A hydrogen peroxide-inactivated virus vaccine elicits humoral and cellular immunity and protects against lethal West Nile virus infection in aged mice

Amelia K. Pinto¹, Justin M. Richner¹, Elizabeth A. Poore⁴, Pradnya P. Patil¹, Ian J. Amanna⁴, Mark K. Slifka⁵, and Michael S. Diamond¹,²,³

Departments of Medicine¹, Molecular Microbiology², Pathology & Immunology³, Washington University School of Medicine, St Louis, MO 63110 USA. Najit Technologies⁴, Beaverton, OR 97006 USA. Oregon National Primate Research Center, Oregon Health & Sciences University⁵, Beaverton, OR 97006 USA.

Corresponding author: Michael S. Diamond, M.D., PhD, Departments of Medicine, Molecular Microbiology and Pathology & Immunology, Washington University School of Medicine, 660 South Euclid Avenue, Box 8051, St Louis, Missouri 63110. Tel: 314-362-2842, Fax: 314-362-9230, Email: diamond@borcim.wustl.edu

Running title: H₂O₂-inactivated vaccine against West Nile virus
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 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 pre-clinical efficacy of a hydrogen peroxide (H$_2$O$_2$)-inactivated WNV-Kunjin strain (WNV-KUNV) vaccine as a candidate for further development. Both young and aged mice vaccinated with H$_2$O$_2$-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 H$_2$O$_2$-inactivated WNV-KUNV vaccine is safe and immunogenic, and may be suitable for protection against WNV infection in vulnerable populations.
INTRODUCTION

In the United States between 1999 and 2012, an estimated 3 million people have been infected with West Nile virus (WNV), resulting in over 780,000 illnesses, greater than 36,500 confirmed cases, and 1,500 deaths ([52] and 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 (42). Advanced age and immunosuppression are risks for the development of severe WNV disease (3, 28, 42, 43). Although the worldwide incidence of WNV infection is increasing (57), there is no specific treatment or vaccine available for use in humans (63).

WNV is a member of the Flaviviridae family of positive-stranded RNA viruses, which includes the globally relevant human pathogens Dengue (DENV), yellow fever (YFV), and Japanese encephalitis (JEV) viruses. 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, 10, 32, 33). Lineage 1 strains are considered emerging and associated with outbreaks of neuroinvasive disease (7, 32); 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 non-virulent strain of WNV circulating in
Australia (5, 32, 33). Lineage 2 strains are usually less pathogenic (8), although variants, including some contemporary strains in Greece and Eastern Europe (49), periodically cause severe neurological disease (50). 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 (4), Russia (36) and India (10).

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 prM and the nonstructural proteins (reviewed in (68)). Multiple reports have shown that WNV-specific T cells also contribute to protection and clearance of WNV from infected hosts (12-14, 64-67). 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 (9, 41). 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 (6).

WNV-Innovator® is a veterinary vaccine administered to horses and exotic zoo animals (44). One potential limitation to this formalin-inactivated vaccine being used in humans is that it is generated from the highly virulent North American WNV New York (WNV-NY) strain. A vaccine based on a virulent WNV-NY strain necessitates the generation of high-titer virus stocks for inactivation in 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 using virulent WNV-NY is to develop a vaccine based on a naturally attenuated strain, such as WNV-Kunjin (WNV-KUNV) (15, 24, 25).

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 (19, 22), and only a very few individuals have developed WNV fever (23). WNV-KUNV is genetically similar to WNV-NY (~98% amino acid identity), with virtually complete conservation of all neutralizing antibody epitopes (33, 61). 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 (24). 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 pre-clinical evaluation of a newly developed H$_2$O$_2$-inactivated WNV-KUNV vaccine in adult and aged mice.
MATERIALS AND METHODS

Mice. Eight to ten week-old C57BL/6 and BALB/c mice were obtained commercially (Jackson Laboratory). Eighteen month-old C57BL/6 were purchased from the National Institutes of Aging breeder colony (Harlan). HLA-A2 transgenic (HHDII) mice have been described (51) 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 (α1/α2 domains), mouse D\(^\text{b}\) (α3 domain), and linker-attached human β\(_2\)-microglobulin (β\(_2\text{m}\)), and were bred in a specific pathogen free facility at Washington University. All mice 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 (3000.0259, 2000, passage 2) (WNV-NY) was described previously (20). WNV-KUNV isolate (CH 16532) 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, 10\(^6\) PFU was injected in 100 μl via an intraperitoneal route (i.p.). For live virus infections in mice with WNV-NY, 10\(^2\) PFU in 50 μl was injected subcutaneously (s.c.) via footpad injection. For intracranial (i.c.) challenges, aged mice were given 10\(^4\) PFU (10,000 x lethal dose (LD)90) and young mice were administered 10\(^6\) PFU (1,000,000 x LD90) in 10 μl.

Vaccine preparation. Serum-free adapted (VP-SFM, Life Technologies) WHO-Vero cells (10-87, ATCC) were grown to confluency 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 hours 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, and 2% sorbitol). Following sterile filtration (0.22 μm) to remove potential aggregates, WNV-KUNV was inactivated with H₂O₂ (Fisher Scientific) at a final concentration of 3.0% for 7 to 8 hours at room temperature, with an additional filtration step performed approximately midway through the inactivation period (4 hours). Post-inactivation, H₂O₂ was removed through further purification by dialysis followed by pelleting of the virus by ultracentrifugation (200,000 x g, 16 hours) or ion-exchange chromatography (cellulose sulfate, JNC Corporation). Mice were immunized i.p. with H₂O₂-WNV-KUNV containing approximately 1 x 10⁷ PFU equivalents/μg protein) that was either adjuvanted with 5 μg monophosphoryl lipid A (MPL, InvivoGen) or 0.1% of aluminum hydroxide gel (alum, Sigma Aldrich). West Nile-Innovator®, a formalin-inactivated crude virus preparation (New York 1999 strain VM2, serial #1666142A), is available commercially (Pfizer) and contains a proprietary oil adjuvant, MetaStim®, as well as excipients. For experiments in this study with West Nile-Innovator®, we used a dose of 100 μl, which is 1/10th 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 (MP Biomedical, final concentration of 12.5 U/mL, 10 minutes at room temperature) 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 (2). 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 4-12% Bis-Tris gels in MOPS 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 minutes, and loaded onto prepared gels. Gels were run in an Xcell SureLock System (Life Technologies) for approximately 45 minutes at a constant voltage of 200V and then stained with the Pierce Silver Stain Kit (Thermo Scientific). Gel electrophoresis for Western blot analysis was carried out similarly, but DTT was omitted to maintain non-reducing conditions. Following electrophoresis, gels were blotted onto PVDF membranes using the iBlot Dry Blotting System (Life Technologies). Post-transfer, membranes were blocked (5% non-fat dry milk in PBS, 0.05% Tween20 [PBS-T]) for 1 hour at room temperature, and probed with the WNV-specific MAb, 7G11, at 1 μg/mL in blocking buffer for 1 hour 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 hour 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 monoclonal antibody (MAb) (53-5.8; rat IgG2b) (BD Bioscience) specific for mouse
CD8β chain according to a previously published protocol (40). A rat IgG1 (Jackson ImmunoResearch) was used as an isotype control. Anti-CD8β or isotype controls MAb (40 μg) were administered to mice via an i.p. injection two days prior to i.c. challenge and an additional 10 μg of anti-CD8β 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-CD8α (Biolegend).

**Flow cytometry.** Intracellular staining of TNF-α and IFN-γ of lymphocytes isolated from the spleen was performed as described previously (54). Briefly, spleens were harvested and homogenized to form a single cell suspension and incubated with 2 μg/ml brefeldin A (Sigma) for six hours at 37°C with 10^-6 M of NS4B 2488–2496, E 347–354, E771-778 (13) (H-2<sup>b</sup> mouse CD8<sup>+</sup> T cells) or SVG9 E720-728 (38) (HLA-A2 transgenic CD8<sup>+</sup> T cells) peptides or 2 μg/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, CD8α, CD44, PD-1, KLRG1, and CTLA-4. D<sup>b</sup>-NS4B tetramer and HLA-A2 SVG9-tetramer were obtained from the NIH tetramer core facility. Cells were washed, fixed, and 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 (BD Bioscience) using FACSDiva 6.1.1 software (BD Bioscience) and analyzed with FlowJo (Treestar). The total numbers of IFN-γ or TNF-α expressing CD8<sup>+</sup> T cells was determined by multiplying the percentage of IFN-γ<sup>+</sup> or TNF-α<sup>+</sup> CD8<sup>+</sup> T cells by the total numbers of splenocytes.
ELISA. WNV-specific IgG levels were determined using an E protein–specific ELISA as described previously (39). Briefly, individual wells of a microtiter plate were coated overnight at 4°C with 1 μg/ml of recombinant WNV E protein. Plates were washed and blocked with 1% bovine serum albumin 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 minutes), serially diluted in Blocking buffer, and added to wells for one hour at room temperature. Following several washes, biotin-conjugated goat anti-mouse IgG (Sigma Aldrich) was added for one hour at room temperature. Plates were then washed and incubated with streptavidin-conjugated horseradish peroxidase (2 μg/ml in Blocking buffer; Vector Labs) for one hour at room temperature. After several washes, plates were developed with tetramethylbenzidine substrate (Dako) and the reaction was stopped with 1 N H2SO4. The OD450 was measured and endpoint dilutions were defined as 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 plaque reduction neutralization assay (11). Neutralization titers of serum from BALB/c mice were carried using a plaque reduction assay as described previously (26). For studies in aged C57BL/6 mice, 50 focus-forming units of WNV was pre-incubated with serial dilutions of heat-inactivated mouse serum at 37°C for one hour in DMEM with 2% FBS, penicillin and streptomycin. Virus-serum mixtures were added in triplicate to individual wells of 96-well tissue culture plates containing Vero cells monolayers at ~ 90% confluency. Virus was incubated with Vero cells for one hour at 37°C, after which wells were overlaid with 1%
carboxymethylcellulose (Sigma) in MEM supplemented with 4% FBS. After culture at 37°C for 24 hours, cells were fixed with 1% paraformaldehyde in PBS (ten minutes at room temperature) and permeabilized with 0.1% saponin and 0.1% BSA in PBS (saponin buffer; five minutes at room temperature). Cells were stained with the humanized E16 anti-WNV MAb (47) (50 μl at 200 ng/ml in saponin buffer) for two hours at room temperature. Following several washes, wells were incubated with horseradish peroxidase-conjugated anti-human IgG antibody (Sigma; 1:5000 in saponin buffer) for one hour at room temperature. Wells were washed and infectious foci were visualized with TrueBlue Substrate (KPL) after a five to ten minute incubation period 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 pre-incubated with serum compared to wells with virus pre-incubation with medium alone) were graphed using Prism software, and EC50 values were calculated.

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

Generation of a H$_2$O$_2$-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 a H$_2$O$_2$-inactivated WNV-NY virus could induce effective neutralizing antibody responses in BALB/c mice (2). Although an inactivated WNV-NY virus 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 (32, 61). To inactivate WNV-KUNV, a previously determined optimal concentration of H$_2$O$_2$ (3.0% v/v) (2) was added to concentrated virus for a period of 7 to 8 hours. Kinetic inactivation experiments demonstrated that under these conditions, WNV-KUNV showed rapid loss of infectivity with a half-life of approximately 2.6 minutes (Fig 1A). Based on these inactivation kinetics, we estimated that 7 hours of inactivation would achieve greater than a 10 log reduction of infectious virus. However, since viable virus dropped below our limit of detection within 90 minutes of the inactivation procedure (Fig 1A), additional co-culture 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 weights of the envelope (E), pre-membrane, and capsid proteins (Fig 1B). The identity of the
dominant 55 kDa band was confirmed by Western blot analysis 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, eight week-old BALB/c (n = 5 for each dose) mice were immunized with a single dose of 2.5, 10, or 40 μg 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-NY in a dose-dependent manner (mean ± standard error of the mean (SEM) for 50% neutralization titers (NT50) were 40 μg: 23,068 ± 6,865; 10 μg: 2,586 ± 1,068; and 2.5 μg: 251 ± 27). At 90 days post-immunization, neutralization titers had increased at all three doses tested (mean ± SEM for NT50 values were 40 μg: 103,602 ± 23,825; 10 μg: 6,958 ± 2,042; and 2.5 μg: 4,506 ± 1,608). Thus, immunization with H₂O₂-WNV-KUNV, analogous to H₂O₂-WNV-NY (2), induced high titers of neutralizing antibodies in mice against WNV-NY that were detected at least three months after single-dose vaccination.

The majority of neutralizing antibodies against WNV are directed against the E protein (7, 45, 47, 60) and inhibit infection by blocking virus attachment, entry, and fusion (reviewed in (53, 58)). To further define the humoral response after vaccination with H₂O₂-WNV-KUNV (10 μg) formulated with alum, we assessed the kinetics of induction of anti-WNV-E specific antibodies in eight 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 post-immunization, and on day 28 half of the mice in each group were
boosted with alum-adjuvanted H$_2$O$_2$-WNV-KUNV. Antibody induction was monitored
using an ELISA that detected antibodies against WNV-NY E protein (Fig 2B). C57BL/6
and BALB/c mice both generated robust anti-WNV E-specific antibody responses by day
14 following a single administration of the vaccine (C57BL/6: 833 ± 146, BALB/c: 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 (2 to 7-fold, $P < 0.05$)
levels of WNV-E specific antibodies were observed on days 14, 42, and 90 in the
C57BL/6 compared to BALB/c mice that received a single dose of vaccine. However, no
differences in the WNV-E specific antibodies were observed into the two strains of mice
after boosting ($P > 0.4$).

Vaccination with H$_2$O$_2$-WNV-NY or H$_2$O$_2$-WNV-KUNV provides 100%
protection against lethal WNV infection following a peripheral route of infection ((2) and
data not shown). To determine the protective activity of the H$_2$O$_2$-WNV-KUNV vaccine
in a highly stringent intracranial (i.c.) challenge model, immunized BALB/c or C57BL/6
mice were infected i.c. with 10$^6$ PFU of WNV-NY (1,000,000 x LD90) 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 mice that received a single
dose of the H$_2$O$_2$-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 H$_2$O$_2$-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
greatest protection.

**Induction CD8$^+$ T cell responses in C57BL/6 and HHDII mice.** We next
determined whether CD8$^+$ T cells contributed to the protection conferred by the H$_2$O$_2$-
WNV-KUNV vaccine. We speculated this was possible with a non-replicating 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 (27, 54). In
C57BL/6 mice, the immunodominant CD8$^+$ T cell epitope is in the NS4B protein (13, 55).
As the H$_2$O$_2$-WNV-KUNV vaccine is highly purified, the non-structural 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 potentially could elicit a CD8$^+$ T cell response (13, 55). To assess this, we measured
the generation of the WNV-specific CD8$^+$ T cell response against NS4B, E347, and E771
peptides in C57BL/6 mice following immunization with H$_2$O$_2$-WNV-KUNV or infection
with live WNV-NY or WNV-KUNV (**Fig 3A-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 H$_2$O$_2$-inactivated WNV.

We speculated that a CD8$^+$ T cell response still might have been generated by
H$_2$O$_2$-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 $\mu$g of H$_2$O$_2$-WNV-KUNV, infected with 10$^5$ PFU of live WNV-NY or 10$^6$ 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 10^6 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 independent of CD8^+ T cell depletion. However, we observed a significant survival decrease (58 versus 14 percent survival, \(P = 0.006\)) in vaccinated mice receiving the CD8β depleting compared to 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 (30, 51). 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 responses against epitopes that are restricted by HLA-A*0201, and have been used previously to demonstrate CD8^+ T cell responses against WNV (30). In WNV infection of humans, the immunodominant HLA-A2-
restricted epitope falls within the E protein (SVG9: SVGGVFTSV) (30, 35, 38) 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 \(10^6\) PFU of WNV-KUNV. At day ten post-infection or vaccination, the spleens of the HHDII mice were harvested and the CD8\(^+\) T cells were characterized functionally and phenotypically (Fig 4A-D). Using a SVG9-specific 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 \(x\) \(10^4\) compared to 8.7 \(x\) \(10^4\) cells, \(P = 0.002\), Fig 4A). SVG9-specific CD8\(^+\) T cells from vaccinated mice also were distinct phenotypically from those generated following infection, as they expressed significantly higher levels of the IL-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 post-infection or vaccination, splenic CD8\(^+\) T cells were restimulated with SVG9 peptide and analyzed for intracellular cytokine levels of IFN-\(\gamma\) and TNF-\(\alpha\). While CD8\(^+\) T cells from H2O2-WNV-KUNV-vaccinated or WNV-KUNV-infected mice both produced IFN-\(\gamma\) and TNF-\(\alpha\), overall, there was a higher percentage and number of SVG9-specific CD8\(^+\) T cells present after live virus infection (Fig 4C, left 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 CD8+ T cell response was equivalent (Fig 4C, 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 protective immune response in the elderly and immunocompromised. However, studies with vaccines against other viruses (e.g., influenza) have revealed that with aging, the ability to induce protective adaptive immunity wanes (reviewed in (37)). To begin to determine the utility of H2O2-WNV-KUNV for this target population, we vaccinated three groups of aged (>18 months) mice with 10 μg of H2O2-WNV-KUNV vaccine complexed with alum, or 100 μl (i.e., 1/10th the equine dose) of WNV-Innovator®, or alum alone. Mice were bled on days 0, 14, 28, 42, 60 and 90 post-vaccination, and the serum IgG 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 both were significantly higher compared 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 West Nile-Innovator® had a higher NT50 value (7,850 versus 818, \( P < 0.002 \)) compared to \( \text{H}_2\text{O}_2\)-WNV-KUNV. To determine if the immune responses generated in the aged mice were protective against i.c. challenge, these animals were infected on day 90 (> 21 months of age) with \( 10^4 \) PFU (10,000 x 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 \( \text{H}_2\text{O}_2\)-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.
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 CNS and prevents WNV encephalitis and death (17, 18). In humans and mice, a large component of the WNV-specific protective antibody response is directed against the viral E protein (48, 56, 68). Here, we demonstrate the protective capacity of a novel H$_2$O$_2$-WNV-KUNV vaccine against lethal WNV challenge. Following vaccination with H$_2$O$_2$-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 H$_2$O$_2$-WNV-KUNV generates a polyfunctional antigen-specific CD$^8^+$ T cell response. Taken together, H$_2$O$_2$-WNV-KUNV induces adaptive immunity to WNV that protects adult and aged mice against lethal infection.

We compared the antibody response of H$_2$O$_2$-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 (44, 65). The vaccine, however, is not purified in a virion-only form, as in C57BL/6 mice it induced a CD$^8^+$ T cell response against the D$^p$-restricted NS4B peptide, which is absent from the virion (65). Although WNV-Innovator® is used effectively in horses (21), 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 (1). 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 differs from a previous study using sucrose gradient-purified H₂O₂-inactivated WNV-NY in BALB/c mice (2). These differences could be due to mouse strain variation (BALB/c vs. C57BL/6), the virus strain used for vaccine production (WNV-NY vs. 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 (13, 55, 64, 65, 69) and secondary challenge (65). We initially set out to assess whether the 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-2b 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 (65), mice that received live WNV or WNV-KUNV and survived initial infection produced higher levels of antibody and survived re-challenge regardless of the presence of CD8$^+$ T cells.

H$_2$O$_2$-WNV-KUNV also induced a robust polyfunctional CD8$^+$ T cell response in the HLA-A2 human class I MHC transgenic mice, wherein the immunodominant CD8$^+$ T cell epitope against WNV maps to the SVG9 peptide in the E protein (38), 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 vaccinated with WNV-KUNV-H$_2$O$_2$. Priming of a potent cellular immune response against a human HLA-restricted WNV epitope suggests that H$_2$O$_2$-WNV-KUNV might stimulate a protective CD8$^+$ T cell response in humans. In support of this, profiling studies from WNV-infected patients has shown that multiple CD8$^+$ T cell epitopes map to the structural proteins of WNV and are presented by different class I MHC alleles (29, 34, 35, 38). 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 H$_2$O$_2$-WNV-KUNV administration.

The elderly and immunocompromised are at the greatest risk of developing severe neurological sequelae as a consequence of WNV infection (46). However, age-related immune defects in both the innate and adaptive immune response create hurdles to the development of an effective vaccine ((31) and references therein). Similar to humans, aged mice are more susceptible to severe WNV disease and thus, a reasonable surrogate for determining vaccine efficacy (12). 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) compared to younger adult mice, and whether each component is durable.

Although a significant fraction of aged mice were protected two 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 a higher 40 μg dose of highly purified H₂O₂-WNV-KUNV could meet or exceed the neutralizing antibody responses elicited by immunization with a 1/10th 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 observed previously in C57BL/6 mice immunized with WNV-Innovator® vaccine (65). 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 cells responses against WNV-specific nonstructural proteins (e.g., NS4B) or possibly, antibodies against other non-structural proteins. For example, passive transfer of anti-NS1 MAbs protects against WNV infection in mice (16) and vaccination of rhesus
24 macaques with NS1 protein from yellow fever virus provided strong protective immunity against lethal virus challenge (62). 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 the WNV infection and disease might wane, the recent epidemic outbreaks in the United States and Europe in 2012 (49, 50, 52, 57, 59) 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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Conflict of interest statement. While the authors M.S.D, A.K.P, and J.R. do not have a conflict of interest to report, M.K.S., I.J.A., P.P.P., and E.A.P. have a financial interest in Najit Technologies, Inc., a company that may have a commercial interest in the results of this research and technology. This potential individual and institutional conflict of interest has been reviewed and managed by OHSU.
REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Inactivation and characterization of a H$_2$O$_2$-WNV-KUNV vaccine.

A. A representative purified preparation of WNV-KUNV (20 mM Tris-HCl [pH = 8.0], 50 mM NaCl, and 2% sorbitol) was inactivated with 3.0% H$_2$O$_2$ at room temperature (25 ± 2°C) for 7 hours. Aliquots of the suspension were removed at the indicated time points and treated with catalase to neutralize residual H$_2$O$_2$ prior to measuring infectious virus titers by plaque assay. The calculated half-life for inactivation is shown. The limit of detection (LOD) is indicated by the dashed line. Empty symbols below the LOD indicate that no viable virus was detected by plaque assay at those time points. The data shown is representative of at least three independent experiments.

B. Following purification, 0.1 μg of representative vaccine antigen was loaded onto a reducing SDS-PAGE gel and protein bands were visualized by Coomassie stain. Bands corresponding to the molecular size of E, prM, and C are denoted.

C. The same vaccine antigen described above was analyzed by SDS-PAGE under non-reducing conditions and probed by western blot with the anti-WNV E protein specific MAb, 7G11. Note, the differences in molecular size of E in panels B and C reflect the presence or absence of a reducing agent, respectively, during SDS-PAGE.

**Figure 2.** The humoral response after vaccination with H$_2$O$_2$-WNV-KUNV in C57BL/6 and BALB/c mice. A. Neutralization titers (NT50) against WNV-NY from serum on days 28 and 90 after immunization with 40 μg, 10 μg, or 2.5 μg of H$_2$O$_2$-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 H$_2$O$_2$-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 H$_2$O$_2$-WNV-KUNV vaccine ($n = 20$ BALB/c and 20 C57BL/6 mice). C. Ninety days after vaccination (with or without boosting) the BALB/c and C57BL/6 mouse groups described in (B) were challenged with 10$^6$ 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.

**Figure 3. CD8$^+$ T cell response in H$_2$O$_2$-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, H$_2$O$_2$-WNV-KUNV-vaccinated, WNV-NY or 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 H$_2$O$_2$-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 six hours ($n = 10$ each group in two independent experiments). C. C57BL/6 mice were infected or vaccinated as described in panel B, and on days 28 and 32 post vaccination or infection 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 10$^6$ PFU of WNV-NY via i.c. route and monitored for survival ($n = 15$ each group from two independent experiment). In this Figure, asterisks indicate comparisons that are statistically different (**, $P < 0.01$; *, $P < 0.05$).
Figure 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 (A) were stained with antibodies against CD127, PD1, and CD62. 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. Flow cytometry contour plots showing (left) TNF-α⁺ CD8⁺ T cells, (middle) IFN-γ⁺ CD8⁺ T cells, and (right) 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. D. 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 six hours (n = 10 for each group.
from two independent experiments). E. Splenocytes from vaccinated H$_2$O$_2$-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). In this Figure, asterisks indicate comparisons that are statistically different (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

**Figure 5. Vaccination and challenge of aged mice.** A. Aged C57BL/6 mice (18 months) were vaccinated with 10 μg of H$_2$O$_2$-WNV-KUNV vaccine adjuvanted with 0.1% of Alum ($n = 15$) 100 μl of WNV Innovator® ($n = 15$), or administered 0.1% Alum alone ($n = 15$), and 28 days after vaccination the mice were boosted. All groups were phlebotomized on days 0, 14, 28, 42, 60 and 90 post-vaccination, and the serum IgG titer against WNV E protein was measured by ELISA. B. On day 90 post immunization neutralization titers in serum from mice described in panel A were measured. C. Vaccinated mice were challenged i.c. with 10$^4$ PFU of WNV-NY on day 90 and monitored for survival. Asterisks indicate comparisons that are statistically different (***, $P < 0.001$; **, $P < 0.01$).
Table 1. Anti-WNV E protein antibody responses after vaccination

<table>
<thead>
<tr>
<th>Mice</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 42</th>
<th>Day 60</th>
<th>Day 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c H\textsubscript{2}O\textsubscript{2}</td>
<td>159 ± 51</td>
<td>1,413 ± 519</td>
<td>3,726 ± 1,117</td>
<td>4,104 ± 1,516</td>
<td>3,444 ± 1,461</td>
</tr>
<tr>
<td>BALB/c H\textsubscript{2}O\textsubscript{2} + boost</td>
<td>237 ± 68</td>
<td>404 ± 151</td>
<td>140356 ± 37818</td>
<td>53460 ± 12036</td>
<td>40500 ± 10246</td>
</tr>
<tr>
<td>C57BL/6 H\textsubscript{2}O\textsubscript{2}</td>
<td>972 ± 193</td>
<td>1844 ± 588</td>
<td>24318 ± 7274</td>
<td>11520 ± 4061</td>
<td>12816 ± 4180</td>
</tr>
<tr>
<td>C57BL/6 H\textsubscript{2}O\textsubscript{2} + boost</td>
<td>660 ± 220</td>
<td>1492 ± 523</td>
<td>201082 ± 43556</td>
<td>73507 ± 16078</td>
<td>55282 ± 14017</td>
</tr>
</tbody>
</table>

The indicated strains of mice were immunized with one or two (indicated by + boost at 28 days) doses of H\textsubscript{2}O\textsubscript{2}-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.
A. 

B. 

C. 

Survival of Vaccination