MAJOR ARTICLE

A West Nile Virus Recombinant Protein Vaccine That Coactivates Innate and Adaptive Immunity

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A chimeric protein West Nile virus (WNV) vaccine capable of delivering both innate and adaptive immune signals was designed by fusing a modified version of bacterial flagellin (STF2Δ) to the EIII domain of the WNV envelope protein. This fusion protein stimulated interleukin-8 production in a Toll-like receptor (TLR)–5–dependent fashion, confirming appropriate in vitro TLR5 bioactivity, and also retained critical WNV-E–specific conformation-dependent neutralizing epitopes as measured by enzyme-linked immunosorbent assay. When administered without adjuvant to C3H/HeN mice, the fusion protein elicited a strong WNV-E–specific immunoglobulin G antibody response that neutralized viral infectivity and conferred protection against a lethal WNV challenge. This potent EIII-specific immune response requires a direct linkage of EIII to STF2Δ, given that a simple mixture of the 2 components failed to induce an antibody response or to provide protection against virus challenge. The presence of a functional TLR5 gene in vivo is also required—TLR5-deficient mice elicited only a minimal antigen-specific response. These results confirm that vaccines designed to coordinately regulate the innate and adaptive immune responses can induce protective immune responses without the need for potentially toxic adjuvants. They also support the further development of an effective WNV vaccine and novel monovalent and multivalent vaccines for related flaviviruses.

West Nile virus (WNV) is one of several flaviviruses known to cause human disease. It was first identified in the West Nile district of Uganda in 1937 and has since spread to more temperate regions that include parts of Europe and North America [1]. Currently, there is no effective treatment for the disease, and the best means of prevention will likely come from an effective vaccine. Members of the flavivirus family are single-stranded positive-sense RNA enveloped viruses whose virions consist of 3 structural proteins [2] that include an envelope (E) protein, a nucleocapsid protein, and a lipid membrane protein. The E protein is associated with the outer viral membrane and plays a major role in virion assembly, receptor binding, and host membrane fusion. This protein is also the major flavivirus immunogen and is the primary target for neutralizing antibodies [3, 4]. As with other flaviviruses, the E protein of WNV has been shown to play a critical role in protective immunity. Immunization of experimental animals with various forms of the E antigen has been shown to induce protective immune response to viral challenge [5–11]. This protection is associated with a virus-neutralizing antibody response that can be passively transferred to naive recipients [11, 12]. T cell helper and effector mechanisms are thought to also play a role in the natural immune response to WNV infection and may play a role in protection (reviewed in [12]). Thus, because of the critical nature of the E protein during virus infection and the host immune response, a vaccine incorporating E protein deter-

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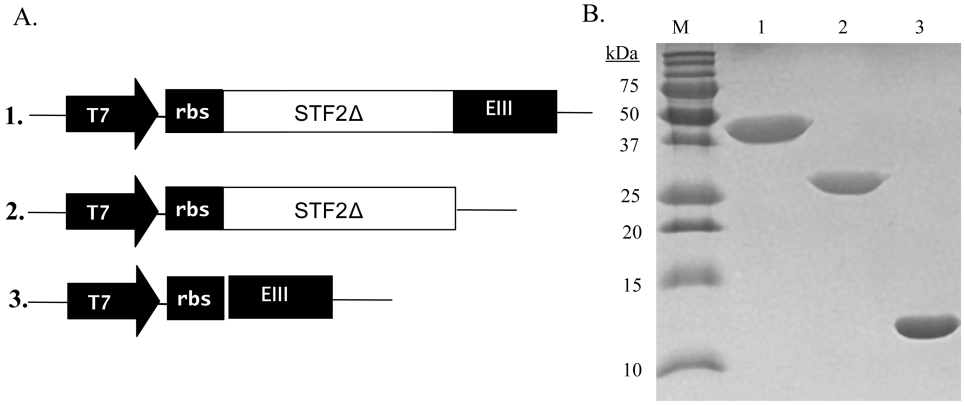

Figure 1. Flagellin (STF2Δ) and West Nile virus (WNV)–EIII proteins. *A,* Schematic representation of STF2Δ.EIII fusion (1), STF2Δ (2), and WNV-EIII (3) expression plasmids. All 3 constructs were designed without affinity tags and were expressed in *Escherichia coli* using the pET/T7 promoter-driven expression system. *B,* SDS-PAGE of purified STF2Δ.EIII fusion, STF2Δ, and EIII proteins. *Lane M,* molecular-weight markers; *lane 1,* 5 μg of STF2Δ.EIII; *lane 2,* 5 μg of STF2Δ; *lane 3,* 5 μg of WNV-EIII. Proteins were visualized by Coomassie staining. rbs, ribosome binding site.

minants has the potential to induce long-lasting protective immunity.

Because E proteins from all flavivirus members are similar, comparative studies using structural data from select flavivirus E proteins have provided much-needed information for rational vaccine design. The E protein exists as a dimer on the surface of mature virus particles and consists of a hydrophobic anchor responsible for the initiation of membrane fusion and 3 distinct domains (EI, EII, and EIII) essential for dimerization and receptor binding [2, 13]. These 3 domains have been defined antigenically using serological means [13] and 3-dimensionally for dengue, tickborne encephalitis, and WNV proteins using x-ray crystallography [3, 14–16]. The amino acid sequences of flavivirus E proteins are also highly homologous and include 12 highly conserved cysteine residues that form 6 disulfide bridges [17]. EI and EII are formed from the folding of noncontiguous amino acid segments. EI is positioned within the center of the protein separating EII and EIII, whereas EII is the principal region of interaction between E protein monomers and is thought to promote dimerization. By contrast, EIII is formed from a single stretch of ~100 aa and contains a fold that is reminiscent of a typical immunoglobulin-like domain. This particular domain is an ideal vaccine target because it is the receptor-binding domain of E and it encodes the majority of the flavivirus type-specific epitopes [13].

To develop a potent WNV vaccine that will ensure a specific and protective immune response, we have designed a recombinant protein that will deliver the EIII domain of the E protein to antigen-presenting cells (APCs) by linking it to the ligand of Toll-like receptor (TLR)–5. TLRs are initiators of the innate immune response and gatekeepers of the adaptive immune response [18–22]. They are the best characterized type of pattern recognition receptor (PRR) expressed on APCs. Engagement of PRRs by their cognate ligands, pathogen-associated molecular patterns (PAMPs), triggers important cellular mechanisms that lead to the expression of costimulatory molecules, the secretion of critical cytokines and chemokines, and efficient processing and presentation of antigens to T cells. To date, a total of 13 TLRs (TLR1–13) have been discovered and the corresponding PAMPs for most of these receptors have been identified. Some well-characterized PAMPs include bacterial cell-wall components (e.g., lipoproteins and lipopolysaccharides), bacterial or viral DNA sequences that contain unmethylated CpG residues, and bacterial flagellin. In the present article, we demonstrate that the fusion of a TLR agonist with a protective domain of the WNV-E protein can elicit protective immunity.

MATERIALS AND METHODS

DNA cloning. Full-length flagellin from *Salmonella typhimurium* fljb (flagellin phase 2), also called "STF2," is encoded by a 1.5-kb gene. A modified version of STF2, designated STF2Δ, was generated by deleting the hypervariable region that spans aa 170–415. The deleted region was replaced with a short flexible linker (GAPVDPASPW) designed to facilitate interactions of the NH2 and COOH terminal regions necessary for TLR5 signaling. The resulting construct, pMT/STF2Δ, was used to generate pET/STF2Δ.EIII and pET/STF2Δ. Proteins were purified using conventional chromatography and required refolding steps because of low solubility in *Escherichia coli.* Under standard growth and induction conditions, STF2Δ.EIII, STF2Δ, and EIII proteins were expressed as insoluble proteins and formed inclusion bodies (IBs). Before the initiation of column chromatography, IBs were washed and processed as described below. STF2Δ.EIII IBs were solubilized with 8 mol/L urea in 100 mmol/L Tris-HCl (pH 8.0) and refolded by rapid dilution into 0.1 mol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, and 1%

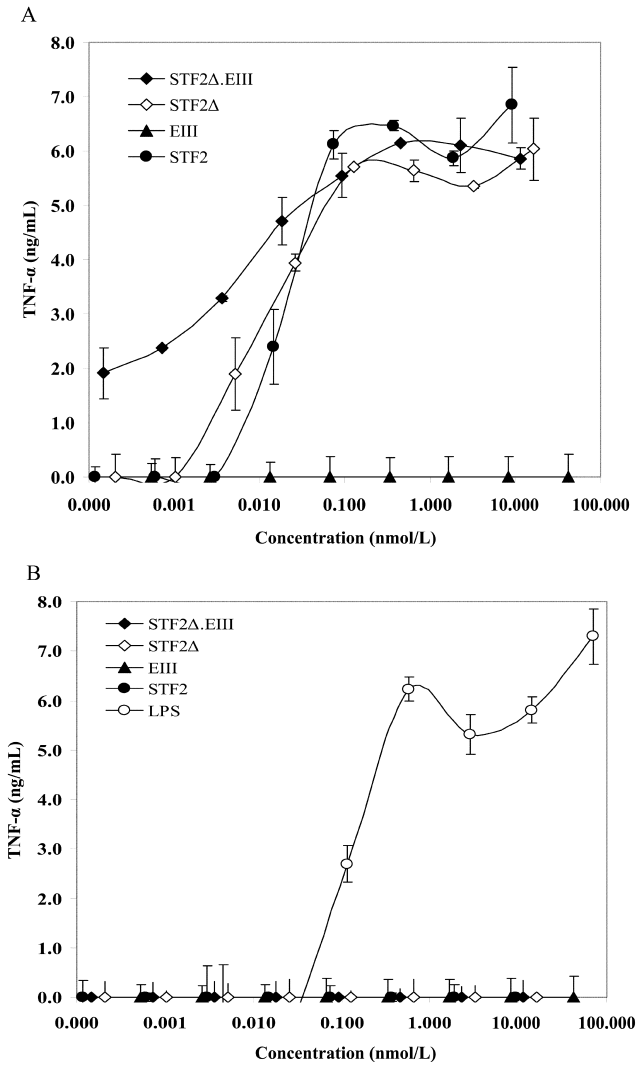

Figure 2. Toll-like receptor (TLR)–5–specific bioactivity of recombinant proteins. Serial dilutions of recombinant flagellin fusion proteins were examined for the ability to activate TLR5-negative RAW264.7 and TLR5-positive RAW/TLR5 cell lines. Supernatants were harvested 16 h after stimulation and were examined for expression of the proinflammatory cytokine tumor necrosis factor (TNF)–α by ELISA by RAW264.7/TLR5 *(A)* and the TLR5-negative parental RAW264.7 *(B)* cell lines. Stimulation with the TLR4 agonist lipopolysaccharide (LPS) is shown as a specificity control.

(wt/vol) glycerol to a final protein concentration of 0.1 mg/mL. Solid ammonium sulfate (AS) was added to the refolded protein to a final concentration of 1 mol/L, and the sample was applied to a butyl sepharose column (GE/Amersham) equilibrated in 25 mmol/L Tris (pH 8.0) and 1 mol/L AS. The bound protein was eluted from the column in 25 mmol/L Tris (pH 8.0). The eluate fractions were extracted 4 times with 1% (wt/vol) Triton X-114 to remove residual endotoxin. The protein was subsequently fractionated on a Superdex 200 gel filtration column (SD200; GE/Amersham Biosciences) to remove detergent and buffer exchange the product into Tris-buffered saline (TBS). STF2Δ IBs were solubilized in 8 mol/L urea in 50 mmol/L Na acetate (pH 4.0). The solubilized protein was captured on SP fast-flow sepharose (GE/Amersham Biosciences) under denaturing conditions and was selectively eluted with 8 mol/L urea and 50 mmol/L Na acetate (pH 4.0) buffer that contained 0.2 mol/L NaCl. The eluted material was pooled and dialyzed against 50 mmol/L Tris-HCl (pH 8.0) and refolded by rapid dilution to 1:10 into 50 mmol/L Tris-HCl (pH 8.0) to a final protein concentration of ~0.1 mg/mL. The refolded SP pool was loaded directly onto Q high-performance sepharose (GE/Amersham), and bound protein was eluted with 20 column volumes of a linear gradient from 0–0.5 mol/L NaCl in 50 mmol/L Tris-HCl (pH 8.0). EIII IBs were solubilized with 8 mol/L urea in 50 mmol/L Na acetate (pH 6.3). The protein was applied to SP fast-flow sepharose (GE/Amersham Biosciences). Bound protein was eluted with 50 mmol/L Na acetate (pH 6.3) and 8 mol/L urea that contained 0.2 mol/L NaCl. SP peak fractions were pooled and dialyzed against 50 mol/L Tris-HCl (pH 8.5). To refold the protein, the dialyzed sample was diluted 1:10 (final protein concentration, ~0.1 mg/mL) in 50 mmol/L Tris-HCl (pH 8.5). The refolded SP pool was loaded directly on Q high-performance sepharose (GE/Amersham). Under these conditions, the majority of EIII did not bind Q and eluted with the flow-through fraction. The Q high-performance flow-through was concentrated to 2 mg/mL (Amicon Ultra-15, 5000 molecular-weight cutoff; Millipore) and applied to size-exclusion chromatography (SD200; GE/Amersham) pre-equilibrated in TBS.

For the isolation of WNV-EIII sequences, a DNA fragment encoding the WNV-E protein (aa 1–406) was subcloned into pMT/BiP/V5-His (Invitrogen) to generate pMT/E. This construct was designed to produce 80% WNV-E protein (without the COOH terminal hydrophobic tail) in *Drosophila* for use as an antigen in serum antibody ELISAs. It also served as a template to isolate the WNV-EIII domain (aa 291–406). The EIII fragment was subcloned separately into pET24a to create pET/EIII and in frame with STF2Δ (fused to the C terminus) to generate pET/STF2Δ.EIII. The STF2Δ sequence of pET/STF2Δ was derived from pMT/STF2Δ. Stable cell pools were expanded as adherent cultures and were adapted to suspension growth in selection medium (*Drosophila* SFM; 18 mmol/L L-glutamine, 1× penicillin/streptomycin, and 25 μg/mL blasticidin). Protein expression was induced with 0.5 mmol/L $CuSO_4$, and E protein was purified by affinity chromatography using nickel NTA (Sigma).

TLR5 bioassay and antibody epitope analysis. To test flagellin fusion proteins for TLR5 bioactivity, a cell-based assay using a modified RAW264.7 cell line that expresses functional TLR5 was used as reported elsewhere [23]. For antibody epitope analysis, the following ELISA procedure was used. Ninety-six–well ELISA plates were coated with serial dilutions (100 μL/

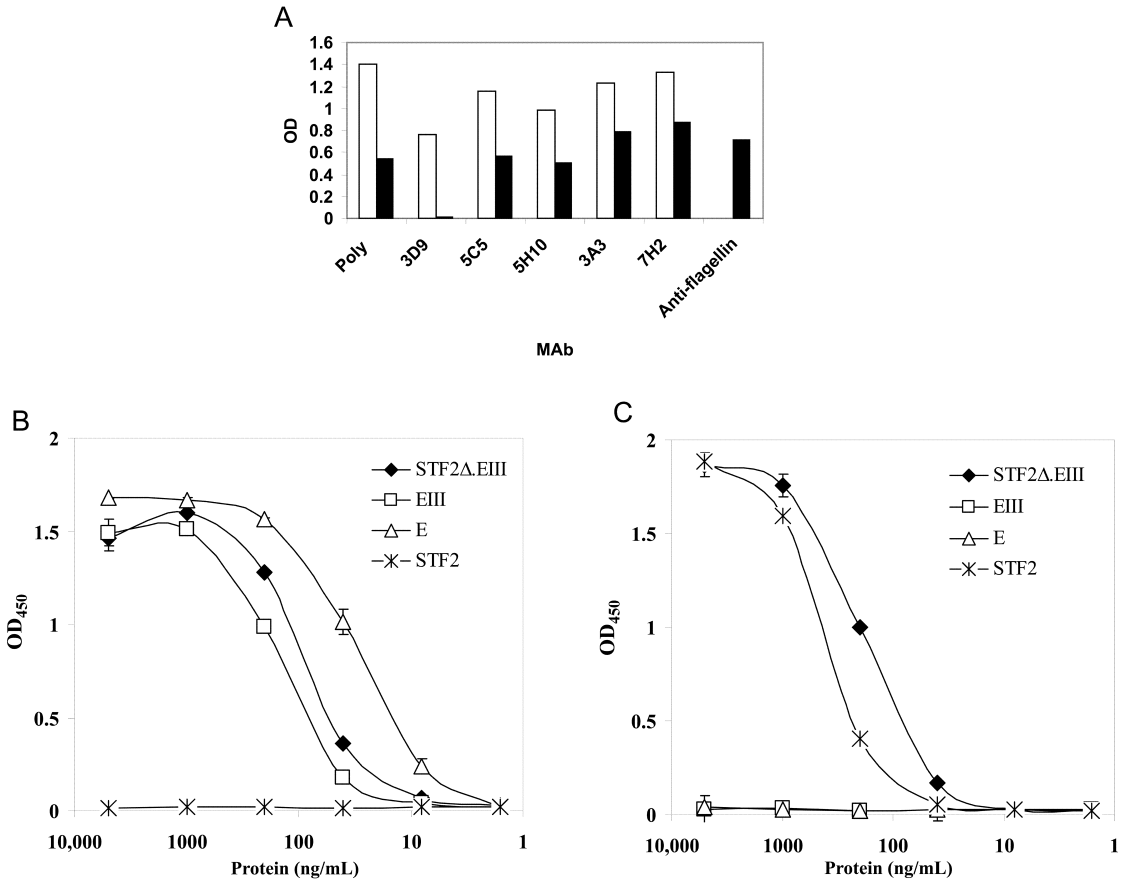

Figure 3. Immunogen antigenicity. *A,* ELISA plates coated with purified West Nile virus (WNV)–E *(white bars)* or STF2Δ.EIII *(black bars)* at 2 μg/mL and incubated with the indicated antibodies. Wells were washed and incubated with horseradish peroxidase–labeled rabbit anti–mouse IgG and developed with 3,3′,5,5′-tetramentylbenzidine substrate. The epitopes for WNV-specific monoclonal antibodies (MAbs) 5C5, 5H10, 3A3, and 7H2 were mapped to the EIII domain of the envelope protein. The epitope for 3D9 resides outside of the EIII domain. A rabbit polyclonal antiserum (Poly) raised against WNV-E protein was used as a control for reactivity with both proteins and the MAb 6H11 (anti-flagellin) was used to assay for flagellin sequences. *B,* Comparison of MAb 5C5 reactivity with WNV-E (E), STF2Δ.EIII, EIII, and STF2 proteins. ELISA plates were coated with serial dilutions of the indicated proteins. *C,* Comparison of flagellin MAb 6H11 reactivity against the same immunogens. WNV-E was produced and purified from stable *Dmel-2* cells that carried the pMT/E construct.

well) of purified STF2Δ-fusion proteins in PBS (2 μg/mL). Plates were blocked with 200 μL/well of assay diluent buffer (ADB; BD Pharmingen) for 1 h at room temperature. Plates were washed 3 times in PBS with 0.01% Tween 20 (PBS-T) and were then incubated with antibodies reactive with flagellin or WNV-E. The expression of flagellin was detected using the monoclonal antibody (MAb) 6H11 (Intotek Pharmaceuticals), whereas the antigenicity of WNV-E proteins was monitored using a panel of MAbs (5C5, 7H2, 5H10, 3A3, and 3D9) purchased from Bioreliance. Antibodies diluted in ADB (100 μL/well) were incubated overnight at 4°C. Plates were washed 3 times with PBS-T. Horseradish peroxidase (HRP)–labeled goat anti–mouse IgG antibodies (Jackson Immunochemical) diluted in ADB were added (100 μL/well), and the plates were incubated for 1 h at room temperature. Plates were washed 3 times with PBS-T. After the addition of 3,3′,5,5′-tetramentylbenzidine Ultra substrate (Pierce Biotechnology) and the monitoring of color development, the A_{450} was measured on a Tecan Farcyte microplate spectrophotometer.

Immunization and viral challenge of mice. C3H/HeN mice, 6–8 weeks old, were purchased form the Jackson Laboratory. Mice were housed and maintained by the Yale University animal facility, and all studies were performed in accordance with the guidelines of the Yale University Institutional Animal Care and Use Committee. TLR5-deficient mice were generated as described elsewhere [24]. Mice were immunized subcutaneously (sc) or intraperitoneally (ip) at 14-day intervals (2 or 3 immunizations, as described in Results). Immunized mice were bled by retro-orbital puncture, and serum was harvested by clotting and centrifugation of the heparin-free blood sam-

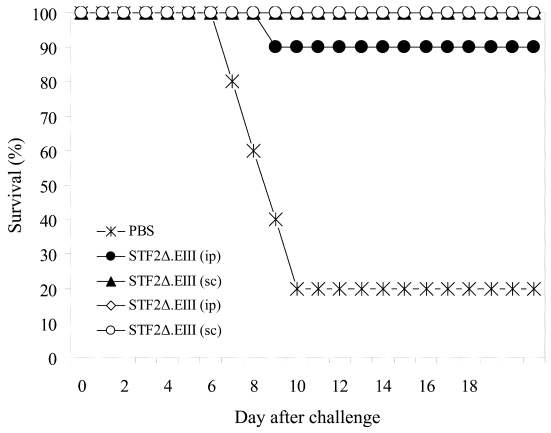

Figure 4. Immunized mice challenged on day 37 with LD_{90} of West Nile virus strain 2741. Survival was monitored for 21 days after challenge. This monitoring period was chosen because survival outcomes beyond 21 days remained constant, making longer evaluation times unnecessary [6, 9, 28]. A graphical representation of the percentage of survivors 21 days after challenge for each immunized group is shown.

ples. For efficacy studies, mice were challenged with a lethal dose (LD_{90}) of WNV strain 2741, and survival was monitored for 21 days after challenge.

Serum antibody determination and reduction in viral plaque number (PRNT) analysis. Serum IgG titers specific for flagellin and for WNV-E were determined by ELISA. Ninety-six–well ELISA plates were coated with 2 μg/mL of purified WNV-E or STF2 protein. Mouse antiserum was serially diluted in PBS and incubated for 1 h at room temperature. Plates were blocked with 200 μL/well of ADB (BD Pharmingen) for 1 h at room temperature and were washed 3 times in PBS-T. Dilutions of the serum in ADB were added (100 μL/well), and the plates were incubated at 4°C overnight. Plates were washed again, HRP-labeled goat anti–mouse IgG antibodies (Jackson Immunochemical) diluted in ADB were added (100 μL/well), and the plates were incubated for 1 h at room temperature. Plates were developed as described above. For PRNT analysis, samples were serially diluted in PBS with 5% gelatin from 1:10 to 1:2560 and processed as described elsewhere [25].

RESULTS

Bioactivity and antigenicity of purified immunogens. To confirm the TLR5 signaling activity of purified immunogens (figure 1), proteins were examined for the ability to activate the TLR5-negative cell line RAW264.7 or RAW264.7 cells transfected with human TLR5 [23]. As a means of comparison, the bioactivity of full-length flagellin protein (STF2) was also determined. This protein was purified under native conditions and was not denatured during purification. As shown in figure 2*A*, nanomolar amounts of STF2 induced significant levels of tumor necrosis factor (TNF)–α (6 ng/mL) with a calculated effective concentration for half-maximal response (EC_{50}) value of 18 nmol/L. Similar TLR5 activity was observed for STF2Δ.EIII and STF2Δ proteins (EC_{50} values of 3 and 10 nmol/L, respectively), which confirmed that removal of the hinge region from flagellin does not affect TLR5 signaling. These data also confirmed that the TLR5 signaling activity of STF2Δ and STF2Δ.EIII is restored after denaturation and refolding of these proteins. As expected, purified EIII, which is not a TLR5 agonist, did not induce TNF-α production in this assay. Finally, none of these proteins induced TNF-α when tested on the parental RAW264.7 cell line, which lacks TLR5 (figure 2*B*). Thus, purified STF2Δ.EIII and STF2Δ have fully functional TLR5 bioactivity.

Because critical domains within EIII are conformation sensitive, it was also necessary to confirm proper folding and epitope display in purified STF2Δ.EIII and EIII by ELISA using a panel of WNV-E polyclonal antibodies and MAbs. For this purpose, we tested several well-characterized E-specific MAbs for reactivity against these purified proteins [26] (BioReliance). As seen in figure 3*A,* all 5 WNV-E MAbs reacted with full-length WNV-E, as expected. This protein contains the majority of the E gene (80%), including the EIII domain, and was produced in *Drosophila* cells as a soluble and folded protein. A comparison of MAb reactivity with STF2Δ.EIII revealed that MAbs 5C5, 5H10, 3A3, and 7H2, but not 3D9, recognized the fusion protein. This pattern of reactivity is consistent with the proposed location of the antibody epitopes within EIII. Polyclonal WNV-E antiserum also reacted with WNV-E and STF2Δ.EIII; however, reactivity with STF2Δ.EIII was somewhat reduced, perhaps because of the reduced number of potential epitopes present in the smaller domain. A flagellin-specific MAb (6H11) was also tested and was shown to specifically react with STF2Δ.EIII but not with WNV-E. Using MAb 5C5, a direct antigenic comparison was made between STF2Δ.EIII and EIII (figure 3*B* and 3*C*), which confirmed that both STF2Δ.EIII and EIII contain critical E-specific conformation-dependent neutralizing epitopes.

Immunogenicity and efficacy of STF2Δ.EIII. The immunogenicity and efficacy of STF2Δ.EIII were tested in an established mouse model for WNV infection [27]. As shown in figure 4, significant levels of WNV-E–specific IgG were detected by day 35 (end-point titers $>10^4$) in mice immunized with STF2Δ.EIII either ip or sc. These levels were also detected after the first boost (day 21; data not shown), which suggests that 2 immunizations might be sufficient to elicit a maximal response. The fusion protein also elicited flagellin-specific IgG with similar kinetics (data not shown) (see figure 5).

Because protection from WNV infection is largely antibody mediated, we tested these same serum samples for their ability

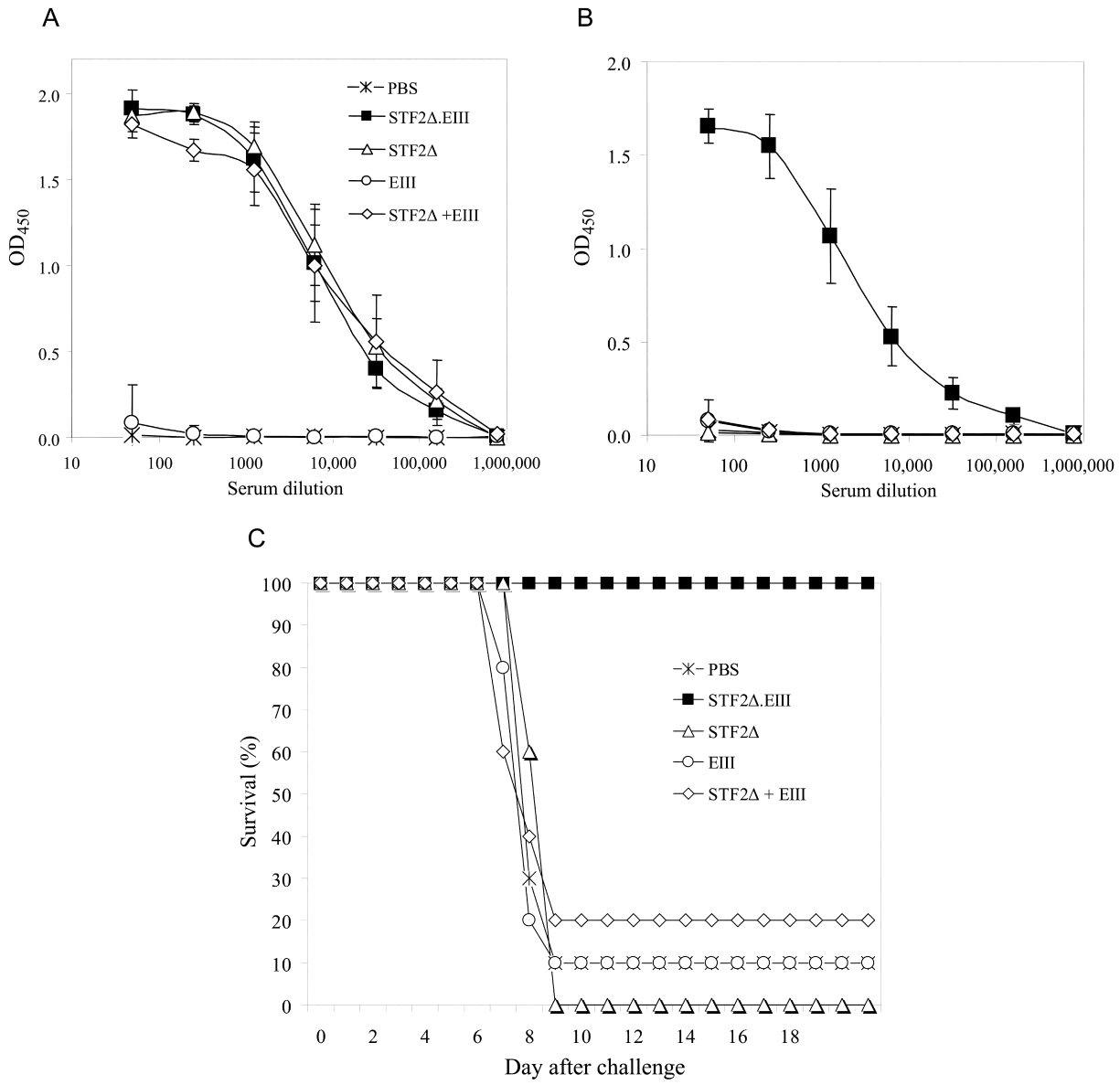

Figure 5. Fusion of the West Nile virus (WNV)–EIII domain to flagellin for a potent and protective EIII-specific immune response in mice. Five groups of C3H/HeN mice (10 mice/group) were immunized on days 0, 14, and 28 subcutaneously with STF2Δ.EIII (25 μg), STF2Δ (18 μg), WNV-EIII (7 μg), or a mixture of STF2Δ (18 μg) and WNV-EIII (7 μg). Doses were chosen to ensure that molar equivalents of each antigen were administered in PBS. On day 35, serum was harvested and tested by ELISA for flagellin *(A)* and WNV-E–specific *(B)* IgG responses. Purified flagellin (STF2) and 80% WNV-E protein were used as antigens for antibody detection. Results are the mean ± SE optical density values at 450 nm obtained from 10 individual mice/group. *C,* Immunized mouse challenge on day 37 with an LD_{90} of WNV strain 2741. Mice monitored for survival for 21 days. Survival curves for each treatment group are shown and expressed as a percentage.

to neutralize virus in vitro. Serum titers that led to $PRNT_{80}$ were recorded. The $PRNT_{80}$ data from 2 independent mouse efficacy studies are presented in table 1. In all cases, pooled serum from mice immunized with STF2Δ.EIII had neutralization titers of ⩾1:40. It has been previously established that neutralization titers of ⩾1:40 typically correlate with protection in vivo [25]. To compare neutralization titers with protection from WNV in vivo, mice were challenged with an LD_{90} (100 pfu/mouse) of WNV. As shown in figure 4 and table 1, only 20% of mice that received PBS alone survived infection. By contrast, ⩾90% survival was observed in groups that were immunized with STF2Δ.EIII. These data are consistent with the neutralization titers measured with serum from these groups. Thus, STF2Δ.EIII administered without adjuvant is capable of eliciting a potent antibody response sufficient to protect immunized mice from a lethal viral challenge.

Requirement for fusion of STF2Δ to EIII. As reported elsewhere [23], the physical linkage of PAMP and antigen, rather

Table 1. West Nile virus (WNV)–E–specific IgG titers and in vitro neutralization activity of antiserum from STF2Δ.EIII-immunized mice.

Study, immunogen	Route	IgG titer (end point)	$PRNT_{80}$, dilution
1			
PBS	ip	ND	<1:10
STF2Δ.EIII	ip	2.35×10^4	1:40
STF2Δ.EIII	sc	2.83×10^4	1:160
2			
STF2Δ.EIII	ip	2.71×10^4	1:80
STF2Δ.EIII	sc	2.93×10^4	1:40

NOTE. Results from 2 independent efficacy studies are shown (1 and 2). C3H/HeN mice (10 mice/group) were immunized 3 times (on days 0, 14, and 28) intraperitoneally (ip) or subcutaneously (sc) with 25 μg of STF2Δ.EIII formulated in PBS. On day 35, serum samples from each group were harvested, assayed for the presence of WNV-E–specific IgG by ELISA, and pooled. Pooled samples were serially diluted and tested for neutralization activity as described in Materials and Methods. WNV-E–specific IgG titers, presented as end-point titers, and virus neutralization potency of pooled serum samples (80% reduction in viral plaque number [$PRNT_{80}$]) are shown. ND, not done.

than a simple mixture of the 2 components, is critical for the elicitation of a potent immune response. This observation was further investigated by comparing the immunogenicity of STF2Δ.EIII with an equimolar amount of STF2Δ and WNV-EIII formulated as a protein mixture. As shown in figure 5*B*, only STF2Δ.EIII elicited measurable WNV-E–specific antibodies, whereas the STF2Δ plus WNV-EIII mixture failed to elicit an E-specific response, even though flagellin antibodies were readily detectable in these immunized mice (figure 5*A*). This pattern of antibody response was also observed after the first boost (day 14), which suggests that a prime-and-single-boost regimen is sufficient to induce a significant antibody response (data not shown; see longevity study below). As expected, immunization with EIII alone did not elicit E-specific antibodies, which demonstrates the poor immunogenicity of this purified antigen. The efficacy of STF2Δ.EIII was demonstrated by challenging these mice with WNV. As shown in figure 5*C*, mice immunized with STF2Δ.EIII were 100% protected. By contrast, no protective advantage over PBS was observed in mice that received STF2Δ or EIII as separate immunogens or as a protein mixture. These results clearly demonstrate that both flagellin and EIII are required for protection and that a physical linkage between them is required to elicit a potent immune response against the WNV-EIII domain. Immunogenicity was also examined in TLR5-deficient mice in a C57BL/6 background [24]. After immunization with STF2Δ.EIII, TLR5-deficient mice exhibited markedly lower WNV-E and flagellin IgG responses than did wild-type mice (figure 6). These studies demonstrate a clear requirement for TLR5 to elicit a significant antigen-specific immune response.

Immune response longevity at different immunogen doses. We examined the longevity and potency of the immune response to STF2Δ.EIII by altering dose and regimen of administration. After immunization, antibody responses were monitored for a prolonged period before virus challenge (figure 7*A* and 7*B*). With 2 immunizations (figure 7*A*), measurable levels of WNV-E–specific IgG and a clear dose response were observed when we compared the groups that received 25 or 2.5 μg of protein. By 10 weeks, although some decay in WNV-E IgG levels was observed, mice immunized with either amount of protein maintained significant levels of circulating antibodies. With 3 immunizations (figure 7*B*), higher antibody titers were detected, and a slightly less pronounced decay in antibody levels was observed at 10 weeks.

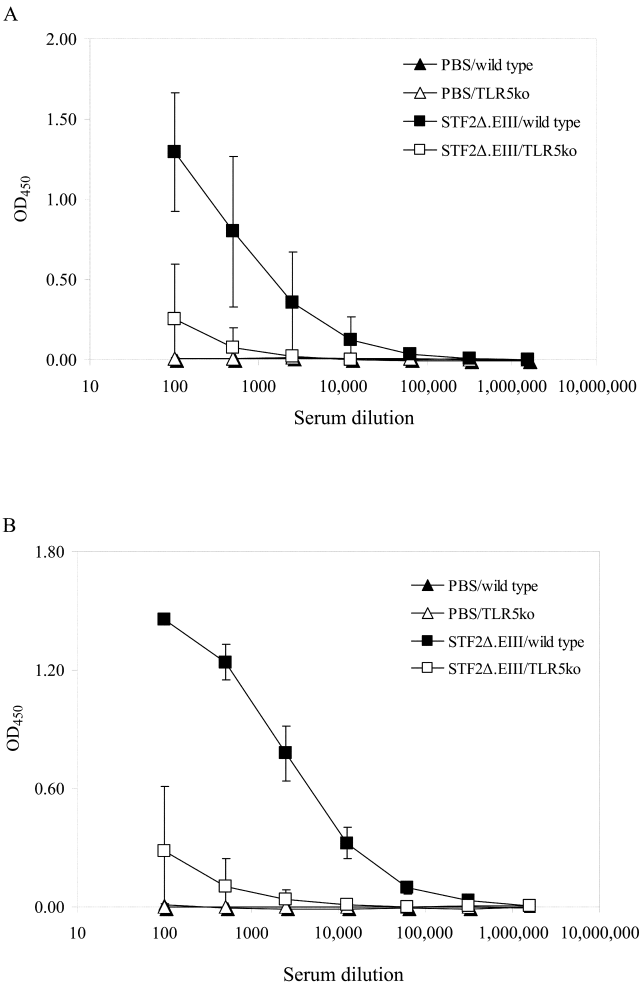

Figure 6. Requirement for Toll-like receptor (TLR)–5 in vivo. IgG responses after immunization of wild-type or TLR5-knockout (ko) C57BL/6 mice with the STF2Δ.EIII fusion protein are shown. Wild-type and TLR5 ko mice (5 mice/group) were immunized with PBS or 25 μg of the STF2Δ.EIII fusion protein subcutaneously on days 0 and 21, and serum was collected on day 28. Anti-flagellin *(A)* and anti–West Nile virus (WNV)–E *(B)* IgG responses were examined by ELISA. The data depict the mean ± SD of 5 individual serum samples/group.

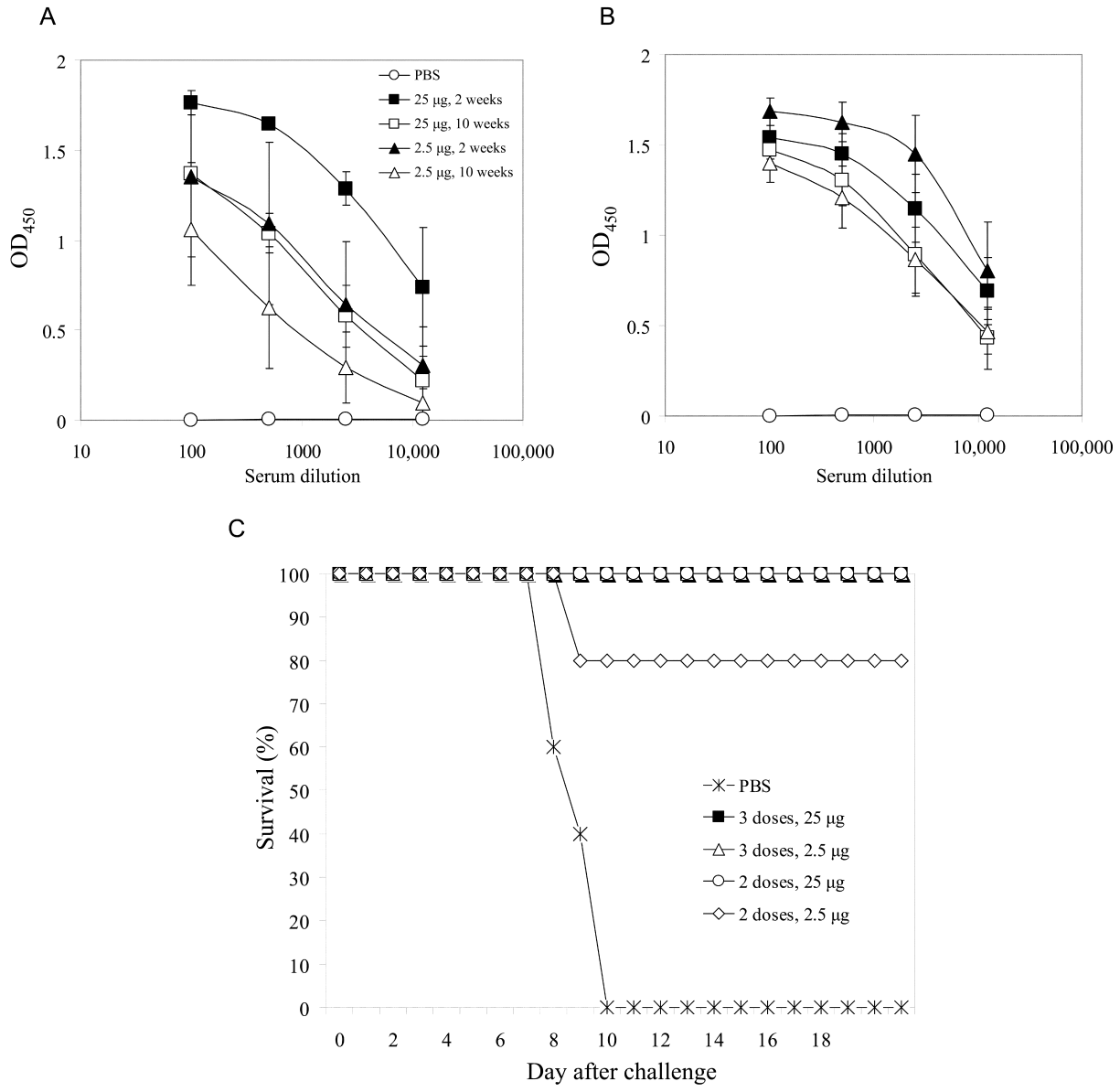

Figure 7. Immune response longevity and potency. Five groups of C3H/HeN mice ($n = 5$ for PBS group and $n = 10$ for remaining groups) were immunized subcutaneously with either 25 or 2.5 μg of STF2Δ.EIII protein 3 times (days 0, 14, and 28) or 2 times (days 14 and 28). Serum was collected 2 and 10 weeks after immunization, and West Nile virus (WNV)–E–specific antibody responses were measured by ELISA. *A,* WNV-E–specific IgG response after 2 immunizations at different immunogen doses. *B,* WNV-E–specific IgG response after 3 immunizations at different immunogen doses. ELISA data are the mean ± SD optical density at 450 nm of individual responses. *C,* Survival of immunized mice after a lethal challenge (LD_{90}) with WNV. Mice shown in panels A and B were challenged 10 weeks after administration of the vaccine.

Ten weeks after the last immunization, all mice in the study were challenged with WNV, and survival was monitored. As shown in figure 7*C*, 100% survival was observed in groups that were immunized 3 times with 25 or 2.5 μg of protein and those that were immunized twice with 25 μg of protein. A survival rate of 80% was observed in mice immunized twice with 2.5 μg of protein. This group of mice had the lowest WNV-E IgG titer before challenge, which might indicate that these mice were at a threshold of circulating protective antibodies. These results indicate that a 2-immunization regimen with ⩾2.5 μg of recombinant protein is sufficient to induce a sustained antibody response and afford protection from WNV infection in mice 10 weeks after immunization.

DISCUSSION

There are several WNV vaccines at various stages of development, but none are currently available for human use. These

include live attenuated chimeric viruses such as a dengue 4–WNV and yellow fever–WNV (Chimerivax-WN02) [29, 30]. These modified flaviviruses have their endogenous preM and E proteins replaced with those from WNV and have been shown to elicit strong immune responses, including neutralizing WNV antibodies in nonhuman primates and humans. Safety and efficacy in human clinical trials are currently being evaluated [30]. Nonreplicating naked-plasmid DNA vaccines are also being developed and have shown promise in animal studies; however, their immunogenicity and efficacy in humans have yet to be demonstrated [31]. Finally, several protein-based subunit vaccines that contain formulations of WNV-NS1 and -E proteins are also in development. The recombinant proteins used in these vaccines are manufactured in heterologous protein expression systems that include *Drosophila melanogastor* cells [25, 32]. In addition, an *E. coli*–expressed recombinant E fusion protein has also shown promise as a potential subunit vaccine [11]. For optimal immunogenicity, these purified protein preparations require the use of alum- and saponin-based adjuvants [25, 32].

In the present article, we describe an *E. coli*–based WNV monovalent subunit protein vaccine that is both immunogenic and efficacious against WNV infection in the absence of an adjuvant. This uniquely designed dual-mode chimeric protein codelivers innate and adaptive immune signals in a single polypeptide. A key feature of this fusion protein is its ability to engage the TLR5 receptor by virtue of bacterial flagellin sequences. Flagellin protein is the major component of the propeller-like flagella that are used by bacteria for motility and are important for host interactions and invasion [33, 34]. The role of flagellin monomers in the induction of proinflammatory responses and their interactions with TLR5 have been well documented [35, 36]. Flagellin-induced activation of the innate response through TLR5 signaling is known to influence the presentation of antigens and the activation of a cellular immune response during the establishment of adaptive immunity [36, 37].

One distinct difference in the design of the fusion protein that we describe (STF2Δ.EIII) is that a modified flagellin protein was used instead of the full-length protein. Bacterial flagellins have highly conserved N- and C-terminal domains that are essential for TLR5 binding and activation [38]. These domains are separated by a hypervariable domain that contains the major antigenic epitopes of the molecule. STFΔ.EIII was designed with a flagellin that lacks this hypervariable region. In its place, a flexible linker was used to join both N- and C-terminal domains. Consistent with previous reports, we have shown that removal of this domain does not affect TLR5 activation in vitro [38]. When fused to the EIII domain of the WNV-E protein, we observed the induction of a potent antibody response to both flagellin and EIII. The kinetics of this antibody response differed from what was described for ovalbumin fusion, in which a full-length flagellin sequence, including the hypervariable region, was used [23]. For STFΔ.EIII, a single immunization was not sufficient to elicit a measurable IgG response. It is not clear whether this difference is associated with the modified flagellin or is intrinsic to the different antigens used in each study. Nevertheless, a potent and protective response was achieved.

It is also clear from these studies that the presence of a functional TLR5 and the physical association of WNV-EIII domain to flagellin (STF2Δ) are critical for generating a significant immune response in the absence of adjuvant. When administered to TLR5 knockout mice as a fusion protein or to wild-type mice as separate protein components, no antigen-specific antibody responses were evident. It is important to note that flagellin antibodies elicited by immunization with flagellin alone or with flagellin fusion proteins do not neutralize the in vitro TLR5 activity of flagellin [23]. Therefore, the presence of these antibodies was not expected to interfere with PAMP activity in vivo. Consistent with this hypothesis, we have shown that the presence of flagellin-specific antibodies does not inhibit primary or boost responses when immunizing with flagellin fusion proteins [23].

Finally, an important attribute of this recombinant WNV vaccine is the use of a subdomain of the WNV-E protein. As mentioned previously, the EIII domain of all WNV is the major target of neutralizing antibodies, and, when it is produced in prokaryotic expression systems, it can fold independently to form a native-like structure that retains its contiguous critical/dominant neutralizing epitopes [26, 39]. In addition, as an independent domain, it is capable of preventing WNV infection in vitro, presumably by blocking interactions between virus and receptor [40]. Moreover, it has been demonstrated that immunizing animals with this domain will elicit neutralizing type-specific antibodies that do not cross-react with E proteins from other flaviviruses [41].

In summary, by linking the WNV-EIII viral domain to flagellin, we have generated a novel fusion protein capable of eliciting a potent and enduring WNV-EIII–specific immune response that is efficacious against WNV infection. On the basis of the immunogenicity and efficacy studies presented here, we believe that this recombinant protein holds great promise as a WNV vaccine and that this flagellin/EIII approach should provide new opportunities for the development of simple, cost-effective vaccines for related flaviviruses and other emerging/reemerging infectious diseases.

While our article was in press, Chu et al. [42] published similar findings regarding the use of the WNV EIII domain as a potential vaccine. Those authors reported that EIII is immunogenic when administered in CpG adjuvant 3 times at high protein doses (100 μg/injection). In that article, pro-

tection from WNV was measured in suckling mice by administering virus mixed with immune serum from immunized mice. This approach tests for the presence of neutralizing antibodies and complements in vitro neutralization assay methodologies. In contrast to those findings, we have reported here that STF2Δ.EIII fusion protein is immunogenic and provides sufficient protection from a direct virus challenge without the use of adjuvant and at significantly lower protein doses (2.5 μg of protein delivered twice). Mice were actively immunized with protein and then challenged with an LD_{90} of virus. The remarkable potency of STF2Δ.EIII as an immunogen in these studies is attributed to the fusion of the EIII domain, which by itself is poorly immunogenic, to the TLR5 agonist flagellin. We found that targeting antigens to APCs through TLRs in this way eliminates the need for adjuvants and minimizes the immunogen dose such that effective subunit protein vaccines can be developed.

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