Supplementary material

Material and methods

Production of WNV envelope protein DIIII in *N. benthamiana* plants

The coding sequence of WNV DIII (amino acid 296-415, Genbank Acc.No. AF196835) was synthesized with an 18-bp sequence coding for the hexa-histidine tag (His_6) added to the 3' terminus of the DIII gene, and subsequently cloned into the plant expression vector. *N. benthamiana* plants were grown and agroinfiltrated with the GV3101 strain containing the DIII-His_6 construct. Agroinfiltrated *N. benthamiana* leaves were harvested 4 days post infiltration, and homogenized in extraction buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCL,1 mM PMSF, tablet protease inhibitor cocktail (Sigma, Germany)). The pH of the clarified extract was adjusted to 5.0, and subjected to centrifugation at 18,000 × *g* for 30 min at 4℃. The supernatant was recovered, pH adjusted back to 8.0, and then subjected to Ni IMAC on a 4-ml His.Bind column in accordance with the manufacturer's instruction (Millipore, USA). The purified WNV DIII was eluted with imidazole and the eluate was dialyzed against PBS.

Plaque reduction neutralization test (PRNT) assay

Serum samples were diluted (1:10 and 1:100) in Opti-MEM serum free medium, while WNV (CT2741) was diluted in Opti-MEM serum free medium (ThermoFisher, NY) to a working concentration of 10^2 PFU per well. Following dilutions, WNV was added to diluted sera and incubated for 1 hr at room temperature. Virus/serum mixture was then transferred to plates containing 90-95% confluent monolayers of Vero cells (ATCC # CCL-81) and incubated for 1 hr at 37°C with 5% CO_2. After removing the virus/serum-containing medium, Vero cells were overlaid with fresh MEM medium containing 1% SeaPlaque™ agarose (Lonza, MD) and further

incubated at 37°C with 5% CO_2 for an additional 72 hr. Plaques were counted after staining with 4% (vol/vol) neutral red. Percent (%) neutralization was calculated as: [(number of WMV plaque per well without test serum) - (number of WNV plaque per well of diluted test serum) / (number of WNV plaque per well without test serum) x 100]. Experiments were repeated twice.

Splenocyte culture and cytokine production

Single-cell suspensions of the spleens from immunized mice were prepared by mechanical dissociation. Cultures of splenocytes at 5×10^6 cells /ml were stimulated with 10 μg/ml of DIII, T cell mitogen Con A (positive control, 5 μg/ml, MilliporeSigma, MA) or culture medium (negative control). The supernatant from splenocyte cultures was collected 24 and 48 hr after stimulation and aliquots were stored at -80°C. Cytokine concentration was quantitated by a multiplex analysis using a custom mouse cytokine kit containing IL-4, IL-6, and IFN-γ (Bio-Rad, CA) following the manufacturer's protocol. Data collection was performed with a Bio-Plex 200 system with Bio-Plex Manager software (version 5.0, Bio-Rad, CA). Each cytokine was measured in triplicate with at least two independent experiments.

Antibody-dependent enhancement Assay

Sera collected from vaccinated mice at week 11 were pooled and total IgG was isolated using IgG purification kits (GE Healthcare, PA). DENV-2 (ATCC#VR-1584) or ZIKV (PRVABC59, ATCC# VR-1843) was mixed with each of eight 3-fold serial dilutions of IgG or an anti-flavivirus E mAb (4G2) (ATCC # HB-112) as positive control, respectively. The antibody-virus mixtures were first incubated at 37°C for 1 hr and then added to FcγRIIA$^+$ K562 cells (ATCC # CCL-2243) at an MOI of 1.0. Infected K562 cells were collected after incubating for 48 hr (DENV-2) or 72 hr (ZIKV) at 37°C with 5% CO_2. K562 cells were then fixed with 4% paraformaldehyde (MilliporeSigma, MA), permeabilized with 0.1% saponin (MilliporeSigma, MA)

and fluorescently labeled with Alexa Fluor488 (Invitrogen, CA). The percentage of infected cells was then determined by flow cytometry.

Results

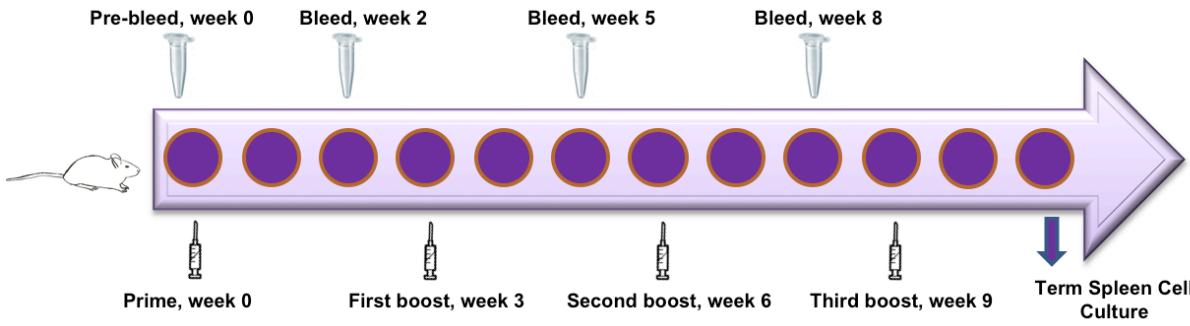

Figure S1. Time course of plant-DIII immunization in mice. BALB/c mice (n = 6 per group) were immunized subcutaneously with four doses of plant-DIII (25 μg per dosage) with alum as adjuvant over an 11-week time period. Antigen was injected on weeks 0, 3, 6 and 9. Blood samples were collected on weeks 0 (preimmune bleed), 2, 5, and 8 (2 weeks after each antigen injection). On day 77 (week 11), mice were humanely euthanized, final blood samples were collected, and the spleens were aseptically removed for *in vitro* splenocyte cultures.

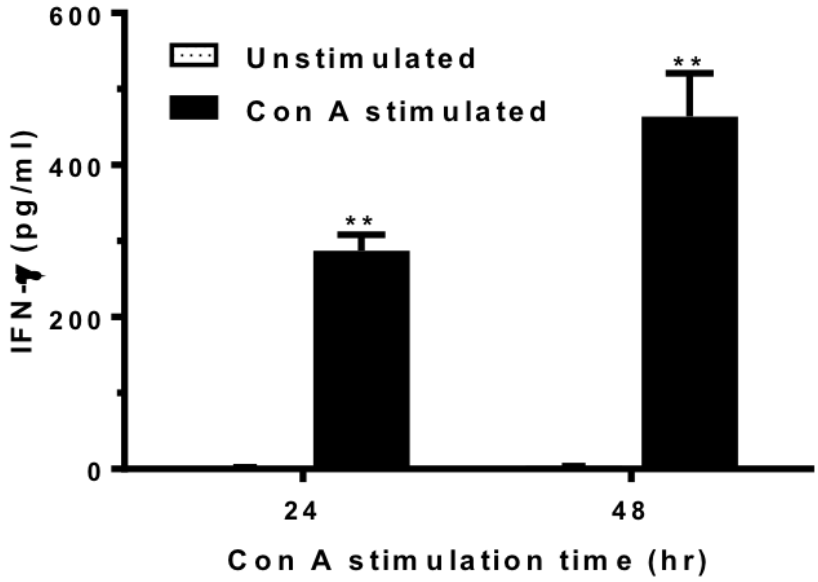

Figure S2. Interferon gamma production from plant-DIII immunized mice.

Splenocytes from mice injected with 25 µg plant-DIII were stimulated *in vitro* with Con A for 24 to 48 hr. The production of IFN-γ was quantitated and the mean concentration (pg/ml) and SD from at least two independent experiments are presented. The induction of IFN-γ by Con A stimulation is observed compared to unstimulated samples ($p < 0.005$).