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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 lentiviral vector (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. 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. 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. Infection during pregnancy has emerged as a threat due to risk of fetal microcephaly and miscarriages. Two 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 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, RNA-based, and lipid-nanoparticle-encapsulated nucleoside-modified mRNA (mRNA-LNP) vaccines. 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.
against infectious diseases.\textsuperscript{13} Antigenic peptides feasible.\textsuperscript{18} Despite the aforementioned benefits, making simultaneous expression of poly-meric and providing rapid immunity as well as long-term protection 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.\textsuperscript{20} 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.\textsuperscript{20} However, for West Nile virus (WNV), a soluble E protein lacking the membrane anchoring region was shown to generate an efficient neutralizing response.\textsuperscript{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 $5 \times 10^6$ 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.\textsuperscript{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 lacking the membrane anchoring region to obviate the risk of mutagenic events.\textsuperscript{24} In this context, we characterized the optimal dose of NILV ZIKV prM-E that provides comparable immunogenicity as $5 \times 10^6$ 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
titer of more than $1 \times 10^4$ was induced by $5 \times 10^7$ TU of NILV, and this antibody titer was comparable to that obtained with $5 \times 10^5$ 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 $\times 10^7$ TU), ILV ZIKV prM-E (5 $\times 10^6$ TU), or ILV GFP (5 $\times 10^6$ 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 $10^7$ plaque forming units (PFU) of the mouse-adapted Zikia 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 $1 \times 10^{10}$ 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 $5 \times 10^6$ 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 (TCID₅₀) 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 Zikia African strain HD78788. Vaccinated mice showed an increase in neutralizing titer from $7 \times 10^4$ to $1 \times 10^5$, 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: $2.3 \times 10^5$ for male-
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 mice during challenge experiments.

An effective vaccine is one that can respond rapidly and provide lifelong immunity. Therefore, we evaluated the neutralizing activity 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 neutralizing antibody titer increased over time, with neutralizing titers of 1 × 10^2, 1.5 × 10^3, 6 × 10^3, and 1.4 × 10^4 (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 5E). 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., where single immunization of 50 μg of a plasmid DNA vaccine encoding ZIKV prM-E
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 platforms9–11 or viral vectors30–33 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
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,5–38 and females have been shown to...
produce a higher quality of antibodies than in male mice. 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, posing a risk for sexual transmission during the period of persistent infection. 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 domains, which are highly cross-reactive and have lower neutralizing capacity. 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. 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. 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. However, this notion remains controversial, as there is a lack of compelling evidence demonstrating association of ADE with ZIKV pathogenicity in humans. 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. 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. A consensus sequence was obtained after alignment with Multalin and was codon optimized. The mamalian codon-optimized sequence coding for prM and E glycoproteins was cloned into the BamHI and Xhol restriction sites of the pFLAPΔU3CMV 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 prM-E sE, which 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.\textsuperscript{53} Vector titers were determined by transducing 293T cells treated with aphidicolin and performing a qPCR as previously described.\textsuperscript{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 10⁶ TU/mouse of ILV or 2 × 10⁷ TU/mouse of NILV without adjuvant via i.p. injection. Phosphate-buffered saline was used to dilute the stocks to the desired concentrations. At various time points after vaccination, mice were challenged via the i.p route with 10⁶ viral particles (10² PFU) ZIKV-HD78788. BALB/c mice purchased from Janvier Laboratories (Saint-Berthevin, France) were vaccinated according to Reed and Muench.\textsuperscript{56} Serum and organ samples 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.,\textsuperscript{54} while primers and probe for ZIKV-PF13-challenged samples were adapted from Lanciotti et al.\textsuperscript{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/μL and ZIKV-PF13 quantification was 200 copies/μL. For ZIKV-HD78788, the following primers sets were used: 5'-AARTACACAT ACCARAAACAAAGTGGT-3', 5'-TCCRCCTCCYCYTGYGGTCTTG-3'; and probes: 5'-6-FAM-TCCRCCTCCYCYTGYGGTCTTG-MGB-3'. For ZIKV-PF13, the following primers sets were used: 5'-CC GTCTCCCAACAAAG-3', 5' CCATACAGCTCTTTTGGCAGAC AT-3'; and probes: 5'-6-FAM-AGCCTACCTTGGACAGCAATCAC MGB-3'.

ZIKV Titration by TCID\textsubscript{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 lysis matrix M (MP Biomedicals, France) in DMEM media containing 2% fetal bovine serum. Approximately 50 μg 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 supernatant 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 TCID\textsubscript{50}/mg was calculated according to Reed and Muench.\textsuperscript{56}

Production of Recombinant Zika EDII Protein

Recombinant EDIII (rEDIII) of Zika was produced using the Drosophila S2 expression system (DES) as previously described.\textsuperscript{53} Synthetic gene was cloned into shuttle vector pHM/T/Bi/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 E DI 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 autoradiography using the SuperSignal West Femto maximum sensitivity substrate (Thermo Fisher Scientific, France) on ChemiDoc XR+ (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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