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A Hydrogen Peroxide-Inactivated Virus Vaccine Elicits Humoral and Cellular Immunity and Protects against Lethal West Nile Virus Infection in Aged Mice

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West Nile virus (WNV) is an emerging pathogen that is now the leading cause of mosquito-borne and epidemic encephalitis in the United States. In humans, a small percentage of infected individuals develop severe neuroinvasive disease, with the greatest relative risk being in the elderly and immunocompromised, two populations that are difficult to immunize effectively with vaccines. While inactivated and subunit-based veterinary vaccines against WNV exist, currently there is no vaccine or therapy available to prevent or treat human disease. Here, we describe the generation and preclinical efficacy of a hydrogen peroxide (H₂O₂)-inactivated WNV Kunjin strain (WNV-KUNV) vaccine as a candidate for further development. Both young and aged mice vaccinated with H₂O₂-inactivated WNV-KUNV produced robust adaptive B and T cell immune responses and were protected against stringent and lethal intracranial challenge with a heterologous virulent North American WNV strain. Our studies suggest that the H₂O₂-inactivated WNV-KUNV vaccine is safe and immunogenic and may be suitable for protection against WNV infection in vulnerable populations.

In the United States between 1999 and 2012, an estimated 3 million people were infected with West Nile virus (WNV), resulting in over 780,000 illnesses, greater than 36,500 confirmed cases, and 1,500 deaths (1; <http://www.cdc.gov/ncidod/dvbid/westnile/index.htm>). While the past few years have been characterized by lower-level endemic transmission to humans, 2012 has witnessed new and intense outbreaks of WNV neuroinvasive disease in several regions of the United States and Europe. While most WNV-infected individuals experience a mild or self-limiting febrile illness, a fraction (~1%) of cases progress to severe neurological manifestations, including high and sustained fever, headache, myalgia, meningitis, encephalitis, or acute flaccid paralysis (2). Advanced age and immunosuppression are risks for the development of severe WNV disease (2–5). Although the worldwide incidence of WNV infection is increasing (6), there is no specific treatment or vaccine available for use in humans (7).

WNV is a member of the *Flaviviridae* family of positive-stranded RNA viruses, which includes the globally relevant human pathogens dengue virus (DENV), yellow fever virus (YFV), and Japanese encephalitis virus (JEV). WNV was originally isolated from a febrile patient in Uganda in 1937, and phylogenetic analysis separates WNV into five distinct but genetically related lineages based on nucleic acid sequence divergence (8–11). Lineage 1 strains are considered emerging and associated with outbreaks of neuroinvasive disease (10, 12); they originate from diverse geographic regions and include WNV New York (WNV-NY), which initiated the WNV epidemic in the United States, and WNV Kunjin (WNV-KUNV), which is a nonvirulent strain of WNV circulating in Australia (10, 11, 13). Lineage 2 strains are usually less pathogenic (8), although variants, including some contemporary strains in Greece and Eastern Europe (14), periodically cause severe neurological disease (15). Less is known about lineage 3, 4, or 5 viruses, as few isolates exist, although symptomatic human cases have been attributed to them in Austria (16), Russia (17), and India (9).

Protection against primary WNV infection or secondary challenge is linked to the induction of protective humoral and cellular immune responses. Most of the protective antibodies generated against WNV bind to the structural envelope (E) protein, with a smaller subset directed against premembrane (prM) and the non-structural proteins (reviewed in reference 18). Multiple reports have shown that WNV-specific T cells also contribute to protection and clearance of WNV from infected hosts (19–25). These studies suggest that an effective WNV vaccine should stimulate both humoral and T cell responses to achieve comprehensive protection against WNV challenge, especially in vulnerable populations. Indeed, live-attenuated replicating vaccines are immunogenic in animals and healthy adults and elicit robust adaptive immune responses (26, 27). Nonetheless, a challenge to their development and licensure is that the main target population in humans (the elderly and immunosuppressed) may be poor candidates for this class of vaccine, unless extensive safety studies are performed (28).

WNV-Innovator is a veterinary vaccine administered to horses and exotic zoo animals (29). One potential limitation to the use of this formalin-inactivated vaccine in humans is that it is generated from the highly virulent North American WNV-NY strain. A vaccine based on a virulent WNV-NY strain necessitates the generation of high-titer virus stocks for inactivation in biosafety level 3 (BSL3) facilities, which adds to production costs, and an absolute requirement for inactivation to prevent transmission of viable virulent virus into vulnerable vaccine recipients. An alternative to

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using virulent WNV-NY is to develop a vaccine based on a naturally attenuated strain, such as WNV-KUNV (30–32).

WNV-KUNV was first isolated in North Queensland, Australia, in 1960. Although 2.5% of the population in northern Australia is seropositive for WNV-KUNV, there has been no documented fatal human infection with this virus (33, 34), and only a very few individuals have developed WNV fever (35). WNV-KUNV is genetically similar to WNV-NY (~98% amino acid identity), with virtually complete conservation of all neutralizing antibody epitopes (11, 36). Indeed, immunization of mice with live and infectious WNV-KUNV, which itself is avirulent in immunocompetent adult mice, completely protects against lethal infection by North American WNV isolates (31). These observations suggest that WNV-KUNV may be a suitable strain for development of a vaccine in humans against the more virulent strains that circulate in the United States and other parts of the globe. Herein, we describe the preclinical evaluation of a newly developed H₂O₂-inactivated WNV-KUNV vaccine in adult and aged mice.

MATERIALS AND METHODS

Mice. Eight- to 10-week-old C57BL/6 and BALB/c mice were obtained commercially (Jackson Laboratory). Eighteen-month-old C57BL/6 mice were purchased from the National Institute on Aging breeder colony (Harlan). HLA-A2 transgenic (HHDII) mice have been described previously (37) and were provided by T. Hansen and B. Carreno (St. Louis, MO) with permission from F. A. Lemonnier (Paris, France). These mice express a chimeric monochain of HLA-A*0201 (α 1/ α 2 domains), mouse D^b (α 3 domain), and linker-attached human β ₂-microglobulin (β ₂m) and were bred in a specific-pathogen-free facility at Washington University. All mouse infections were performed in an A-BSL3-accredited facility at Washington University in accordance with federal guidelines and approval of the Washington University Animal Studies Committee.

Viruses. The WNV lineage I New York isolate (WNV-NY; 3000.0259, 2000, passage 2) was described previously (38). The WNV-KUNV isolate (CH16532) was a generous gift of R. Tesh (World Reference Center of Emerging Viruses and Arboviruses, Galveston, TX). For live virus infections in mice with WNV-KUNV, 10⁶ PFU was injected in 100 μ l via the intraperitoneal (i.p.) route. For live virus infection in mice with WNV-NY, 10² PFU in 50 μ l was injected subcutaneously (s.c.) via footpad injection. For intracranial (i.c.) challenges, aged mice were given 10⁴ PFU (10,000 times the 90% lethal dose [LD₉₀]) and young mice were administered 10⁶ PFU (1,000,000 times the LD₉₀) in 10 μ l.

Vaccine preparation. Serum-free-adapted (VP-SFM medium; Life Technologies) WHO-Vero cells (10-87; ATCC) were grown to confluence in flat-stock cell culture or on Cytodex-1 microcarrier beads (GE Healthcare) in a WAVE bioreactor (GE Healthcare). Cells were infected with WNV-KUNV strain CH16532 (multiplicity of infection, 0.1) and harvested approximately 48 h later. Supernatants were concentrated and initially purified by tangential flow filtration followed by diafiltration into inactivation buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2% sorbitol). Following sterile filtration (pore size, 0.22 μ m) to remove potential aggregates, WNV-KUNV was inactivated with H₂O₂ (Fisher Scientific) (H₂O₂-WNV-KUNV) at a final concentration of 3.0% for 7 to 8 h at room temperature, with an additional filtration step performed approximately midway through the inactivation period (4 h). After inactivation, H₂O₂ was removed through further purification by dialysis, followed by pelleting of the virus by ultracentrifugation (200,000 \times g, 16 h) or ion-exchange chromatography (cellulose sulfate; JNC Corporation). Mice were immunized i.p. with H₂O₂-WNV-KUNV containing approximately 1 \times 10⁷ PFU equivalents/ μ g protein that was adjuvanted with either 5 μ g monophosphoryl lipid A (MPL; InvivoGen) or 0.1% aluminum hydroxide gel (alum; Sigma-Aldrich). WNV-Innovator, a formalin-inactivated crude virus preparation (New York 1999 strain VM2, serial number 1666142A),

is available commercially (Pfizer) and contains a proprietary oil adjuvant, MetaStim, as well as excipients. For experiments in this study with WNV-Innovator, we used a dose of 100 μ l, which is 1/10 of the dose administered to horses.

Virus inactivation studies. To determine the kinetics of virus inactivation, WNV-KUNV that was concentrated by tangential flow filtration was treated with a final concentration of 3.0% H₂O₂ and monitored over time. At specific time points, aliquots were sampled from the bulk suspension and treated with catalase (final concentration, 12.5 U/ml; 10 min at room temperature; MP Biomedical) to remove residual H₂O₂. This procedure was performed twice on each sample to ensure complete removal of H₂O₂. Following this, standard plaque assays were performed on Vero cells as previously described (39). Of note, catalase treatment alone had no impact on the infectivity of WNV-KUNV (data not shown); thus, virus inactivation was due to exposure to 3.0% H₂O₂.

Gel electrophoresis. Reducing SDS-PAGE was performed with NuPAGE 4 to 12% bis-Tris gels in MOPS (morpholinepropanesulfonic acid) running buffer per the manufacturer's instructions (Life Technologies). Vaccine antigen samples were diluted into NuPAGE SDS sample buffer supplemented with a final concentration of 50 mM dithiothreitol (DTT), heated at 80°C for 10 min, and loaded onto prepared gels. Gels were run in an Xcell SureLock system (Life Technologies) for approximately 45 min at a constant voltage of 200 V and then stained with a Pierce silver stain kit (Thermo Scientific). Gel electrophoresis for Western blot analysis was carried out similarly, but DTT was omitted to maintain nonreducing conditions. Following electrophoresis, gels were blotted onto polyvinylidene difluoride membranes using an iBlot dry blotting system (Life Technologies). After transfer, membranes were blocked (5% nonfat dry milk in phosphate-buffered saline [PBS], 0.05% Tween 20 [PBS-T]) for 1 h at room temperature and probed with the WNV-specific monoclonal antibody (MAb) 7G11 at 1 μ g/ml in blocking buffer for 1 h at room temperature. Membranes were washed five times with PBS-T and probed with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody at an optimal dilution for 1 h at room temperature. Following a final series of washes with PBS-T, blots were developed with the Pierce ECL Western blotting substrate (Thermo Scientific) according to the manufacturer's recommendations and exposed to Biomax XAR film (Kodak).

CD8⁺ T cell depletion experiments. CD8⁺ T cells were depleted from mice with a rat MAb (53-5.8; rat IgG2b; BD Bioscience) specific for the mouse CD8 β chain according to a previously published protocol (40). A rat IgG1 antibody (Jackson ImmunoResearch) was used as an isotype control. Anti-CD8 β or isotype control MAb (40 μ g) was administered to mice via an i.p. injection 2 days prior to i.c. challenge, and an additional 10 μ g of anti-CD8 β or isotype control MAb was administered intravenously (i.v.) on the day of virus challenge. Two days after infection, the efficiency of depletion of CD8⁺ T cells was assessed by flow cytometry after staining peripheral blood mononuclear cells with anti-CD8 α (Biolegend).

Flow cytometry. Intracellular staining for tumor necrosis factor alpha (TNF- α) and gamma interferon (IFN- γ) of lymphocytes isolated from the spleen was performed as described previously (41). Briefly, spleens were harvested and homogenized to form a single-cell suspension and incubated with 2 μ g/ml brefeldin A (Sigma) for 6 h at 37°C with 10⁻⁶ M of peptide NS4B from residues 2488 to 2496, E from residues 347 to 354, E from residues 771 to 778 (20) (H-2^b mouse CD8⁺ T cells), or SVG9 E from residues 720 to 728 (42) (HLA-A2 transgenic CD8⁺ T cells) or 2 μ g/ml anti-CD3 (145-2C11) (BD Biosciences). After incubation, the cells were stained with directly labeled antibodies (all from Biolegend, unless indicated) against CD3, CD4, CD11a, CD19, CD27, CD43, CD62L, CD69, CD122, CD127, CD8 α , CD44, programmed cell death protein 1 (PD-1), killer cell lectin-like receptor subfamily G member 1 (KLRG1), and cytotoxic T lymphocyte antigen 4 (CTLA-4). D^b-NS4B tetramer and HLA-A2 SVG9 tetramer were obtained from the NIH tetramer core facility. Cells were washed, fixed, permeabilized with FixPerm buffer (eBioscience), and stained intracellularly for anti-IFN- γ , anti-TNF- α , (eBioscience), or anti-granzyme B (Invitrogen). Lymphocytes were processed

on an LSRII flow cytometer (BD Bioscience) using FACSDiva (version 6.1.1) software (BD Bioscience) and analyzed with FlowJo software (Treestar). The total numbers of IFN- γ - or TNF- α -expressing CD8⁺ T cells was determined by multiplying the percentage of IFN- γ -positive (IFN- γ ⁺) or TNF- α -positive (TNF- α ⁺) CD8⁺ T cells by the total numbers of splenocytes.

ELISA. WNV-specific IgG levels were determined using an E-protein-specific enzyme-linked immunosorbent assay (ELISA) as described previously (43). Briefly, individual wells of a microtiter plate were coated overnight at 4°C with 1 μ g/ml of recombinant WNV E protein. Plates were washed and blocked with 1% bovine serum albumin (BSA) in PBS supplemented with 0.05% Tween 20 (blocking buffer). Serum samples from naïve, immunized, or infected mice were heat inactivated (56°C for 60 min), serially diluted in blocking buffer, and added to wells for 1 h at room temperature. Following several washes, biotin-conjugated goat anti-mouse IgG (Sigma-Aldrich) was added for 1 h at room temperature. Plates were then washed and incubated with streptavidin-conjugated horseradish peroxidase (2 μ g/ml in blocking buffer; Vector Laboratories) for 1 h at room temperature. After several washes, plates were developed with tetramethylbenzidine substrate (Dako) and the reaction was stopped with 1 N H₂SO₄. The optical density at 450 nm was measured, and end-point dilutions were defined as those that were twice above the background average (BSA-coated wells).

Neutralization assay. The neutralizing activity of serum antibodies against WNV was assessed using either a focus or a plaque reduction neutralization assay (44). Neutralization titers of serum from BALB/c mice were determined using a plaque reduction assay as described previously (45). For studies in aged C57BL/6 mice, 50 focus-forming units of WNV were preincubated with serial dilutions of heat-inactivated mouse serum at 37°C for 1 h in Dulbecco modified Eagle medium with 2% fetal bovine serum (FBS), penicillin, and streptomycin. Virus-serum mixtures were added in triplicate to individual wells of 96-well tissue culture plates containing Vero cell monolayers at ~90% confluence. Virus was incubated with Vero cells for 1 h at 37°C, after which wells were overlaid with 1% carboxymethyl cellulose (Sigma) in minimal essential medium supplemented with 4% FBS. After culture at 37°C for 24 h, cells were fixed with 1% paraformaldehyde in PBS (10 min at room temperature) and permeabilized with 0.1% saponin and 0.1% BSA in PBS (saponin buffer; 5 min at room temperature). Cells were stained with the humanized E16 anti-WNV MAb (46) (50 μ l at 200 ng/ml in saponin buffer) for 2 h at room temperature. Following several washes, wells were incubated with horseradish peroxidase-conjugated anti-human IgG antibody (1:5,000 in saponin buffer; Sigma) for 1 h at room temperature. Wells were washed and infectious foci were visualized with TrueBlue substrate (KPL) after a 5- to 10-min incubation at room temperature. Wells were rinsed with water and dried prior to analysis with a Biospot counter (Cellular Technology) using Immunocapture software. The percent reduction in spot numbers in samples preincubated with serum compared to the numbers in wells with virus preincubation with medium alone were graphed using Prism software, and 50% effective concentration (EC₅₀) values were calculated.

Statistical analysis. For survival analysis, Kaplan-Meier curves were analyzed by the log rank test. The statistical significance of viral burden, antibody titers, and number of activated T cells was analyzed by the Mann-Whitney test. All statistical analyses were performed using Prism software (GraphPad Prism).

RESULTS

Generation of an H₂O₂-inactivated WNV-KUNV vaccine. Our goal was to generate an inactivated immunogenic vaccine that elicited protective immune responses against WNV and could be used safely in at-risk populations. Previously, members of our group demonstrated that an H₂O₂-inactivated WNV-NY could induce effective neutralizing antibody responses in BALB/c mice (39). Although inactivated WNV-NY might be a suitable veterinary vaccine, for humans, given the possible safety concerns and

manufacturing issues associated with large-scale cultivation of a BSL3 virus, we decided to use the attenuated WNV-KUNV strain, as it has 98% amino acid identity to WNV-NY (10, 36). To inactivate WNV-KUNV, a previously determined optimal concentration of H₂O₂ (3.0%, vol/vol) (39) was added to concentrated virus for a period of 7 to 8 h. Kinetic inactivation experiments demonstrated that under these conditions, WNV-KUNV showed a rapid loss of infectivity, with a half-life of approximately 2.6 min (Fig. 1A). Based on these inactivation kinetics, we estimated that 7 h of inactivation would achieve a greater than 10-log-unit reduction of infectious virus. However, since the amount of viable virus dropped below our limit of detection within 90 min of the inactivation procedure (Fig. 1A), additional coculture assays in Vero cells, using up to 5% of the final purified vaccine, confirmed complete inactivation (data not shown). Following inactivation, the vaccine material was purified further by ultracentrifugation and ion-exchange chromatography. Analysis by SDS-PAGE of the final purified, inactivated vaccine demonstrated that it contained three bands at approximately 55, 20, and 15 kDa, which correspond to the predicted molecular masses of the envelope (E), pre-membrane, and capsid (C) proteins, respectively (Fig. 1B). The identity of the dominant 55-kDa band was confirmed by Western blotting with an anti-WNV E-protein-specific MAb (Fig. 1C). Representative vaccine lots were screened for recognition by conformationally sensitive MAbs against WNV and indicated that the H₂O₂-inactivated virus remains antigenically intact (data not shown).

Induction of antibody responses. To assess the potential of the H₂O₂-WNV-KUNV vaccine to generate a neutralizing antibody response, 8-week-old BALB/c mice ($n = 5$ for each dose) were immunized with a single dose of 2.5, 10, or 40 μ g of vaccine complexed with 0.1% alum. Neutralization assays were performed with serum harvested from animals at days 28 and 90 after vaccination (Fig. 2A). By day 28, H₂O₂-WNV-KUNV induced neutralizing antibody responses against WNV-KUNV in a dose-dependent manner (mean \pm standard error of the mean [SEM] 50% neutralization titers [NT₅₀], 23,068 \pm 6,865 for a dose of 40 μ g, 2,586 \pm 1,068 for 10 μ g, and 251 \pm 27 for 2.5 μ g). At 90 days postimmunization, neutralization titers had increased at all three doses tested (mean \pm SEM NT₅₀ values, 103,602 \pm 23,825 for a dose of 40 μ g, 6,958 \pm 2,042 for 10 μ g, and 4,506 \pm 1,608 for 2.5 μ g). Thus, immunization with H₂O₂-WNV-KUNV, analogous to H₂O₂-WNV-NY (39), induced high titers of neutralizing antibodies against WNV in mice that were detected at least 3 months after single-dose vaccination.

The majority of neutralizing antibodies against WNV are directed against the E protein (12, 46–48) and inhibit infection by blocking virus attachment, entry, and fusion (reviewed in references 49 and 50). To further define the humoral response after vaccination with H₂O₂-WNV-KUNV (10 μ g) formulated with alum, we assessed the kinetics of induction of anti-WNV-E-specific antibodies in 8-week-old BALB/c and C57BL/6 mice with or without boosting. In this series, serum was collected on days 0, 14, 28, 42, 60, and 90 days postimmunization, and on day 28, half of the mice in each group were boosted with alum-adjuvanted H₂O₂-WNV-KUNV. Antibody induction was monitored using an ELISA that detected antibodies against WNV-NY E protein (Fig. 2B). Both C57BL/6 and BALB/c mice generated robust anti-WNV E-specific antibody responses by day 14 following a single administration of the vaccine (for C57BL/6 mice, 833 \pm 146 reciprocal

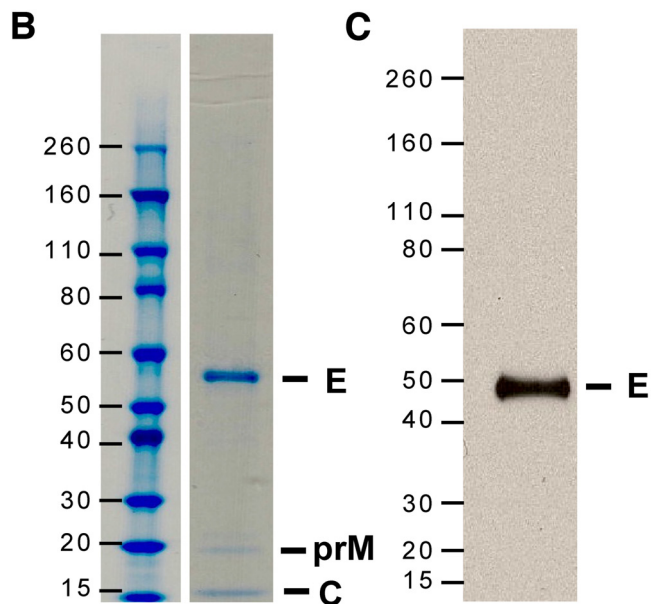
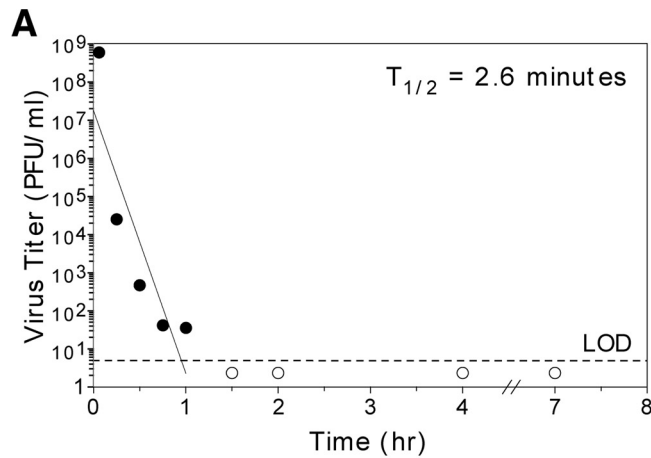


FIG 1 Inactivation and characterization of an H₂O₂-WNV-KUNV vaccine. (A) A representative purified preparation of WNV-KUNV (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 2% sorbitol) was inactivated with 3.0% H₂O₂ at room temperature (25 ± 2°C) for 7 h. Aliquots of the suspension were removed at the indicated time points and treated with catalase to neutralize residual H₂O₂ prior to measuring infectious virus titers by plaque assay. The calculated half-life ($T_{1/2}$) for inactivation is shown. The limit of detection (LOD) is indicated by the dashed line. Empty symbols below the limit of detection indicate that no viable virus was detected by plaque assay at those time points. The data shown are representative of at least three independent experiments. (B) Following purification, 0.1 µg of representative vaccine antigen was loaded onto a reducing SDS-polyacrylamide gel and protein bands were visualized by Coomassie stain. Bands corresponding to the molecular sizes of E, prM, and C are denoted. (C) The same vaccine antigen described above was analyzed by SDS-PAGE under nonreducing conditions and probed by Western blotting with the anti-WNV E-protein-specific MAb 7G11. Note that the differences in molecular sizes of E in panels B and C reflect the presence or absence of a reducing agent, respectively, during SDS-PAGE. The numbers to the left of the gels in panels B and C are molecular masses (in kilodaltons).

dilution of serum; for BALB/c mice, 198 ± 43 reciprocal dilution of serum), and substantially increased titers were observed on days 42, 60, and 90 in the mice that were boosted (Table 1). Higher levels (2- to 7-fold, $P < 0.05$) of WNV-E-specific antibodies were observed on days 14, 42, and 90 in the C57BL/6 mice than BALB/c

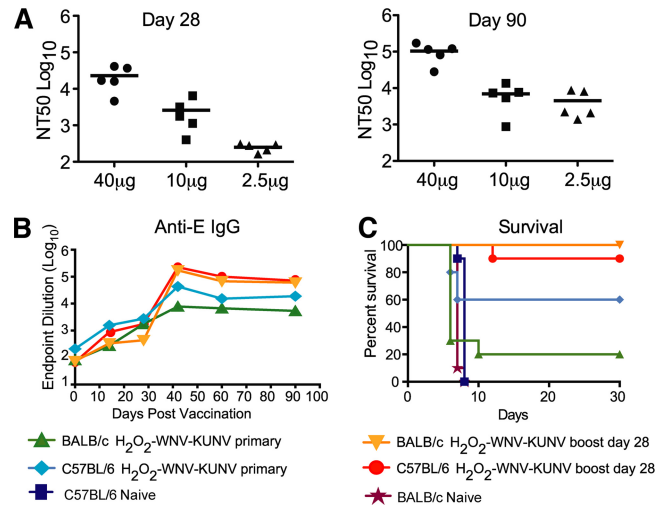


FIG 2 Humoral response after vaccination with H₂O₂-WNV-KUNV in C57BL/6 and BALB/c mice. (A) Neutralization titers (NT_{50}) against WNV-NY from serum on days 28 and 90 after immunization with 40 µg, 10 µg, or 2.5 µg of H₂O₂-WNV-KUNV vaccine formulated with 0.1% alum in BALB/c mice. Data are pooled from 2 independent experiments. (B) WNV E-protein-specific ELISA comparing H₂O₂-WNV-KUNV vaccination in BALB/c and C57BL/6 mice immunized with 10 µg on days 0, 14, 28, 42, 60, and 90. On day 28, half of the mice in each group were boosted with the H₂O₂-WNV-KUNV vaccine ($n = 20$ BALB/c mice and 20 C57BL/6 mice). (C) Ninety days after vaccination (with or without boosting), the BALB/c and C57BL/6 mouse groups described in panel B were challenged with 10⁶ PFU of WNV-NY via an i.c. route and monitored for survival. In addition, naïve BALB/c or C57BL/6 mice were challenged i.c. as controls.

mice that received a single dose of vaccine. However, no differences in the WNV E-specific antibodies were observed in the two strains of mice after boosting ($P > 0.4$).

Vaccination with H₂O₂-WNV-NY or H₂O₂-WNV-KUNV provides 100% protection against lethal WNV infection following a peripheral route of infection (39) (data not shown). To determine the protective activity of the H₂O₂-WNV-KUNV vaccine in a highly stringent i.c. challenge model, immunized BALB/c or C57BL/6 mice were infected i.c. with 10⁶ PFU of WNV-NY (1,000,000 times the LD₅₀) on day 90 after initial vaccination (Fig. 2C). As expected, all unimmunized BALB/c and C57BL/6 mice succumbed to i.c. infection. While only one (10%) BALB/c mouse that received a single dose of the H₂O₂-WNV-KUNV vaccine survived the challenge, 60% of C57BL/6 mice were protected ($P = 0.05$). In comparison, BALB/c (10 of 10) and C57BL/6 (9 of 10) mice that had been boosted on day 28 were protected against the i.c. challenge on day 90. Thus, the H₂O₂-WNV-KUNV vaccine induces sufficient immunity to protect mice from a highly lethal direct infection of WNV in the brain, although boosting was required for the greatest protection.

Induction of CD8⁺ T cell responses in C57BL/6 and HHDII mice. We next determined whether CD8⁺ T cells contributed to the protection conferred by the H₂O₂-WNV-KUNV vaccine. We speculated that this was possible with a nonreplicating vaccine, as a substantial fraction of the anti-WNV CD8⁺ T cell response associated with live virus infection requires CD8-α dendritic cells and antigen cross-presentation (41, 51). In C57BL/6 mice, the immunodominant CD8⁺ T cell epitope is in the NS4B protein (20, 52). As the H₂O₂-WNV-KUNV vaccine

TABLE 1 Anti-WNV E-protein antibody responses after vaccination^a

Mice	Antibody titer				
	Day 14	Day 28	Day 42	Day 60	Day 90
BALB/c H ₂ O ₂	159 ± 51	1,413 ± 519	3,726 ± 1,117	4,104 ± 1,516	3,444 ± 1,461
BALB/c H ₂ O ₂ + boost	237 ± 68	404 ± 151	140,356 ± 37,818	53,460 ± 12,036	40,500 ± 10,246
C57BL/6 H ₂ O ₂	972 ± 193	1,844 ± 588	24,318 ± 7,274	11,520 ± 4,061	12,816 ± 4,180
C57BL/6 H ₂ O ₂ + boost	660 ± 220	1,492 ± 523	201,082 ± 43,556	73,507 ± 16,078	55,282 ± 14,017

^a The indicated strains of mice were immunized with one dose or two doses (indicated by + boost, at 28 days) of H₂O₂-WNV-KUNV adjuvanted with alum. Serum was collected at the indicated days after vaccination and analyzed for the titer of anti-E protein antibodies by ELISA. The results reflect *n* = 10 mice per group.

is highly purified, the nonstructural NS4B protein is absent from the vaccine preparation. Nonetheless, there are two subdominant epitopes (E347 and E771) that are encoded by the WNV E protein and conserved in WNV-KUNV that could potentially elicit a CD8⁺ T cell response (20, 52). To assess this, we measured the generation of the WNV-specific CD8⁺ T cell response against the NS4B, E347, and E771 peptides in C57BL/6 mice following immunization with H₂O₂-WNV-KUNV or infection with live WNV-NY or WNV-KUNV (Fig. 3A and B). While we detected a CD8⁺ T cell response to the dominant and subdominant epitopes following infection with the live viruses, we did not identify a CD8⁺ T cell response to any of the epitopes following vaccination with purified H₂O₂-inactivated WNV.

We speculated that a CD8⁺ T cell response still might have been generated by H₂O₂-WNV-KUNV but was below the threshold level of detection by our flow cytometry assay. To test this hypothesis, we evaluated the effect of CD8⁺ T cell depletion on secondary challenge following vaccination (Fig. 3C). Groups of 16 mice were immunized with 10 μg of H₂O₂-WNV-KUNV, infected with 10² PFU of live WNV-NY or 10⁶ PFU of live WNV-KUNV, or left unvaccinated. Twenty-eight days later, all mice received 40 μg of a CD8β-depleting or isotype control MAb. Two days later, the mice were administered a second dose of depleting or isotype control MAb and concurrently challenged with 10⁶ PFU of WNV-NY via a stringent i.c. route. As expected, all naïve control mice succumbed to i.c. infection regardless of the presence or absence of CD8⁺ T cells. In comparison, mice receiving live WNV (WNV-NY or WNV-KUNV) survived the challenge independently of CD8⁺ T cell depletion. However, we observed a significant survival decrease (58 versus 14% survival, *P* = 0.006) in vaccinated mice receiving the CD8β-depleting MAb compared to that in mice receiving the isotype control MAb. Thus, although WNV-specific CD8⁺ T cells in vaccinated animals were below our limits of detection by flow cytometry, a response was generated and protected mice against a stringent lethal virus challenge.

To confirm the potential of H₂O₂-WNV-KUNV for generating a CD8⁺ T cell response and begin to address its applicability for humans, we repeated immunization studies in HHDII mice (37, 53). These mice are on an isogenic C57BL/6 background and express the α1 and α2 domains of HLA-A*0201 linked to the α3 domain of mouse D^b with a linker-attached human β₂m. These mice are genetically deficient in mouse β₂m and H2-D^b and have very low surface expression of H2-K^b. Due to the absence of mouse β₂m and mouse neonatal FcR (FcRn), HHDII mice have much lower levels of circulating antibody and are highly susceptible to WNV infection, making them less useful as a pathogenesis

or vaccine model in which humoral responses are important. However, they are a suitable model for assessing CD8⁺ T cell responses against epitopes that are restricted by HLA-A*0201 and have previously been used to demonstrate CD8⁺ T cell responses against WNV (53). In WNV infection of humans, the immunodominant HLA-A2-restricted epitope falls within the E protein (SVG9 [SVGGVFTSV]) (42, 53, 54) and is conserved between members of the *Flaviviridae* family, including WNV-NY and WNV-KUNV (data not shown).

To test for the development of HLA-A2-restricted WNV-specific CD8⁺ T cell responses, HHDII mice either were immunized with 40 μg of H₂O₂-WNV-KUNV or infected with 10⁶ PFU of WNV-KUNV. At day 10 postinfection or postvaccination, the spleens of the HHDII mice were harvested and the CD8⁺ T cells were characterized functionally and phenotypically (Fig. 4A to D). Using an SVG9-specific major histocompatibility complex (MHC) class I tetramer, we identified a WNV-specific CD8⁺ T cell population in both vaccinated and infected animals, although the percentage and total number of SVG9-specific CD8⁺ T cells was lower in vaccinated mice (5% compared to 11%, *P* = 0.04; 3.6 × 10⁴ compared to 8.7 × 10⁴ cells, *P* = 0.002; Fig. 4A). SVG9-specific CD8⁺ T cells from vaccinated mice were also distinct phenotypically from those generated following infection, as they expressed significantly higher levels of interleukin-7Rα (CD127, *P* = 0.008); PD-1 (*P* = 0.03), a negative regulator of immune responses; and CD62L (*P* = 0.04), a cell adhesion molecule that regulates T cell homing (Fig. 4B).

We next characterized functionally the antigen-specific CD8⁺ T cells after vaccination or infection. At day 8 postinfection or postvaccination, splenic CD8⁺ T cells were restimulated with SVG9 peptide and analyzed for intracellular levels of the cytokines IFN-γ and TNF-α. While CD8⁺ T cells from both H₂O₂-WNV-KUNV-vaccinated and WNV-KUNV-infected mice produced IFN-γ and TNF-α, overall, there were a higher percentage and a higher number of SVG9-specific CD8⁺ T cells present after live virus infection (Fig. 4C, left four panels). Nonetheless, we observed no difference in the relative proportion of antigen-specific CD8⁺ T cells that produced IFN-γ, TNF-α, or IFN-γ and TNF-α (double-positive cells) when comparing samples from vaccinated and infected mice, which suggests that the polyfunctionality of the CD8⁺ T cell response was equivalent (Fig. 4C, far right panel). To assess their relative avidity, antigen-specific CD8⁺ T cells from H₂O₂-WNV-KUNV-vaccinated or WNV-KUNV-infected mice were stimulated *ex vivo* with a dose titration of the SVG9 peptide, and the production of IFN-γ and TNF-α was analyzed (Fig. 4D). No difference in the functional avidity of the SVG9-specific CD8⁺ T cells was observed after vaccination and infection.

Protection of aged C57BL/6 mice against intracranial WNV challenge. An ideal WNV vaccine would elicit a durable and pro-

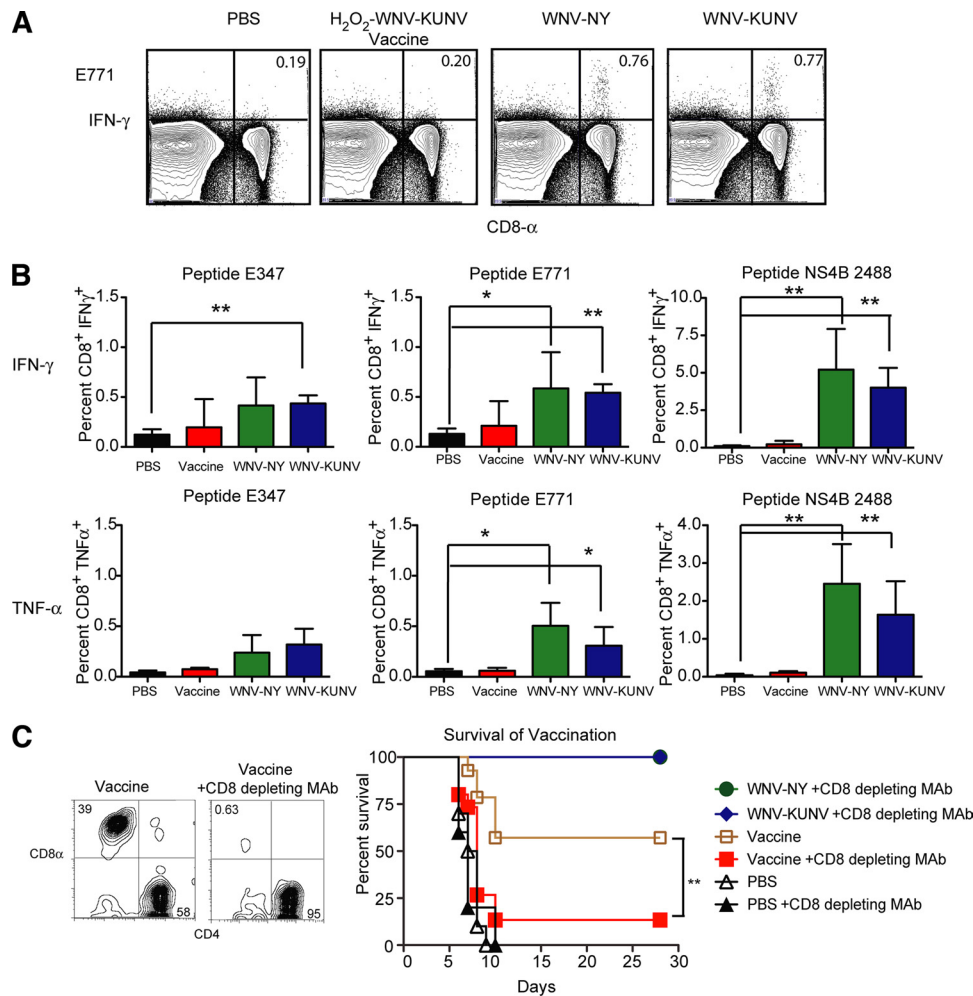


FIG 3 CD8⁺ T cell response in H₂O₂-WNV-KUNV-vaccinated C57BL/6 mice. (A) Flow cytometry contour plots showing IFN-γ⁺ CD8⁺ T cells after restimulation of cells with the E771 K^b-restricted peptide. From left to right are examples from PBS-treated, H₂O₂-WNV-KUNV-vaccinated, WNV-NY-infected, and WNV-KUNV-infected mice at day 8. (B) Summary of intracellular TNF-α and IFN-γ intracellular staining to identify WNV-specific CD8⁺ T cells following vaccination with 40 μg of H₂O₂-WNV-KUNV vaccine adjuvanted with 5 μg of MPL or infection with WNV-NY or WNV-KUNV. Eight days after vaccination or infection, splenocytes were harvested and stimulated with WNV-peptides E347, E771, and NS4B in the presence of brefeldin A for 6 h (*n* = 10 mice in each group in two independent experiments). (C) C57BL/6 mice were infected or vaccinated as described for panel B, and on days 28 and 32 postvaccination or postinfection, the mice received either 40 μg of anti-CD8β or an isotype control MAb. (Left) Flow cytometry contour plots show the efficiency of the depletion of CD8⁺ T cells; (right) on day 32, all mice were challenged with 10⁶ PFU of WNV-NY via the i.c. route and monitored for survival (*n* = 15 mice in each group from two independent experiment). Asterisks indicate comparisons that are statistically significantly different (**, *P* < 0.01; *, *P* < 0.05).

tective immune response in the elderly and immunocompromised. However, studies with vaccines against other viruses (e.g., influenza virus) have revealed that with aging, the ability to induce protective adaptive immunity wanes (reviewed in reference 55). To begin to determine the utility of H₂O₂-WNV-KUNV for this target population, we vaccinated three groups of aged (age, >18 months) mice with 10 μg of H₂O₂-WNV-KUNV vaccine complexed with alum, 100 μl (i.e., 1/10 the equine dose) of WNV-Innovator, or alum alone. Mice were bled on days 0, 14, 28, 42, 60, and 90 postvaccination, and the serum IgG response against WNV-NY E protein was measured by ELISA (Fig. 5A). On day 28, the groups were boosted with 10 μg of H₂O₂-WNV-KUNV, 100 μl of WNV-Innovator, or alum, respectively. Notably, we observed no difference (*P* > 0.8) in the anti-E-protein antibody response between the H₂O₂-WNV-KUNV and WNV-Innovator vaccines at any of the time points, whereas the responses to both

vaccines were significantly higher than the response to alum alone. Thus, H₂O₂-WNV-KUNV and WNV-Innovator both induced a robust E-protein-specific humoral response in aged C57BL/6 mice.

To assess the functional quality of the antibody response in immunized aged mice, we assessed neutralizing activity (Fig. 5B). By day 90, both vaccines had induced strongly neutralizing responses, although WNV-Innovator had a higher NT₅₀ value than H₂O₂-WNV-KUNV (7,850 versus 818, *P* < 0.002). To determine if the immune responses generated in the aged mice were protective against i.c. challenge, these animals were infected on day 90 (age, >21 months) with 10⁴ PFU (10,000 times the LD₉₀) of WNV-NY (Fig. 5C). All aged mice that had received alum alone succumbed to the WNV-NY challenge, with a mean survival time of 8 days. Aged mice that received WNV-Innovator were protected (*P* < 0.0001). In comparison, mice that received H₂O₂-

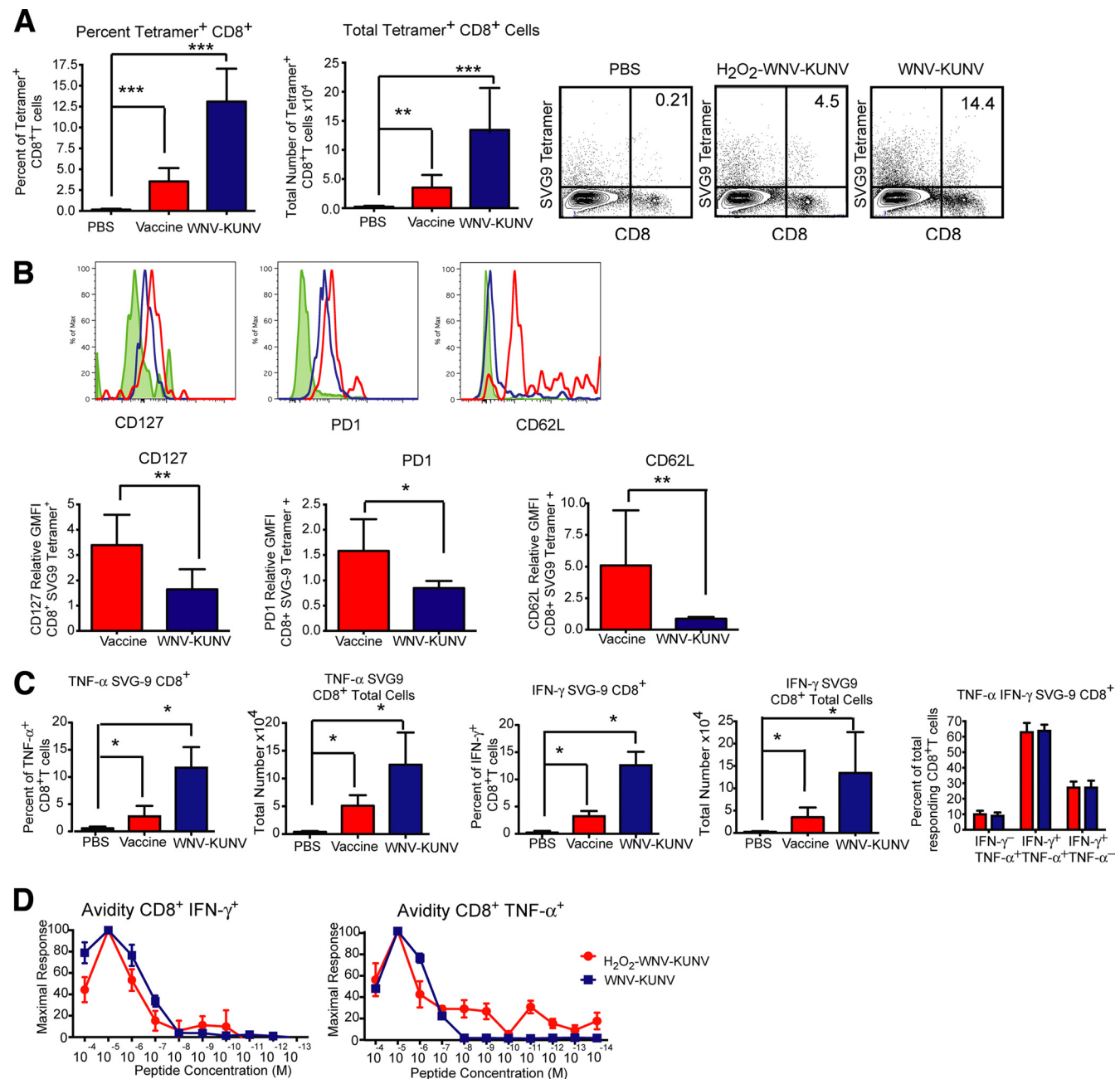


FIG 4 CD8⁺ T cell response in HHDII mice after vaccination with H₂O₂-WNV-KUNV. (A) Eight days following immunization with 40 μg of H₂O₂-WNV-KUNV vaccine adjuvanted with 5 μg of MPL or infection with 10⁴ PFU of WNV-KUNV, splenocytes were stained with an SVG9-specific MHC class I tetramer (n = 9 per group from three independent experiments). (Left) Percentage of tetramer-positive CD8⁺ T cells; (middle) number of tetramer-positive CD8⁺ T cells; (right) representative examples of SVG9 tetramer staining of lymphocytes from H₂O₂-WNV-KUNV-vaccinated or WNV-KUNV-infected HHDII mice. Some background staining (0.18 to 0.2%) of the SVG9 tetramer was observed in cells of the CD8-negative gate from PBS-treated, vaccinated, or WNV-infected mice. This level corresponded to that (0.21%) seen in the CD8⁺ T cell gate from PBS-treated animals. (B) The tetramer-positive SVG9-specific CD8⁺ T cells from panel A were stained with antibodies against CD127, PD1, and CD62L. The filled green histograms represent staining of naïve CD8⁺ T cells and are shown as a negative control. The relative geometric mean fluorescence intensity (GMFI) reflects data pooled from several independent experiments after normalization. (C) Summary of flow cytometry data showing TNF-α⁺ CD8⁺ T cells, IFN-γ⁺ CD8⁺ T cells, and IFN-γ⁺ TNF-α⁺ CD8⁺ T cells after restimulation of cells with the SVG9 peptide. Representative examples shown are from H₂O₂-WNV-KUNV-vaccinated or WNV-KUNV-infected mice at day 8. Far right panel, summary of intracellular TNF-α and IFN-γ intracellular staining to identify WNV-specific CD8⁺ T cells following vaccination with 40 μg of H₂O₂-WNV-KUNV vaccine adjuvanted with 5 μg of MPL or infection with WNV-KUNV. Eight days after infection or vaccination, splenocytes were harvested and stimulated with SVG9 in the presence of brefeldin A for 6 h (n = 10 mice for each group from two independent experiments). (D) Splenocytes from vaccinated H₂O₂-WNV-KUNV- or WNV-KUNV-infected HHDII mice were stimulated *ex vivo* with different doses of SVG9 peptide, and the production of IFN-γ and TNF-α was analyzed (n = 8 mice). Asterisks in this figure indicate comparisons that are statistically significantly different (***, P < 0.001; **, P < 0.01; *, P < 0.05).

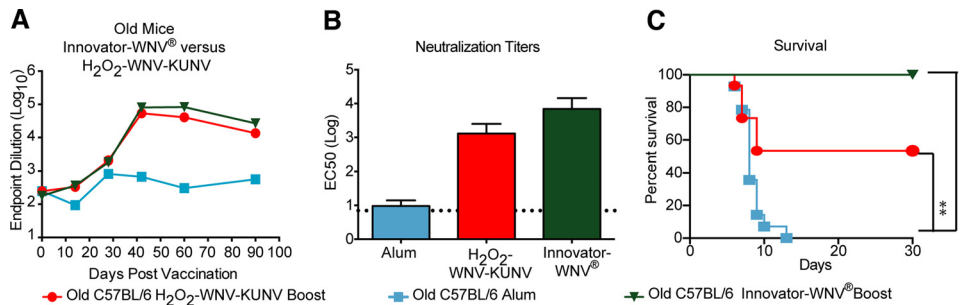


FIG 5 Vaccination and challenge of aged mice. (A) Aged C57BL/6 mice (age, 18 months) were vaccinated with 10 μ g of H₂O₂-WNV-KUNV vaccine adjuvanted with 0.1% alum ($n = 15$), vaccinated with 100 μ l of WNV-Innovator ($n = 15$), or administered 0.1% alum alone ($n = 15$), and at 28 days after vaccination, the mice were boosted. All groups were phlebotomized on days 0, 14, 28, 42, 60, and 90 postvaccination, and the titer of serum IgG against WNV E protein was measured by ELISA. (B) On day 90 postimmunization, neutralization titers in serum from the mice described in panel A were measured. (C) Vaccinated mice were challenged i.c. with 10⁴ PFU of WNV-NY on day 90 and monitored for survival. Asterisks indicate comparisons that are statistically significantly different (***, $P < 0.001$; **, $P < 0.01$).

WNV-KUNV also showed significant protection (53% survival, $P = 0.005$ compared to alum alone) from i.c. challenge. Thus, both inactivated vaccines induced immunity in aged animals, and the WNV-Innovator vaccine provided greater protection in the i.c. challenge model.

DISCUSSION

The development of a WNV-specific humoral immune response is an important criterion for the development of an effective vaccine. Passive transfer of serum containing WNV-specific antibodies protects against virus dissemination into the central nervous system and prevents WNV encephalitis and death (56, 57). In humans and mice, a large component of the WNV-specific protective antibody response is directed against the viral E protein (18, 58, 59). Here, we demonstrate the protective capacity of a novel H₂O₂-WNV-KUNV vaccine against lethal WNV challenge. Following vaccination with H₂O₂-WNV-KUNV in both young and old mice, we observed a significant WNV E-protein-specific and neutralizing antibody response that was enhanced by boosting and remained elevated for the duration of the study. Moreover, using human HLA-A2 transgenic and wild-type C57BL/6 and BALB/c adult mice, we showed that the H₂O₂-WNV-KUNV vaccine generates a polyfunctional antigen-specific CD8⁺ T cell response. Taken together, H₂O₂-WNV-KUNV induces adaptive immunity to WNV that protects adult and aged mice against lethal infection.

We compared the antibody response of H₂O₂-WNV-KUNV to that of a commercially available veterinary vaccine (WNV-Innovator), which is used in horses and exotic birds but not in humans. The inactivated WNV-Innovator formulation is produced after formalin inactivation of WNV-NY and complexing with MetaStim, a proprietary adjuvant (23, 29). The vaccine, however, is not purified in a virion-only form, as in C57BL/6 mice it induced a CD8⁺ T cell response against the D^b-restricted NS4B peptide, which is absent from the virion (23). Although WNV-Innovator is used effectively in horses (60), there are regulatory barriers to using unpurified vaccines in humans. Moreover, as the viral backbone is derived from the virulent WNV-NY 1999 strain, this raises the safety threshold for complete virus inactivation before immunization of humans, especially those at risk for severe disease (61). While a single administration of purified H₂O₂-WNV-KUNV induced a strong WNV-specific antibody response

in C57BL/6 mice, the antibody titer was lower than that observed with WNV-Innovator. This finding differs from that of a previous study using sucrose gradient-purified H₂O₂-inactivated WNV-NY in BALB/c mice (39). These differences could be due to mouse strain variation (BALB/c versus C57BL/6), the virus strain used for vaccine production (WNV-NY versus WNV-KUNV), or the relative purity of the vaccine preparation. Following boosting, however, comparable WNV-specific E antibody titers were observed with H₂O₂-WNV-KUNV and WNV-Innovator. Regardless, the H₂O₂-WNV-KUNV vaccine provided significant protection in young and aged mice from a stringent i.c. challenge with WNV-NY.

CD8⁺ T cells contribute to protection against and clearance of primary WNV infection (20, 22, 23, 52, 62) and secondary challenge (23). We initially set out to assess whether H₂O₂-WNV-KUNV could stimulate both a protective antibody and CD8⁺ T cell response. Due to the purity of the vaccine preparation, which contains virions composed of prM/M, E, and C proteins and the viral RNA, the H-2^b immunodominant epitope in the NS4B protein was absent, making it difficult to detect an antigen-specific CD8⁺ T cell response in C57BL/6 mice. Nonetheless, depletion studies followed by i.c. challenge revealed that the H₂O₂-WNV-KUNV vaccine induced a CD8⁺ T cell response in C57BL/6 mice. While the vaccine response generated a robust neutralizing antibody response, it was not sufficient to prevent lethal infection after direct introduction of WNV into the brain; protection required the presence of CD8⁺ T cells. In comparison, and as reported previously (23), mice that received live WNV or WNV-KUNV and survived initial infection produced higher levels of antibody and survived rechallenge regardless of the presence of CD8⁺ T cells.

H₂O₂-WNV-KUNV also induced a robust polyfunctional CD8⁺ T cell response in the HLA-A2 human MHC class I transgenic mice, wherein the immunodominant CD8⁺ T cell epitope against WNV maps to the SVG9 peptide in the E protein (42) and, thus, is present in the purified vaccine. We observed no difference in the relative avidity of SVG9-specific CD8⁺ T cells from mice receiving live WNV-KUNV in comparison to those from mice vaccinated with H₂O₂-WNV-KUNV. Priming of a potent cellular immune response against a human HLA-restricted WNV epitope suggests that H₂O₂-WNV-KUNV might stimulate a protective CD8⁺ T cell response in humans. In support of this, profiling

studies from WNV-infected patients have shown that multiple CD8⁺ T cell epitopes map to the structural proteins of WNV and are presented by different MHC class I alleles (42, 54, 63, 64). Future immunization studies in other HLA transgenic mice and, ultimately, humans will be required to define the extent of antigen-specific CD8⁺ T cell responses generated after H₂O₂-WNV-KUNV administration.

The elderly and immunocompromised are at the greatest risk of developing severe neurological sequelae as a consequence of WNV infection (65). However, age-related immune defects in both the innate and adaptive immune response create hurdles to the development of an effective vaccine (see reference 66 and references therein). Similar to humans, aged mice are more susceptible to severe WNV disease and, thus, a reasonable surrogate for determining vaccine efficacy (19). Our observation that aged mice developed strong WNV-specific humoral responses and were protected after immunization and boosting with H₂O₂-WNV-KUNV is a first step in demonstrating vaccine efficacy in a highly susceptible population. Further studies are needed to assess whether the mechanism of protection in aged mice is similar in its relative composition (antibody versus T cell mediated) to that in younger adult mice and whether each component is durable.

Although a significant fraction of aged mice were protected 2 months after boosting with a 10-μg dose of H₂O₂-WNV-KUNV formulated with alum, complete protection was generated only by the formalin-inactivated WNV-Innovator vaccine formulated with a strong veterinary adjuvant, MetaStim. It is unclear how much WNV protein (soluble and intact virion protein) is in the WNV-Innovator vaccine. It is possible that the response to a higher, 40-μg dose of highly purified H₂O₂-WNV-KUNV could meet or exceed the neutralizing antibody responses elicited by immunization with a 1/10 horse dose of WNV-Innovator vaccine, and this could potentially improve protection against robust i.c. challenge. Alternatively, since the WNV-Innovator vaccine is not purified, it may contain other uncharacterized factors, such as the immunogenic NS4B protein (a nonstructural protein not found in purified H₂O₂-WNV-KUNV), that could enhance the efficacy and/or immunogenicity of the vaccine; indeed, robust NS4B-specific CD8⁺ T cell responses were previously observed in C57BL/6 mice immunized with the WNV-Innovator vaccine (23). At present, it is unclear if the protective advantage of the WNV-Innovator vaccine observed in aged C57BL/6 mice is due to CD8⁺ T cell responses against WNV-specific nonstructural proteins (e.g., NS4B) or, possibly, antibodies against other nonstructural proteins. For example, passive transfer of anti-NS1 MAbs protects against WNV infection in mice (67), and vaccination of rhesus macaques with NS1 protein from yellow fever virus provided strong protective immunity against lethal virus challenge (68). To improve the immunogenicity of H₂O₂-WNV-KUNV, in future studies, we plan to optimize the dose, prime-boost sequence, adjuvant, and route of administration to create an inactivated vaccine that confers the greatest level of protection in susceptible populations. Although in recent years it appeared as if WNV infection and disease might wane, the recent epidemic outbreaks in the United States and Europe in 2012 (1, 6, 14, 15, 69) suggest that this trend may be changing, highlighting a need for the development of a safe and effective WNV vaccine that can be used in at-risk populations.

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