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# A Two-dose Intranasal Vaccine Containing West Nile Virus Envelope Domain III Adjuvanted with a Mast Cell Activating Cationic Peptide Mastoparan 7 and CpG Induces Protective Anti-West Nile Virus Immunity in Mice

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## Abstract

West Nile Virus (WNV) causes serious neurologic disease in humans yet no human effective vaccine is currently available. Domain III of the WNV envelope protein (EDIII) has been shown to induce protective WNV-neutralizing antibodies in animal models, hence a potential candidate for human WNV vaccine. In addition, nasal vaccines can be administered rapidly and this would be beneficial against WNV outbreaks. The goal of this study was to determine if a two-dose nasal WNV vaccine would induce long-term protective anti-WNV immunity in a mice. Female BALB/c mice were given two or three intranasal doses of 15-60 µg EDIII combined with mastoparan 7 peptide (M7-OH) and CpG between days 0-21. Immune serum was tested for anti-EDIII antibody concentration, antibody avidity, virus neutralization, T cell responses and protection of mice against WNV strain NY99 challenge. In this study, two-dose 60 µg EDIII with combined adjuvants protected 100% of mice challenged on day 60. At day 189, three-dose regimen of 15 µg EDIII with combined adjuvants provided 93% protection while two-doses of 45 µg or 60 µg EDIII provided 87% survival. The protection were significant when compared to naïve mice or mice immunized with EDIII without adjuvants. In conclusion, 60 µg EDIII with combination of M7-OH and CpG is an effective vaccine using a short immunization regimen of days 0 and 14. The study has also shown that intranasal use of 45 or 60 µg of EDIII with combined adjuvants M7-OH and CpG are good candidates for induction of rapid and long-term protective immunity against WNV NY99 in mice.

**Key words:**
Mastoparan 7, CpG, two-dose, EDIII, intranasal, vaccine

## 1. Introduction

West Nile Virus is a single-stranded positive-polarity RNA virus belonging to the family Flaviviridae [1, 2]. It was first identified in Uganda in 1937 [3], and is endemic in Africa, Europe, the Middle East and Asia [4]. First cases of WNV in the USA were reported in 1999 [5], and later reported in South America and the Caribbean [5, 6]. WNV circulates between mosquitoes and birds with humans and horses as incidental hosts [7]. Symptoms occur in approximately 20% of infected humans as febrile illness [8], with severe neurological complications in about 1% [8, 9]. Immunosuppressed and elderly individuals are more at risk to develop severe disease [7, 10, 11]. Between 1999-2012 about 36,000 cases and 1,500 deaths were reported in USA alone [12]. Currently there is no approved vaccine or treatment for WNV in humans.

Studies in mice using WNV EDIII as vaccine [13] have demonstrated induction of protective immunity against WNV [14, 15]. WNV EDIII is however, poorly immunogenic and therefore use of adjuvants could enhance its immune activation [16, 17]. Several vaccine adjuvants with immunostimulatory effects including CpG have been identified [18, 19], and the most recent ones are the cationic molecule adjuvants [20]. It has also been shown that combination of cationic adjuvants with CpG can be a novel strategy to amplify and diversify immune responses [21].

The goal of this study was to develop an immunization strategy that could use a rapid I.N regimen to induce long-term protective immunity using a mouse model. The rationale was that a short immunization schedule would be administered rapidly in the event of an outbreak, and the use of I.N route is needle-free and requires minimal technical knowledge to administer the vaccine. Since I.N delivery is needle-free the safety risks associated with injectable vaccines are eliminated, yet they have been shown to generate immune responses with similar magnitude and quality to injected vaccines [22].

## 2.1 Materials

Female BALB/c mice were purchased from Frederick Cancer Research, Frederick, MD, USA. WNV EDIII protein (sequence 298GMQLKGTTYGVCSKAFKFLGTPADTGHGTVVLELQYTGTDGPCKVPISSVASLNDLT PVGRLVTVNPFVSVATANAKVLIELEPPFGDSYIVVGRGEQQINHHWHKSGSSIGKAFTTTLKGALE415) was purchased from GenScript, Piscataway, NJ, USA while CpG (ODN 1826) was purchased from InvivoGen, San Diego, CA, USA. Mastoparan analog M7-OH (sequence INLKALAALAKALL-OH) was purchased from CPC scientific Inc. Sunnyvale, CA, while Vero cells and WNV NY99 strain 35262-11 (cat#NR-677) were purchased from ATTC (Manassas, VA, USA). Goat anti-mouse IgG (Cat#1030-04), IgG1 (Cat#1070-04) and IgG2a (1080-04) were purchased

from Southern Biotech (Birmingham, AL, USA), and ammonium thiocyanate ($NH_4SCN$, Cat#1762-95-4) was purchased from Avantor Performance Materials, Inc. Center Valley, PA.

## 2.2 Immunizations, sample collection and mice challenge

Mice were immunized I.N as previously described [23] using between 15 µg- 60 µg EDIII antigen, either alone or adjuvanted with 5 nanomoles M7-OH and 10 µg CpG. The 15 µg EDIII antigen adjuvanted with M7-OH and CpG was administered on days 0, 7 and 21 while two-dose regimens comprising between 30-60 µg EDIII antigen adjuvanted with M7-OH and CpG were administered on days 0 and 7 or 0 and 14 or 0 and 21. In addition, 30 or 60 µg EDIII antigen alone were given on days 0, 7 and 21 as negative controls. Blood samples were obtained from mice on days 42 or 182 and serum was tested antigen specific antibody and avidity testing, and for determination of virus neutralization. To determine antiviral prevention, WNV NY99 was propagated in Vero cells as previously described [18] and $1x10^5$ ffu were inoculated I.P per mouse on day 60 or 189. Then inoculated mice were monitored for weight loss and survival over a period of two weeks.

## 2.3 Antibody and avidity ELISA, and virus neutralization assays

Serum anti-EDIII-specific IgG antibody titers and antibody avidity were determined using ELISA as previously described [24], using 2 µg/ml EDIII protein as plate coating antigen. Serum anti-EDIII antibody titers were expressed as the reciprocals of the last dilution factors with ELISA units three-fold above that of same dilution of pre-immune serum. The antibody avidity were determined as previously described [25] with minor modifications as follows. After serum incubation on the antigen-coated plate, between 0-4 molar $NH_4SCN$ was added and incubated for 15 minutes and then followed by similar procedure to antibody ELISA [24]. The avidity indices were determined using ELISA units expressed as percent of units from wells with zero molar $NH_4SCN$. The virus neutralization assay was performed using plaque reduction neutralization test (PRNT) in 48-well plates as previously described [26].

## 2.4 Splenocyte cultures and cytokine assay

Spleens were obtained from mice of different vaccine groups using sterile technique on day 49. The spleen cells were then collected as previously described [24], counted and suspended at 1.0 $x10^7$/ml of culture media. From the cells suspension, 250 µl were cultured per well using 48-well plates (Falcon, cat#353078, BD, Franklin Lakes, NJ USA) and added with either 250 µl media or containing 5 µg/ml EDIII antigen for and incubated for 60 hours. At the end of culture, supernatants were tested for different TH1, TH2 and TH17 type cytokines using a multiplex bead assay (Bio-Rad laboratories, Cat#171-30407).

## 2.5 Statistical analysis

Antibody titers, cytokines and weights were analyzed using 1-way ANOVA with Tukey's multiple comparison test, while survival rates were analyzed by Chi-Square test. Differences were considered significant when $p < 0.05$ (Graph Pad Prism version 5).

## 3.0. Results

### 3.1 A three-dose intranasal immunization using 15 µg EDIII antigen adjuvanted with M7-OH and CpG induces superior antigen specific antibody responses than a two-dose immunization using 30 µg EDIII antigen adjuvanted with M7-OH and CpG.

Our previous preliminary studies have shown that I.N administration of three immunizations of 15 µg EDIII antigen adjuvanted with M7-OH and CpG induces protective immunity against WNV infection in mice. On the basis of this data, our next goal was to develop a 2-dose I.N regimen that could induce rapid protective immunity against WNV infection. This study used a regimen of days 0, 7 and 21 as guide, to administer 2-dose vaccine administrations that included days 0 and 7; 0 and 14; and 0 and 21. Since fewer immunizations were utilized, a higher antigen dose was expected in order to develop an effective 2-dose regimen, and therefore 30 µg EDIII antigen was used instead of 15 µg.

Results from this study have demonstrated induction of significantly high serum anti-WNV EDIII IgG titers (GMT) in mice immunized with either three- or two-dose vaccine regimens using 15 µg EDIII antigen adjuvanted with M7-OH and CpG given on days 0, 7 and 21 ($1.9 \times 10^7$ GMT), or 30 µg EDIII antigen adjuvanted with M7-OH and CpG given on days 0 and 7 ($1.4x10^4$ GMT), or 0 and 14 ($1.99 \times 10^6$ GMT), or 0 and 21 ($9.6 \times 10^2$ GMT) compared to 30 µg EDIII antigen without adjuvants (3.2 x 10 GMT) (Figure 1A). The three-dose regimen using adjuvanted 15 µg EDIII antigen induced significantly greater anti-EDIII IgG1 ($7.9 \times 10^5$ GMT) and IgG2a ($4.1 \times 10^7$ GMT) antibody titers than 30 µg EDIII antigen without adjuvants, IgG1 (3.2 x 10 GMT) and IgG2a (3.4 x 10 GMT) (Figure 1B). Similarly, significantly high IgG1 antibody titers were observed among two-dose regimens that used the schedules of days 0 and 7 ($1.9 \times 10^4$ GMT) or 0 and 14 ($4.97 \times 10^4$ GMT), but not of days 0 and 21. The IgG2a antibody titers however, were significantly increased with all the three schedules that used two-dose regimen, days 0 and 7 ($2.0 \times 10^4$ GMT), or 0 and 14 ($2.3 \times 10^5$ GMT), or 0 and 21 ($1.2 \times 10^3$ GMT) (Figure 1B). Despite the minor differences, IgG1 and IgG2a antibody titers that were induced by two-dose schedules were more comparable whereas three-dose regimen induced significantly higher IgG2a than IgG1 with a fold-difference of about 52 (Figure 1B). Overall, IgG antibody titers that were induced by two-dose schedules were significantly lower compared to the three-dose schedule.

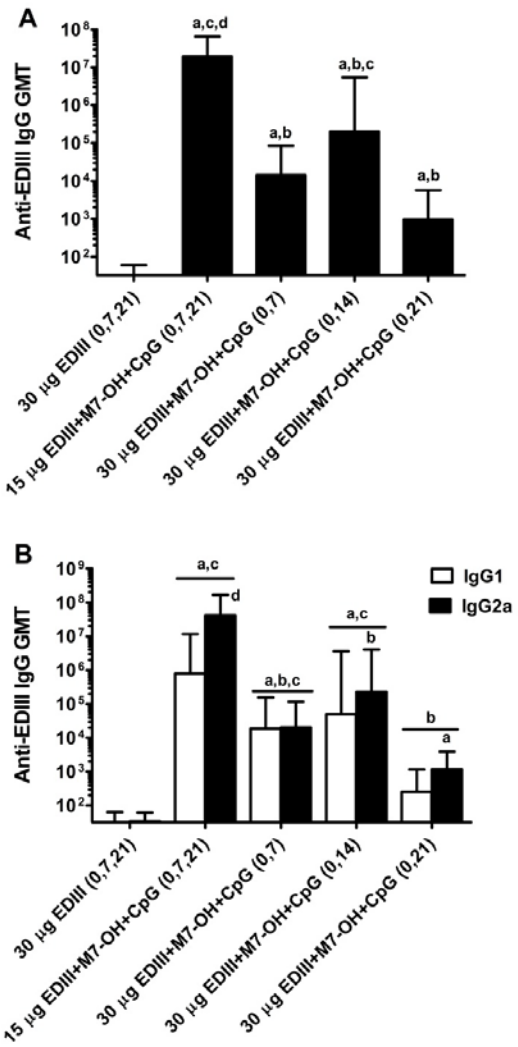


**Figure 1.** A **three-dose intranasal immunization with 15 µg EDIII antigen adjuvanted with M7-OH and CpG induces superior antigen specific antibody responses than two-dose intranasal immunization with 30 µg EDIII antigen adjuvanted with M7-OH and CpG.** Female BALB/c mice were immunized I.N with either 30 µg EDIII antigen without adjuvant (n = 10) or with 30 µg EDIII antigen combined with 10 µg CpG and 5 nanomoles M7-OH. Two-dose immunizations were administered in three different schedules on either days 0 and 7 (n = 10,) or 0 and 14 (n = 5) or 0 and 21 (n = 10). The three-dose immunizations were administered on days 0, 7 and 21 (n =10 for each group). Animals were bled on day 42 and their serum samples tested for anti-EDIII antigen specific antibodies by ELISA method. (**A**) Serum anti-EDIII antigen IgG, (**B**) Serum anti-EDIII antigen specific IgG1 and IgG2a. (**a** > 30 µg EDIII antigen without adjuvant administered on days 0, 7 and 21, **b** < 15 µg EDIII antigen adjuvanted with CpG and M7-OH given on days 0, 7 and 2, **C** > 30 µg EDIII antigen adjuvanted with CpG and M7-OH given on days 0 and 21, **d** > 30 µg EDIII antigen adjuvanted with CpG and M7-OH given on days 0 and 7. In parenthesis are the immunization days. The data was analyzed using PRISM software 1 way ANOVA, with Tukey's Multiple Comparison Test. Differences were considered significant when $p < 0.05$.

### 3.2 Two-dose intranasal immunizations using 45 µg or 60 µg EDIII antigen adjuvanted with M7-OH and CpG induce EDIII antigen-specific antibody responses that are comparable to three-dose immunization using 15 µg EDIII antigen adjuvanted with M7-OH and CpG.

After observing that two-dose immunizations with adjuvanted 30 µg EDIII antigen could not induce comparably high anti-EDIII antigen specific antibodies to three-dose immunization using adjuvanted 15 µg EDIII antigen, the EDIII antigen dose was increased from 30 µg to 45 µg or 60 µg. Two-dose immunizations using 45 µg EDIII antigen adjuvanted with M7-OH and CpG induced significantly high serum anti-EDIII IgG antibodies using schedules of days 0 and 7 ($1.7 \times 10^5$ GMT), or 0 and 21 ($1.99 \times 10^5$ GMT) compared to 60 µg EDIII antigen without adjuvants. In the contrary, two-dose schedule using 60 µg EDIII antigen adjuvanted with M7-OH and CpG induced significantly high anti-EDIII antigen-specific IgG antibodies when administered on days 0 and 14 ($1.7 \times 10^5$ GMT) only (Figure 2A). Analyses of antigen-specific IgG subtypes showed that anti-EDIII antigen-specific IgG1 antibody titers were enhanced by 45 µg EDIII antigen adjuvanted with M7-OH and CpG when given on days 0 and 21 ($1.7 \times 10^5$ GMT), or 60 µg EDIII antigen adjuvanted with M7-OH and CpG given on days 0 and 7 ($1.3 \times 10^5$ GMT), or 0 and 14 ($8.6 \times 10^4$ GMT) (Figure 2B). On the other hand anti-EDIII antigen specific IgG2a antibodies increased when 45 µg of EDIII antigen adjuvanted with M7-OH and CpG was administered on days 0 and 7 ($9.9 \times 10^4$ GMT), or 0 and 14 ($4.97 \times 10^4$ GMT) or 0 and 21 ($3.5 \times 10^5$ GMT) (Figure 2B). Similarlyy, using 60 µg EDIII antigen adjuvanted with M7-OH and CpG was effective at inducing IgG2a when administered on days 0 and 7 ($1.7 \times 10^5$ GMT) or 0 and 14 ($5.7 \times 10^4$ GMT) but not on days 0 and 21 (Figure 2B). We also observed that anti-EDIII antigen specific IgG and IgG1 antibody titers were comparable between the three-dose and two-dose regimens, while IgG1 and IgG2a titers were only comparable within each vaccine group (Figure 2A, B).

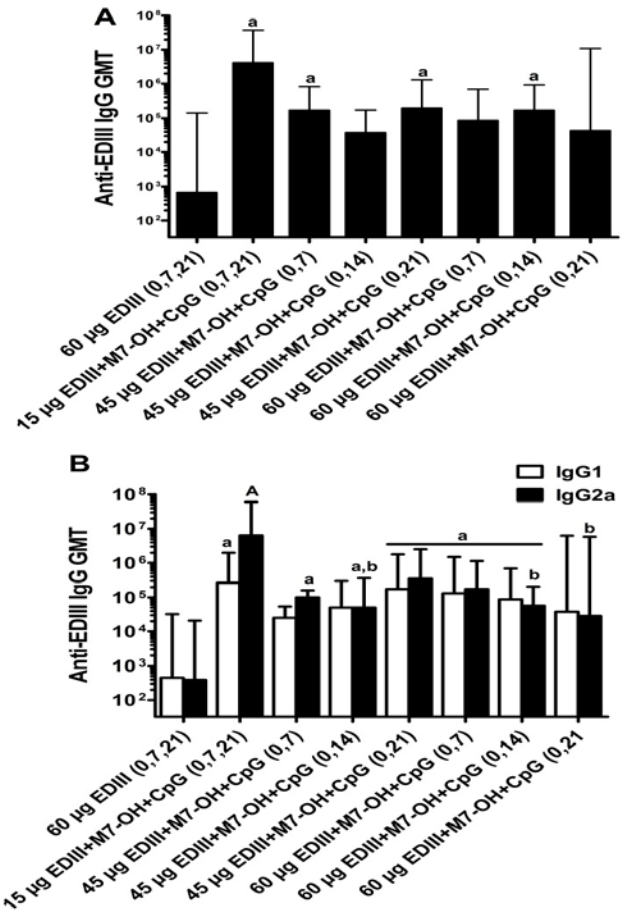


**Figure 2. Two-dose intranasal immunizations with 45 µg or 60 µg EDIII antigen adjuvanted with M7-OH and CpG induces comparable antibody responses to three-dose immunization with 15 µg EDIII antigen adjuvanted with CpG and M7-OH**. Female BALB/c mice were immunized I.N with either 60 µg EDIII antigen without adjuvant (n=5) or 15 µg EDIII antigen adjuvanted with CpG and M7-OH (n=5) on days 0, 7 and 21 or with 45 µg (n = 5) or 60 µg (n = 5) EDIII antigen adjuvanted with 10 µg CpG and 5 nanomoles M7-OH. Two-dose immunizations were administered on days 0 and 7 or 0 and 14 or 0 and 21. Blood samples were collected from mice on day 42 and their serum tested for anti-EDIII antigen specific antibodies using ELISA test. (**A**) Serum anti-EDIII IgG, (**B**) Serum anti-EDIII IgG1 and IgG2a (**a, A** > 60 µg EDIII antigen without adjuvants given on days 0, 7 and 21, **b** < 15 µg EDIII antigen adjuvanted with CpG and M7-OH given on days 0, 7 and 21"A". In parenthesis are the immunization days. The data was analyzed using PRISM software 1 way ANOVA, with Tukey's Multiple Comparison Test.Differences were considered significant when $p < 0.05$.

### 3.3 Two-dose intranasal immunizations using 45 or 60 µg EDIII antigen adjuvanted with M7-OH and CpG induced long-term protective anti-WNV immunity.

Since we observed that increasing the EDIII antigen dose to 45 or 60 µg adjuvanted with M7-OH and CpG induced significantly high serum antibody titers compared to 60 µg EDIII antigen without adjuvants, we next determined the effectiveness of the immune response to protect against WNV infection. To determine the efficacy mice were immunized using 45 µg or 60 µg EDIII antigen adjuvanted with M7-OH and CpG on days 0 and 14 and challenged with WNV NY99. After immunization, first, blood samples were collected on day 42 and serum tested for anti-EDIII antigen specific antibodies. Significantly high anti-EDIII IgG (6.3 x $10^5$ GMT), IgG1 (9.1 x $10^5$ GMT) and IgG2a (7.9 x $10^4$ GMT) antibodies were induced by 45 µg EDIII antigen adjuvanted with M7-OH and CpG. Similarly, 60 µg EDIII antigen adjuvanted with M7-OH and CpG induced significantly high anti-EDIII antigen specific IgG (1.0 x $10^6$ GMT), IgG1 (1.1 x $10^6$ GMT) and IgG2a (1.2 x $10^5$ GMT) antibodies (Figures 3A, B) compared to 60 µg EDIII antigen without adjuvants. Surprisingly, the three-dose regimen using adjuvanted 15 µg EDIII antigen induced significantly high IgG (3.2 x $10^7$ GMT), IgG1 (4.4 x $10^7$ GMT) and IgG2a (1.6 x $10^7$ GMT) antibodies compared to two-dose regimens using adjuvanted 45 or 60 µg EDIII antigen (Figure 3A, B). Comparison of IgG antibody subjects showed that two-doses of adjuvanted 45 or 60 µg EDIII antigen induced significantly higher anti-EDIII antigen IgG1 antibodies than IgG2a (Figure 3B). Immunized mice were challenged with WNV NY99 strain 35262-11 on day 60. Mice were monitored for two weeks and it was observed that all mice that were immunized with two doses using 60 µg EDIII antigen adjuvanted with M7-OH and CpG survived (100%, p=0.02), and were also protected from lossing body weight (98%; p= 0.02) compared to naïve mice (Figure 3C, D). The mice that received three-doses of 15 µg EDIII antigen with adjuvants preserved 96% of prechallenge body weight while 93% of the mice survived upto 2 weeks after challenge. The preservation of body weight and the survival rates were not significant in the three-dose group when compared to naïve mice who had 92% and 60% body weight and survival respectively. Similarly, mice that received a two-dose immunization of 45 µg EDIII antigen adjuvanted with M7-OH and CpG preserved 95% of body weight and had 86% survival rate but were not significantly different from those of naïve mice (Figure 3C, D)

Long-term efficacy of the immune induction by the two-dose schedule developed was also evaluated. To determine presence of long-term immunity, mice were immunized as described above using either 45 or 60 µg EDIII antigen adjuvanted with M7-OH and CpG including the 60 µg EDIII antigen only control and the adjuvanted 15 µg EDIII antigen positive control. Mice were allowed to stay for a longer period after immunization, and blood samples collected on 182 to determine serum anti-EDIII antigen antibody titers. The mice were then challenged a week later with WNV NY99 strain 35262-11 on day 189, and followed up for 2 weeks to monitor body weight and survival rates. Analyses of day 182 blood samples showed that the three-dose regimen using adjuvanted 15 µg EDIII antigen induced significantly higher serum anti- EDIII IgG (6.12 x $10^7$ GMT), IgG1 (2.2 x $10^7$ GMT) and IgG2a (9.6 x $10^6$ GMT) antibodies compared to mice immunized with 60 µg EDIII antigen without adjuvants (Figures 4A and B). Similarly, two-dose regimen of adjuvanted 45 µg EDIII antigen induced significantly high anti-EDIII antigen specific IgG (8.0 x $10^6$

GMT), IgG1 (1.2 x $10^7$ GMT) and IgG2a (4.2 x $10^5$ GMT) as well as adjuvanted 60 µg EDIII antigen anti-EDIII IgG (4.12 x $10^5$ GMT), IgG1 (4.4 x $10^5$ GMT), and IgG2a (3.4 x $10^4$ GMT) compared to anti-EDIII IgG (4.4 x 10 GMT), IgG1 (3.7 x 10 GMT) and IgG2a (2.3 x 10 GMT) in mice immunized with 60 µg EDIII antigen without adjuvants (Figures 4A and B). Anti-EDIII antigen specific antibody subtype analysis showed that the three-dose schedule using adjuvanted 15 µg EDIII antigen induced significantly high IgG, IgG1 and IgG2a antibodies compared to adjuvanted 60 µg EDIII antigen ($p < 0.05$). Efficacy analysis showed that mice that were immunized with three-doses of adjuvanted 15 µg EDIII antigen or two-doses of adjuvanted 45 µg EDIII antigen were protected from weight loss (> 90%) compared to mice that received 60 µg EDIII antigen without adjuvants (87%), $p = 0.003$ (Figure 4C). On the other hand, survival rates showed that adjuvanted three-dose 15 µg EDIII antigen and both two-dose regimens of adjuvanted 45 and 60 µg EDIII antigen induced long-term protective immunity in mice that conferred >80% survival rate, significantly higher compared to naïve mice (40%) and mice that were immunized with non-adjuvanted 60 µg EDIII antigen (33%), $p = 0.011$ (Figure 4D). This study has demonstrated that a 2 dose WNV EDIII nasal vaccine administered on days 0 and 14 is able to provide rapid protection (at day 60) using 60 µg EDIII antigen adjuvanted with M7-OH and CpG, and also long-term protection (at day 189) using either 45 µg or 60 µg EDIII antigen combined with M7-OH and CpG adjuvants.

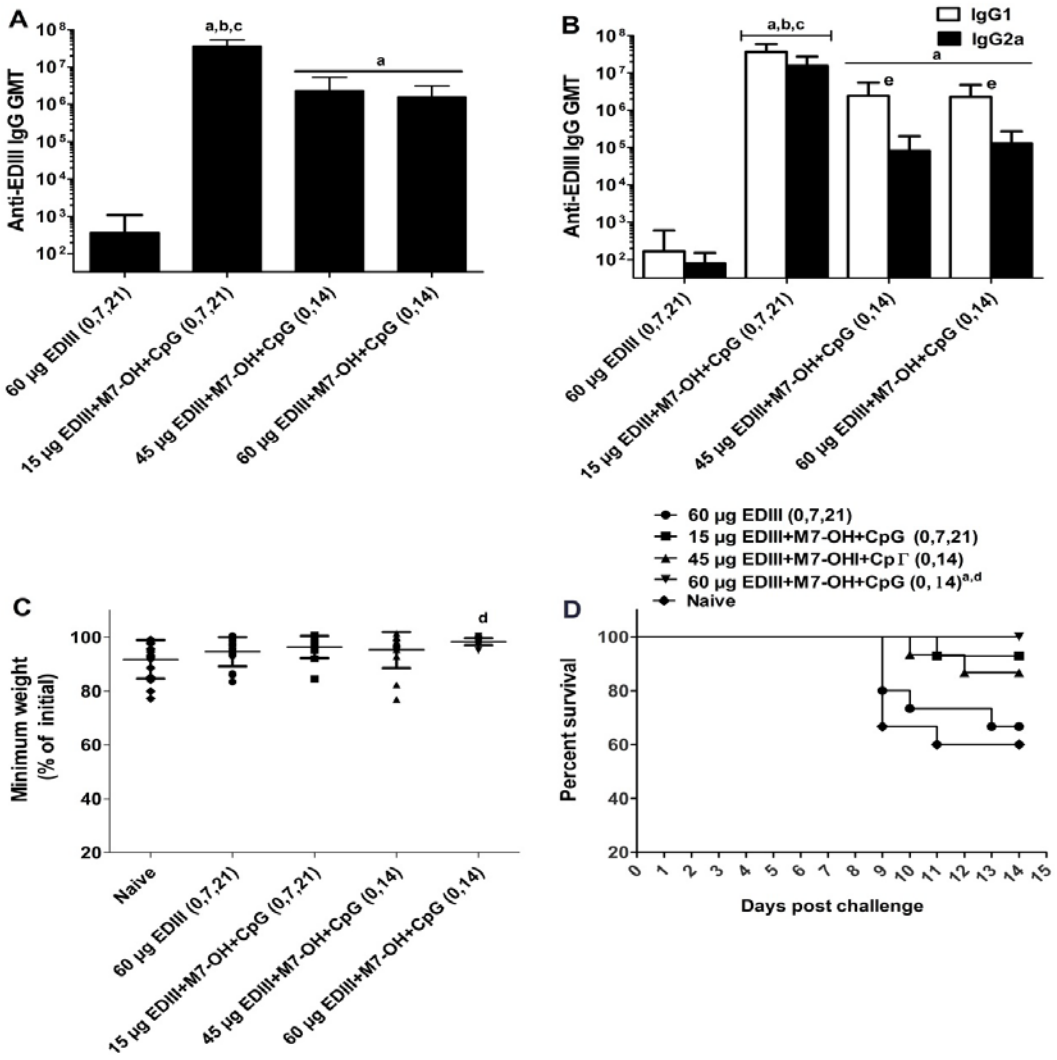


**Figure 3. Two-dose intranasal immunization with 60 µg EDIII antigen adjuvanted with CpG and M7-OH induces protective immune responses in mice against WNV challenge**. Female BALB/c mice were immunized I.N with either 60 µg EDIII antigen without adjuvant (n = 15) or 15 µg EDIII antigen adjuvanted with CpG and M7-OH (n= 15) on days 0, 7 and 21 or with 45 µg (n= 15) or 60 µg (n= 15) EDIII antigen adjuvanted with 10 µg CpG and 5 nanomoles M7-OH on days 0 and 14. Mice were bled on day 42 and serum samples were tested for anti-EDIII antigen specific antibodies using ELISA test. Mice were then challenged on day 60 with 1.0 x 10^5 ffu WNV NY 99 and monitored for morbidity and/or lethality for averagely two weeks. (**A**) Serum anti-EDIII antiugen specific IgG, (**B**) Serum anti-EDIII antigen specific IgG1 and IgG2a, (**C**) Minimum body weight after challenge expressed as percent of pre-challenge body weight, (**D**) Percent survival after challenge with WNV NY99. **a** > 60 µg EDIII without adjuvant given on days 0, 7 and 21, **b** > 45 µg EDIII antigen adjuvanted with CpG and M7-OH, **c** > 60 µg EDIII antigen adjuvanted with CpG and M7-OH, **d** > naïve mice, **e** > IgG2a in the same vaccine group. In parenthesis are the immunization days. Antibody titers and body weights were analyzed

using 1 way ANOVA, with Tukey's Multiple Comparison Test while ssurvival proportions were analyzed using Chi-square Test (PRISM software). Differences were considered significant when $p < 0.05$.

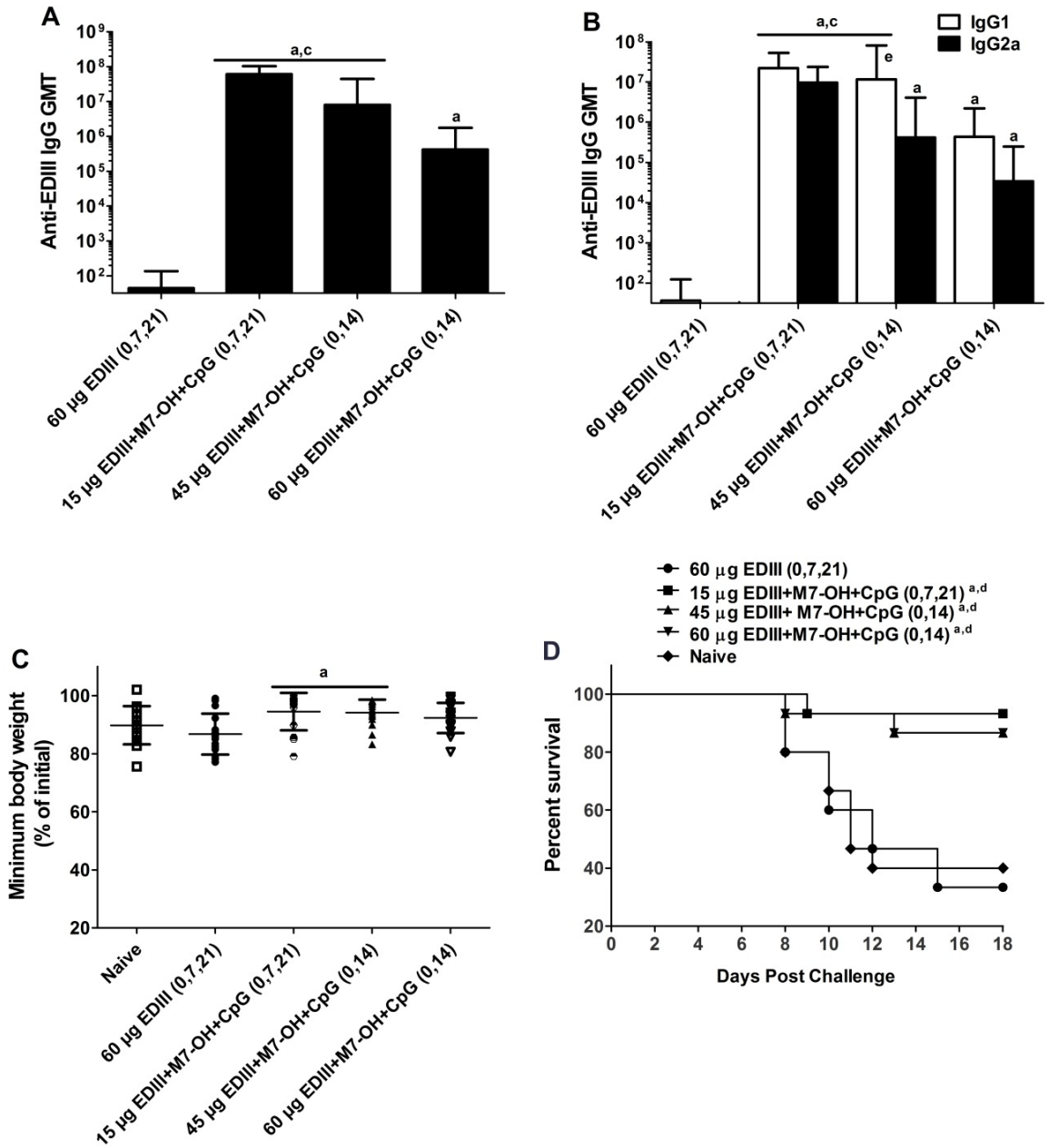


**Figure 4. Two-dose intranasal immunizations with 45 or 60 µg EDIII antigen adjuvanted with CpG and M7-OH induce effective and long-term anti-WNV immunity in mice.**

Female BALB/c mice were immunized I.N with either 60 µg EDIII antigen without adjuvant (n= 15) or with 15 µg EDIII antigen adjuvanted with 10 µg CpG and 5 nanomoles M7-OH (n= 15) on days 0, 7 and 21. Additional mice were given two-dose immunizations of 45 µg (n= 15) or 60 µg (n= 15) EDIII antigen adjuvanted with CpG and M7-OH on days 0 and 14. Mice were bled on 182 and their sera tested for anti-EDIII antigen-specific antibodies by ELISA method. Mice were later challenged on day 189 with 1.0 x 10^5 ffu of WNV NY99 and monitored for signs of morbidity and/or lethality for about two weeks. (**A**) Day 182 serum anti-EDIII antigen specific IgG, (**B**) Day 182 serum anti-EDIII antigen specific IgG1 and IgG2a, (**C**) Minimum body weight after challenge, expressed as percent of pre-challenge body weight, (**D**) Percent survival after challenge. **a** > 60 µg EDIII antigen without adjuvant given on days 0, 7 and 21, **c** > 60 µg EDIII antigen adjuvanted with CpG and M7-OH, **d >** naïve mice, **e** > IgG2a in the same vaccine group. In parenthesis are the immunization days. Antibody titers and body weight data were analyzed by 1 way ANOVA with Tukey's Multiple Comparison Test, while survival was analyzed using Chi-square Test**.** Differences were considered significant when $p < 0.05$.

### 3.4 Two-dose intranasal immunizations using 45 or 60 µg EDIII antigen adjuvanted with M7-OH and CpG induce IgG antibodies with comparable avidity to three-dose immunization with 15 µg EDIII antigen adjuvanted with M7-OH and CpG.

Since the antibody binding avidity to specific antigen has been shown to influence its protective capacity of the antibody [27, 28], the avidity of serum antibody binding to WNV EDIII was determined.. Day 42 and day 182 serum samples were tested antibody avidity using ELISA. It was observed that, at a lower concentration of 1 molar $NH_4SCN$, the avidity index of day 42 serum IgG from three-dose immunization using adjuvanted 15 µg EDIII antigen, and two-dose immunization using adjuvanted 60 µg EDIII antigen were greater than the avidity index of antibodies induced by adjuvanted 45 µg EDIII antigen (Figure 5A). Avidity test on day182 serum showed a higher avidity index for serum from three-dose schedule with adjuvanted 15 µg EDIII antigen compared to two-dose immunization with adjuvanted 60 µg EDIII antigen (Figure 5B) at 1 molar $NH_4SCN$. Further evaluation at using high concentration of 2 or 4 molar $NH_4SCN$, avidity index of serum for days 42 and 182 IgG were not significantly different between three-dose immunization using adjuvanted 15 µg EDIII antigen, and two-dose immunizations with either adjuvanted 45 or 60 µg EDIII antigen (Figure 5A, B). Comparison of avidity indices between day 42 and 182 serum IgG tested at 2 molar $NH_4SCN$ did not show any differences within each vaccine formulation (Figure 5C).

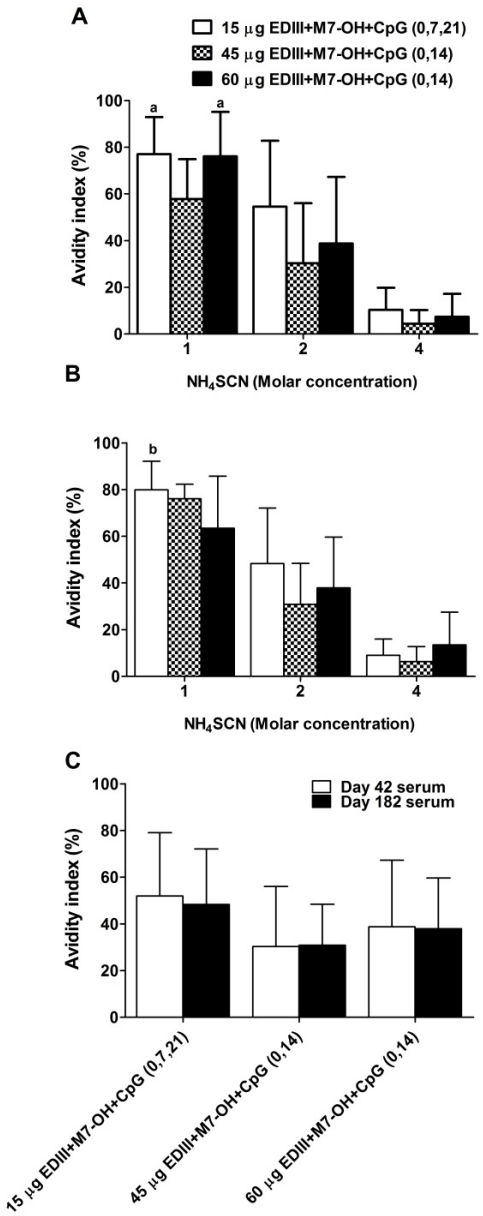


**Figure 5. Two-dose intranasal immunizations with 45 or 60 µg EDIII antigen adjuvanted with CpG and M7-OH induce anti-WNV specific IgG with similar avidity index to the three-dose immunization regimen using 15 µg EDIII antigen adjuvanted with CpG and M7-OH.**
Female BALB/c mice were immunized I.N as described in Figure 3 and Figure 4. Days 42 and 182 sera obtained as described in figures 3 and 4 were tested for IgG binding avidity to EDIII antigen using $NH_4SCN$ elution ELISA test. The ELISA relative light units were expressed as percent of zero molar $NH_4SCN$ treatment of serum samples, and then expressed as avidity indices. **(A)** Day 42 serum IgG binding avidity indices, **(B)** Day 182 serum IgG binding avidity indices, (**C**) Comparison of day 42 and day 182 avidity indices using 2 molar $NH_4SCN$. **a** > two-dose immunization with 45 µg EDIII antigen adjuvanted with CpG and M7-OH at 1 molar concentration ammonium thiocyanate, **b** > two-dose immunization with 60 µg EDIII antigen adjuvanted with CpG and M7-OH at 1 molar ammonium thiocyanate concentration (n=10-15 mice per group). Percent avidity was analyzed using ANOVA and Tukey's multiple comparison test (PRISM Software). Differences were considered significant when $p < 0.05$.

### 3.5 Two-dose intranasal immunizations using 45 or 60 µg EDIII antigen adjuvanted with M7-OH and CpG induce EDIII antigen-specific IFN-γ and IL-17.

After demonstrating that the developed two-dose regimen could induce effective immune responses against WNV infection, and having observed induction of antigen-specific antibody responses, we then evaluated if T cells responses were also induced. Mice were immunized intranasal using either three-dose regimen with 15 µg EDIII antigen adjuvanted with M7-OH and CpG or using a two-dose regimen with adjuvanted 45 or 60 µg EDIII antigen. Splenocytes were obtained from mice on day 49 and stimulated in culture with EDIII antigen to determine EDIII specific responses. On analysis of the supernatant, our results have shown that three-dose regimen using adjuvanted 15 µg EDIII antigen can induce high IL-4 (73.75 ± 53.1 pg/ml, $p < 0.0037$) production compared to naive cells (Figure 6A). The two-dose regimen with either 45 or 60 µg EDIII with adjuvants did not induce IL-4 production. However, two-dose immunizations with either 45 or 60 µg EDIII antigen with adjuvants induced high IFN-γ (145.72 ± 193.35 pg/ml, 45 µg; 265.31 ± 276.07 pg/ml, 60 µg) production than naïve cells, although they were significantly lower than that induced by the three-dose regimen of adjuvanted 15 µg EDIII antigen (1389+1053 pg/ml) (Figure 6B). We also observed that all the three vaccine formulations, the three-dose immunization with adjuvanted 15 µg EDIII antigen and the two-dose schedules using 45 or 60 µg EDIII antigen with adjuvants induced significantly high levels of IL-17 compared to naïve mice and those that were immunized with 60 µg EDIII antigen without adjuvants. The three-dose regimen induced 3905.44 ± 3134.63 pg/ml of IL-17 while the two-dose regimens of 45 µg EDIII with adjuvants and 60 µg EDIII with adjuvants induced 1166.13 ± 1007.40 pg/ml and 2332.38 ± 1782.63 pg/ml IL-17 respectively. Naïve mice responded with very low IL-17 of 18±16 pg/ml while those that received the 60 µg EDIII antigen without adjuvants responded with 71±78 pg/ml IL-17 (Figure 6C). Therefore, IL-17 production was comparable between the three-dose regimen and two-dose regimens.

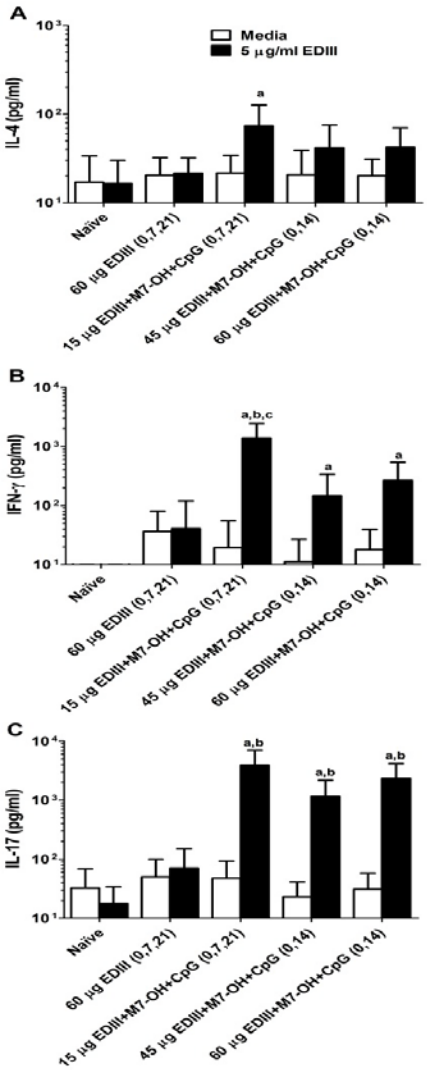


**Figure 6. Intranasal two-dose immunizations with 45 or 60 µg EDIII antigen adjuvanted with CpG and M7-OH induce anti-WNV specific IFN-γ and IL-17 cytokines in mice.**
Female BALB/c mice were immunized I.N with either 60 µg EDIII antigen without adjuvant (n = 10) or 15 µg EDIII antigen adjuvanted with CpG and M7-OH (n= 10) on days 0, 7 and 21 or with 45 µg (n= 10) or 60 µg (n= 10) EDIII antigen adjuvanted with 10 µg CpG and 5 nanomoles M7-OH on days 0 and 14. Naïve mice (n=10) were housed with the immunized mice for the entire period for splenocyte harvest. Splenocytes were harvested on day 49 and cultured with media or re-stimulated with EDIII antigen (5 µg/ml) for 60 hours. Supernatants were collected and tested for cytokine concentrations of IL-4, IFN-γ and IL-17 using a multiplex bead assay (BioPlex). **(A)** IL-4, **(B)** IFN-γ, **(C)** IL-17; **a** > Naïve mice, **b** > 60 µg EDIII antigen without adjuvant, **c** > 45 µg EDIII antigen adjuvanted with CpG and M7-OH. In parenthesis are the immunization days. This data is from one experiment (n=9-10 mice per group). Cytokine concentrations were log10 transformed and analyzed using ANOVA and Tukey's multiple comparison test (PRISM Software). Differences were considered significant when $p < 0.05$.

## 4. Discussion

In this study we have demonstrated that a two-dose vaccine immunization that can be completed rapidly in two weeks (day 0 and 14) can induce protective immunity to an infectious flavivirus. The study has shown that a rapid needle-free I.N immunization using 60 µg EDIII antigen with M7-OH and CpG adjuvants on days 0 and 14 can give 100% protection to mice against WNV (day 60) (Figure 3D). In addition, it has been shown that a two-dose quick immunization using 45 µg or 60 µg EDIII antigen with adjuvants can provide long-term protection to mice when exposed later to WNV infection as observed on day 189 challenge (Figure 4D). Protection at day 189 indicated that two immunizations could generate long-term immunity. These observations are interesting because short immunization schedules are advantageous because they minimize cases of missed immunizations [29] and can lead to high rates of vaccine coverage. A two-dose schedule that can be completed within two weeks is most suitable for combating re-emerging infections such as WNV that require rapid intervention measures.

Other studies have also reported induction of protective immune responses using EDIII antigen, although by a subcutaneous route of administration. The study used 25 µg EDIII antigen with CpG adjuvant administered ones on day 0 and boosted by oil-emulsion on day 14, and demonstrated significant protection to C57BL/6 mice. When challenged on day 42 with WNV NY99, 80% survived compared to naïve mice [13]. This study compares well with out study when

mice were challenged on day 60. Mice that had received two-doses of 60 µg EDIII antigen with adjuvants on days 0 and 14 were protected from infection by WNV (Figure 3D). Our study presents additional advantage because it uses a pain-free I.N route of vaccine administration as opposed to subcutaneous route that is painful and with higher safety risks, and also requires highly trained personnel to administer the vaccine. The use of CpG as adjuvant has been shown to augment effective immune responses to WNV infection. In a related study where they used a S.C vaccine, significant protection against WNV infection was also reported in C3H/HeN mice using a single immunization. The study used 2.5 µg WNV envelope protein encapsulated with CpG nanoparticle adjuvant [26]. These two studies support our findings that have demonstrated the induction of WNV protective immunity with WNV envelope proteins combined with CpG. In our studies, CpG was only effective as an adjuvant in the presence of M7-OH cationic peptide through the intranasal route of administration. Our study has demonstrated that the combination of cationic M7-OH and CpG is required for effective intranasal adjuvant activity. Previous efforts towards WNV vaccine development have also employed a live attenuated chimeric vaccine (Chimerivax-WN02), hat contains the pre-membrane and envelope proteins of WNV NY99 in a yellow fever 17D vaccine virus. The vaccine was administered as a single S.C injection and induced WNV neutralizing antibodies in >90% of human vaccinees [30]. The disadvantage of this vaccine is that although it required only one delivery, the fact that it is injectable and live attenuated increases the potential for being unsafe compared to I.N delivered recombinant protein used with cationic M7-OH and CpG in our studies.

In this study, intraperitoneal infection of naïve BALB/c mice with WNV strain NY99 35262-11 caused 40% and 60% mortality at days 60 and 189 respectively (Figures 3D and 4D). Compared to other studies, there is a lot of variability in the vulnerability of naïve mice to WNV infections similar to our observations. For example, studies by Qiao M et al reported 20 % mortality [31], while Liu and others reported 60% mortality after intraperitoneal infection of BALB/c mice with WNV NY99 [32]. Therefore, it is not uncommon that we observed low mortality rates in non-immune BALB/c mice in our studies. However, the lack of complete mortality in non-immunized mice is challenging to vaccine evaluation studies in determining the benefits of vaccines especially if it is partially protective and not 100%.

Some vaccine studies have reported that the failure by a vaccine to induce sufficiently mature antibodies can lead to lack of protection [33]. This implies that induction of high avidity antibodies by vaccines is desired because it can be used as indicator of antibody maturity [34, 35]. In our study, we demonstrated presence of similar serum anti-EDIII IgG avidity index between three-dose and two-dose immunizations regimens when tested at 2 or 4 molar $NH_4SCN$ (Figure 5). We also failed to see any differences in IgG avidity index between short-term (day 42) and long-term (182) (Figure 5C) immune serum, which implied that antibody quality and strength was preserved for over 6 months through day189. The preservation of antibody quality was demonstrated with significant protection of mice upon challenge with WNV on day 189.

Effective protection against virus challenge may indicate neutralization of the virus by the immune system. Some studies have demonstrated that generation of measurable virus neutralizing antibodies correlate with protection against the virus [36]. In our current study however, we did not detect virus neutralization in serum samples from all vaccine groups (data not shown). The fact that we observed in vivo protection against WNV challenge despite lack of in vitro virus neutralization activity is a clear indication that additional immune factors may play a role in determining antibody-neutralizing capacity and in vivo protection. Other studies have shown that in some cases epitopes available in vivo may not be available in sufficient amounts on mature virions in vitro to allow neutralization [37], which may explain the presence of in vivo protection in the absence of in vitro virus neutralization. Some factors that may also influence in vitro virus neutralization include lack of epitope accessibility or their poor surface distribution [37, 38]. On the other hand, in vivo protection against virus challenge may be enhanced by other factors such as antibody opsonization and T cell responses [39].

This study has also demonstrated that increasing the antigen dose is one strategy that can be used to reduce the number of immunization doses. When the EDIII antigen was increased from 15 µg to 45 µg or 60 µg, protective anti-WNV immunity was generated that was greater than three-dose immunizations with 15 µg EDIII antigen at day 60. However, for long-term protection, the two-dose regime generated protective immune responses comparable to three-dose regimen with 15 µg EDIII antigen (Figures 3D and 4D). This observation is also supported by previous studies which showed that I.P viomycin antigen with FCA/FICA induced maximum antibody responses when the optimum antigen dose of 10 µg was used with less booster immunizations than when a lower dose of 3 µg was used [40]. This observation shows a three-fold increase in the antigen (3 to 10 µg) for maximum antibody responses, which is similar to our study where 15 µg was increased, 3-fold (45 µg) or 4-fold (60 µg) to induce maximum immune responses with fewer immunizations.

Findings from this study also shown that a lower dose of 45 µg EDIII antigen can induce greater anti-EDIII antigen-specific IgG and IgG1 antibodies compared to 60 µg EDIII (Figure 4A and B). Although such an observation was surprising and unexpected because both antigen doses were administered using the same schedule, another has also shown that cytomegalovirus glycoprotein B adjuvanted with MF59 induced higher antibody titers with a lower antigen dose compared to a higher antigen dose [41]. Similarly, studies using viomycin showed that a higher antigen dose than the optimum can lead to lower antibody responses [40]. In this regard, there is an optimum antigen dose required for immune activation, and excess antigen may lead to immune tolerance.

In addition to the antigen dose, studies have shown that the interval between the primary and booster immunizations may influence the magnitude of immune responses especially if less than optimum antigen dose is used [40]. In our study, two-dose immunization with 30 µg EDIII antigen with M7-OH and CpG adjuvants induced high IgG antibody titers when administered on days 0 and 14 than when given days 0 and 21 (Figure 1). In a related study, it has been shown that I.N meningococcal subtype B vaccine given on days 0, 7, 28 and 56 elicited immune responses with bactericidal activity that were greater than elicited by a day 0, 28 and 56 schedule [42]. The observation in the meningococcal vaccine implied that the addition of day 7 in the schedule that shortened the interval between the primary and first booster immunization might have enhanced induction of bactericidal antibody production. In our study we have shown that even with a lower dose (15 µg) a day 0, 7 and 21 regimen induced higher IgG antibody responses that day 0 and 21 using 3-fold (45 µg) or 4-fold (60 µg) increased antigen dose (Figure 3A). Therefore, our study as the meningococcal vaccine study suggests that early boosting may enhance the magnitude of immune responses.

The adjuvant combination of M7-OH and CpG led to induction of both $Th_1$ and $Th_2$ type humoral immune responses characterized by IgG2a and IgG1 respectively (Figures 1B, 2B, 3B, and 4B). In this adjuvant combination, CpG and M7-OH could be responsible for inducing Th1 and Th2 type responses respectively, because previous studies have shown CpG to generate predominantly $Th_1$ [18], while cationic peptides induced Th2 type responses [21, 43]. Studies using cationic peptide HH2 or the polymer (PLGA) combined with CpG adjuvants have also shown induction of IgG1 and IgG2a antibodies [21], demonstrating that combination of cationic and CpG adjuvants can generate both Th1 and Th2 type antibodies. On the other hand predominantly $Th_1$ and $Th_{17}$ T cell responses characterized by IFN-γ and IL-17 were observed with two-dose immunization, while in addition to $Th_1$ and $Th_{17}$, $Th_2$ (IL-4) type response was observed with three-dose immunization schedule (Figure 6). Previous studies with combination adjuvant IC31 that comprises oligodeoxynucleotide (ODNa1) and cationic $KLKL_5KLK$ peptide have shown induction of T and B cell responses with $Th_1$ dominance [44], and also a $Th_1$ cellular and, $Th_1$ and $Th_2$ humoral responses [45], indicating diversity of the immune responses by combined adjuvants. T cell responses including IFN-γ have been shown to protect against WNV infection [46, 47], but the role of IL-17 in protection is not yet clear [48, 49]. In our study, like others we have shown that combination of a cationic peptide and TLR ligand forms a potent adjuvant platform for vaccines to induce both $Th_1$ and $Th_2$ type responses. Induction of immune responses from both $Th_1$ and $Th_2$ arms improves protective capacity of the generated immunity because different pathogens may respond differently to either type of the immune response.

Our study has also shown that two-dose I.N immunizations using 45 or 60 µg EDIII antigen with M7-OH and CpG adjuvants can induce long-term protective immunity against WNV (Figure 4). Induction of durable immunity is desirable because it eliminates the need for booster immunizations. Previous studies link the use of vaccine adjuvants to the induction of long-term immunity [50]. Therefore, in our study, the inclusion of M7-OH and CpG adjuvants may have contributed to the induction of long-term protective immunity observed after challenge of mice on day 189. We observed that mice immunized with EDIII antigen and adjuvants had greater immune responses than mice immunized with EDIII antigen alone. We observed that adjuvanted 45 µg or 60 µg EDIII antigen provided significant protection to mice when challenged with WNV on day 189.

In conclusion, by 3-fold or 4-fold increase of EDIII antigen from 15 µg to 45 µg or 60 µg, and combining with mast cell activating cationic peptide M7-OH and immunostimulatory molecule CpG adjuvants, we have developed a two-dose intranasal WNV vaccine regimen that induces maximum and protective immunity using a day 0 and 14 schedule. In addition, this formulation and regimen induces rapid anti-WNV immunity that is durable (day 189). This study has therefore developed M7-OH and CpG as a combination adjuvant platform for intranasal vaccines to induce both immediate and long-term immune responses that can be evaluated for other vaccine antigens.

## Conflicts of Interest

The authors declare that they do not have competing interests.

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