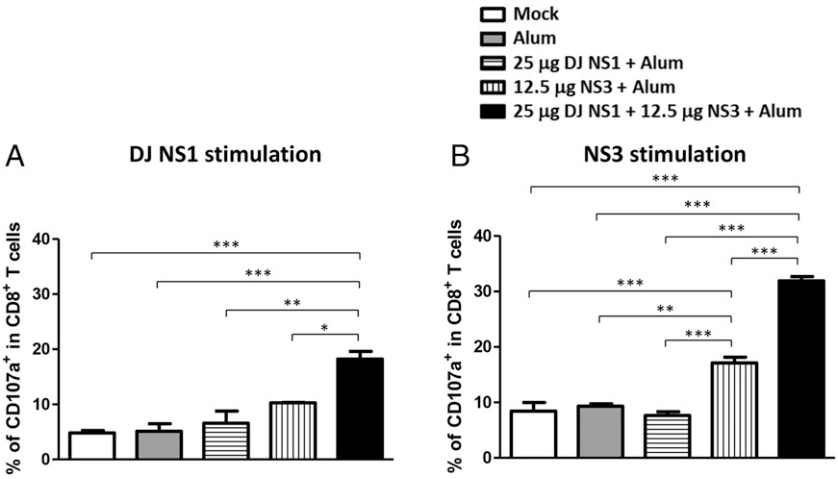

FIGURE 3. Increased expression of CD107a, a marker for cytotoxic $CD8^+$ T cell activity, in the DJ NS1- and NS3-immunized mice after DJ NS1 or NS3 Ag stimulation ex vivo. C3H/HeN mice were immunized s.c. with different doses of immunogen (i.e., 25 μg of DJ NS1 plus 12.5 μg of NS3 per mouse, 25 μg of DJ NS1 per mouse, 12.5 μg of NS3 per mouse, alum alone, or PBS) at day 0 and boosted at day 14 ($n = 4$ per group; data are representative of two independent experiments). Mice were sacrificed at day 21, and LN cells were collected and restimulated with 5 μg/ml DJ NS1 (**A**) or NS3 (**B**) proteins for 3 d and then incubated with anti-CD3/CD28 Abs, monensin, and brefeldin A for the final 4 h to perform surface staining of CD107a expression in $CD8^+$ T cells by flow cytometry. The averages of each group ± SD are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, as determined by one-way ANOVA with Tukey post hoc test.

prolonged bleeding time in mice 48 h post DENV infection alone or with alum. Although there was a trend of reduced DENV-induced prolonged bleeding time in active immunization with DJ NS1 or NS3, only active immunization with DJ NS1 plus NS3 significantly reduced DENV-induced prolonged bleeding time compared with DENV infection alone and alum control (Fig. 5A).

Next, we observed vascular changes in the mouse peritoneum. The results showed that active immunization with DJ NS1 plus NS3 significantly reduced DENV-induced vascular leakage compared with DENV infection alone or alum immunization followed by DENV infection (Fig. 5B, Supplemental Fig. 4A). In contrast, active immunization with DJ NS1 or NS3 alone did not significantly reduce DENV-induced vascular leakage compared with DENV infection alone or alum immunization followed by DENV infection.

The mouse sera were tested for their inhibitory effects on vascular permeability. The results were similar with the finding in Fig. 5B that sera collected from mice after active immunization with DJ NS1 alone or DJ NS1 plus NS3, but not NS3 alone, significantly inhibited NS1-induced vascular leakage in the endothelial cell line (Supplemental Fig. 4B). These results indicate that active immunization with DJ NS1 or DJ NS1 plus NS3 provide better protective effects against vascular leakage in vitro (Fig. 6).

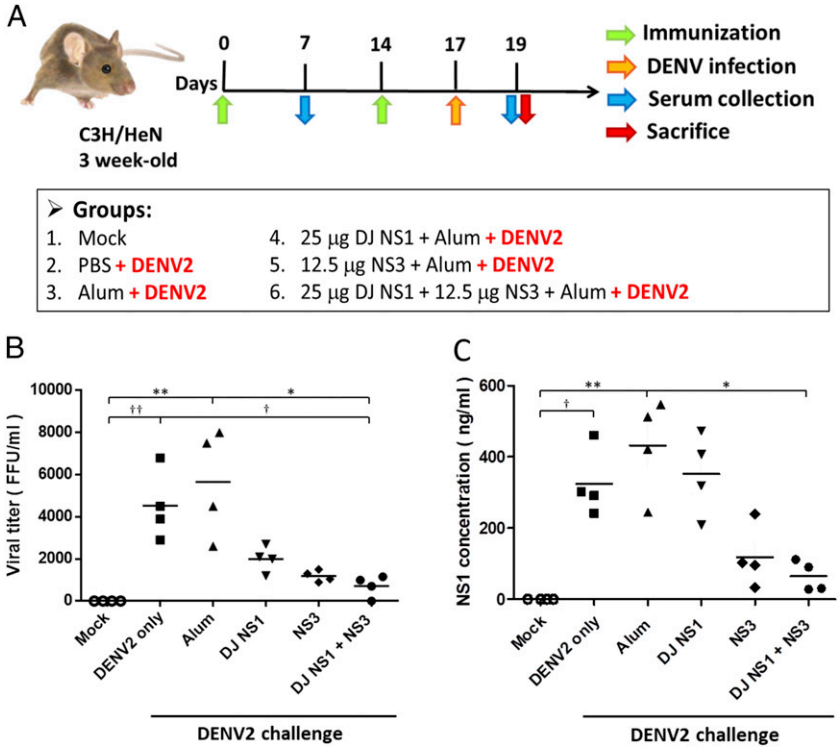

FIGURE 4. Active immunization with DJ NS1 and NS3 reduces viral titers and sNS1 levels in the plasma of DENV-infected mice. (**A**) Experimental design of mouse model of immunization is shown ($n = 4$ per group; data are representative of three independent experiments). C3H/HeN mice were inoculated i.v. with 1×10^8 PFU DENV2 strain 454009A at day 17. (**B**) Mice were sacrificed at day 19, and plasma samples were collected to determine the viral titers by fluorescent focus assay. (**C**) The NS1 levels in plasma were determined by ELISA. The averages of each group ± SD are shown. Cross (†) and asterisk (*) symbols indicate statistical significance at $p < 0.05$ when compared with the DENV-only group and the alum control group, respectively. † or $*p < 0.05$, †† or $**p < 0.01$, as determined by Kruskal-Wallis test with Dunn posttest.

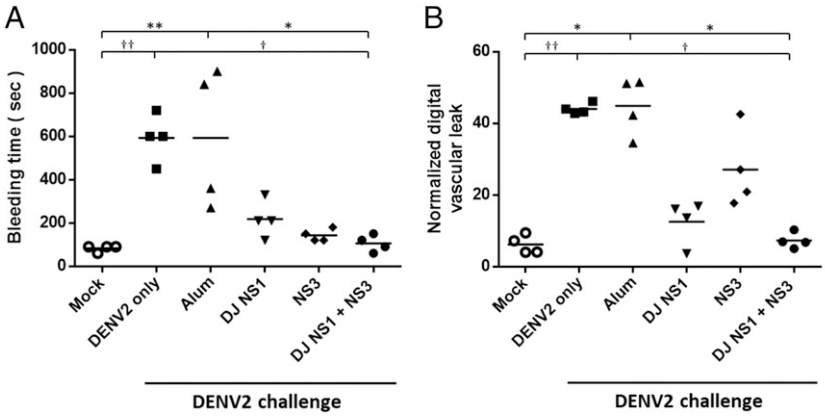

FIGURE 5. Active immunization with DJ NS1 and NS3 reduces DENV-elicited bleeding tendency and vascular change. Experimental design of the mouse model of immunization is shown in Fig. 4 ($n = 4$ per group; data are representative of three independent experiments). Mice were inoculated i.v. with 1×10^8 PFU DENV2 strain 454009A at day 17. (**A**) The tail bleeding time was determined at 2 d postinfection. (**B**) Mice were sacrificed at day 19, the tissues were collected, and the clinical scores of vascular leakage were quantified as digital severity by ImageJ. The averages of each group ± SD are shown. Cross (†) and asterisk (*) symbols indicate statistical significance at $p < 0.05$ when compared with the DENV-only group and the alum control group, respectively. † or *$p < 0.05$, †† or **$p < 0.01$, as determined by Kruskal-Wallis test with Dunn posttest.

Discussion

In this study, we used modified NS1 combined with NS3 in a novel vaccine strategy and investigated the mechanisms of the observed protective effects against DENV in mice. Nonreplicating recombinant protein–based subunit vaccines are considered to be safer and can be easily reformulated. In an effort to solve a common problem for subunit vaccine, namely that certain highly purified Ags are poorly immunogenic, we combined NS1 protein with immunodominant NS3 protein. Our results show that NS3 protein can synergistically induce more effective immune responses, including not only generating NS3-specific CTL responses but also promoting NS1-specific T cell responses and Ab titers. Active immunization with DJ NS1 plus NS3 exhibited better performance in both immune responses and protection compared with DJ NS1 alone or NS3 alone (Fig. 6).

Previous studies showed that Th1-biased responses dominated by IFN-γ are associated with less severe secondary DENV infection, whereas T cell production of TNF-α is associated with more severe infection (38, 39). Other studies have confirmed that the recombinant DENV2 NS3 protein induced Th1 responses with a higher IFN-γ/TNF-α (40). However, a previous clinical study demonstrated the association of robust T cell responses with dengue hemorrhagic fever, particularly against NS3 (41). This discrepancy needs to be further investigated. In our study, active immunization with DJ NS1 plus NS3 induces CD107a expression on the surface of $CD8^+$ T cells in response to DJ NS1 stimulation, revealing that combination of DJ NS1 with NS3 promotes development of cytotoxic activity against DJ NS1. In agreement with the results from CTL assay, active immunization with DJ NS1 plus NS3 induced cytotoxicity against not only NS3-expressing L929 but also DJ NS1-expressing L929 cells. A previous study indicated that the concomitant production of IFN-γ and CD107a expression with new synthesis of perforin protein can promote cytotoxicity in Ag-specific $CD8^+$ T cells (42). However, the detailed mechanisms of cytotoxic action remain to be elucidated.

Because active immunization with NS3 also induces high titers of anti-NS3 IgM and IgG, we cannot exclude the possible role of anti-NS3 Abs in our infection model. Previous studies showed the protective effects by Abs directed against NS3 (24, 25). Although anti-NS3 Abs, which are not neutralizing Abs, have been detected in sera of patients with primary and secondary DENV infection (43, 44), the role of anti-NS3 Abs remains to be further investigated.

The clinical symptoms of DENV infection range from asymptomatic, classic dengue fever, to severe life-threatening dengue

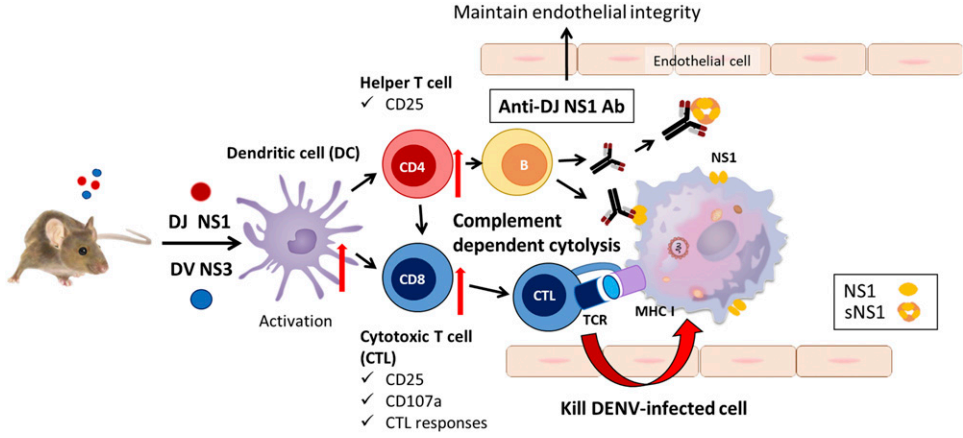

FIGURE 6. Schematic model of immune responses and protective effects induced by active immunization with DJ NS1 and NS3. The incorporation of DJ NS1 and NS3 proteins for immunization can synergistically induce more effective immune responses as demonstrated by generating NS3-specific CTL responses and high CD107a expression as well as promoting NS1-specific T cell responses and Ab titers. The protective mechanisms include direct killing of DENV-infected cells by CTLs and neutralization of NS1-induced pathogenic effects by anti-NS1 Abs.

hemorrhagic fever, which are mainly characterized by increased vascular leakage, leading to hemorrhage, hypovolemia, hypotension, and also shock syndrome. In our DENV infection model, mice presented with viremia, prolonged bleeding time, vascular change, and hemorrhage. Prolonged bleeding time may reflect coagulation abnormalities, including thrombocytopenia and disruption of the coagulation system. In adults, platelet counts are significantly associated with bleeding manifestations of DENV infection (45). The mechanisms involved in thrombocytopenia and bleeding during DENV infection are not fully understood, although several hypotheses have been suggested (46). In our DENV infection model, active immunization with either DJ NS1 or NS3 can shorten DENV-associated prolonged bleeding time (Fig. 5A). DJ NS1 induces NS1-neutralizing Abs that directly block the pathogenesis of sNS1 (Supplemental Fig. 4B) and also cause complement-mediated cytolysis with anti-NS1 Abs against DENV-infected cells (37). In contrast, active immunization with NS3 induces NS3-specific CTL to reduce viral titers as well as sNS1 levels in serum to shorten the prolonged bleeding time. Collectively, active immunization with DJ NS1 plus NS3 can significantly shorten DENV-associated prolonged bleeding time through multiple mechanisms.

The pathogenesis of and protection against vascular leakage and hemorrhage are complex. sNS1 can direct complement against endothelial cells and induce endothelial cell apoptosis (11, 19). Recent studies showed that sNS1 can bind to TLR4 and then lead to cell release of proinflammatory cytokines that contribute to vascular leakage (12, 13). sNS1 has also been shown to activate TLR2 and TLR6, leading to increased proinflammatory cytokine production (14). In addition, macrophage migration inhibitory factor induced by DENV infection or NS1 may enhance DENV replication through autophagy, which may also contribute to vascular leakage by disrupting endothelial cell tight junctions both in humans and in mice (18, 47, 48). NS1 can also directly disrupt the endothelial glycocalyx, leading to hyperpermeability (17, 18). Thus, sNS1 can be a major factor directly or indirectly contributing to vascular leakage and hemorrhage. As shown in our study, active immunization with DJ NS1 alone or DJ NS1 plus NS3 significantly prevented DENV-associated vascular permeability change. In contrast, active immunization with NS3 alone did not prevent vascular change (Fig. 5B). These results highlight the essential roles of NS1-neutralizing Abs in preventing vascular leakage and hemorrhage.

Based on clinical observations that dengue disease is more severe in secondary heterotypic infections, any successful vaccine would need to induce a protective and durable immune response to all four DENV serotypes simultaneously to avoid Ab-dependent enhancement (ADE) (49). Our vaccine candidate, DJ NS1 plus NS3, will not cause ADE. Moreover, we have demonstrated that active immunization with DJ NS1 plus NS3 induced specific $CD8^+$ T cell responses, which recently were shown to be able to be protective against heterotypic DENV reinfection (35, 50). Following an emerging Zika virus (ZIKV) infection, several studies showed that previous DENV infection can cause ADE of ZIKV infection by DENV-specific Abs (51, 52). In contrast, another study demonstrated that five DENV epitope–specific $CD8^+$ T cells confer cross-protection against subsequent ZIKV infection (53). Our candidate vaccine NS3 component contains one of the five epitopes that can mediate cross-protection against subsequent ZIKV infection and may avoid ADE of ZIKV. However, cross-protection against ZIKV infection provided by our vaccine candidate needs to be further investigated.

In conclusion, our work explores a novel DENV vaccine strategy combining DJ NS1 and NS3 proteins, which provides enhanced protection against DENV challenge and associated pathological effects.

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Disclosures

The authors have no financial conflicts of interest.

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