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Immunogenicity of RepliVAX WN, a Novel Single-Cycle West Nile Virus Vaccine

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Summary

We recently reported that immunization with RepliVAX WN, a single-cycle West Nile virus (WNV) vaccine, protected mice against WNV challenge. We have extended these studies by characterizing the RepliVAX WN-elicited antibody and T cell responses. WNV-specific IgG antibody responses comprised predominantly of IgG_{2c} and IgG_{2b} subclasses were detected 8 months after immunization. Vigorous WNV-specific CD4⁺ and CD8⁺ T cell responses directed at both structural and nonstructural WNV proteins were detected which were characterized by cytolytic activity and secretion of IFN- γ and TNF- α . Importantly, RepliVAX WN immunization resulted in vigorous CD8⁺ memory T cell responses detected at 8 months after immunization.

Keywords

West Nile Virus; Single-cycle virus; CD8⁺ T cell

1. Introduction

West Nile virus (WNV) is a member of the *Flavivirus* genus which contains a number of arthropod-borne human pathogens. WNV naturally infects a number of bird species and is transmitted to humans primarily by mosquitoes that become infected after feeding on viremic birds. In 1999 WNV was introduced into the United States in an outbreak in New York that resulted in 62 cases of human disease with 7 fatalities. Subsequently, WNV has spread across the continent producing a sustained epidemic that has resulted in over 11,000 cases of neurologic disease with approximately 1,100 fatalities [1,2]. WNV continues to spread with a range that now includes Canada, Latin America and the Caribbean. Most infected humans develop either a subclinical infection or West Nile fever, a nonspecific

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febrile illness. However, infection can also result in severe disease including meningitis or encephalitis and death. There is also evidence of long-term neurologic sequelae for those who experience neurologic disease and survive. Age is the most common risk factor for acquiring WNV disease, with elderly individuals at greatly increased risk of developing West Nile encephalitis. Administration of an effective WN vaccine would be an effective method of protection for these individuals.

Vigorous adaptive immune responses are elicited by WNV infection and numerous studies have demonstrated the protective capacities of specific immune components. Consistent with the role of antibody in protection, mice deficient of B cells or secreted antibodies are more susceptible to West Nile disease than intact animals [3–5] and protection against infection in B cell-deficient mice can be partially restored by passive transfer of immune serum [4]. Mechanisms of antibody-mediated protection against WNV include virus neutralization by mechanisms interfering with attachment, internalization, or post-internalization events [6–8], Fc gamma receptor-dependent mechanisms [9], and complement activation [10]. The E protein, which covers the entire surface of the virion, appears to be the primary target of neutralizing antibodies [11]. The nonstructural protein NS1 is secreted from infected cells and is not associated with the virion, yet antibodies directed against this protein have been shown to protect against infection by mechanisms both dependent and independent of complement components or Fc gamma receptors [12].

Cell-mediated responses are also elicited during WNV infection and have been shown to play an important role in limiting or clearing infection. Recent work has shown that mice with defects in CD8⁺ T cell responses exhibit a reduced ability to clear WNV infections [13,14] while transfer of WNV-specific CD8⁺ lymphocytes [15,16] protects recipients from lethal challenge. Likewise, WNV-specific CD4⁺ T cells with cytotoxic function have been detected in WNV-infected mice and have been shown to play a role in protection against infection of the central nervous system [17,18].

We have previously reported the development of a novel live attenuated virus (RepliVAX WN) composed of single-cycle WNV particles [19–21]. To produce RepliVAX WN, the WNV capsid (C) gene was deleted from the WNV genome rendering RepliVAX WN unable to produce infectious particles in vaccinated animals. However, RepliVAX WN can be replicated in stable cell lines that express the WNV C protein which becomes incorporated into the RepliVAX WN particle. Thus, the initial particle can infect cells in the same manner as wild type virus. Although normal cells infected by RepliVAX WN *in vitro* or *in vivo* produce all WNV proteins other than C, the lack of vaccine encoded C protein precludes formation of more infectious particles. Therefore, RepliVAX WN is safe and does not spread or cause disease. Immunization of mice and hamsters with the single-cycle RepliVAX WN vaccine elicited a strong neutralizing antibody response and protection against challenge with fully virulent WNV [19–22]. In the current studies, we extended our previous results to examine the characteristics and specificity of the WNV-specific IgG antibody response and, given the important role of T cells in protection against WNV, we examined the epitope specificity, magnitude, and functionality of the WNV-specific T cell response elicited by immunization with RepliVAX WN. The results of these studies demonstrate the development of vigorous WNV-specific T cell responses characterized by predominant production of IFN- γ and TNF- α and WNV-specific cytotoxic T cell activity. Together, these results demonstrate that this novel immunization platform is effective for eliciting durable and multifunctional WNV-specific antibody and cell-mediated immune responses.

2. Materials and methods

2.1 Mice

C57BL/6 (B6) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions in the AAALAC-approved University of Texas Medical Branch vivarium. All procedures were approved by the UTMB Institutional Animal Use and Care Committee with oversight of staff veterinarians.

2.2 Vaccine

The construction and characterization of RepliVAX WN has been described previously [21]. Briefly, C-deleted WNV RNA [19] was propagated in BHK(VEErep/Pac-Ubi-C*) cells [23], which provide the C protein in trans for production of single-cycle virus particles. RepliVAX WN stocks were titrated on Vero cell monolayers and stored at -80°C . Quantification of the infectious units (IU) of RepliVAX WN in working stocks was performed as described previously [19,21]. Briefly, Vero cell monolayers were infected with serial dilutions of RepliVAX WN working stock. Cells were fixed at 28 hours and infected cells were detected by immunohistochemical staining with polyclonal mouse hyperimmune ascites fluid specific for WNV. Mice were immunized by intraperitoneal (i.p.) inoculation with 10^4 or 10^6 IU RepliVAX WN in L-15 medium supplemented with 10mM HEPES and 0.5% FBS.

2.3 WNV Antigen Generation

WNV truncated E and WNV NS1 antigens were obtained from clarified culture fluids harvested from cultures of VEErep-bearing BHK cells as previously described [21]. WNV subviral particles (SVPs) were generated from Vero cell cultures inoculated with RepliVAX WN as previously described [21]. Clarified cell culture supernatant containing SVPs were concentrated using 100K NMWL centrifugal filters (Amicon Ultra, Millipore) according to manufacturer's protocol and purified on a 40-10% sucrose gradient prior to use. All antigens were titrated by ELISA to identify the optimal antigen concentrations for antibody capture.

2.4 ELISA

WNV-specific IgG was quantified on 96-well plates coated with WNV NS1, truncated E protein, or WNV SVPs. Serial dilutions of immune and normal serum were added to the plate and incubated overnight at 4°C . The plates were washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG (Southern Biotech) overnight at 4°C . Endpoint titers of individual IgG subclasses were determined using biotinylated IgG subclass-specific antibodies (Southern Biotech) followed by addition of streptavidin-peroxidase (Sigma-Aldrich, St. Louis, MO). Plates were developed by addition of o-phenylenediamine dihydrochloride plus hydrogen peroxide (Sigma-Aldrich). The OD_{490} for each experimental sample was determined on a Thermo Max microplate reader (Molecular Devices, Sunnyvale, CA). The endpoint dilution was defined as the reciprocal of the final dilution resulting in an $\text{OD}_{490} > 0.1$ and at least 3 standard deviations greater than the OD_{490} values obtained from normal serum controls. Results were expressed as the mean of the reciprocal of the endpoint dilution ($\log_{10}$) for each immunization group. A 1:50 serum dilution was the lowest dilution tested and was considered the lower limit for all serum ELISAs.

2.5 ELISPOT

For IgG antibody secreting cell (ASC) detection, filter membrane assay plates (Millipore) were coated overnight at 4°C with optimal dilutions of SVPs, NS1 antigen, or ovalbumin (OVA) as a control. The plates were washed and blocked with 2.5% bovine serum albumin

(BSA) in PBS before the addition of cells. Splenocytes or bone marrow cells harvested from RepliVAX WN vaccinated animals were plated in serial dilutions and incubated at 37°C for 15 hours. Plates were washed and HRP-conjugated IgG antibodies (Southern Biotech) were added and incubated overnight at 4°C. Plates were developed using 3-amino-9-ethylcarbazole, dimethylformamide and hydrogen peroxide in sodium acetate buffer (Sigma).

For T cell IFN- γ ELISPOTs, filter plates were coated overnight at 4°C with purified anti-mouse IFN- γ (BD Pharmingen) then blocked. Serial dilutions of splenocytes from naïve or RepliVAX WN immunized animals were plated with mitomycin C-treated B6 splenocytes previously pulsed with immunogenic WNV peptides, and incubated at 37°C for 40 hours. The following immunogenic peptides representing WNV CD4⁺ and CD8⁺ T cell epitopes have been reported previously [15,16,18] and were synthesized (New England Peptide, Gardner, MA) and used for stimulation: CD8⁺ T cell epitopes; E protein amino acid (AA) residues 347–354, RSYCYLAT (E₃₄₇); NS4B protein AA residues 2488–2496, SSVWNATTA (NS4B₂₄₈₈) or CD4⁺ T cell epitopes; E protein AA residues 431–445, IFVHGPTTVESHGNY (E₄₃₁); E protein AA residues 641–655, PVGRLVTVPFVSVA (E₆₄₁); NS3 protein AA residues 1616–1630, TKPGVFKTPEGEIGA (NS3₁₆₁₆); NS3 protein AA residues 2066–2080, RRWCFDGPRTNTILE (NS3₂₀₆₆). After incubation of stimulation cultures, plates were washed and incubated overnight at 4°C with biotinylated anti-mouse IFN- γ (BD Pharmingen), washed again, and incubated with streptavidin-peroxidase (Sigma-Aldrich) for 1 h at 37°C. Plates were developed as described above for IgG ASC ELISPOT. WNV IgG ASC or IFN- γ -secreting cells were quantified using an ImmunoSpot reader and data were analyzed with ImmunoSpot software (Cellular Technology Ltd, Cleveland, OH).

2.6 In Vivo Cytotoxic T Lymphocyte Assay

B6 mice were immunized by i.p. injection with 1×10^6 IU RepliVAX WN. On selected days after immunization, 2×10^7 target cells were adoptively transferred i.v. to immunized animals and naïve controls. To generate target cells, naïve B6 splenocytes were divided into two fractions: one fraction was pulsed with immunogenic WNV peptides for 45 minutes at 37°C and then labeled with 2.5 μ M of CFSE and the second fraction was incubated with media only, for use as an internal control, and labeled with 0.25 μ M of CFSE. NS4B₂₄₈₈, or E₃₄₇ peptide-coated targets were utilized in CD8⁺ CTL assays and E₆₄₁ plus NS3₂₀₆₆ peptide-coated targets were utilized in CD4⁺ CTL assays. Equal numbers of immunogenic peptide-pulsed targets and control targets were mixed and delivered by i.v. injection. Splenocytes from recipient mice were harvested and single-cell suspensions made after 4 hours for CD8⁺ CTL assays and after 16 hours for CD4⁺ CTL assays. Red blood cells were removed by centrifugation over histopaque (Sigma), the lymphocyte layer was fixed with 1% formaldehyde and CFSE labeled cell populations were quantified by flow cytometry. Data were acquired on a BD FACSCanto (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). The percent specific lysis was calculated as $(1 - \text{ratio for naïve mice} / \text{ratio for RepliVAX WN immunized mice}) \times 100$, where the ratio = $\%CFSE_{\text{low}} / \%CFSE_{\text{high}}$.

2.7 Intracellular Cytokine Staining (ICS)

Whole splenocyte populations were harvested from RepliVAX WN-vaccinated mice and restimulated by culture with 1 μ M immunogenic peptides or PMA/ionomycin (Sigma) for 2 hours prior to addition of brefeldin A for the remaining 4 hours of culture. Restimulated cells were harvested, blocked with anti-Fc γ RII/III mAb and then stained with fluorochrome-conjugated antibody to CD8 α or CD4. Cells were permeabilized using a Cytotfix/Cytoperm kit and stained with fluorochrome-conjugated mAb for IFN- γ (FITC), TNF- α (PE-Cy 7) and

IL-2 (PE). All flow cytometry reagents were purchased from BD Pharmingen. Data were acquired on a BD FACSCanto and analyzed using FlowJo software (Tree Star).

2.8 Cytokine ELISA

Detection of IL-4, TNF- α , or IFN- γ production by CD4⁺ effector T cell populations was accomplished as described previously [24]. Briefly, OD₄₉₀ values of culture supernatants were obtained and compared to the linear portion of the cytokine standard curve. Cytokine concentrations were calculated using SoftMax software (Molecular Devices).

2.9 Statistics

GraphPad Prism (GraphPad Software, San Diego, CA) was used to graph and analyze data. Student *t*-test or one-way analysis of variance (ANOVA) with the Bonferroni correction for multiple groups were used where appropriate. *P* values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1 Development of WNV-specific antibody responses following RepliVAX WN immunization

We previously reported that immunization of mice, hamsters, and non-human primates with RepliVAX WN resulted in vigorous neutralizing antibody responses and provided protection against WNV challenge [21,22,25]. Here, we have extended these observations by examining the development and duration of WNV-specific antibody responses and characterizing the IgG subclass profile of serum antibodies elicited by RepliVAX WN immunization. B6 mice were immunized by i.p. inoculation with either 10⁴ or 10⁶ IU RepliVAX WN and the kinetics and magnitude of the resulting WNV-specific IgG ASC response were determined by ELISPOT. As shown in Figure 1A, B cells secreting IgG antibody specific for the WNV NS1 protein were detected in immunized animals by day 8 post immunization. In animals immunized with 10⁴ IU RepliVAX WN, the number of NS1-specific IgG ASC peaked on day 8 and subsequently declined through day 22 post immunization. In mice immunized with 10⁶ IU RepliVAX WN, the number of NS1-specific IgG ASC detected on day 8 was comparable to that in the lower dose group. However, ASC numbers continued to increase and peaked at day 11 at which time they were significantly greater than those in the lower dose group. Subsequently, the number of NS1-specific IgG ASC in the 10⁶ IU group also gradually tapered off through day 22 post immunization. The same response pattern was detected for B cells secreting antibody reactive with SVPs (Fig. 1B). The SVP-reactive IgG ASC response peaked on day 11 in the 10⁶ IU RepliVAX WN group and the number of cells secreting SVP-reactive IgG antibody was significantly higher than in the 10⁴ IU RepliVAX WN group on days 11, 14, and 22 post immunization. Long-lived plasma cells maintained in bone marrow are thought to be responsible for maintenance of serum antibody levels and are indicative of a durable antibody response [26]. NS1- and SVP-specific IgG ASC were detected in the bone marrow on day 22 (Fig. 1C) and were maintained as long-lived plasma cells through 8 months post immunization (Fig. 1D). The magnitude of the WNV-specific ASC response in the bone marrow was reflective of the immunization dose with significantly higher numbers of NS1- and SVP-specific ASC detected in the 10⁶ IU immunization group. The long-term presence of these cells correlated with high levels of WNV NS1- and E-specific antibody as well as SVP-reactive IgG antibodies in serum 8 months after RepliVAX WN immunization (Fig. 2). Immunization with 10⁶ IU RepliVAX WN resulted in significantly higher titers of viral antigen-specific IgG compared to the 10⁴ IU group for all 3 antigens assayed. Endpoint titers of SVP-reactive IgG antibody were significantly higher than titers of NS1- and E-specific antibody (*P* < 0.05, ANOVA, Bonferroni correction) whereas there was no significant difference

between NS1- and E- specific IgG titers. The IgG subclass profile of the elicited antibody specific for all antigens tested was predominated by IgG_{2b} and IgG_{2c} antibodies while extremely low levels of WNV antigen-specific IgG₁ antibody were detected. Together, the presence of high titer serum IgG antibody and long-lived plasma cells in bone marrow were indicative of a durable vaccine-elicited antibody response. Further, the IgG subclass analysis results were indicative of a strong Th1-type immune response.

3.2 CD8⁺ T cell response elicited by RepliVAX WN immunization

RepliVAX WN immunization elicited a dose-dependent, WNV-specific CD8⁺ T cell response in B6 mice (Fig. 3A, B). Mice immunized with RepliVAX WN mounted a strong response to an immunodominant epitope in the WNV nonstructural protein NS4B (NS4B₂₄₈₈; Fig. 3A) and additionally to a WNV E glycoprotein epitope (E₃₄₇; Fig. 3B) [15, 16]. Significant numbers of IFN- γ -secreting NS4B₂₄₈₈ - and E₃₄₇ -specific T cells were detected in spleens of B6 mice immunized with 10⁴ IU RepliVAX WN ($P < 0.05$ compared to splenocytes from naïve mice, ANOVA); however, immunization with 10⁶ IU RepliVAX WN resulted in significantly greater numbers of epitope-specific T cells compared to the 10⁴ IU dose ($P < 0.05$, ANOVA). The time course of the CD8⁺ T cell response to RepliVAX WN immunization was established by quantification of WNV epitope-specific T cells by ELISPOT. IFN- γ secreting, NS4B₂₄₈₈-specific CD8⁺ T cells were detected at 4 days post immunization, the response peaked at day 6, and began to decline by day 8 post immunization (Fig. 3C). The response to the E₃₄₇ epitope was similar except the response remained high on day 8, although not significantly different than day 6 levels. The effector function of vaccine elicited CD8⁺ T cells was examined by characterization of cytotoxic T cell activity and antigen-specific cytokine production. RepliVAX WN immunized mice displayed high levels of specific cytolytic activity for both the NS4B₂₄₈₈ and E₃₄₇ CD8⁺ T cell epitopes (Fig. 3D) as early as day 4 post immunization ($P < 0.0001$ compared to naïve mice, Student t test). The response against both epitopes peaked on day 6 and declined by day 10 post immunization. Cytokine production was measured to further assess effector function of vaccine-elicited CD8⁺ T cells. As anticipated, RepliVAX WN-elicited CD8⁺ T cells produced IFN- γ and TNF- α in response to stimulation with immunogenic WNV NS4B₂₄₈₈ (Table 1). Approximately 2.1 % of CD8⁺ T lymphocytes from spleens of RepliVAX WN-immunized mice produced IFN- γ alone, 1.1% produced both IFN- γ and TNF- α , and less than 0.1% produced only TNF- α . Stimulation of cells from immunized mice with the E₃₄₇ peptide resulted in similar levels of cytokine production (data not shown) whereas control stimulation of immune splenocytes or WNV peptide stimulation of splenocytes from non-immune mice resulted in only background cytokine levels.

3.3 CD4⁺ T cell response to RepliVAX WN immunization

A dose-dependent effect was also observed on the magnitude of the RepliVAX WN-elicited CD4⁺ T cell response (Fig. 4A, B). Significantly higher numbers of CD4⁺ T cells recognizing 2 previously described immunodominant CD4⁺ T cell epitopes [18] were detected in mice immunized with 10⁶ IU compared to 10⁴ IU RepliVAX WN ($P < 0.05$, ANOVA). The time course of the CD4⁺ T cell response to RepliVAX WN immunization was measured by ELISPOT. Significant responses to 4 previously described immunodominant CD4⁺ T cell epitopes [18] on the NS3 protein and the E glycoprotein were detected (Fig 4C). The epitope dominance profile of RepliVAX WN-elicited cells was identical to that previously reported for WNV-infected B6 mice with the highest response directed at the NS3₂₀₆₆ epitope and lesser responses to the NS3₁₆₁₆, E₆₄₁, and E₄₃₁ epitopes. The peak response against all CD4⁺ T cell epitopes was detected at day 13 post inoculation and the response contracted through day 22. Previous reports have described CD4⁺ T cells with cytolytic activity resulting from infection of mice with fully virulent WNV [18]. WNV epitope-specific cytotoxic CD4⁺ T cells were also detected in the spleens of mice

immunized with the single-cycle WNV vaccine (Fig. 4D). Specific cytolysis of NS3₂₆₀₆ peptide-pulsed targets was detected on day 6 post immunization ($P < 0.0001$, compared to naïve mouse group, Student *t* test), peaked on day 8 and declined on day 11 post immunization. The E₆₄₁ response was detected on day 8 and also declined by day 11 post immunization. Overall the peak CD4⁺ cytotoxic T cell response elicited by RepliVAX WN immunization (approximately 20% specific lysis for the NS3₂₆₀₆ CD4⁺ T cell epitope) was of lower magnitude than the vaccine-elicited CD8⁺ cytotoxic T cell response, (greater than 90% for the NS4B₂₂₄₈ CD8⁺ T cell epitope, Fig. 3D). CD4⁺ T cells from RepliVAX WN-immunized mice produced predominantly IFN- γ at 48 and 72 hours following stimulation with WNV E₆₄₁ and NS3₂₆₀₆ peptides (Table 2). Small quantities of TNF- α were detected in 72 hr culture supernatants although levels in peptide stimulated cultures were not significantly higher than in unstimulated cultures. IL-4 levels fell below the level of detection at both the 48 and 72 hour time points. The secretion of high levels of IFN- γ correlated well with the presence of high quantities of IgG_{2c} antibodies in the serum of RepliVAX WN-immunized mice (Fig. 2).

3.4 RepliVAX WN immunization elicits WNV-specific, CD4⁺ and CD8⁺ memory T cells

B6 mice were immunized with either 10⁴ or 10⁶ IU RepliVAX WN and antigen-specific memory T cells from immune and naïve mice were quantified eight months later by ELISPOT. As shown in Figure 5A WNV NS4B₂₂₄₈- and E₃₄₇-specific CD8⁺ memory T cells were detected in spleens of mice immunized with 10⁴ or 10⁶ IU RepliVAX WN, however the response was directed primarily at the WNV NS4B₂₂₄₈ epitope. Consistent with the magnitude of the primary T cell response (Fig. 3), significantly higher numbers of WNV NS4B₂₄₈₈ - and E₃₄₇ -specific memory cells were detected in the 10⁶ IU immunization group compared to mice immunized with 10⁴ IU ($P < 0.05$ and 0.0005, respectively, ANOVA). Similarly, CD4⁺ memory T cells were detected following stimulation of splenocytes from the 10⁶ IU RepliVAX WN immunization group with immunogenic E₆₄₁ and NS3₂₆₀₆ peptides (Fig. 5B; $P < 0.005$ compared to naïve mouse group, ANOVA) although the response was of a much lower magnitude than for CD8⁺ memory T cells. Together, these results demonstrate that RepliVAX WN immunization elicits demonstrable memory T cell responses which were particularly vigorous for the CD8⁺ T cell sub-population.

4. Discussion

Effective vaccine-mediated protection against WNV disease will most likely require both virus-specific antibody and cell-mediated immune responses. Many groups have reported flavivirus vaccine candidates that can efficiently induce virus-specific antibody responses, but the induction of appropriate T cell responses, particularly CD8⁺ T cell responses, has been less frequently reported. In this regard, live attenuated vaccines have been shown to be very effective for inducing high magnitude, virus-specific T cell responses. We previously described the construction and characterization of a novel and safe, single-cycle WNV vaccine [19,21]. A single immunization with WNV RepliVAX WN was shown previously to induce protective immunity in mice, hamsters, and non-human primates [21,22,25]. Given that RepliVAX WN particles are capable of only a single round of replication, the antigen-specificity, magnitude, and durability of the WNV-specific T and B cell responses elicited by immunization was of interest. The results of the current study demonstrate that a single immunization with RepliVAX WN elicits a durable IgG antibody response and vigorous WNV-specific T cell responses, including strong WNV-specific CD8⁺ memory T cell responses. Moreover, the IgG subclasses of the serum antibody response and the effector functions of the elicited T cells are consistent with responses appropriate for control and elimination of challenge virus.

Antibody-mediated protection against WNV generally requires antibody directed against the E glycoprotein or nonstructural protein NS1 and may involve classical virus neutralization or non-neutralizing mechanisms, including Fc gamma receptor-dependent mechanisms. Serum IgG antibody and IgG antibody secreting cells from RepliVAX WN immunized mice reacted with these antigens and remained detectable up to 8 months after immunization. Large quantities of NS1 are released from flavivirus-infected cells and can be detected in both the serum of infected individuals and associated with cell surface membranes [27–30]. The results of the current study confirm that animals infected with the single-cycle vaccine RepliVAX WN also produce and release sufficient quantities of NS1 to elicit a strong antibody response to this nonstructural protein. Flavivirus-infected cells also release both fully infectious virions and small non-infectious SVPs containing only the E glycoprotein and prM/M protein in the absence of capsid protein or viral genome [31–33] that can serve as a potent source of E protein for stimulation of the adaptive immune response. Similarly, expression of the E and prM gene products in the absence of the capsid protein by cells infected with RepliVAX WN results in formation and release of non-infectious SVPs [21]. High levels of cells secreting IgG antibody reactive with WNV SVPs were also detected and were presumably specific for either E or prM/M proteins. The ability of a vaccine capable of only a single round of replication to elicit such a high magnitude and durable antibody response is significant and may be influenced by the duration of antigen expression by RepliVAX WN infected cells. Viral gene expression can be detected at the site of inoculation in mice inoculated subcutaneously with firefly luciferase-expressing WNV single-cycle particles for approximately 2 weeks (D. Widman, manuscript in preparation). The prolonged release of SVPs and secretion of NS1 from cells initially infected with RepliVAX WN may thus have important ramifications for continued stimulation of the WNV-specific antibody response in immunized mice. Importantly, this stimulation drove the production of high numbers of WNV-specific ASC and successfully drove the differentiation of these cells to become long-lived plasma cells. Both NS1-specific and SVP-reactive IgG antibody-secreting cells were maintained in the bone marrow up to 8 months after immunization and were most likely responsible for the maintenance of the serum IgG response [26]. Together, these results demonstrate the utility of this single-cycle flavivirus vaccine platform for generating vigorous, durable, virus-specific antibody responses.

A single immunization with RepliVAX WN also resulted in development of virus-specific T cell responses in a dose-dependent manner. The epitope-specificity of RepliVAX WN-elicited CD8⁺ T cells mirrored that reported previously for wild type WNV [15,16] and effector mechanisms required for efficient termination of viral infections were expressed by these cells including epitope-specific cytolytic activity, and IFN- γ and TNF- α production. Interestingly, although nearly equivalent numbers of NS4B₂₄₈₈ and E₃₄₇-specific cells were detected during the primary T cell response to RepliVAX WN immunization, the CD8⁺ memory T cell response was predominated by cells recognizing the NS4B₂₄₈₈ epitope. The reason for this preferential selection is currently unclear but it is possible it may reflect subtle differences in the kinetics of activation and cytokine milieu at the time of activation for the 2 different epitope-specific T cell populations such that there is a preferential drive towards memory T cell differentiation for the NS4B₂₄₈₈-specific cells. A vaccine-elicited CD4⁺ T cell response lower in magnitude than the vaccine-elicited CD8⁺ T cell response was detected that also expressed cytolytic activity and Th1 cytokine production. Given the single-cycle nature of the RepliVAX WN infection, the epitope-specificity of vaccine-elicited CD4⁺ T cells was of interest. As discussed previously, RepliVAX WN-infected cells at the site of inoculation release SVPs over the course of several days prolonging the availability of the E glycoprotein for uptake by antigen presenting cells. Therefore it was not surprising to detect RepliVAX WN-elicited CD4⁺ T cells recognizing previously reported epitopes in the E glycoprotein. However, it was not clear whether CD4⁺ T cells specific for non-structural proteins would be detected in RepliVAX Wn-immunized animals.

Specifically, epitopes on the NS3 protein, including an immunodominant epitope, have been shown to be recognized by CD4⁺ T cells during a wild type WNV infection [18]. The same WNV NS3 epitopes were recognized by T cells elicited by RepliVAX WN immunization suggesting that sufficient NS3 antigen was produced and processed to elicit CD4⁺ T cell recognition of this protein.

The results of the present studies clearly show a dose-dependent effect of RepliVAX WN immunization on the magnitude of the resulting WNV-specific B and T cell responses. Previous challenge studies demonstrated that mice immunized with either 10⁶ or 4 × 10⁴ IU RepliVAX WN were fully protected against mortality and disease symptoms following WNV challenge [21]. Taken together, these results suggest that fully protective immune responses are generated by immunization with 10⁴ IU RepliVAX WN. Given the diminutive WNV-specific CD4⁺ memory T cell response elicited by immunization with this dose, it seems likely that the CD4⁺ T cell component made only a modest contribution to protection. While it is not possible to identify completely the correlates of immunity from these studies, vigorous WNV-specific antibody and CD8⁺ memory T cell responses were detected in mice immunized with this dose of RepliVAX WN and likely contributed more substantially to protection.

Candidate live attenuated vaccines against WNV have been described involving either deletion mutants of small portions of the WNV C protein [34] or chimeric viruses in which the WNV prM and E genes have been switched onto either a dengue virus [35] or yellow fever virus [36] backbone and all have been shown to elicit strong neutralizing antibody responses. Unlike the antibody response to RepliVAX WN which is directed against both E and NS1 proteins, chimeric-based vaccine-elicited protective antibody would be directed primarily to the WNV E glycoprotein. The antibody response to C protein deletion mutant vaccine candidates would presumably be directed towards the entire array of WNV proteins although characterization of antibody responses to this vaccine did not assess the protein specificity of the response [34]. Very little data are available regarding the T cell responses to other candidate live attenuated WNV vaccines with the exception that a chimeric WNV vaccine given to humans elicited E protein specific IFN-γ-secreting T cells in a clinical study [36]. Many WNV-specific T cell responses are directed against non-structural proteins which would not be elicited by current chimeric WNV vaccines. In contrast, T cell responses to RepliVAX WN were extremely similar to those characterized following infection with WNV [15,16,18] and targeted both structural and non-structural WNV proteins.

Taken together, the results of the current studies suggest that a single immunization with RepliVAX WN elicited vigorous and durable WNV-specific T and B cell responses. In addition to our previous results demonstrating the development of strong neutralizing antibody responses following RepliVAX WN immunization [21,22], the current results demonstrate the development of durable virus-specific antibody responses predominated by IgG subclass responses known to facilitate protection against viral disease and spread of infection. Importantly, RepliVAX WN immunization elicited vigorous T cell responses to both structural and nonstructural proteins with effector phenotypes commensurate with the ability to eliminate viral infection.

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Fig. 1.

Development of the WNV-specific IgG antibody secreting cell (ASC) response to RepliVAX WN immunization. Dose dependent ASC response to NS1 (A) and SVP (B) antigens. B6 mice (n= 5/group) were immunized with 10⁴ or 10⁶ IU RepliVAX WN and antigen-specific ASC were quantified by ELISPOT on the indicated days as described in Methods. The results shown are from one experiment of 2 performed. (** *P* < 0.0001, * *P* < 0.05 compared to naïve mice; Student *t*-test). Bars indicate comparison between 10⁴ and 10⁶ IU immunization groups. NS1 and SVP-reactive ASC were detected in the bone marrow of RepliVAX WN immunized mice 22 days (C) and 8 months (D) after immunization. The results shown are compiled from 2 separate experiments (n=5–10/group). For panel C, * *P* < 0.005, ** *P* < 0.0005 compared to naïve mice. For panel D, * *P* < 0.05, ** *P* < 0.0005 compared to naïve mice.

Fig. 2.

Characterization of the WNV-specific serum IgG response to RepliVAX WN immunization. B6 mice were immunized with 10^4 or 10^6 IU RepliVAX WN and serum was collected from immune ($n=14$ /group) and naïve ($n=8$ /group) mice 8 months later. Serial dilutions of immune or naïve sera from individual mice were plated on assay plates coated with WNV NS1, WNV SVP, or WNV truncated E antigen and developed to detect IgG, IgG₁, IgG_{2b}, or IgG_{2c} antibody as described in Methods. Results are expressed as the mean endpoint titer ($\log_{10} \pm$ standard error of the mean (SEM)). The lowest dilution tested was 1:50 ($1.69 \log_{10}$) and no WNV reactive antibody was detected in naïve serum at this dilution. Therefore this was regarded as the lower limit of detection for these experiments. (** $P < 0.001$, * $P < 0.05$; ANOVA compared to naïve). Lines indicate comparisons between 10^4 and 10^6 IU RepliVAX WN immunization groups.

Fig 3.

Antigen-specificity and effector function of CD8⁺ T cells to RepliVAX WN immunization. Detection of WNV NS4B₂₂₄₈- (A) and E₃₄₇-specific (B) CD8⁺ T cells after RepliVAX WN immunization of B6 mice. Groups of 5 B6 mice were immunized with 10⁴ or 10⁶ IU RepliVAX WN and the IFN-γ secreting cell response was quantified by ELISPOT as described in Methods. Comparisons were made between immunized and naïve mice: * $P < 0.05$; ANOVA. (C) Kinetics of CD8⁺ T cell IFN-γ response. B6 mice (n=5/group) were immunized with 10⁶ IU RepliVAX WN and WNV-specific T cells quantified by ELISPOT on the indicated days. Immune group values were compared to the non-immune mice (day 0) response to the same peptide. *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$; Student *t*-test. (D) Kinetics of the CD8⁺ cytotoxic T lymphocyte response to RepliVAX WN immunization. B6 mice were immunized with RepliVAX WN and WNV-peptide pulsed target cells were injected into immunized and naïve mice on the indicated days and the per cent specific lysis was calculated as described in Methods. Comparisons were made between immune and naïve mice injected with the same target cells. ** $P < 0.0001$, * $P < 0.005$; Student *t*-test; n= 5–8 mice/group from 3 separate experiments.

Fig. 4.

Antigen-specificity and effector function of CD4⁺ T cells to RepliVAX WN immunization. Effect of immunization with 10⁴ or 10⁶ IU RepliVAX WN on induction of a WNV NS3₂₀₆₆ - (A) and E₃₄₇ -specific (B) CD4⁺ T cell response. (* $P < 0.05$; ANOVA, versus naïve controls). (C) Kinetics and epitope-specificity of the CD4⁺ T cell response following RepliVAX WN immunization. B6 mice were immunized with 10⁶ IU RepliVAX WN and splenocytes were stimulated with the indicated WNV peptide and the number of IFN- γ secreting cells from 5 mice/group quantified by ELISPOT on the indicated day. Epitope-specific values from immunized mice were compared to values from non-immune cells (day 0) stimulated with the same peptide. ** $P < 0.001$, * $P < 0.05$; Student t -test. Results of a representative experiment of 2 performed are shown. (D) Kinetics of the CD4⁺ cytotoxic T lymphocyte response following RepliVAX WN immunization of B6 mice. RepliVAX WN immunized mice (n=5) were assessed for cytolytic activity on the indicated day post-immunization. Preparation of targets and detection and calculation of cytotoxicity were performed as described in Methods. Comparisons were made between immune and naïve mice injected with the same target cells. ** $P < 0.0001$, * $P < 0.005$; Student t -test. Results are compiled from 2 separate experiments; n=6/group.

Fig. 5.

Immunization of mice with RepliVAX WN elicits WNV-specific CD4⁺ and CD8⁺ memory T cell responses. Mice were immunized with 10⁴ or 10⁶ IU WNV RepliVAX WN. Eight months later, immune or non-immune splenic lymphocytes were stimulated with mitomycin C-treated splenocytes pulsed with immunogenic WNV peptides representing known CD8⁺ (A) and CD4⁺ (B) T cells. Results are expressed as the number of IFN-γ-secreting cells/spleen and are pooled from 3 separate experiments for a total of 10–15 mice/group. For panel A, **P* < 0.005 compared to Naïve mice, ** *P* = 0.0008 compared to media-stimulated cultures, *** *P* < 0.0001 compared to media-stimulated cultures, **** *P* = 0.0032 compared to media stimulated cultures. For panel B, * *P* < 0.005 compared to naïve mice, ** *P* = 0.0063 compared to media-stimulated cultures.

Table 1**Cytokine Production by RepliVAX WN-Elicited CD8⁺ T Lymphocytes**

Immunization	Stimulation	% of Total CD8 ⁺ T lymphocytes		
		IFN γ ⁺	IFN γ ⁺ + TNF α ⁺	TNF α ⁺
RepliVAX WN	NS4B ₂₄₈₈	2.1 ± 0.2 ^{a,b}	1.1 ± 0.09 ^{c,d}	0.07 ± 0.02
RepliVAX WN	Media	0.15 ± 0.16	0.09 ± 0.07	0.01 ± 0.03
None	NS4B ₂₄₈₈	0.01 ± 0.01	0.04 ± 0.01	0.05 ± 0.03
None	Media	0.01 ± 0.02	0.02 ± 0.02	0.01 ± 0.05

Splenocytes from RepliVAX WN-immunized (n=5) or non-immunized mice (n=2) were stimulated with the immunodominant NS4B₂₄₈₈ peptide or media as a control. Cytokine production was assessed by intracellular cytokine staining as described in Methods.

^a $P=0.0004$ compared to media-stimulated immune splenocytes.

^b $P<0.0001$ compared to peptide-stimulated non-immune splenocytes

^c $P<0.0001$ compared to media-stimulated immune splenocytes.

^d $P=0.0007$ compared to peptide-stimulated non-immune splenocytes.

Table 2Cytokine Production by RepliVAX WN-Elicited CD4⁺ T Lymphocytes

Stimulation ^a	Culture	Concentration (pg/mL) ± SEM		
		IFN γ	TNF α	IL-4
Peptides	48 h	3,310 ± 1,115 ^b	ND ^c	ND
None	48 h	ND	ND	ND
Peptides	72 h	12,192 ± 2,992 ^d	156 ± 48	ND
None	72 h	64 ± 64	95 ± 49	ND

^aLymphocytes from immunized mice (n= 6) were stimulated with E₆₄₁ plus NS32066 peptides and the cytokine concentration determined by ELISA as described in Methods.

^b*P*= 0.014 compared to 48 h media-stimulated culture (Student *t* test).

^cND= Not detectable. Based on the standard curve values, the limit of detection for these cytokine assays was considered to be 50 pg/ml.

^d*P*= 0.002 compared to 72 h media-stimulated culture (Student *t* test).

Cytokine concentration values for lymphocytes of naïve mice fell below the limit of detection for all cytokines at all time points.