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A Single Dose of NILV-Based Vaccine Provides Rapid and Durable Protection against Zika Virus

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Zika virus, a member of the *Flaviviridae* family, is primarily transmitted by infected *Aedes* species mosquitoes. In 2016, Zika infection emerged as a global health emergency for its explosive spread and the remarkable neurological defects in the developing fetus. Development of a safe and effective Zika vaccine remains a high priority owing to the risk of re-emergence and limited understanding of Zika virus epidemiology. We engineered a non-integrating lentiviralvector(NILV)-based Zika vaccine encoding the consensus pre-membrane and envelope glycoprotein of circulating Zika virus strains. We further evaluated the immunogenicity and protective efficacy of this vaccine in both immunocompromised and immunocompetent mouse models. A single immunization in both mouse models elicited a robust neutralizing antibody titer and afforded full protection against Zika challenge as early as 7 days post-immunization. This NILV-based vaccine also induced a long-lasting immunity when immunized mice were challenged 6 months after immunization. Altogether, our NILV Zika vaccine provides a rapid yet durable protection through a single dose of immunization without extra adjuvant formulation. Our data suggest a promising Zika vaccine candidate for an emergency situation, and demonstrate the capacity of lentiviral vector as an efficient vaccine delivery platform.

INTRODUCTION

Zika virus (ZIKV) is one of the *Flaviviridae* family members, an enveloped, single-stranded RNA virus, transmitted via infected *Aedes* species mosquito. The genome of ZIKV encodes three structural proteins (the capsid, pre-membrane/membrane, and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). ZIKV was first identified in the Zika forest of Uganda in 1947, but it was not until 2015 that ZIKV started to capture researchers' attention due to an association between ZIKV and Guillain-Barré syndrome (GBS) as well as congenital birth defects.[1,2] Due to the biological plausibility of ZIKV infection, ZIKV emerged as a global health concern, leading to the declaration of a public health emergency by the World Health Organization (WHO) in 2016. Although the incidence of ZIKV infection substantially decreased in 2017 and 2018, the risk of re-emergence or re-introduction of ZIKV into the population should not be underestimated provided that 61 countries globally have established evidence of competence of local *Aedes aegypti* vectors.[3] The risk of re-emergence therefore places an additional demand on a ZIKV vaccine to confine the disease.

ZIKV is transmitted sexually between the sexes, and, once infected, ZIKV can persist long term in body fluids.[4] Infection during pregnancy has emerged as a threat due to risk of fetal microcephaly and miscarriages.[2,5,6] These serious clinical manifestations of ZIKV infection place pregnant women, as well as men and women of reproductive age, at high priority for protection. The development of Guillain-Barré syndrome and neurological disease in adults[7] puts additional emphasis of vaccination on other age groups. Major ZIKV-affected areas have significant healthcare barriers, making these susceptible populations difficult to reach and hard to track for subsequent vaccine booster administration. Multiple Zika vaccine candidates have shown promising results in controlling ZIKV infection in preclinical models, including DNA-based,[8–10] RNA-based,[11] and lipid-nanoparticle-encapsulated nucleoside-modified mRNA (mRNA-LNP) vaccines.[12] However, these candidates required adjuvant and multiple-dose administration, therefore falling short in addressing the aforementioned constraints. Hence, a key attribute to an emergency Zika vaccine is to generate effective and rapid protection response via a single-dose vaccination. With the single-dose format, higher vaccination opportunities and improved vaccine safety could be assured under unhygienic conditions in ZIKV epidemic areas.

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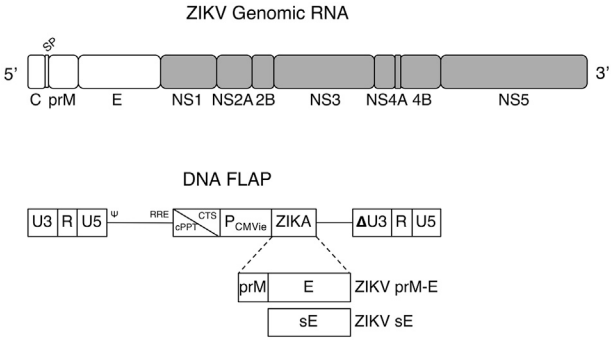

Figure 1. ZIKV Vaccine Development

Organization of ZIKV genomic RNA consists of structural proteins (white boxes) and non-structural proteins (gray boxes) (top), and schematic representation of lentiviral vectors backbone, LV DNA FLAP (bottom), encoding two different ZIKV antigen candidates: the prM signal peptide followed by the full-length Zika envelope (prM-E), and the soluble Zika envelope (sE). C, capsid; SP, signal peptide; TMD, transmembrane domain; RRE, rev response element; cPPT, central polypurine tract; CTS, central termination sequence; P_{CMVie}, cytomegalovirus immediate early promoter.

Lentiviral vector (LV) is a promising vaccine vector to deliver and express engineered antigens to induce adaptive immunity and fight against infectious diseases.[13–15] LV targets non-dividing cells such as dendritic cells and leads to continued antigen expression throughout the lifetime of the transduced cells.[14,16,17] The sustained antigen presentation in immune cells is suggested to contribute to the robust humoral and cellular immune response observed in a spectrum of LV vaccines. LV is beneficial for vaccine delivery owing to the lack of pre-existing immunity in humans as well as its high encoding capacity for transgenes, making simultaneous expression of polyantigenic peptides feasible.[18] Despite the aforementioned benefits, the integrative nature of LV has raised considerable safety concerns in human application. To avoid the risk of insertional mutagenesis, we engineered a non-integrating LV (NILV), which carries a defective HIV-1 integrase mutant.

Herein, we propose a safe NILV Zika vaccine that is highly immunogenic and provides rapid immunity as well as long-term protection upon immunization, while requiring only a single dose of administration without adjuvantation. Our Zika vaccine encodes the pre-membrane and envelope (prM-E) proteins, and it induces a robust protective antibody response that strongly suppresses viral replication in immunocompromised mice and results in sterilizing immunity in immunocompetent mice. Altogether, this NILV ZIKV prM-E fulfills the WHO recommendations for Zika vaccine and represents a promising vaccine candidate against ZIKV infection.

RESULTS

ZIKV Vaccine Design

The envelope (E) protein of flaviviruses is the main target of neutralizing antibodies.[19] The proper folding of E protein requires the co-expression of both prM and E proteins, which had been demonstrated for Japanese encephalitis virus (JEV), another virus in the genus *Flavivirus*.[20] However, for West Nile virus (WNV), a soluble E protein lacking the membrane anchoring region was shown to generate an efficient neutralizing response.[21,22] Therefore, we constructed two versions of integrating LV (ILV) ZIKV vaccine, encoding either the prM signal peptide followed by the full-length envelope (prM-E) or the soluble envelope (sE) immunogens (Figure 1) based on the consensus sequence of circulating ZIKV strains (Figures S1 and S2). First, we compared the immunogenicity of ILV encoding either prM-E or sE in C57BL/6 mice by intraperitoneal (i.p.) immunization with 5×10^6 transducing units (TU) of ILV ZIKV prM-E, ILV ZIKV sE, or ILV green fluorescent protein (GFP) as control. Three weeks after the first immunization, we performed a subsequent booster immunization. Detection of anti-E antibodies was performed using recombinant envelope domain III (EDIII) from ZIKV.[23] After the first immunization, both ZIKV constructs induced comparable envelope-specific immunoglobulin G (IgG) antibody titers. Following the second immunization, a 10-fold increase in antibody response was observed in ILV ZIKV sE-immunized mice. However, ILV ZIKV prM-E-immunized mice experienced no changes in antibody titers between the first and second immunization, indicating that the prime-boost regimen has no effect in increasing the antibody titer of ILV ZIKV prM-E immunization (Figure 2A). To determine whether antibodies from immunized mice can neutralize ZIKV *in vitro*, we performed focus reduction neutralization tests (FRNTs) 4 weeks after immunization. We analyzed the neutralizing ability of these antibodies on both ZIKV strains PF13 and HD78788, which represent the Asian and African ZIKV lineages, respectively. Despite higher antibody titers, sera from ILV ZIKV sE-immunized mice weakly neutralized the ZIKV strain PF13 after prime-boost immunization, when compared to the ILV ZIKV prM-E counterpart (Figure 2B). Interestingly, antibodies elicited by ILV ZIKV prM-E can efficiently cross-neutralize both ZIKV strains (Figures 2B and 2C). The differences observed in the neutralizing ability of ILV ZIKV sE and prM-E toward ZIKV could be due to the differences in avidity of the antibodies. The ability to neutralize both ZIKV strains makes prM-E a better candidate for a prophylactic vaccine.

A Single Dose of NILV ZIKV prM-E Induced High Cross-Neutralizing Antibody Titers

The use of integrating viral vector often poses risk of mutagenic events, and immunization with ILV-based vaccine is not ideal in a target population that includes pregnant women. To obviate the risk of mutagenic events, we eliminated the viral integration by using an NILV carrying a catalytically dead integrase with a point mutation in the integrase catalytic domain. The defective integrase leads to accumulation of NILV-harbored transgenes as episomal elements in the nucleus, and this defectiveness does not inhibit the sustained transgene expression.[24] However, NILV suffers a slight reduction in immunogenicity that can be compensated with an increased dose.[25] In this context, we characterized the optimal dose of NILV ZIKV prM-E that provides comparable immunogenicity as 5×10^6 TU of ILV ZIKV prM-E in C57BL/6 mice. We titrated several doses of NILV ZIKV prM-E, and sera were collected for antibody titer analysis at 3 weeks post-immunization. A high EDIII-specific IgG antibody

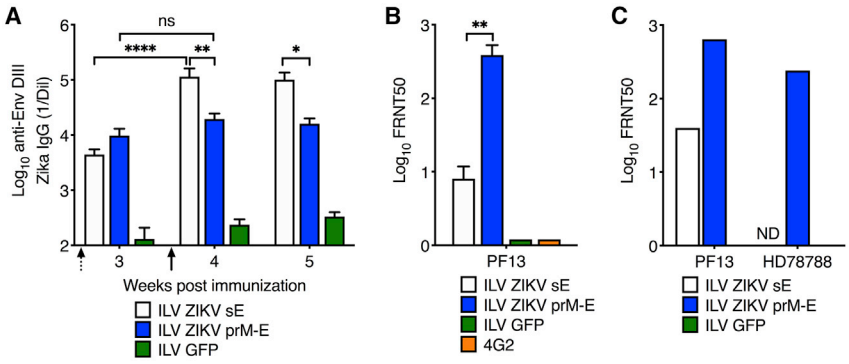

Figure 2. ILV ZIKV prM-E Rather Than sE Elicits High Neutralizing Activity

C57BL/6 mice (n = 5) were injected i.p. with 5×10^6 TU of ILV ZIKV sE (white), ILV ZIKV prM-E (blue), or ILV GFP (green). (A) EDIII-specific IgG antibody titers were determined by ELISA after the initial priming (dotted arrow) and boosting (black arrow) with the respective ILV vaccines. (B) Neutralization activity of serum samples against ZIKV 4 weeks post-immunization. The FRNT50 titers were determined by a FRNT assay using the pan-flavivirus non-neutralizing monoclonal antibody 4G2 as control and serially diluted sera from immunized C56BL/6 mice in the presence of ZIKV PF13. Results are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. (C) Sera from immunized mice was collected and pooled at 4 weeks post-immunization to determine the FRNT50 titer for ZIKV PF13 and HD78788.

titer of more than 1×10^4 was induced by 5×10^7 TU of NILV, and this antibody titer was comparable to that obtained with 5×10^5 TU of ILV (Figure 3A). With the aim of performing a lethal ZIKV challenge, we also monitored the immunogenicity of our vaccine in interferon α/β receptor knockout (A129) mice, a mouse model susceptible to Zika infection. Groups of A129 mice (n = 6/group) were injected i.p. with a single dose of NILV ZIKV prM-E (2×10^7 TU), ILV ZIKV prM-E (5×10^6 TU), or ILV GFP (5×10^6 TU), and EDIII-specific IgG antibody titers were measured at 2 and 3 weeks post-immunization. Both ILV and NILV ZIKV prM-E induced a high level of EDIII-specific IgG antibody titers at all time points, with no statistical differences between the two vectors (Figure 3B). We also found no statistical difference in the neutralizing titer of mice immunized with ILV or NILV ZIKV prM-E when examining the neutralizing capacity on ZIKV strain HD78788 (Figure 3C). We further showed that antibodies from NILV ZIKV prM-E-immunized mice can neutralize both HD78788 and PF13 ZIKV strains (Figure 3D). FRNT50 titers were 2.64 logs (FRNT90 of 1.68 logs) and 2.98 logs (FRNT90 of 2.02 logs) for HD78788 and PF13 ZIKV strains, respectively. We also showed that *in vitro* NILV ZIKV prM-E transduction induced release of prM-E virus-like particles (VLPs), suggesting that prM-E VLPs could partly contribute to the high neutralizing antibody response after *in vivo* NILV immunization (Figure 3E).

NILV ZIKV prM-E Provides Robust Protection against Lethal Challenge in the A129 Mouse Model

We evaluated the protective efficacy of the NILV ZIKV prM-E in equal number of male and female A129 mice (n = 6/group). These immunocompromised mice are highly susceptible to ZIKV, and ZIKV infection in these mice causes high lethality.[26,27] At 4 weeks post-immunization with a single dose of NILV ZIKV prM-E, mice were challenged i.p. with 10^2 plaque forming units (PFU) of the mouse-adapted Zika African strain HD78788. Mortality, body weight, temperature, and viremia level were monitored daily for a period of 28 days after infection. As expected, mice immunized with the control vector ILV GFP did not survive (Figure 4A), as they developed 6 days of high viremia with a peak viral load of 1×10^{10} copies/mL at day 3 after infection (Figure 4B). Alternatively, all recipients of NILV ZIKV prM-E survived the infection with minimal fluctuation in weight and temperature (Figure 4A). Viral replication was strongly suppressed in vaccinated mice, as indicated by a 5-log reduction in viremia level in immunized mice when compared to GFP-immunized mice (Figure 4B). We also observed similar protective efficacy in mice immunized with 5×10^6 TU of ILV ZIKV prM-E, which was performed simultaneously with NILV ZIKV prM-E using the same experimental control (Figures S3A and S3B). Of note, we observed a different protective outcome according to the sex of immunized mice. Vaccinated female mice were fully protected, while male mice experienced a small blip of viremia at a mean value of 10^5 RNA copies/mL at 3 days after challenge (Figure S3B). Although male mice showed a small blip in viremia, they still experienced 4-log lower viremia than did the control ILV-GFP group. When checking for presence of virus in organs, we detected no infectious viral particles in NILV ZIKV prM-E-immunized mice using a median tissue culture infectious dose ($TCID_{50}$) assay (Figure 4C). When using qRT-PCR for viral RNA detection, NILV ZIKV prM-E-immunized mice demonstrated up to a 7-log reduction of viral load in organs of immunized-mice (Figure S3C). The residual viral RNA in organs of immunized mice does not constitute a threat to transmission, and viral RNA has been shown to persist in organs even after viral clearance.[28,29] To examine whether ZIKV challenge boosted immune responses, neutralizing activity was measured at 0, 5, 14, and 29 days after challenge with the Zika African strain HD78788. Vaccinated mice showed an increase in neutralizing titer from 7×10^2 to 1×10^4, which proved that the neutralizing ability of the antibodies elicited by NILV were further improved upon ZIKV infection (Figure 4D).

A Single Dose of NILV ZIKV prM-E Vaccine Induces an Early and Durable Protection against Challenge

Due to previous observation that a small blip of viremia was observed in male- but not in female-immunized mice, we analyzed the kinetics of EDIII-specific IgG and IgM antibodies, taking into account the sex of the immunized mice. We immunized i.p. A129 mice containing an equal number (n = 7) of male and female mice with NILV ZIKV prM-E and analyzed EDIII-specific antibodies at 1, 2, 3 and 4 weeks post-immunization. At 1 week post-immunization, we detected a high level of EDIII-specific IgG antibody titers: 2.3×10^4 for male-

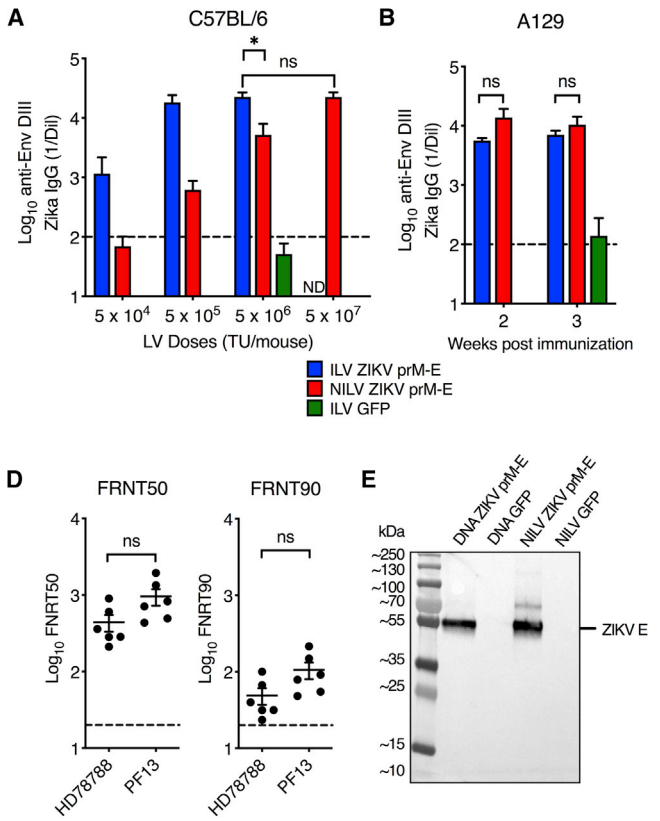

Figure 3. NILV ZIKV prM-E Elicits High Antibody Levels with Neutralizing Activity

(A) C57BL/6 mice (n = 6) were injected i.p. once with a range of doses of ILV ZIKV prM-E (5×10^4 to 5×10^6 TU) (blue), NILV ZIKV prM-E (5×10^4 to 5×10^7 TU) (red), or ILV GFP (5×10^6 TU) (green) and EDIII-specific IgG antibody titers were evaluated 3 weeks later. (B) A129 mice (n = 6) were injected i.p. with a single dose of 2×10^7 TU of NILV ZIKV prM-E or ILV GFP as negative control. Sera were collected at 2 and 3 weeks post-immunization to determine the EDIII-specific IgG antibody titers. (C) The neutralizing antibody titers were determined at 4 weeks post-immunization by FNRT as in Figure 1. Each symbol stands for one mouse. (D) The neutralizing ability of sera collected from A129 mice after 4 weeks post-immunization on ZIKV PF13 and HD78788. Black dotted lines represent the initial serum dilution. Data were expressed as means ± SEM. *$p < 0.05$. (E) HEK293T cells were transfected with DNA ZIKV prM-E (positive control) and DNA GFP (negative control), or transduced with NILV ZIKV prM-E and NILV GFP (negative control). VLP-containing supernatants were revealed using 4G2 antibody via a western blot. The size of the protein ladder is indicated on the left, and the black line on the right indicated the position of the ZIKV E protein.

immunized mice and 4.2×10^4 for the female counterpart. The level of EDIII-specific IgG antibody doubled at 2 weeks post-immunization in both male- and female-immunized mice to 5×10^4 and 8×10^4, respectively. At 3 and 4 weeks post-immunization, the level of EDIII-specific IgG antibody plateaued and remained comparable to antibody titers at 2 weeks post-immunization (Figure 5A, left panel). Alternatively, the level of EDIII-specific IgM antibody peaked at 1 week post-vaccination, but the level started to decrease significantly at 2 weeks post-vaccination. The level of EDIII-specific IgM antibody was barely detectable at 3 weeks post-vaccination (Figure 5A, right panel). We also noticed that the average EDIII-specific IgG antibody level in immunized female mice, although not statistically significant, was always higher than that in male mice regardless of the time points after immunization (Figure 5A). The higher amount of antibody observed in female mice compared to male mice could account for the better protection observed in female than male mice during challenge experiments.

An effective vaccine is one that can respond rapidly and provide lifelong immunity. Therefore, we evaluated the neutralizing ability and the protective efficacy of the NILV ZIKV prM-E in A129 mice at various durations after immunization. In another independent experiment, we looked into the neutralizing ability of the total antibodies at 1, 2, 3, and 24 weeks post-immunization. We noticed that the neutralization antibody titer increased over time, with neutralizing titers of 1×10^2, 1.5×10^2, 6×10^2, and 1.4×10^3 (Figure 5B). At 1, 2, 3, and 24 weeks post-vaccination, mice were challenged with the mouse-adapted Zika African strain HD78788. All vaccinated mice survived the infection with no detectable viremia in sera, except for mice challenged 24 weeks after immunization, where a small blip of viremia was detected at 6 days after ZIKV challenge (3,790 RNA copies/mL) (Figure 5C). We checked the presence of infectious virus in testes, ovary, and brain of challenged A129 mice. As expected, the placebo animal experienced a mean level of 10^5 $TCID_{50}$ value per milligram of tissue in the testes and brain while vaccinated animals had no detectable infectious virus in the testes and brain 1 month after challenge (Figure 5D). Vaccinated animals experienced a 4-log reduction in infectious viral load, which is similar to viral RNA detection using qRT-PCR showing a 5- to 6-log viral load reduction (Figure S4). This demonstrated that NILV ZIKV prM-E vaccine confers long-lasting protection not only up to 24 weeks after vaccination, but also as early as 7 days after vaccination.

The NILV ZIKV prM-E Is More Efficient Than a DNA prM-E Vaccine

We further validated the efficacy of our NILV ZIKV prM-E vaccine by performing a comparison study with a DNA prM-E vaccine in immunocompetent BALB/c mice challenged with an Asian strain of ZIKV (PF13). We chose to use the DNA flap plasmid backbone containing cytomegalovirus (CMV) promoter and prM-E antigen as plasmid DNA vaccine (Figure 1, bottom). Therefore, the DNA vaccine encodes identical promoter and prM-E sequence as that of the NILV vaccine (Figure S2). The experimental setup performed for DNA vaccination was similar to Larocca et al.,[9] where single immunization of 50 μg of a plasmid DNA vaccine encoding ZIKV prM-E

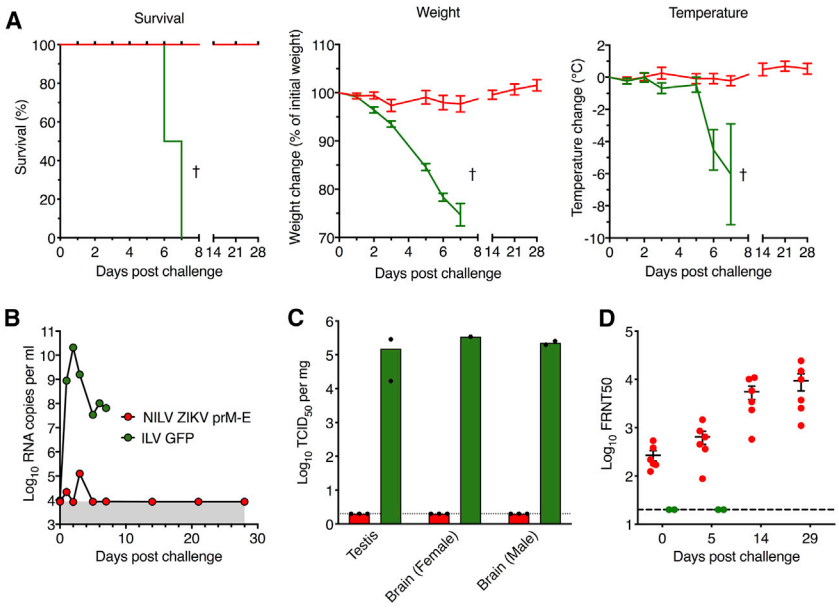

Figure 4. NILV ZIKV prM-E Strongly Suppressed Viremia Replication in A129 Mice

A129 mice with equal numbers of males and females (n = 6) were immunized with a single dose of 2×10^7 TU of NILV ZIKV prM-E (red) or ILV GFP (green) as control vector. Immunized mice were challenged at 4 weeks post-immunization via the i.p. route with 10^2 PFU of ZIKV strain HD78788. (A) The survival (left panel), weight (middle panel), and temperature (right panel) of mice after challenge were monitored for a period of 28 days. (B) Viremia was determined by qRT-PCR. (C) Viral loads in testis and brain were quantified 28 days post-challenge using a $TCID_{50}$ assay. Each dot represents one mouse. (D) Post-challenge neutralizing antibodies were determined by FRNT using ZIKV HD78788. Each symbol stands for one mouse.Data were expressed as means ± SEM.

was introduced via the intramuscular (i.m.) route while comparing to mice receiving a single immunization of 2×10^7 TU of NILV ZIKV prM-E or ILV GFP via the i.p. route. Immunized mice were assessed for EDIII-specific IgG antibody and ZIKV-specific neutralizing capacity 4 weeks after immunization. Mice receiving NILV ZIKV prM-E elicited 40-fold higher EDIII-specific antibody titers than did mice that received the DNA prM-E (Figure 6A). Consistent with the antibody titer, the neutralizing antibody titers of NILV ZIKV prM-E-vaccinated mice were significantly higher than those in DNA prM-E-vaccinated mice (Figure 6B). To access the protective efficacy, mice previously immunized with DNA or NILV vaccines were infected intravenously with 10^2 PFU Asian strain of ZIKV (PF13) at week 4. All vaccinated mice and control mice survived the infection with no significant weight and temperature change, as BALB/c mice are resistant to ZIKV infection (data not shown). Although there were no changes in weight and temperature, the presence of viral RNA in the sera of BALB/c immunized with control ILV GFP vector was detected with a mean peak viral load of 5×10^3 copies/mL (Figure 6C). The NILV ZIKV prM-E vaccine provided complete protection with a 25-fold reduction in viremia level in BALB/c mice against Zika Asian strain PF13, but DNA prM-E-vaccinated mice experienced 6 days of viremia breakthrough with only a 10-fold reduction of viremia level (Figure 6C). These data suggested that NILV ZIKV prM-E induced a higher antibody response and provided better protection than did DNA ZIKV prM-E.

DISCUSSION

The unprecedented ZIKV epidemic, combined with the devastating Zika-associated birth defects, emphasizes the urgent need for a preventive vaccine. The main priority of any vaccine is to effectively trigger an immune response to abolish disease manifestation. However, a balance between efficacy, durability of protection, dose regimen, safety, and reactogenicity of a vaccine is essential for maximizing the intended beneficial effects for vaccinated individuals. The NILV platform that we introduced in the present study appears to achieve such balance. The NILV platform is non-replicative and safe, regardless of prior serological status. After a single dose of vaccination, the NILV ZIKV prM-E induced a high level of immunity that significantly suppressed viral replication upon challenge in both A129 and BALB/c mice.

The NILV platform has several advantages, including more effective immune response, genetic stability, non-replicative, expression of multigene antigens, and a single-dose format. A ZIKV vaccine with a single-dose format is particularly important, especially when periodic boosting in low- and middle-income ZIKV risk areas is unpractical. In addition to our NILV-based ZIKV prM-E, several single-dose ZIKV vaccine candidates based on nucleic acid platforms[9–11] or viral vectors[30–33] have been reported. While the neutralization titer was significantly higher than those induced by plasmid DNA, mRNA, or recombinant adenoviral vector in the immunocompetent model, the protective efficiency of these vaccines might be overestimated, as validation was performed in immunocompetent mouse models or/and non-human primates, where ZIKV infection is non-fatal. The A129 mouse model represents a better model than the immunocompetent model for vaccine validation, as ZIKV infection is fatal due to suppression of the immune response,[32] providing a clear-cut and conclusive readout to vaccine efficacy.

Previous Zika vaccine studies provided excellent data on the short-term protective efficacy of their vaccine candidates at peak immunity.[9,30,32] Although the short-term efficacy of a vaccine is critical, it is also crucial that a Zika vaccine be fast acting in providing adequate immune response for immediate protection and that it also has the ability to offer long-term immunity. We demonstrated that our vaccine can fully protect vaccinated animals within a week of immunization and induces a high level of neutralizing antibodies titer up to 24 weeks after immunization. The induction of high neutralizing

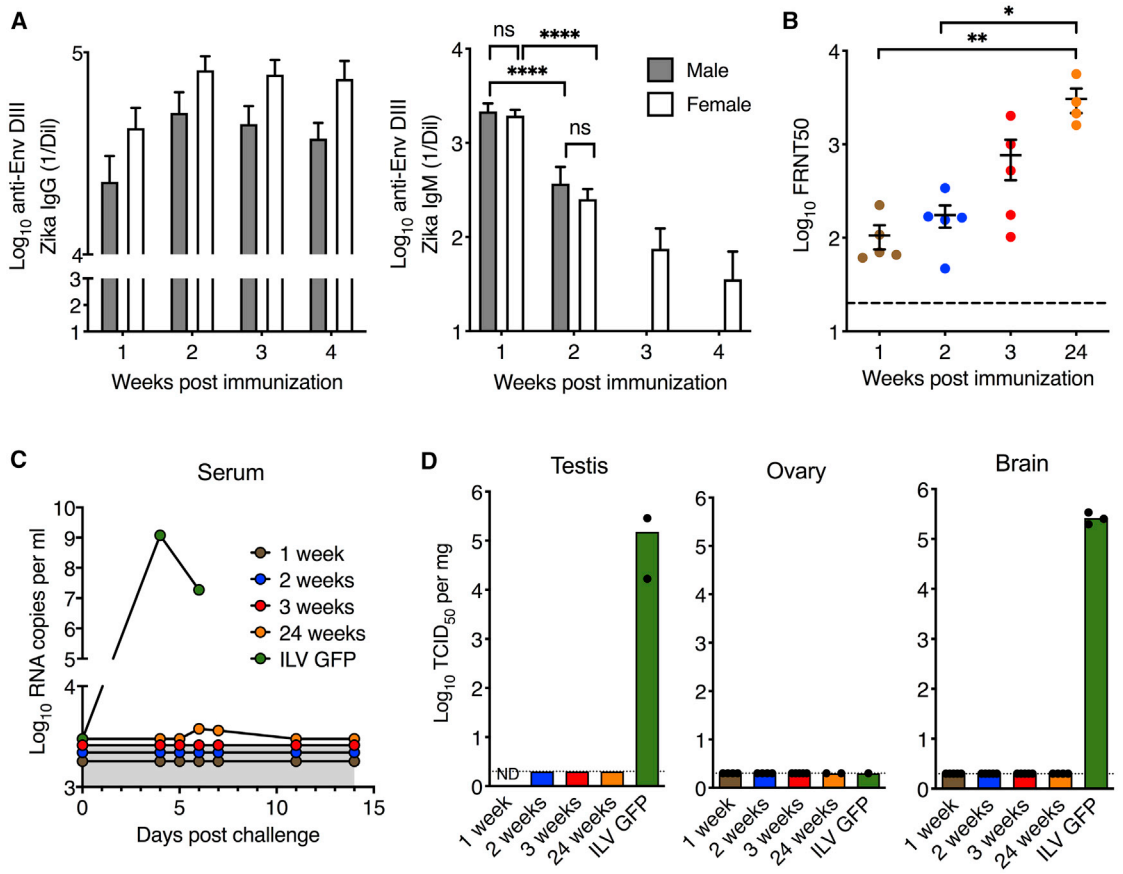

Figure 5. NILV ZIKV prM-E Provides Early Protection and Long-Lasting Immunity

(A) A129 male (n = 7) and female (n = 7) mice were immunized with a single dose of NILV ZIKV prM-E or ILV GFP as control vector, and anti-EDIII Zika IgG (left) and IgM (right) antibodies were measured at 1, 2, 3, and 4 weeks post-immunization. (B) A129 mice ($n \geq 4$) were challenged i.p. with 10^2 PFU of ZIKV strain HD78788 after immunization for 1, 2, 3, and 24 weeks and the FRNT50 neutralizing titers prior to challenge of ZIKV were measured. Each symbol represents one mouse. Results are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. (C) Viremia level in the serum was quantified using qRT-PCR. (D) Viral loads in testis, ovary, and brain were quantified 1 month after challenge using a $TCID_{50}$ assay. Each dot represents one mouse.

antibodies responses could partly be credited to the release of VLPs after *in vivo* immunization with NILV. Typically, antibody responses decline over time after vaccination, but our NILV ZIKV prM-E vaccine induced high neutralizing antibody titers that steadily increased over 24 weeks after a single injection, with the total EDIII-specific IgG titer remaining stable after 2 weeks post-immunization. We hypothesized that the increase of neutralizing antibody titers over time after vaccination could be due to affinity maturation, a process in which antibodies progressively increase in binding strength toward specific epitopes due to repeated exposure of the same antigen.[34] Although the NILV platform does not integrate into the host genome, the persistence of antigen for a duration of at least 7 days (unpublished data) and the possible release of VLPs *in vivo* upon NILV immunization could potentially lead to affinity maturation.

To critically evaluate the efficacy of NILV ZIKV prM-E, we further compared our vaccine to a DNA vaccine platform in an immunocompetent mice model based on the immunization protocol of Larocca et al.[9] We achieved high neutralizing antibody titers in NILV-immunized mice that correlate with full protection in BALB/c mice, while DNA-immunized mice demonstrated low neutralizing titers with weak protection. We observed a discrepancy in the neutralizing antibody titers and protective efficacy between our results and the study performed by Larocca et al.[9] In our study, the DNA-immunized group only triggered a weak neutralizing antibody titer of 1:10 dilution with mild suppression of viral replication, while their report indicated that their DNA vaccine can afford full protection in BALB/c mice against ZIKV-Brazil and ZIKV-Puerto Rico with a neutralizing titer of 1:20 dilution. Discrepancies observed between these two studies could be attributed to a slightly different design of the prM-E antigen in these vaccines.

The NILV ZIKV prM-E strongly suppresses viral replication in a lethal ZIKV challenge, but a sex bias toward the protective efficacy was observed. This sex bias toward vaccine efficacy has been documented in several vaccine studies,[35–38] and females have been shown to

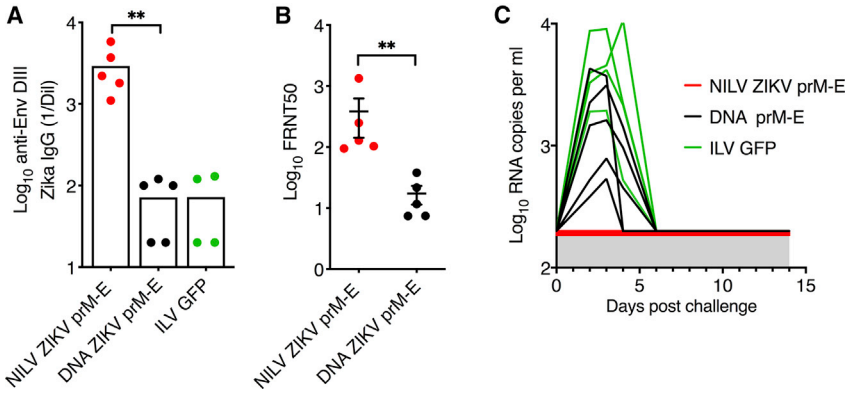

Figure 6. NILV ZIKV prM-E Provides Better Protection Than DNA Vaccination

BALB/c mice (n = 5) were immunized with a single dose of NILV ZIKV prM-E (red), DNA prM-E (black), or ILV GFP (green) as control vector before being challenged intravenously with 10^2 PFU of ZIKV strain PF13, 4 weeks post-immunization. (A) EDIII-specific antibody titers of immunized BALB/c mice before ZIKV challenge. Each symbol represents one mouse. (B) Pre-challenge neutralizing antibodies determined by FNRT50. Data are expressed as means ± SEM. and significant differences are indicated. **$p < 0.01$. (C) Viremia levels in the serum of individual immunized mice quantified using qRT-PCR for a period of 14 days.

produce a higher quality of antibodies than in male mice.[39,40] In line with the latter description, we also observed a higher amount of antibody in immunized female mice as compared to male mice. The reduced responsiveness toward vaccine in males complicates Zika vaccine development because the male reproductive tract represents a reservoir for prolonging viral shedding,[41] posing a risk for sexual transmission during the period of persistent infection.[42] Due to the risk of sexual transmission, men at reproductive age are prioritized for receiving Zika vaccine. Although with the reduced quality of antibodies induced in male A129 mice, these male mice presented a marked decrease in viremia level of 4 logs, and this drastic restriction of viremia levels in the lethal model demonstrated the protective efficacy of our vaccine in the vaccinated-male group. NILV ZIKV prM-E is thus a promising candidate against ZIKV infection that deserves further in-depth evaluation.

The E protein is primarily the antigenic target for neutralizing antibodies and thereby is a target for vaccine design. The E protein of ZIKV and dengue virus (DENV) show structural homology, and they share 35%, 51%, and 29% identity in the EDI, EDII, and EDIII of the respective E monomers.[43] Antibodies targeting ZIKV EDIII are ZIKV-specific and highly neutralizing, unlike antibodies against the EDI and EDII domains, which are highly cross-reactive and have lower neutralizing capacity.[43] Such cross-reactivity, coupled with low neutralizing ability of antibodies, poses the risk of antibody-dependent enhancement (ADE). The ADE model hypothesizes that antibodies specific to the first encountered virus are developed and bind, but incompletely neutralize, closely related viral species. The incompletely neutralized viruses remain invasive and are overlooked by the immune system owing to the antibody-bound status.[44] Besides antibodies against E protein, antibodies against prM have also been described to be poorly neutralizing with high cross-reactivity, therefore facilitating ADE in DENV infection.[45,46] However, to date, the role of anti-prM antibodies and ADE in ZIKV infection remains inconclusive.

Several important concerns remain for the clinical development of Zika vaccine. First, the possibility of ADE that causes devastating effects upon subsequent encounter of viruses closely related to ZIKV is of concern.[44] However, this notion remains controversial, as there is a lack of compelling evidence demonstrating association of ADE with ZIKV pathogenicity in humans.[47,48] Given the potential risk of ADE, our team is working on a T cell-based vaccine to target and clear ZIKV via a T cell-mediated route, independent of the antibody-neutralizing paradigm. Another important concern in the development of Zika vaccine is the ability to protect pregnant women and prevent maternal-to-fetus transmission. Several reported Zika vaccines have been shown to prevent vertical transmission, and this with similar neutralizing antibody titers compared to titers induced by NILV ZIKV prM-E.[49] Although this parameter was not tested in our experiment, we speculate that the NILV ZIKV prM-E could protect pregnant mice and their fetuses considering the high neutralizing antibody titers obtained. In brief, we have developed a NILV-based ZIKV prM-E vaccine candidate that confers rapid and durable protection with a single immunization. Given the lack of integration and pre-existing immunity in the human population, the NILV platform represents a safe and promising platform for vaccine delivery.

MATERIALS AND METHODS

LV Construction and Production

For the construction of recombinant LVs expressing ZIKA proteins, 14 sequences of full-length –prM-E and E spanning from amino acids 1 to 408, derived from Asian and African strains of ZIKV, were downloaded from GenBank.[50] A consensus sequence was obtained after alignment with Multalin[51] and was codon optimized. The mammalian codon-optimized sequence coding for prM and E glycoproteins was cloned into the BamHI and XhoI restriction sites of the pFLAP-ΔU3CMV plasmid, to generate pFLAPΔU3CMV/ZIKV prM-E. A Kozak sequence and a virus leader sequence were included. The optimized sequence was further modified by PCR mutagenesis to generate pFLAPΔU3CMV/ZIKV sE that contains the gene encoding to a soluble E protein. In the resulting vectors pFLAP/prM-E and pFLAP/sE, the CMV immediate early promoter drives the constitutive expression of recombinant envelope proteins. Plasmids were produced with maxiprep kits (Macherey-Nagel, Düren, Germany). Sequences were confirmed by double-stranded sequencing. Lentiviral particles were produced by transient calcium phosphate co-transfection of HEK293T cells with the vector plasmid pTRIP/sE, a VSV-G envelope

protein of serotype New Jersey expression plasmid (pHCMV-G) and an encapsidation plasmid (p8.74 or pD64V for the production of integration-proficient or integration-deficient vectors respectively), as previously described.[52] Vector titers were determined by transducing 293T cells treated with aphidicolin and performing a qPCR as previously described.[53] ILV and NILV vectors harboring GPF were used as controls.

Viral Stocks

The African ZIKV strain HD78788 was obtained from the Institut Pasteur collection and the Asian strain Zika PF13 (strain H/PF/2013; GenBank: KJ776791), isolated from a patient during a ZIKV outbreak in French Polynesia in 2013, was obtained through the DENFREE (FP7/2007–2013) consortium. Viral stocks were prepared from supernatants of infected Vero cells. Low passage number Vero cells were infected at a MOI of 0.01 plaque-forming units (PFU)/cell, and virus was harvested 2 days later. Culture supernatants were clarified by centrifugation, and fetal bovine serum was added to 20% final concentration (v/v) and stored at −80°C. The concentration and infectivity of the stocks were determined by RT-PCR and PFU assays. The ratio of viral particles to PFU of both stocks was approximately 250.

Animals

A129 mice were obtained from colonies maintained under specific pathogen-free conditions at Institut Pasteur. Mice were vaccinated either with 10^6 TU/mouse of ILV or 2×10^7 TU/mouse of NILV without adjuvant via i.p. injection. Phosphate-buffered saline was used to dilute the stocks to the desire concentrations. At various time points after vaccination, mice were challenged via the i.p route with 10^6 viral particles (10^2 PFU) ZIKV-HD78788. BALB/c mice purchased from Janvier Laboratories (Saint-Berthevin, France) were vaccinated either with 50 μg of DNA vaccine via the i.m. route or 2×10^7 TU/mouse of NILV i.p. in a 50-μL vol of saline without adjuvant. Four weeks later, mice were challenged with 10^2 PFU of ZIKV PF13. Mice were monitored for signs of illness (lethargy, ruffled fur, hunched posture, and neurological signs and symptoms, such as paralysis and tremors). Weights and temperatures were regularly recorded. Mice were considered moribund when they did not respond to stimuli, had neurological disease (partial paralysis, tremors, unsteady gait, and/or falling), or lost more than 20% of their initial weight. Blood was collected and serum samples were frozen at −80°C before titration. All mice were maintained at the Institut Pasteur animal facility under specific pathogen-free conditions. All protocols were reviewed by the Institut Pasteur competent authority for compliance with the French and European regulations on Animal Welfare and with Public Health Service recommendations (CETEA no. 2016.0028).

ZIKV Titration by Quantitative Real-Time PCR

Serum and organ samples from mice challenged with ZIKV-HD78788 and ZIKV-PF13 were tested for the presence of ZIKV RNA using qRT-PCR targeting the NS5 and envelope protein, respectively. Approximately 50 mg of a frozen tissue sample suspended in TRIzol was homogenized twice using the FastPrep-24 (VWR, France) each cycle for 30 s at 4.0 m/s. Primers and probe for ZIKV-HD78788-challenged samples were adapted from Faye et al.,[54] while primers and probe for ZIKV-PF13-challenged samples were adapted from Lanciotti et al.[55] Standard curves with serial dilutions of known copies of pCDNA3.1 encoding the ZIKV NS5 gene and pCDNA5 encoding the ZIKV envelope gene were used to quantify viral loads in the samples. RNA was extracted from 25 μL (ZIKV-HD78788 challenged) and 100 μL (ZIKV-PF13 challenged) of serum by a QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Extracted RNA was reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase, and amplification was done on a QuantStudio 12K Flex real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Assay sensitivity for ZIKV-HD78788 quantification was 9,600 copies/mL and ZIKV-PF13 quantification was 200 copies/mL. For ZIKV-HD78788, the following primers sets were used: 5′-AARTACACAT ACCARAACAAAGTGGT-3′, 5′-TCCRCTCCCYCTYTGGTCTTG -3′; and probes: 5′-6-FAM-TCCRCTCCCYCTYTGGTCTTG-MGB-3′. For ZIKV-PF13, the following primers sets were used: 5′-CC GCTGCCCAACACAAG-3′, 5′ CCACTAACGTTCTTTTGCAGAC AT-3′; and probes: 5′-6-FAM-AGCCTACCTTGACAAGCAATCAG ACACTCAA-MGB-3′.

ZIKV Titration by $TCID_{50}$ Assay

To determine the infectious ZIKV titer, 6,000 Vero cells were seeded in 100 μL in a 96-well flat-bottom plate and incubated overnight. ZIKV samples from organs of immunized animals were collected on days of killing and frozen at –80°C, until homogenization with lysing matrix M (MP Biomedical, France) in DMEM media containing 2% fetal bovine serum. Approximately 50 mg of frozen tissue samples was homogenized twice using the FastPrep-24 (VWR, France) each cycle for 30 s at 4.0 m/s. The supernatant of homogenized tissue samples was collected after centrifugation to remove debris. The supernatants were titrated 10-fold, and 100 μL of each sample was used for incubation with Vero cells. The titration of ZIKV samples was performed in triplicates, and TCID50/mg was calculated according to Reed and Muench.[56]

Production of Recombinant Zika EDIII Protein

Recombinant EDIII (rEDIII) of Zika was produced using the *Drosophila* S2 expression system (DES) as previously described.[23] Synthetic gene was cloned into shuttle vector pMT/BiP/HisA (Life Technologies, Carlsbad, CA, USA) in which the SNAP-tag sequence had been initially inserted as a stabilizing protein. Resulting plasmid encoding chimeric protein SNAP-EDIII was transfected into S2 cells to establish stable cell lines S2/Zika EDIII according to the manufacturer's recommendations. After a 7-day cadmium induction of the stable S2 cell lines, cell supernatant was recovered and secreted soluble His-tagged recombinant EDIII protein was diafiltered and purified on immobilized metal affinity chromatography (IMAC) and size-exclusion chromatography columns.

ELISA

Serum samples were repeatedly obtained from vaccine-immunized mice at different time points post-injection. ELISA was used to

quantify the antibodies in the sera before and after immunization. Briefly, Nunc PolySorp plates (Fisher Scientific, France) were coated overnight at 4°C with 1 μg/mL of the recombinant Zika EDIII in carbonate buffer (pH 9.6). Plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 10% fetal calf serum for 1.5 h. Subsequently, serial dilutions of sera were added to the plates for 2 h. A peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Europe, Cambridgeshire, UK) or IgM antibody (Jackson ImmunoResearch Europe, Cambridgeshire, UK) was added for 1 h before developing the plates using an *o*-phenylenediamine substrate (Sigma, P8787). Plates were analyzed at 450 nm/620 nm on a PR3100 reader (Bio-Rad, France). Antibody titers were determined by serial endpoint dilutions and were defined as the highest serum dilution that resulted in an absorbance value 2-fold greater than that of non-immune serum.

Neutralization Assay

The neutralization potential of serum samples was determined by FRNT. Briefly, serially 2-fold diluted heat-inactivated sera were incubated with a previously titrated amount of virus (1,500–2,500 ffu) of ZIKV for 2 h at 37°C. Vero cell monolayers in 96-well plates were subsequently infected with the mixture for 2 h at 37°C. Then, the inoculum was removed and an overlay containing 1.6% (w/v) methylcellulose (Sigma, M0512-2506) was added to the cells. After incubation for 2 days, cells were fixed with paraformaldehyde for 0.5 h at room temperature and washed three times with PBS. Cells were then permeabilized with 0.5% Triton X-100 for 15 min and washed with PBS. Foci were stained using the pan-flavivirus monoclonal antibody 4G2 (Institut Pasteur) for 1.5 h followed by horseradish peroxidase (HRP)-linked anti-mouse IgG (Bio-Rad, Hercules, CA, USA) for 1.5 h and developed using an AEC (3-amino-9-ethylcarbazole) peroxidase substrate kit (Vector VIP; Vector Laboratories, Burlingame, CA, USA). Plates were developed in the dark and dried before counting pink foci. Foci were imaged using a CTL Immunospot S6 Micro Analyzer (Cell Signaling Technology, Cleveland, OH, USA). The endpoint titer was determined as the concentration required for 3-fold the background signal of naive serum. The ZIKV neutralization activity of samples was determined by FRNT, and the FRNT50 titer was determined by a log mid-point linear regression analysis using Prism 6 software (GraphPad). The FRNT50 was calculated as the dilution factor of the sample that neutralized $\geq$50% of the virus. Seropositivity was defined as a titer $\geq$1:10.

Production of VLP

5×10^6 HEK293T cells were transfected with DNA ZIKV prM-E and DNA GFP (negative control) in a 10-cm dish, while 1×10^6 HEK293T cells were used for NILV ZIKV prM-E or NILV GFP (negative control) transduction at an MOI of 5 in six-well plates. One day after transduction, cells were trypsinized and plated onto a 10-cm dish for further propagation. Cells were cultured in DMEM supplemented with 1% penicillin/streptomycin and 2% fetal calf serum. Supernatants of cell culture were harvested 72 h after transfection or transduction and partially purified via ultracentrifugation with a 20% sucrose cushion at 26,000 rpm at 4°C in a Beckmann SW28 rotor for 3 h. The pellet after ultracentrifugation was resuspended in 100 μL of PBS.

Western Blot

Detection of ZIKV virus-like particles was conducted via western blotting. Partially purified supernatants were resolved on non-reducing 4%–12% precast Criterion XT Bis-Tris polyacrylamide gel (Bio-Rad, France) and then transferred onto a nitrocellulose membrane (Bio-Rad, France). The nitrocellulose membrane was blocked in 5% non-fat milk in 0.5% Tween 2-PBS for 2 h at room temperature and probed overnight with 4G2 primary antibody at 1:1,000 dilution. HRP-conjugated anti-mouse secondary antibodies were incubated for 1 h at room temperature before visualization with enhanced chemiluminescence using the SuperSignal West Femto maximum sensitivity substrate (Thermo Fisher Scientific, France) on ChemiDoc XRS+ (Bio-Rad, France). A PageRuler Plus prestained protein ladder was used as the size reference.

Statistical Analysis

Error bars in data represent mean ± SEM. For statistical analyses, Mann-Whitney tests were performed for comparing independent groups two by two, and Kruskal-Wallis tests followed by Tukey's multiple comparisons were performed to compare multiple groups using GraphPad Prism 8 statistical software. Data were considered significant when p values were less than 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2020.05.016.

AUTHOR CONTRIBUTIONS

Study concept and design: M.B. and P.C. Acquisition of data: M.W.K., F.A., P.S., and M.B.. Analysis and interpretation of data: M.W.K., F.A., P.S., and M.B. Drafting of the manuscript: M.W.K., M.B., and P.C.. Statistical analysis: M.W.K. and M.B. Technical or material support: S.P., M.P., and E.S.-L.

DECLARATION OF INTERESTS

P.C. is the founder and CSO of TheraVectys. The remaining authors declare no competing interests.

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