Development and immune correlates of Zika virus vaccines

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UNIVERSITY OF AMSTERDAM

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DEVELOPMENT AND IMMUNE CORRELATES OF ZIKA VIRUS VACCINES

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INTRODUCTION

ADAPTED FROM: ZIKA VIRUS VACCINES

Peter Abbink, Kathryn E. Stephenson and Dan H. Barouch

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The recent epidemic of Zika virus (ZIKV) in the Americas has revealed the devastating consequences of ZIKV infection, particularly in pregnant women. Congenital Zika syndrome, characterized by malformations and microcephaly in neonates as well as developmental challenges in children, highlights the need for the development of a safe and effective vaccine. Multiple vaccine candidates have been developed and have shown promising results in both animal models and phase I clinical trials. However, important challenges remain for clinical development of these vaccines. In this Progress, we discuss recent preclinical studies and lessons learned from first in-human clinical trials with ZIKV vaccines.

Introduction

Zika virus (ZIKV), a flavivirus of the family *Flaviviridae,* was first isolated in 1947 in the Zika forest in Uganda[1]. ZIKV is an enveloped, positive-sense single-stranded RNA virus. Its 11kb genome encodes a single polyprotein which is cleaved into individual proteins. Structural proteins capsid (C), precursor-membrane (prM) — which is cleaved to the mature membrane protein (M) — and envelope (ENV) are assembled in virus particles (Figure 1). The non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are involved in replication and control host cell processes to favor virus production. Until recently, infection with ZIKV was generally regarded as a self-limited, mild illness with rash, headache, myalgia and conjunctivitis, and few ZIKV infections were reported globally[2]. In 2007, ZIKV was recognized as the cause of an outbreak in the Yap Islands, Federated States of Micronesia[3] followed in 2013 by an outbreak in French Polynesia[4] before spreading to the Americas in 2015[5] via Easter Island, Chile[6]. As a result of the sudden rise in congenital abnormalities and occurrences of Guillain-Barré syndrome, the scientific community established a causal association between ZIKV infection and these neurological adverse outcomes[7-9]. This led the WHO to declare ZIKV and its suspected link to birth defects[10,11] a Public Health Emergency of International Concern in February 2016[12].

Research on this virus then markedly increased [13-16]. Studies resolved structures of the virion and the proteins that contribute to pathogenicity [17-20] and defined candidate entry receptors and cell tropism[21,22]. Neuroprogenitor cells have been described as a preferred target for ZIKV,

leading to apoptosis of these cells and congenital Zika syndrome (CZS), including microcephaly and other brain malformations[23,24]. The AXL receptor (also known as Tyrosine-protein kinase receptor UFO)[25-27], which is highly expressed on human radial glial cells, astrocytes and microglia in the developing human cortex, has been hypothesized to account for the observed neurotropism and the related congenital malformations. However, the role of AXL as an entry receptor for ZIKV remains unknown[28].

The close relationship between ZIKV and other well-studied flaviviruses, such as West Nile virus (WNV), Japanese encephalitis virus (JEV), dengue virus (DENV) and tick-borne encephalitis virus (TBEV), has facilitated ZIKV research and the development of vaccines[29-31]. Experience gained over 2 decades of research on these flaviviruses guided vaccine design and suggested protection against ZIKV may be achieved by antibodies that bind ENV[25]. Currently, several vaccine candidates are under development (Table 1). These include DNA vaccines, purified inactivated viruses (PIVs), live attenuated viruses (LAVs), mRNA vaccines and viral vectored vaccines (modified Vaccinia virus Ankara (MVA), measles virus (MV) and adenovirus vectors (Ad)). These efforts from multiple laboratories have led to the unprecedented pace of ZIKV vaccine development.

In this Progress we discuss recent advances in animal models and the results from first-in-human phase I clinical trials of ZIKV vaccine candidates. In addition, we address potential challenges for late stage development of ZIKV vaccine candidates.

CZS and developmental problems

With the rapid spread of ZIKV through the Americas, many detrimental effects on fetuses and neonates were observed following infection in pregnant women[32,33]. In Brazil, potential confounders, such as the insecticide pyriproxyfen and the tetanus, diphtheria and pertussis (Tdap) vaccine did not correlate with the increased incidence of birth defects, whereas ZIKV confirmed by RT-PCR or antibody detection did correlate, suggesting that ZIKV was the causative agent of CZS[8,34]. Furthermore, animal studies have shown that ZIKV infection impacts fetal development[35,36]. Moreover, severe developmental problems have been observed in follow-up studies of children born with microcephaly to women confirmed to have been infected with ZIKV during pregnancy[37]. Developmental problems are

also likely to occur in children infected during pregnancy without microcephaly, although detailed studies have not yet been completed[38-40].
The confirmation of ZIKV as the causative agent for CZS, combined with the severe developmental problems of neonates born with CZS, emphasizes the urgent need for a preventative vaccine. Lessons learned from congenital rubella syndrome further support that an effective vaccine might drastically reduce the incidence of infection and prevent birth defects[41]. However, until a vaccine is available, education and other preventative measures need to be implemented to prevent CZS[42], including the development of antiviral medications[43,44].

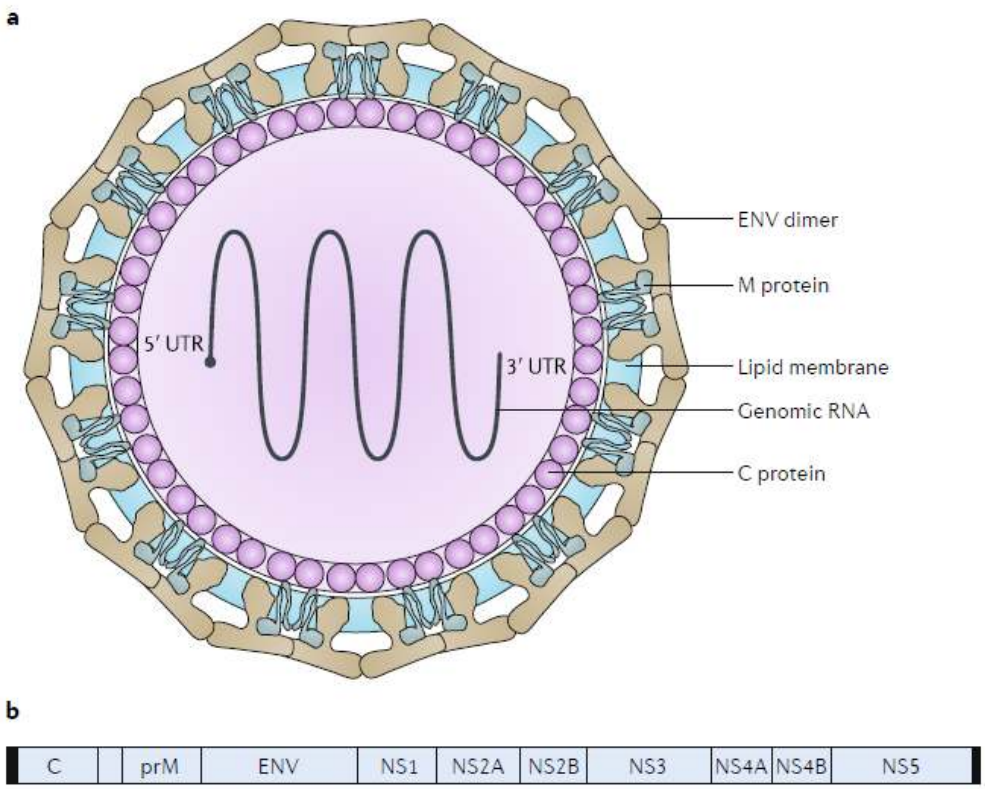

Figure 1. The Zika virus particle and genome. A single positive- strand RNA copy is packaged in an enveloped virus particle that is assembled by the structural proteins (part a). The non- structural proteins are involved in viral replication and immune evasion. Structural proteins capsid (C), precursor membrane (prM) and envelope (ENV) and non- structural proteins (NS1, NS2A , NS2B, NS3, NS4A , NS4B and NS5) are flanked by 5′ and 3′ UTRs (black boxes) (part b). The primary target of neutralizing antibodies is the envelope, which together with the membrane protein is properly folded to display binding epitopes. M protein, mature membrane protein.

THESIS SCOPE

No vaccines to Zika are currently available; however recent outbreaks have exposed the urgent need for prevention and treatment of infection. Devastating congenital defects after Zika infection of pregnant women have led to a massive increase in the search for a protective vaccine.

The aim of the work described in this thesis was to develop an effective vaccine against Zika virus infection and assess immune correlates of protection. Work done on related flavivirus vaccines such as Dengue virus, West Nile virus and Japanese encephalitis virus provided insights on potential targets of immune responses. We set out to engage the immune system in mounting an immune response to the envelope protein of Zika virus which could lead to block of infection. In **chapter 2**, we established a wild type mouse model which closely mirrors human course and duration of Zika infection. We developed purified inactivated virus vaccine and recombinant DNA vaccines expressing various forms of Zika premembrane-envelope immunogens to be tested in the mouse model. First assessments of immune correlates were also established. In **chapter 3**, we set out to extend our findings from the mouse model to a non-human primate model. In addition, we expanded our vaccine candidates to include a recombinant adenoviral vaccine vector. Our understanding of immune correlates of protection of our vaccine candidates was further expanded. In **chapter 4**, we aimed to assess durability of vaccine induced protective immune responses. In addition, immune correlates were further defined. Finally, in **chapter 5**, purified neutralizing antibodies against Zika were assessed for their ability to be used as therapeutic and prophylactic treatment of Zika infection.

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2

VACCINE PROTECTION AGAINST ZIKA VIRUS FROM BRAZIL

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ABSTRACT

Zika virus (ZIKV) is a flavivirus that is responsible for the current epidemic in Brazil and the Americas[1,2]. ZIKV has been causally associated with fetal microcephaly, intrauterine growth restriction, and other birth defects in both humans[3,4,5,6,7,8] and mice[9,10,11]. The rapid development of a safe and effective ZIKV vaccine is a global health priority[1,2], but very little is currently known about ZIKV immunology and mechanisms of immune protection. Here we show that a single immunization with a plasmid DNA vaccine or a purified inactivated virus vaccine provides complete protection in susceptible mice against challenge with a strain of ZIKV involved in the outbreak in northeast Brazil. This ZIKV strain has recently been shown to cross the placenta and to induce fetal microcephaly and other congenital malformations in mice[11]. We produced DNA vaccines expressing ZIKV pre-membrane and envelope (prM-Env), as well as a series of deletion mutants. The prM-Env DNA vaccine, but not the deletion mutants, afforded complete protection against ZIKV, as measured by absence of detectable viraemia following challenge, and protective efficacy correlated with Env-specific antibody titers. Adoptive transfer of purified IgG from vaccinated mice conferred passive protection, and depletion of CD4 and CD8 T lymphocytes in vaccinated mice did not abrogate this protection. These data demonstrate that protection against ZIKV challenge can be achieved by single-shot subunit and inactivated virus vaccines in mice and that Env-specific antibody titers represent key immunologic correlates of protection. Our findings suggest that the development of a ZIKV vaccine for humans is likely to be achievable.

MAIN

The World Health Organization declared the clusters of microcephaly and neurological disorders and their association with ZIKV infection to be a global public health emergency on February 1, 2016. ZIKV is believed to cause neuropathology in developing fetuses by crossing the placenta and targeting cortical neural progenitor cells[9,10,11,12,13,14], leading to impaired neurogenesis and resulting in microcephaly and other congenital malformations. ZIKV has also been associated with neurologic conditions in adults, such as Guillain-Barré syndrome[15].

Vaccines have been developed for other flaviviruses, including yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, and dengue viruses, but no vaccine currently exists for ZIKV. To develop preclinical challenge models for candidate ZIKV vaccines, we obtained low-passage ZIKV isolates from northeast Brazil (Brazil/ZKV2015; University of São Paulo)[11] and Puerto Rico (PRVABC59; US Centers for Disease Control and Prevention) (Extended Data Fig. 1). We expanded these viruses in Vero cells to generate preclinical challenge stocks, which we termed ZIKV-BR and ZIKV-PR, respectively. These ZIKV strains are part of the Asian ZIKV lineage16 and differ from each other by five amino acids in the polyprotein (Extended Data Fig. 2). The Brazil/ZKV2015 strain has also recently been reported to recapitulate key clinical manifestations, including fetal microcephaly and intrauterine growth restriction, in wild-type SJL mice[11]. Similarly, the related French Polynesian H/PF/2013 strain has been shown to induce placental damage and fetal demise in Ifnar$^{-/-}$ C57BL/6 mice as well as in wild-type C57BL/6 mice following IFN-α receptor blockade10.

We designed ZIKV prM-Env immunogens based on the Brazil BeH815744 strain (Extended Data Fig. 2) and optimized them for increased antigen expression. We also designed deletion mutants lacking prM and/or lacking the transmembrane region (ΔTM) or the full stem (Δstem) of Env (Fig. 1a). Plasmid DNA vaccines encoding these antigens were produced, and transgene expression was verified by western blot (Fig. 1b). To assess the immunogenicity of these vaccines, groups of Balb/c mice (n = 5–10 per group) received a single immunization of 50 μg of each DNA vaccine by the intramuscular (i.m.) route at week 0. Env-specific antibody responses were evaluated at week 3 by ELISA. The prM-Env DNA vaccine elicited higher Env-specific antibody titers than did the Env DNA vaccine and all of the ΔTM and Δstem deletion mutants (Fig. 1c), indicating the importance of including prM as well as the full-length Env sequence. No prM-specific antibody responses were detected (Extended Data Fig. 3). The prM-Env DNA vaccine also induced ZIKV-specific neutralizing antibodies after a single immunization (Table 1), as measured by a virus-specific microneutralization assay17. In addition, the prM-Env DNA vaccine induced Env-specific CD8+ and CD4+ T-lymphocyte responses, as assessed by IFNγ ELISPOT and multiparameter intracellular cytokine staining assays (Fig. 1d, e).

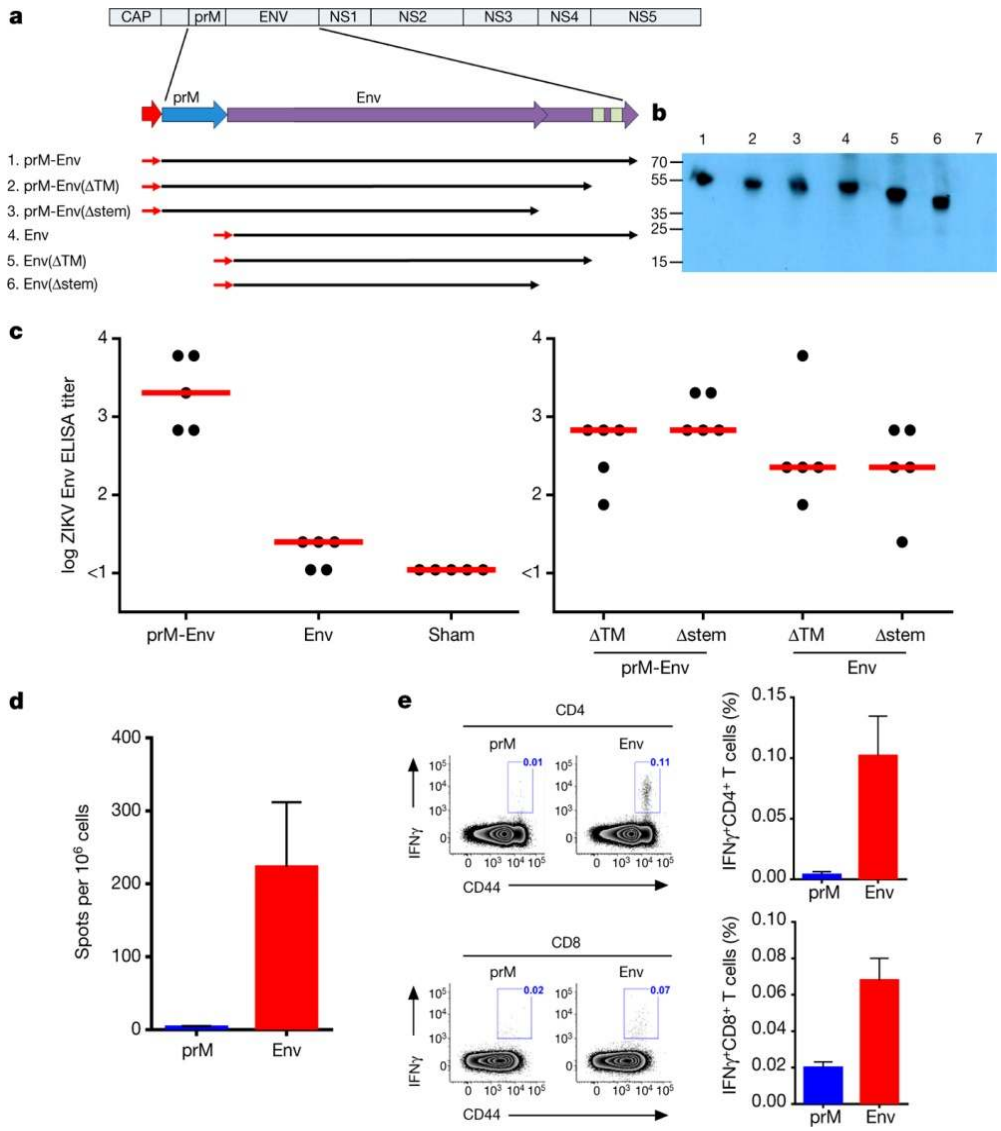

Figure 1. Construction and immunogenicity of DNA vaccines. a, Schema of ZIKV prM-Env immunogens and deletion mutants. **b**, Western blot of transgene expression from (1) prM-Env, (2) prM-Env(ΔTM), (3) prM-Env(Δstem), (4) Env, (5) Env(ΔTM), (6) Env(Δstem), and (7) sham DNA vaccines transfected in 293T cells. Balb/c mice (n = 5 per group) received a single immunization with 50 μg of these DNA vaccines by the i.m. route. **c**, Humoral immune responses were assessed at week 3 following vaccination by Env-specific ELISA. Red bars reflect medians. **d**, **e**, Cellular immune responses were assessed by IFNγ ELISPOT assays (**d**) and multi-parameter intracellular cytokine staining assays (**e**). Error bars reflect s.e.m.

To assess the protective efficacy of these DNA vaccines against ZIKV challenge, we infected vaccinated or sham control Balb/c mice at week 4 by the intravenous (i.v.) route with 10^5 viral particles (10^2 plaque-forming units (PFU)) of ZIKV-BR or ZIKV-PR. Viral loads following ZIKV challenge were quantitated by RT–PCR[18]. Sham-vaccinated mice inoculated with ZIKV-BR developed approximately 6 days of detectable viraemia with a mean peak viral load of 5.42 log copies per ml (range 4.55–6.57 log copies per ml; $n = 10$) on day 3 after challenge (Fig. 2a). In contrast, a single immunization with the prM-Env DNA vaccine provided complete protection against ZIKV-BR challenge with no detectable viraemia (<100 copies per ml) at any time point ($n = 10$). Complete protection was also observed when vaccinated mice were challenged at week 8 (data not shown). The prM-Env DNA vaccine also afforded complete protection against ZIKV-PR challenge ($n = 5$). ZIKV-PR replicated to slightly lower levels (mean peak viral load 4.96 log copies per ml; range 4.80–5.33 log copies per ml; $n = 5$) than did ZIKV-BR in sham controls. In contrast with the prM-Env DNA vaccine, the DNA vaccines lacking prM as well as the ΔTM and Δstem deletion mutants did not provide complete protection against ZIKV-BR challenge, although viral loads were still reduced in these animals as compared with sham controls (Fig. 2b).

The varying degrees of protection obtained with this set of DNA vaccines allowed for an analysis of immune correlates of protection. Protective efficacy correlated with Env-specific binding antibody titers ($P = 0.0005$ comparing protected versus infected animals; Fig. 2c) as well as ZIKV-specific neutralizing antibody titers >10 (Table 1). In addition, peak viral loads on day 3 were inversely correlated with antibody titers ($P < 0.0001$, $R^2 = 0.72$; Fig. 2d). These data suggest that Env-specific antibodies were critical for the protective efficacy of DNA vaccines against ZIKV-BR challenge. Mice that received two immunizations with the prM-Env DNA vaccine at week 0 and week 4 developed high neutralizing antibody titers of 1,022 at week 8 (Table 1) and were also protected against ZIKV-BR challenge (data not shown).

The prM-Env DNA vaccine also provided complete protection against ZIKV-BR challenge in SJL mice (Extended Data Fig. 4) and against both ZIKV-BR and ZIKV-PR challenge in C57BL/6 mice (Extended Data Figs 5and 6). ZIKV-BR replicated efficiently in SJL mice, consistent with a previous study[11],

although at slightly lower levels (mean peak viral load 4.70 log copies per ml; range 3.50–5.92 log copies per ml; $n = 5$) than in Balb/c mice (Fig. 2a). In contrast, both ZIKV-BR and ZIKV-PR replicated poorly in C57BL/6 mice (Extended Data Fig. 5), also consistent with previous reports, potentially as a result of robust IFN-α-mediated innate immune restriction in this strain of mice[10,11,19,20].

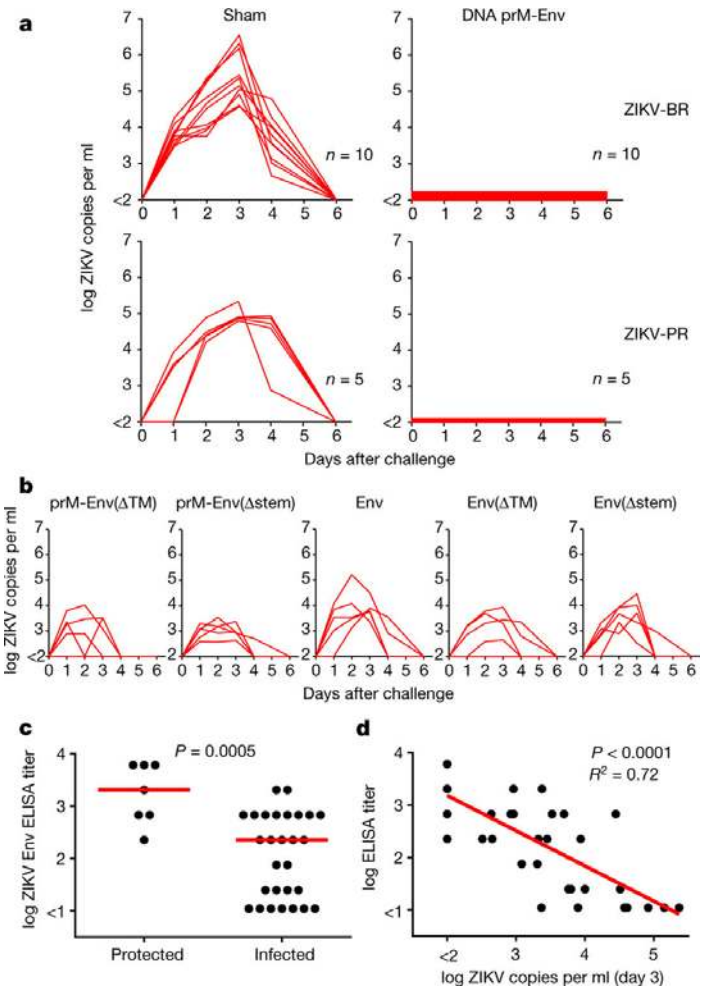

Figure 2. Protective efficacy of DNA vaccines. a, Balb/c mice ($n = 5$ or 10 per group) received a single immunization by the i.m. route with 50 μg prM-Env DNA vaccine or a sham vaccine and were challenged at week 4 by the i.v. route with 10^5 viral particles (10^2 PFU) ZIKV-BR or ZIKV-PR. Serum viral loads are shown. **b**, Mice ($n = 5$ per group) received a single immunization with 50 μg of various DNA vaccines and were challenged with ZIKV-BR. **c**, **d**, Correlates of protective efficacy (**c**) and day 3 viral loads (**d**) are shown. Red bars reflect medians. *P* values and R^2 values reflect *t*-tests and Spearman rank-correlation tests.

To investigate the immunological mechanism of protection against ZIKV-BR challenge, we purified IgG from serum from Balb/c mice vaccinated with prM-Env DNA. Passive infusion of varying quantities of purified IgG by the i.v. route resulted in median Env-specific log serum antibody titers of 2.82 (high), 2.35 (mid) and 1.87 (low) in recipient mice following adoptive transfer (Fig. 3a). All recipient mice with log serum antibody titers of 2.35 or higher were protected against ZIKV-BR hallenge (Fig. 3b, c), demonstrating that protection can be mediated by vaccine-elicited IgG alone and confirming that the magnitude of Env-specific antibody titers correlates with protective efficacy ($P < 0.0001$, Fig. 3b). In contrast, only 1 out of 5 recipient mice that received low levels of Env-specific IgG were protected, although they still exhibited reduced viral loads compared with sham controls (Extended Data Fig. 7). These data define the minimum threshold of Env-specific antibody titers required for protection in this model.

We next depleted $CD4^+$ and/or $CD8^+$ T lymphocytes in prM-Env-vaccinated mice on day −2 and day −1 before challenge (>99.9% efficiency; Extended Data Fig. 8). Depletion of these T-lymphocyte subsets did not detectably abrogate the protective efficacy of the prM-Env DNA vaccine against ZIKV-BR challenge (Fig. 3d). These data indicate that Env-specific T-lymphocyte responses were not required for protection in this model, although these findings do not exclude the possibility that ZIKV-specific cellular immune responses may be beneficial in other settings.

To extend these observations to a vaccine platform that has historically provided clinical efficacy against other flaviviruses, we explored the immunogenicity and protective efficacy of a ZIKV purified inactivated virus (PIV) vaccine derived from the Puerto Rico PRVABC59 strain. Groups of Balb/c mice ($n = 5$ per group) received a single immunization of 1 μg of the PIV vaccine with alum or alum alone by the i.m. or subcutaneous (s.c.) routes. Antibody titers were higher in the group that received the PIV vaccine by the i.m. route rather than by the s.c. route, as compared by ELISA (Fig. 4a). The PIV vaccine by both routes also induced ZIKV-specific neutralizing antibodies after a single immunization (Table 1). At week 4, all mice were i.v. challenged with ZIKV-BR as described above. Complete protection was observed in the group that received the PIV vaccine by the i.m. route (Fig. 4b, c). Two mice that received the PIV vaccine by the s.c.

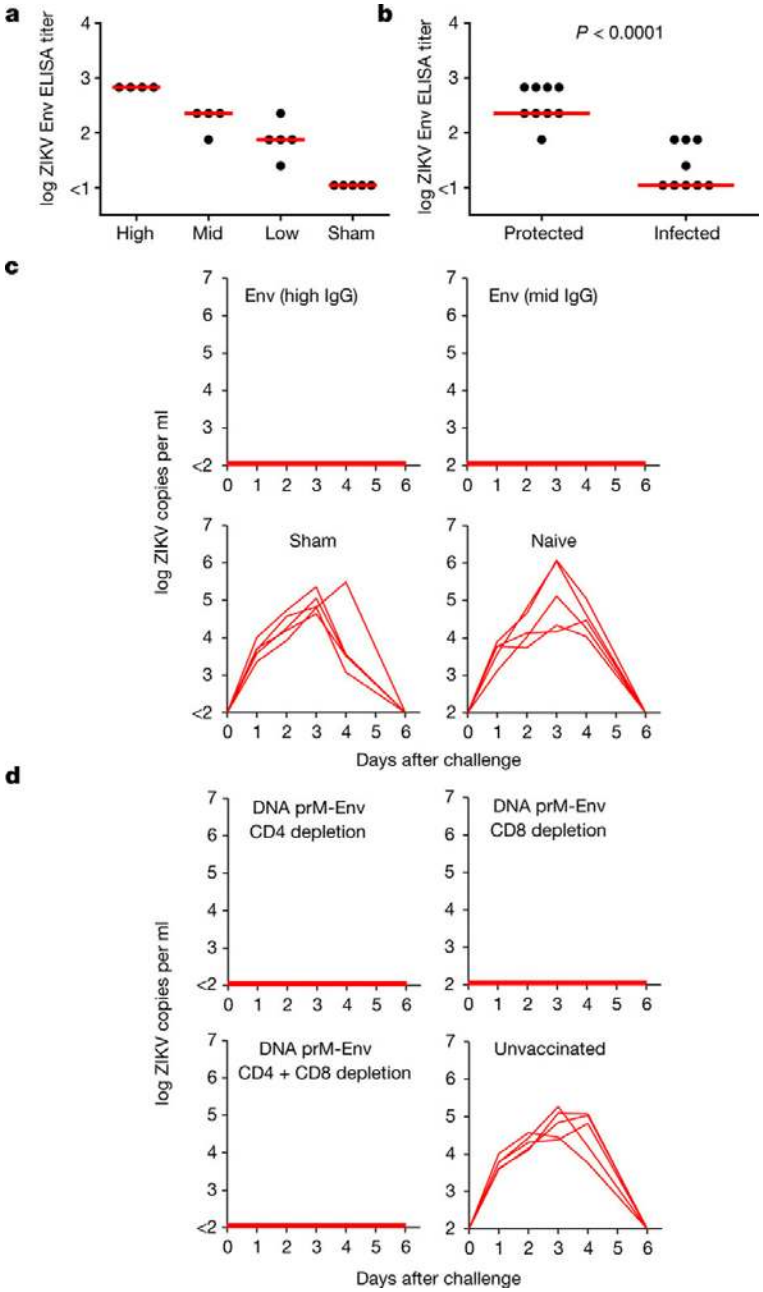

Figure 3. Mechanistic studies. a, Env-specific serum antibody titers in recipient Balb/c mice (*n* = 5 per group) following adoptive transfer of varying amounts (high, mid, low) of IgG purified from serum from mice vaccinated with prM-Env DNA or naive mice (sham). **b**, Correlates of protective efficacy. **c**, Serum viral loads in mice that received adoptive transfer of purified IgG from vaccinated mice and were challenged with ZIKV-BR. **d**, Serum viral loads in prM-Env-DNA-vaccinated mice that were depleted of $CD4^+$and/or $CD8^+$ T lymphocytes before challenge with ZIKV-BR. Red bars reflect medians. *P* values reflect *t*-tests.

route showed brief low levels of viraemia (Fig. 4c), potentially consistent with the lower Env-specific binding antibody titers in this group (Fig. 4b).

Our data demonstrate that a single immunization with a DNA vaccine or a PIV vaccine provided complete protection against parenteral ZIKV challenge in mice. The prM-Env DNA vaccine afforded protection in three strains of mice and against both ZIKV-BR and ZIKV-PR challenges, suggesting the generalizability of these observations. Protective efficacy was mediated by vaccine-elicited Env-specific antibodies, as evidenced by (1) statistical analyses of immune correlates of protection (Figs 2c, d), (2) adoptive transfer studies with purified IgG from vaccinated mice (Fig. 3a–c), and (3) T-lymphocyte depletion studies in vaccinated mice (Fig. 3d). The adoptive transfer studies also defined the threshold of Env-specific antibody titers required for protection in this model.

It is difficult to extrapolate directly the results from these vaccine studies in mice to potential clinical efficacy in humans. Nevertheless, the robust protection observed in the present studies and the clear immune correlates of protection suggest a path forward for ZIKV vaccine development in humans. Of note, similar antibody-based correlates of protection, including neutralizing antibody titers >10, have been reported for other flavivirus vaccines, including yellow fever virus, tick-borne encephalitis virus, and Japanese encephalitis virus[21,22,23]. Moreover, the ZIKV-BR challenge isolate used in the present study has been shown in wild-type SJL mice to recapitulate certain key clinical findings of ZIKV infection in humans, including fetal microcephaly and intrauterine growth retardation[11]. ZIKV-BR did not lead to a fatal outcome in wild-type Balb/c and SJL mice, as has been observed in *Ifnar*$^{-/-}$ C57BL/6 mice[10,19,20], but the magnitude and duration of viraemia in Balb/c and SJL mice appear comparable with that in humans[2], suggesting the potential relevance of this model. It is notable that ZIKV-BR replicated efficiently in wild-type Balb/c and SJL mice (Fig. 2a, Extended Data Fig. 4), but replicated poorly in wild-type C57BL/6 mice (Extended Data Fig. 5), which is consistent with previous observations[10,11] and indicates important strain-specific differences for ZIKV infectivity. Further investigation into the immunologic mechanisms underlying these differences may lead to insights into innate immune control of ZIKV. Moreover, further characterization of the susceptible Balb/c and SJL murine models may facilitate future studies of ZIKV pathogenesis

and the development of antiviral interventions. Future studies will also need to address the potential relevance of cross-reactive antibodies against dengue virus and other flaviviruses on ZIKV vaccine immunogenicity and protective efficacy.

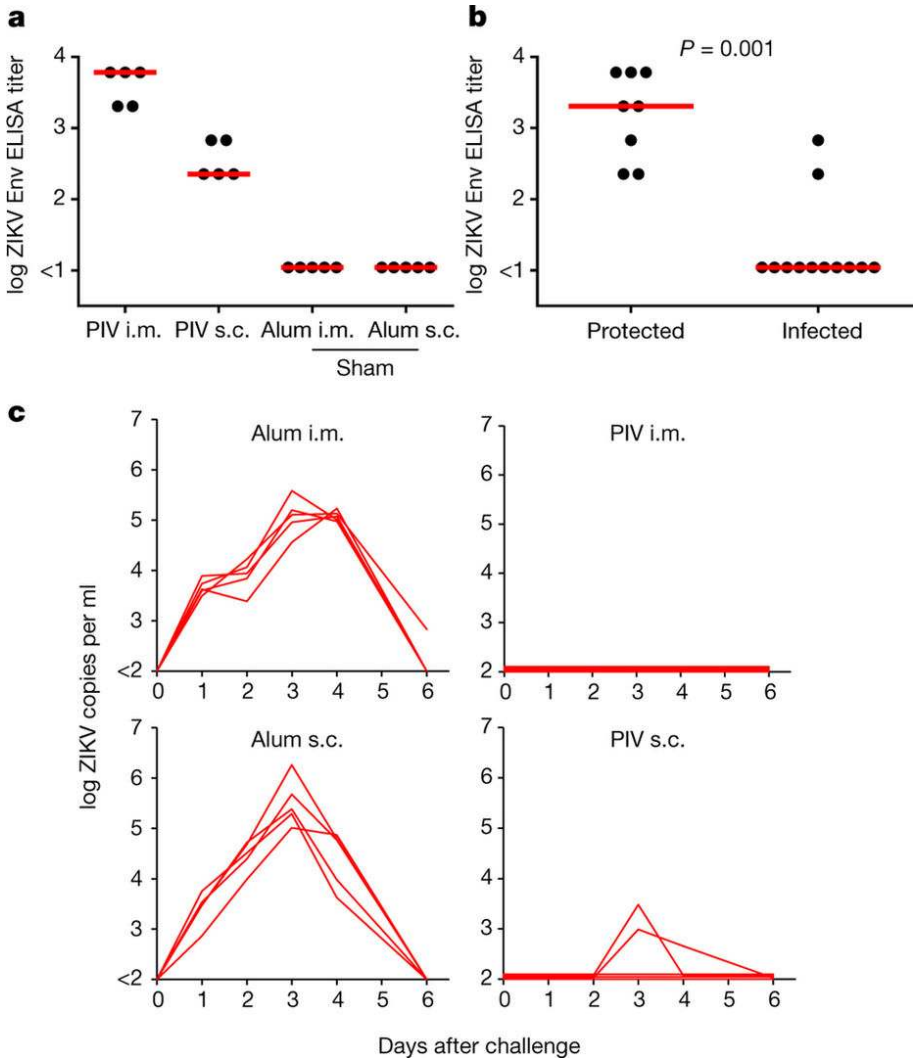

Figure 4. Immunogenicity and protective efficacy of the PIV vaccine. Balb/c mice (*n* = 5 per group) received a single immunization by the i.m. or s.c. route with 1 µg PIV vaccine with alum, or alum alone, and were challenged at week 4 by the i.v. route with 10^5 viral particles (10^2 PFU) ZIKV-BR. **a**, Humoral immune responses were assessed at week 3 following vaccination by Env-specific ELISA. **b**, Correlates of protective efficacy. **c**, Serum viral loads are shown following ZIKV-BR challenge. Red bars reflect medians. *P* values reflect *t*-tests.

The epidemiology of the current ZIKV outbreak[1,2] and the clinical consequences for fetuses in pregnant women who become infected[3,4,5,6,7,8] necessitate the urgent development of a ZIKV vaccine. Our data demonstrate that complete protection against ZIKV challenge was reliably and robustly achieved with both DNA vaccines and purified inactivated virus vaccines in susceptible mice. These vaccine platforms have previously been used at comparable doses to develop vaccines for other flaviviruses, including West Nile virus[24,25], dengue viruses[26,27], tick-borne encephalitis virus[28,29], and Japanese encephalitis virus30, and may offer safety advantages over live attenuated and replicating flavivirus vaccines, particularly for pregnant women. Moreover, the magnitude of Env-specific antibody titers that provide complete protection against ZIKV challenge in mice should be readily achievable by DNA vaccines and purified inactivated virus vaccines in humans. Taken together, our findings provide substantial optimism that the development of a safe and effective ZIKV vaccine for humans will probably be feasible.

METHODS

Animals

Balb/c, SJL, and C57BL/6 female mice at 6–8 weeks of age were purchased from Jackson Laboratories (Bar Harbour). Mice were vaccinated with 50 µg DNA vaccine in saline without adjuvant by the i.m. route or with 1 µg PIV vaccines with 100 µg alum (Alhydrogel; Brenntag Biosector, Denmark) adjuvant by the i.m. or s.c. routes in a 100 µl volume and were then challenged at week 4 by the i.v. route with 10^5 viral particles (10^2 plaque-forming units (PFU)) ZIKV-BR or ZIKV-PR. Animals were randomly allocated to groups. Immunologic and virologic assays were performed blinded. Sample size was determined to achieve 80% power to detect significant differences in protective efficacy. All animal studies were approved by the BIDMC Institutional Animal Care and Use Committee (IACUC).

DNA vaccines

ZIKV strain BeH815744 (accession number KU365780) was used to design transgenes, which were produced synthetically. Sequences were

optimized for enhanced transgene expression. Pre-membrane and envelope (prM-Env; defined as amino acids 216–794 of the polyprotein) or Env alone were cloned into the mammalian expression plasmid pcDNA3.1^{+} (Invitrogen). Deletion mutants lacked the transmembrane (ΔTM) or stem (Δstem) regions of Env. A Kozak sequence and the Japanese encephalitis virus leader sequence were included[24]. Plasmids were produced with Machery-Nagel endotoxin-free gigaprep kits. Sequences were confirmed by double-stranded sequencing.

PIV vaccine

The ZIKV purified inactivated virus (PIV, also termed ZPIV) vaccine was produced at the Pilot Bioproduction Facility, Walter Reed Army Institute of Research, Silver Spring, MD, USA. The PIV vaccine was based on the Puerto Rican PRVABC59 isolate, which was obtained from the US Centers for Disease Control and Prevention, Fort Collins, CO, USA. The Vero cells used for passage and vaccine production were a derivative of a certified cell line manufactured at The Salk Institute, Swiftwater, PA. After inoculation, virus was collected on days 5 and 7, clarified by centrifugation and depth filter (0.45–0.2 μm), and treated with benzonase. The viral harvest was concentrated with an ultrafilter followed by purification using Captocore chromatography resin. The purified ZIKV was then inactivated with formalin (0.05%) at 22 °C for 7 days. Following inactivation, formalin was removed by dialysis, and the antigen concentration was adjusted. The final PIV vaccine was assessed for infectivity by passage in Vero cells followed by plaque assays to demonstrate inactivation.

ZIKV challenge stocks

ZIKV stocks were provided by University of São Paulo, Brazil (Brazil ZKV2015; ZIKV-BR[11]) and the US Centers for Disease Control and Prevention, USA (Puerto Rico PRVABC59; ZIKV-PR). Both strains were passage number 3. Low-passage-number Vero cells were then infected at a multiplicity of infection (MOI) of 0.01 PFU per cell. Supernatant was screened daily for viral titers and collected at peak growth. 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 viral particle to PFU ratio of both stocks was approximately 1,000.

RT–PCR

Cap genes of available ZIKV genomes were aligned using Megalign (DNAstar), and primers and probes to a highly conserved region were designed using primer express v3.0 (Applied Biosystems). Primers were synthesized by Integrated DNA Technologies (Coralville) and probes by Biosearch Technologies (Petaluma). To assess viral loads, RNA was extracted from serum with a QIAcube HT (Qiagen). Reverse transcription and RT–PCR were performed as previously described[18]. The wild-type ZIKV BeH815744 *Cap* gene was used as a standard and was cloned into pcDNA3.1+ vector, and the AmpliCap-Max T7 High Yield Message Maker Kit was used to transcribe RNA (Cellscript). RNA was purified using the RNA clean and concentrator kit (Zymo Research), and RNA quality and concentration was assessed by the BIDMC Molecular Core Facility. Log dilutions of the RNA standard were reverse-transcribed and included with each RT–PCR assay. Viral loads were calculated as virus particles per ml. Assay sensitivity was 100 copies per ml. The infectivity of virus in peripheral blood from ZIKV challenged mice was confirmed by PFU assays.

PFU assay

Vero WHO cells were seeded in a MW6 plate to reach confluency at day 3. Cells were infected with log dilutions of ZIKV for 1 h and overlayed with agar. Cells were stained after 6 days of infection by neutral red staining. Plaques were counted, and titers were calculated by multiplying the number of plaques by the dilution and divided by the infection volume.

Western blot

To assess transgene expression from DNA vaccines, cell lysates obtained 48 h following lipofectamine 2000 (Invitrogen) transient transfection of 293T cells were mixed with reducing sample buffer, heated for 5 min at 100 °C, cooled on ice, and run on a precast 4–15% SDS–PAGE gel (Biorad). Protein was transferred to PVDF membranes using the iBlot dry blotting system (Invitrogen), and the membranes were blocked overnight at

4 °C in PBS-T (Dulbeco's phosphate buffered saline + 0.2% V/V Tween 20 + 5% W/V non-fat milk powder). Following overnight blocking, the membranes were incubated for 1 h with PBS-T containing a 1:5,000 dilution of mouse anti-ZIKV Env monoclonal antibody (BioFront Technologies). Membranes were then washed 3 times with PBS-T and incubated for 1 h with PBS-T containing a 1:1,000 dilution of rabbit anti-mouse horseradish peroxidase (HRP) (Jackson ImmunoResearch). Membranes were then washed 3 times with PBS-T and developed using the Amersham ECL plus western blotting detection system (GE Healthcare).

ELISA

Mouse ZIKV Env ELISA kits (Alpha Diagnostic International) were used to determine endpoint antibody titers using a modified protocol. 96-well plates coated with ZIKV Env protein were first equilibrated at room temperature with 300 µl of kit working wash buffer for 5 min. 6 µl of mouse serum was added to the top row, and threefold serial dilutions were tested in the remaining rows. Samples were incubated at room temperature for 1 h, and plates washed 4 times. 100 µl of anti-mouse IgG HRP-conjugate working solution was then added to each well and incubated for 30 min at room temperature. Plates were washed 5 times, developed for 15 min at room temperature with 100 µl of 3,3',5,5'–tetramehylbenzidine (TMB) substrate, and stopped by the addition of 100 µl of stop solution. Plates were analysed at 450 nm / 550 nm on a VersaMax microplate reader using Softmax Pro 6.0 software (Molecular Devices). ELISA endpoint titers were defined as the highest reciprocal serum dilution that yielded an absorbance >2-fold over background values.

Neutralization assay

A high-throughput ZIKV microneutralization (MN) assay was developed for measuring ZIKV-specific neutralizing antibodies as a modified version of a qualified dengue virus microneutralization assay used in clinical dengue vaccine trials[17]. Briefly, serum samples were serially diluted threefold in 96-well micro-plates, and 100 µl of ZIKV-PR containing 100 PFU were added to 100 µl of each serum dilution and incubated at 35 °C for 2 h. Supernatants were then transferred to microtiter plates containing confluent Vero cell monolayers (World Health Organization, NICSC-

011038011038). After incubation for 4 d, cells were fixed with absolute ethanol: methanol for 1 h at −20 °C and washed three times with PBS. The pan-flavivirus monoclonal antibody 6B6-C1 conjugated to HRP (6B6-C1 was a gift from J. T. Roehrig, CDC) was then added to each well, incubated at 35 °C for 2 h, and washed with PBS. Plates were washed, developed with 3,3',5,5'–tetramethylbenzidine (TMB) substrate for 50 min at room temperature, stopped with 1:25 phosphoric acid, and absorbance was read at 450 nm. For a valid assay, the average absorbance at 450 nm of three non-infected control wells had to be ≤ 0.5, and virus-only control wells had to be ≥ 0.9. Normalized absorbance values were calculated, and the MN50 titer was determined by a log mid-point linear regression model. The MN50 titer was calculated as the reciprocal of the serum dilution that neutralized ≥ 50% of ZIKV. Seropositivity was defined as a titer ≥ 1:10.

ELISPOT

ZIKV-specific cellular immune responses were assessed by IFNγ ELISPOT assays using pool of overlapping 15-amino-acid peptides covering the prM or Env proteins (JPT). 96-well multiscreen plates (Millipore) were coated overnight with 100 µl per well of 10 µg ml^{-1} anti-mouse IFNγ (BD Biosciences) in endotoxin-free Dulbecco's PBS (D-PBS). The plates were then washed three times with D-PBS containing 0.25% Tween 20 (D-PBS-Tween), blocked for 2 h with D-PBS containing 5% FBS at 37 °C, washed three times with D-PBS-Tween, rinsed with RPMI 1640 containing 10% FBS to remove the Tween 20, and incubated with 2 µg ml^{-1} of each peptide and 5×10^5 mouse splenocytes in triplicate in 100 µl reaction mixture volumes. Following 18 h incubation at 37 °C, the plates were washed nine times with PBS-Tween and once with distilled water. The plates were then incubated with 2 µg ml^{-1} biotinylated anti-mouse IFNγ (BD Biosciences) for 2 h at room temperature, washed six times with PBS-Tween, and incubated for 2 h with a 1:500 dilution of streptavidin-alkaline phosphatase (Southern Biotechnology Associates). Following five washes with PBS-Tween and one with PBS, the plates were developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate chromogen (Pierce), stopped by washing with tap water, air dried, and read using an ELISPOT reader (Cellular Technology Ltd). The numbers of spot-forming cells (SFC) per 10^6 cells were calculated. The medium background levels were typically <15 SFC per 10^6 cells.

Intracellular cytokine staining

ZIKV-specific $CD4^+$ and $CD8^+$ T-lymphocyte responses were assessed using splenocytes and analysed by flow cytometry. Cells were stimulated for 1 h at 37 °C with 2 μg ml^{-1} of overlapping 15-amino-acid peptides covering the prM or Env proteins (JPT). Following incubation, brefeldin-A and monensin (BioLegend) were added, and samples were incubated for 6 h at 37 °C. Cells were then washed, stained, permeabilized with Cytofix/Cytoperm (BD Biosciences). Data was acquired using an LSR II flow cytometer (BD Biosciences) and analysed using FlowJo v.9.8.3 (Treestar). Monoclonal antibodies included: CD4 (RM4-5), CD8α (53-6.7), CD44 (IM7), and IFNγ (XMG1.2). Antibodies were purchased from BD Biosciences, eBioscience, or BioLegend. Vital dye exclusion (LIVE/DEAD) was purchased from Life Technologies.

IgG purification and adoptive transfer

Serum was collected from prM-Env DNA-vaccinated mice or naive mice, and polyclonal IgG was purified using protein G purification kits (Thermo Fisher Scientific). Varying amounts of purified IgG was infused by the i.v. route into naive recipient mice before ZIKV challenge.

$CD4^+$ and $CD8^+$ T-lymphocyte depletion

Anti-CD4 (GK1.5) and/or anti-CD8 (2.43) (Bio X Cell) monoclonal antibodies were administered at doses of 500 μg per mouse to prM-Env DNA vaccinated mice by the i.p. route on day −2 and day −1 before ZIKV challenge. Antibody depletions were >99.9% efficient as determined by flow cytometry.

Statistical analyses

Analysis of virologic and immunologic data was performed using GraphPad Prism version 6.03 (GraphPad Software). Comparisons of groups was performed using *t*-tests and Wilcoxon rank-sum tests. Correlations were assessed by Spearman rank-correlation tests.

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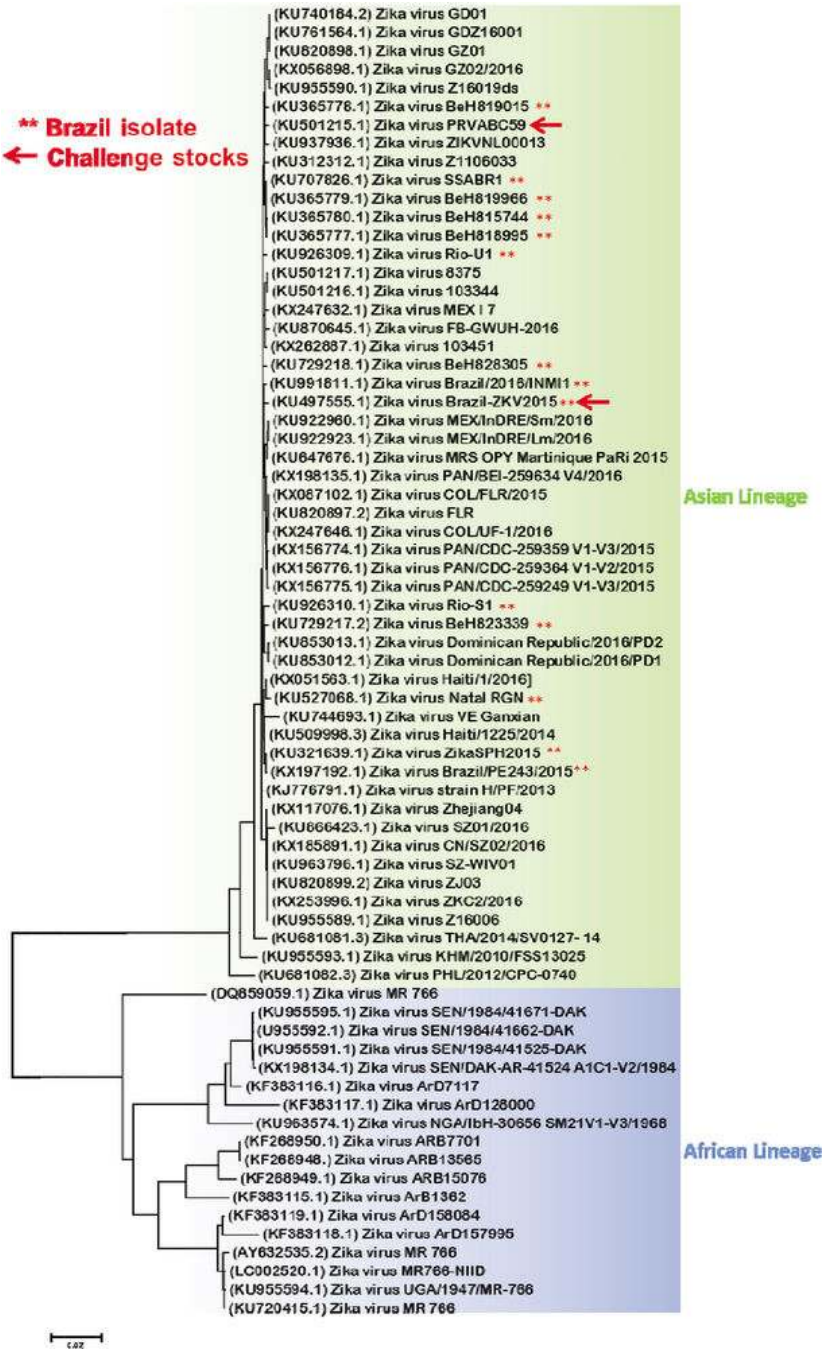
SUPPLEMENTARY MATERIALS



Extended Data Figure 1. ZIKV maximum likelihood phylogenetic tree. The ZIKV-BR and ZIKV-PR challenge isolates are depicted with red arrows.

- Brazil ZKV2015 (Brazil strain; ZIKV-BR challenge stock)
- PRVABC59 (Puerto Rico strain; ZIKV-PR challenge stock)
- BeH815744 (Brazil strain; immunogen design)
- H PF 2013 (French Polynesian strain)
- MR766 (African strain)

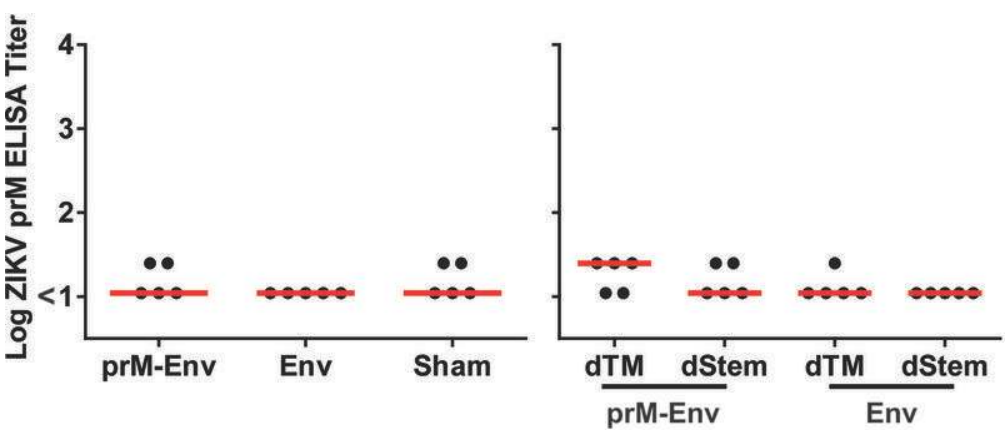
AA identical

# AA divergent	Brazil ZKV2015	PRVABC59	BeH815744	H PF 2013	MR766
Brazil ZKV2015		3418	3419	3419	3294
PRVABC59	5		3420	3420	3295
BeH815744	4	3		3421	3296
H PF 2013	4	3	2		3298
MR766	125	124	123	121	

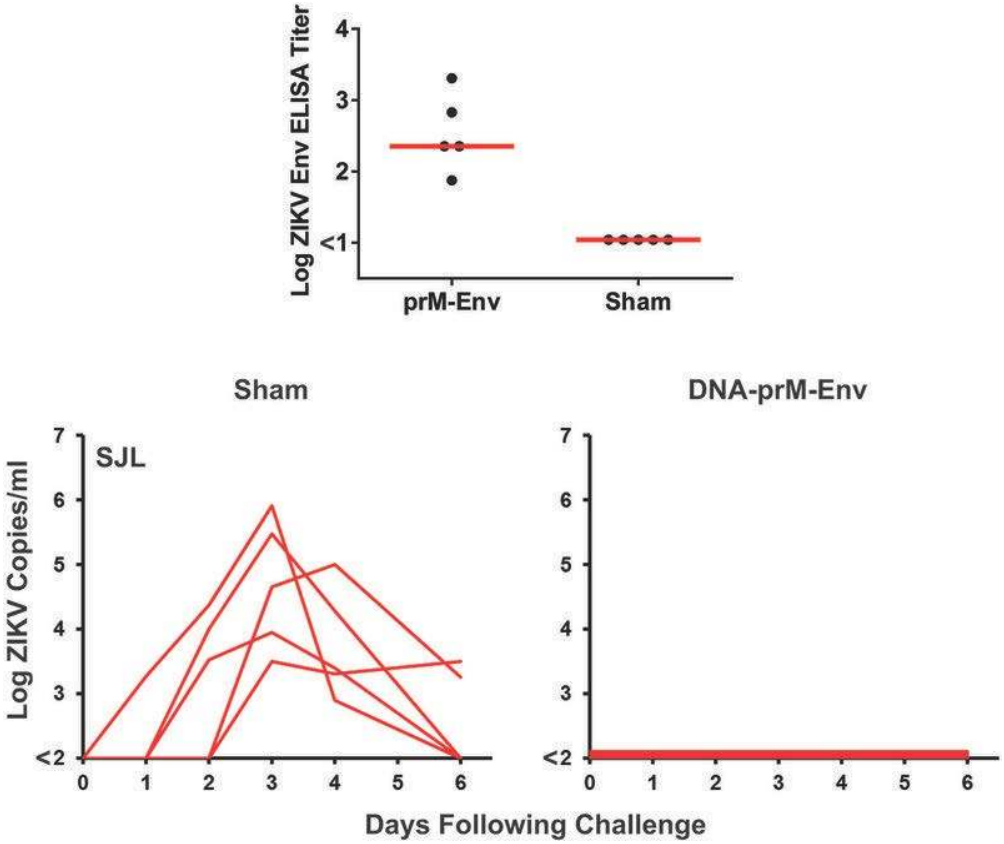
Percent identity

Divergence	Brazil ZKV2015	PRVABC59	BeH815744	H PF 2013	MR766
Brazil ZKV2015		99.9	99.9	99.9	96.5
PRVABC59	0.1		99.9	99.9	96.5
BeH815744	0.1	0.1		99.9	96.5
H PF 2013	0.1	0.1	0.1		96.6
MR766	3.6	3.6	3.6	3.5	

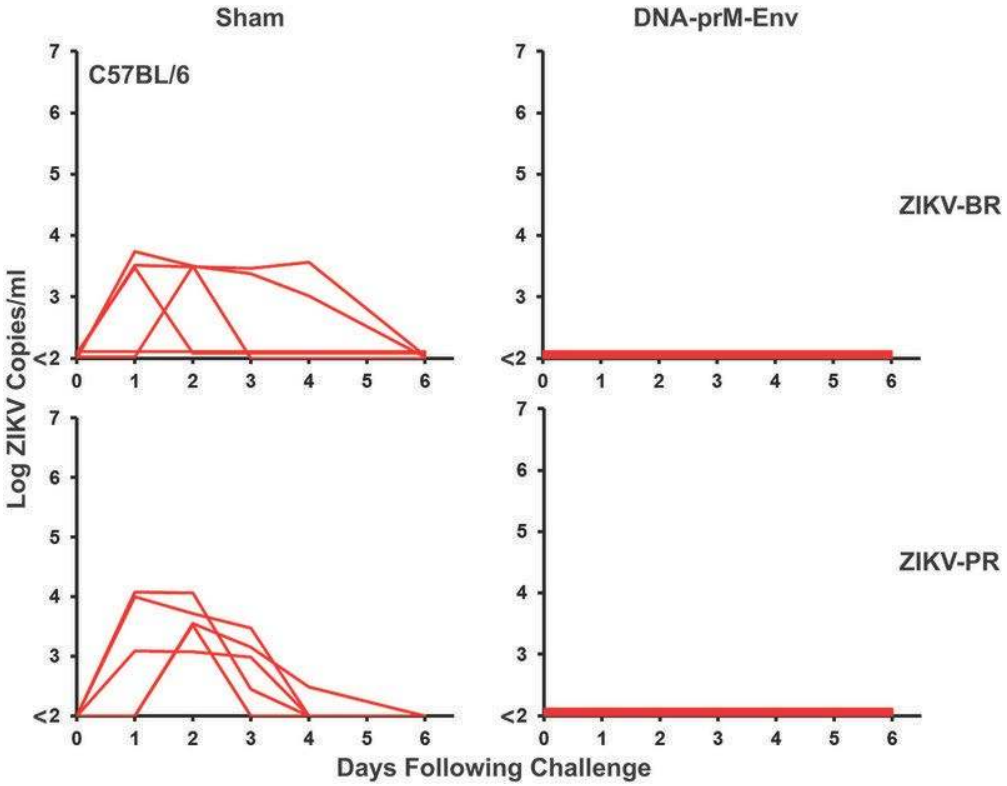
Extended Data Figure 2. **ZIKV amino acid sequence comparisons.** Number of and percentage of amino acid differences in the polyprotein are shown for the following ZIKV isolates: Brazil/ZKV2015 (Brazil strain; ZIKV-BR challenge stock), PRVABC59 (Puerto Rico strain; ZIKV-PR challenge stock), BeH815744 (Brazil strain; immunogen design), H/PF/2013 (French Polynesian strain), and MR766 (African strain).



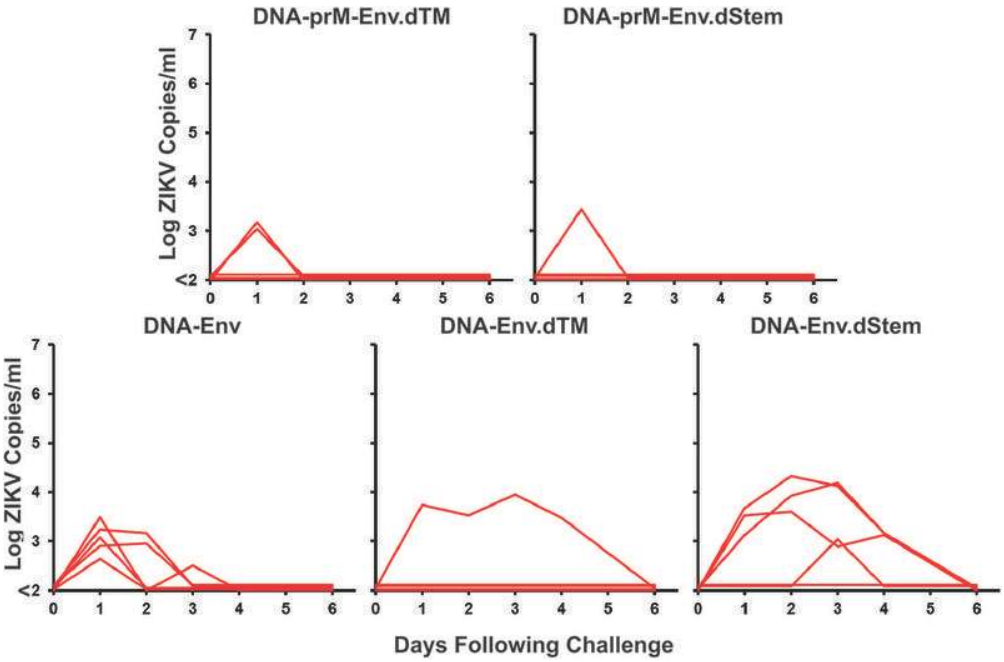
Extended Data Figure 3. prM-specific antibody responses in DNA-vaccinated mice. In the experiment described in Fig. 2, humoral immune responses were assessed at week 3 following vaccination by prM-specific ELISA. Red bars reflect medians.



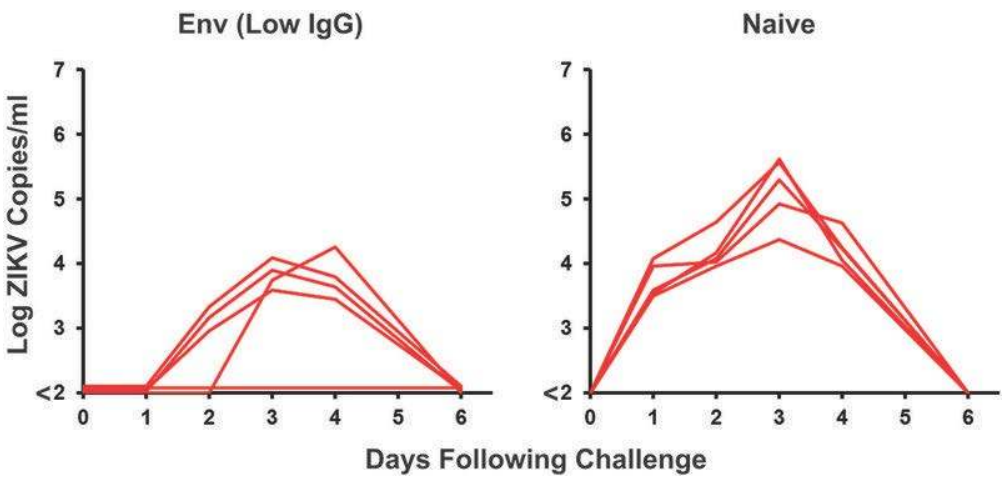
Extended Data Figure 4. Immunogenicity and protective efficacy of prM-Env DNA vaccine in SJL mice. SJL mice (*n* = 5 per group) received a single immunization by the i.m. route with 50 µg prM-Env DNA vaccine or a sham vaccine and were challenged at week 4 by the i.v. route with 10^5 viral particles (10^2 PFU) ZIKV-BR. Humoral immune responses were assessed at week 3 after vaccination by Env-specific ELISA (top). Red bars reflect medians. Serum viral loads are shown following ZIKV-BR challenge (bottom).



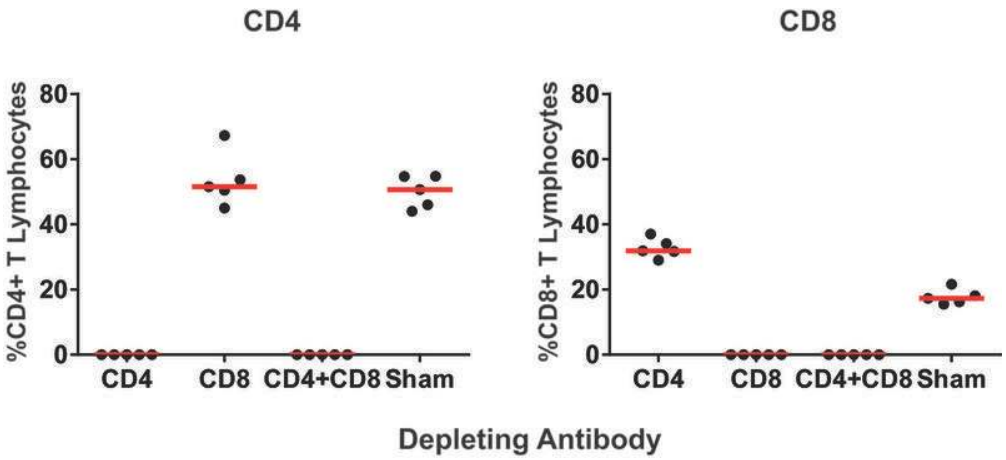
Extended Data Figure 5. Protective efficacy of prM-Env DNA vaccine in C57BL/6 mice. C57BL/6 mice (*n* = 5 per group) received a single immunization by the i.m. route with 50 µg prM-Env DNA vaccine or a sham vaccine and were challenged at week 4 by the i.v. route with 10^5 viral particles (10^2 PFU) ZIKV-BR or ZIKV-PR. Serum viral loads are shown following challenge.



Extended Data Figure 6. Protective efficacy of various DNA vaccines in C57BL/6 mice. C57BL/6 mice (*n* = 5 per group) received a single immunization by the i.m. route with 50 µg of various DNA vaccines and were challenged at week 4 by the i.v. route with 10^5 viral particles (10^2 PFU) ZIKV-BR. Serum viral loads are shown following challenge.



Extended Data Figure 7. Adoptive transfer of low titers of Env-specific IgG. Serum viral loads in mice that received adoptive transfer of low titers of Env-specific IgG (as defined in Fig. 3a) and were then challenged with ZIKV-BR.



Extended Data Figure 8. CD4$^+$ and CD8$^+$ T-lymphocyte depletion. CD4$^+$ and/or CD8$^+$ T-lymphocyte depletion following monoclonal antibody treatment of Balb/c mice vaccinated with prM-Env DNA.

3

PROTECTIVE EFFICACY OF MULTIPLE VACCINE PLATFORMS AGAINST ZIKA VIRUS CHALLENGE IN RHESUS MONKEYS

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ABSTRACT

Zika virus (ZIKV) is responsible for a major ongoing epidemic in the Americas and has been causally associated with fetal microcephaly. The development of a safe and effective ZIKV vaccine is therefore an urgent global health priority. Here we demonstrate that three different vaccine platforms protect against ZIKV challenge in rhesus monkeys. A purified inactivated virus vaccine induced ZIKV-specific neutralizing antibodies and completely protected monkeys against ZIKV strains from both Brazil and Puerto Rico. Purified immunoglobulin from vaccinated monkeys conferred passive protection in adoptive transfer studies. A plasmid DNA vaccine and a single-shot recombinant rhesus adenovirus serotype 52 vector expressing ZIKV prM-Env also elicited neutralizing antibodies and completely protected monkeys against ZIKV challenge. These data support the rapid clinical development of ZIKV vaccines for humans.

The explosive and unprecedented ZIKV outbreak in the Americas (1, 2) prompted the World Health Organization to declare this epidemic a public health emergency of international concern. ZIKV has been causally associated with fetal microcephaly, intrauterine growth retardation, and other congenital malformations in both humans (3–6) and mice (7–9), and has also been linked with neurologic disorders such as Guillain-Barre syndrome (10). Several reports have shown that ZIKV can infect placental and fetal tissues, leading to prolonged viremia in pregnant women (11) and nonhuman primates (12). ZIKV also appears to target cortical neural progenitor cells (7–9, 13, 14), which likely contributes to neuropathology.

We recently reported the protective efficacy of a ZIKV purified inactivated virus (PIV) vaccine from strain PRVABC59 and a DNA vaccine expressing an optimized pre-membrane and envelope (prM-Env) immunogen from strain BeH815744 against ZIKV challenges in mice (15). These studies utilized ZIKV challenge strains from both Brazil (ZIKV-BR; Brazil/ZKV2015) (9) and Puerto Rico (ZIKV-PR; PRVABC59). ZIKV replication in mice was dependent on the mouse strain (15) and may be less extensive than in nonhuman primates (12). We therefore evaluated the immunogenicity and protective efficacy of inactivated virus, gene-based, and vector-based vaccines in ZIKV challenge studies in rhesus monkeys.

ZIKV PIV vaccine study

We first immunized 16 rhesus monkeys by the subcutaneous route with 5 μg ZIKV PIV vaccine with alum (N=8) or sham vaccine (alum only) (N=8) at weeks 0 and 4 (fig. S1). All PIV vaccinated animals developed ZIKV Env-specific binding antibodies by ELISA as well as ZIKV-specific neutralizing antibodies by microneutralization (MN50) assays at week 2 following initial immunization. Median log antibody titers at week 2 were 1.87 by ELISA (Fig. 1A) and 2.27 by MN50 assays (Fig. 1B). Following the week 4 boost immunization, median log antibody titers increased substantially to 3.54 by ELISA (Fig. 1A) and 3.66 by MN50 assays (Fig. 1B) at week 6. In contrast, sham control monkeys did not develop detectable ZIKV-specific antibody responses (fig. S2). Binding antibody titers correlated with neutralizing antibody titers in the PIV vaccinated animals ($P<0.0001$, $R=0.88$, Spearman rank correlation test; fig. S3), although only minimal antibody-dependent cellular phagocytosis responses were observed. The majority of PIV vaccinated monkeys (Fig. 1, C and D) but not sham control animals (fig. S4) also developed modest cellular immune responses, primarily to Env, as measured by interferon (IFN)-γ ELISPOT assays.

To assess the protective efficacy of the PIV vaccine against ZIKV challenge, we infected PIV immunized and sham control monkeys by the subcutaneous route with 10^6 viral particles (VP) [10^3plaque-forming units (PFU)] of ZIKV-BR or ZIKV-PR (N=4/group) (*15*). Viral loads following ZIKV challenge were quantitated by RT-PCR (*15*), and viral infectivity was confirmed by growth in Vero cells. ZIKV-specific MN50 titers increased following challenge, particularly in the sham controls (fig. S5). Sham control monkeys exhibited 6-7 days of detectable viremia with median peak viral loads of 5.82 log copies/ml (range 5.21-6.29 log copies/ml; N=8) on day 3-5 following challenge (Fig. 2A). Virus was also detected in the majority of sham control animals in urine and cerebrospinal fluid (CSF) on day 3, as well as in colorectal secretions and cervicovaginal secretions on day 7 (Fig. 2, B to E).

In contrast, PIV vaccinated monkeys showed complete protection against ZIKV challenge, as evidenced by no detectable virus (<100 copies/ml) in blood, urine, CSF, colorectal secretions, and cervicovaginal secretions in all animals following challenge (N=8; P=0.0002, Fisher's exact test comparing PIV vaccinated animals vs. sham controls). We were unable to assess ZIKV in

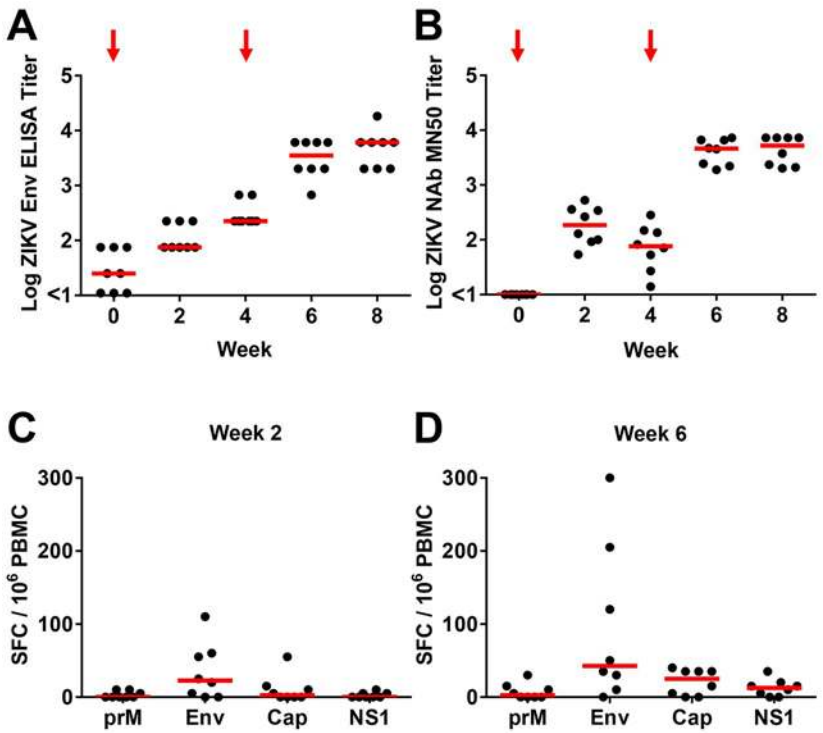

Fig. 1. Immunogenicity of the ZIKV PIV vaccine. (**A**) Env-specific ELISA titers and (**B**) ZIKV-specific microneutralization (MN50) titers following immunization of rhesus monkeys by the s.c route with 5 µg ZIKV PIV vaccine at weeks 0 and 4 (red arrows). The maximum measurable log MN50 titer in this assay was 3.86. Cellular immune responses by IFN-γ ELISPOT assays to prM, Env, Cap, and NS1 at (**C**) week 2 and (**D**) week 6. Red bars reflect medians.

semen in the male animals in this study due to inadequate sample volumes. No major differences in plasma viral loads were observed between the sham controls that received ZIKV-BR vs. ZIKV-PR (fig. S6).

Adoptive transfer studies

We next explored the mechanism of the observed protection by adoptive transfer studies. We purified IgG from plasma from ZIKV PIV vaccinated monkeys at week 8 by protein G affinity chromatography. Vaccine-elicited, ZIKV-specific IgG was then infused into four groups of naïve Balb/c mice (N=5/group) by 5-fold serial dilutions of the purified IgG preparation, which had a log ELISA titer of 3.30 and a log MN50 titer of 3.30.

Following infusion, these groups of recipient mice (designated I, II, III, IV) had median log ELISA titers of 2.83, 2.35, 1.40, and <1.00 (Fig. 3A) and median log MN50 titers of 2.93, 1.77, 1.14, and <1.00 (Fig. 3B). Mice were then challenged by the intravenous route with 10^5 VP (10^2 PFU) of ZIKV-BR, as we previously described (*15*). The higher two doses of purified IgG provided complete protection following ZIKV challenge, whereas the lower two doses of purified IgG resulted in reduced viremia as compared with sham infused control mice (Fig. 3, C to E).

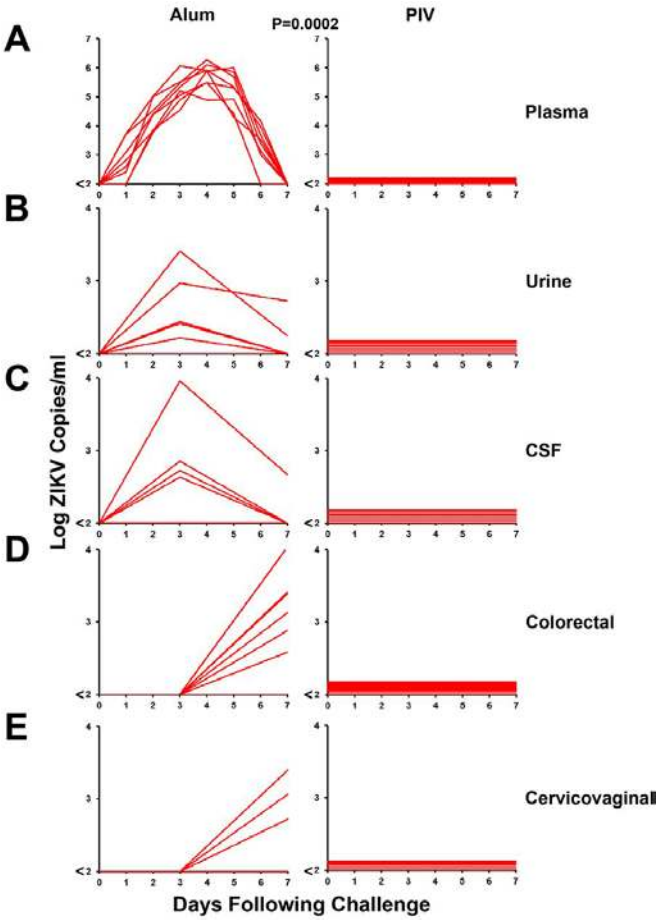

Fig. 2. Protective efficacy of the ZIKV PIV vaccine. PIV vaccinated and sham control rhesus monkeys (*n* = 8/group) were challenged by the s.c route with 10^6 VP (10^3 PFU) ZIKV-BR or ZIKV-PR. Each group contained 6 female and 2 male animals. Viral loads are shown in (**A**) plasma, (**B**) urine, (**C**) CSF, (**D**) colorectal secretions, and (**E**) cervicovaginal secretions. Viral loads were determined on days 0, 1, 2, 3, 4, 5, 6, 7 for the plasma samples (A) and on days 0, 3, 7 for the other samples [(B) to (E)]. Data are shown for all 8 animals in each panel, except for the 6 females for cervicovaginal secretions in (E). *P* value reflects Fisher's exact test.

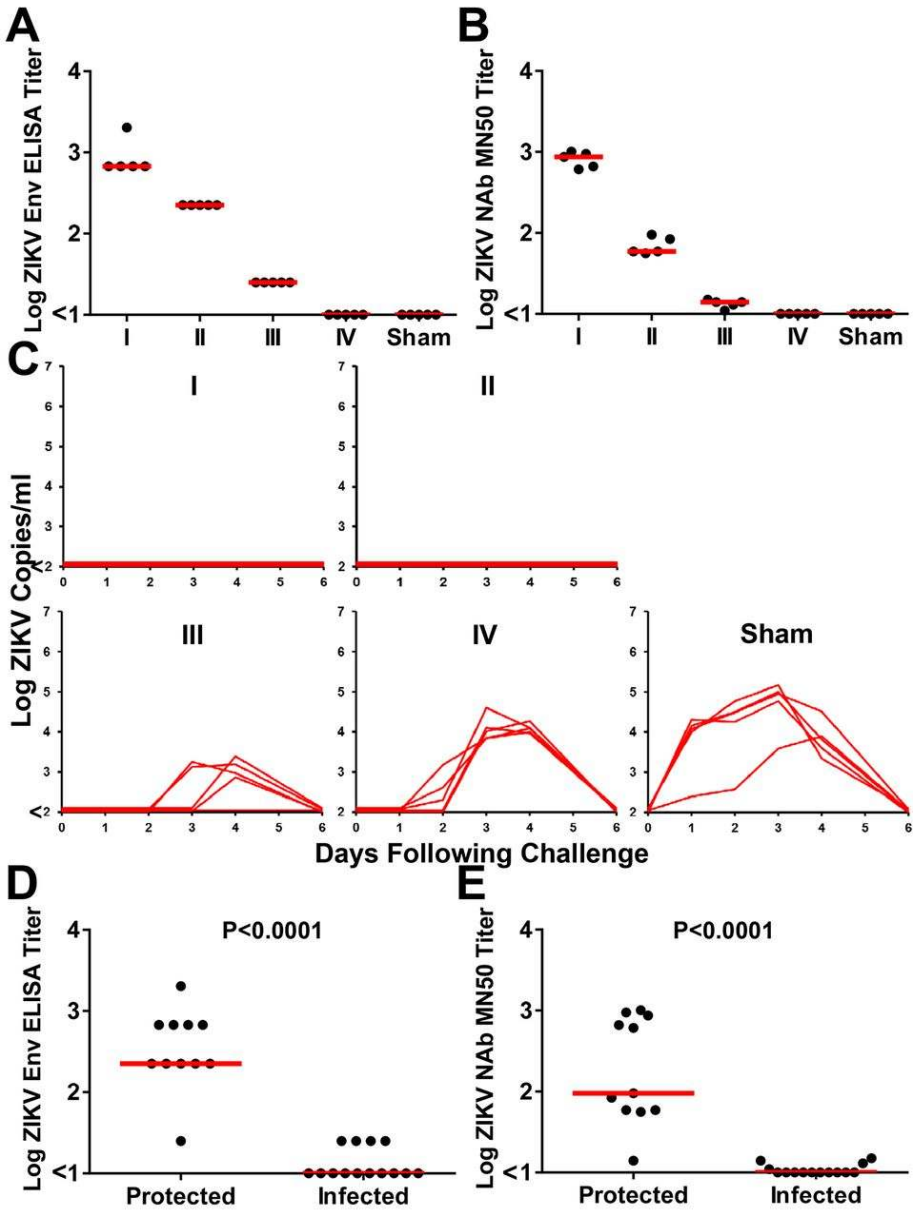

Fig. 3 Adoptive transfer studies in mice. (**A**) Env-specific serum ELISA titers and (**B**) ZIKV-specific microneutralization (MN50) titers in serum from recipient Balb/c mice (*n* = 5/group) 1 hour following adoptive transfer of 5-fold serial dilutions (Groups I, II, III, IV) of IgG purified from PIV vaccinated rhesus monkeys or sham controls. (**C**) Plasma viral loads in mice following challenge with 10^5 VP (10^2 PFU) ZIKV-BR. (**D** and **E**) Immune correlates of protection. Red bars reflect medians. *P* values reflect *t* tests.

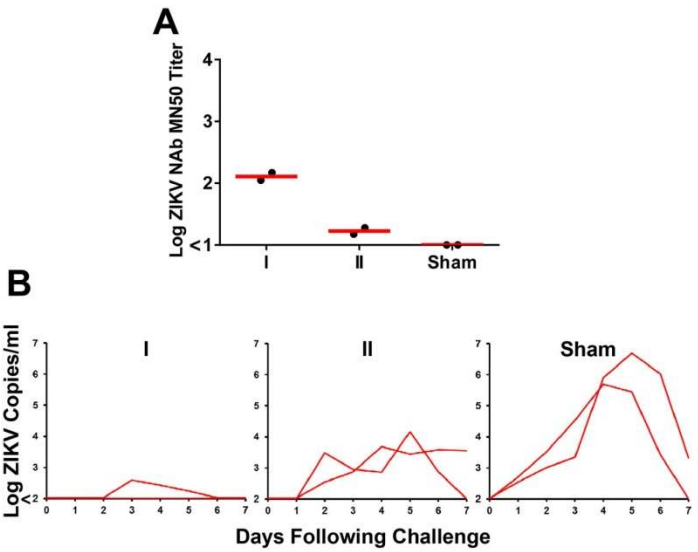

Fig. 4 Adoptive transfer studies in rhesus monkeys. (**A**) ZIKV-specific microneutralization (MN50) titers in serum from recipient rhesus monkeys (*n* = 2/group) 1 hour following adoptive transfer of 5-fold dilutions (Groups I, II) of IgG purified from PIV vaccinated rhesus monkeys or sham controls. (**B**) Plasma viral loads in rhesus monkeys following challenge with 10^6VP (10^3 PFU) ZIKV-BR. Red bars reflect medians.

Vaccine-elicited, ZIKV-specific IgG was also infused into two groups of naïve rhesus monkeys (N=2/group). Following infusion, these groups of recipient monkeys (designated I, II) had median log MN50 titers of 2.11 and 1.22 (Fig. 4A). Monkeys were then challenged with 10^6 VP (10^3 PFU) of ZIKV-BR. In the animals that received the higher IgG dose, one animal was completely protected and the other showed a blip of viremia on days 3-5 (Fig. 4B). No enhancement of viral replication was observed at subtherapeutic IgG concentrations. Taken together, these data demonstrate that purified IgG from ZIKV PIV vaccinated rhesus monkeys provided passive protection following adoptive transfer in both rodents and primates.

ZIKV DNA and RhAd52 vaccine study

To evaluate the immunogenicity and protective efficacy of gene-based and vector-based ZIKV vaccines, we immunized 12 rhesus monkeys with a plasmid DNA vaccine (*15*) or a rhesus adenovirus serotype 52 (RhAd52) vector-based vaccine (*16*) (fig. S1). Monkeys were immunized by

the intramuscular route with 5 mg DNA vaccine expressing prM-Env at weeks 0 and 4 (N=4), a single immunization of 10^{11} VP RhAd52 vector expressing prM-Env at week 0 (N=4), or sham vaccine (N=4). The DNA-prM-Env vaccine induced ZIKV-specific neutralizing antibody titers in all animals after the week 4 boost immunization, although only minimal MN50 titers were detected after the initial priming immunization (Fig. 5A). In contrast, the RhAd52-prM-Env vaccine induced ZIKV-specific neutralizing antibody responses in all animals at week 2 after the initial priming immunization (Fig. 5A). Moreover, the RhAd52 vector induced substantial breadth of antibody responses against linear ZIKV Env epitopes by peptide microarray assays as compared to the other vaccines tested (*17*) (fig. S7).

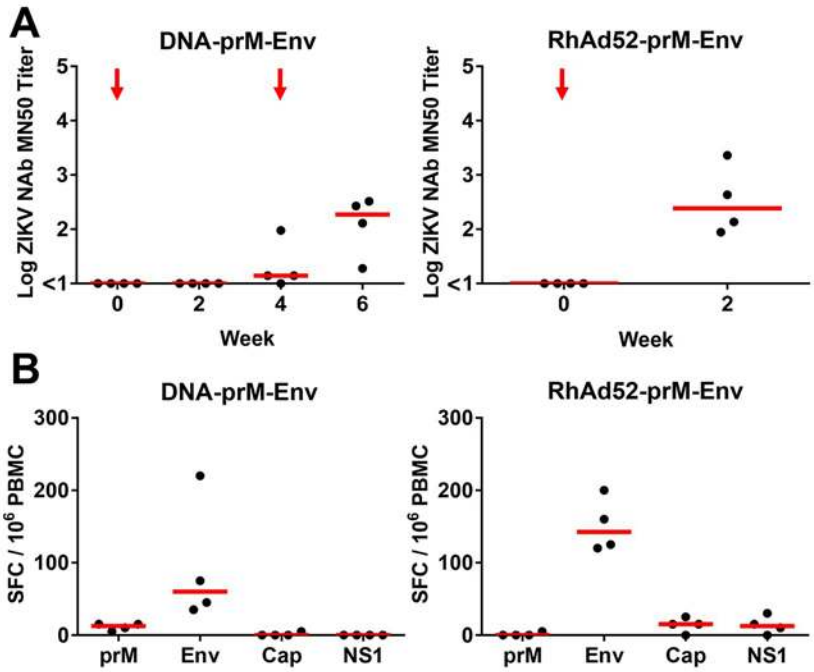

Fig. 5 Immunogenicity of the ZIKV DNA-prM-Env and RhAd52-prM-Env vaccines. (**A**) ZIKV-specific microneutralization (MN50) titers following immunization of rhesus monkeys by the i.m. route with 5 mg DNA-prM-Env vaccine at weeks 0 and 4 (red arrows) or a single immunization with 10^{11} vp RhAd52-prM-Env at week 0. (**B**) Cellular immune responses by IFN-γ ELISPOT assays to prM, Env, Cap, and NS1 at week 6 for the DNA vaccine or at week 4 for the RhAd52 vaccine. Red bars reflect medians.

The DNA-prM-Env vaccine also induced detectable Env-specific IFN-γ ELISPOT responses after the week 4 boost immunization, and the RhAd52-prM-Env vaccine induced Env-specific cellular immune responses after the initial week 0 priming immunization (Fig. 5B). Monkeys were challenged 4 weeks after the final vaccination, and both the DNA and RhAd52 vaccines provided complete protection against subcutaneous challenge with 10^6VP (10^3 PFU) of ZIKV-BR as measured by plasma viral loads (Fig. 6).

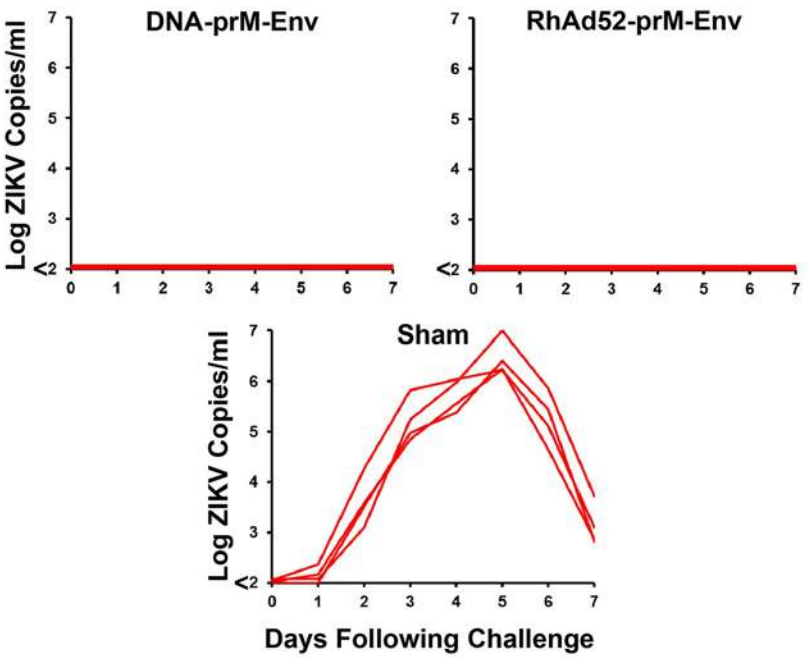

Fig. 6 Protective efficacy of the ZIKV DNA-prM-Env and RhAd52-prM-Env vaccines. DNA vaccinated, RhAd52 vaccinated, and sham control rhesus monkeys (*n* = 4/group) were challenged by the s.c route with 10^6 VP (10^3 PFU) ZIKV-BR or ZIKV-PR. Plasma viral loads are shown.

DISCUSSION

In this study, we demonstrate that three different vaccine platforms provided complete protection against ZIKV challenge in rhesus monkeys. No specific clinical safety adverse effects related to the vaccines were observed. We recently reported the protective efficacy of the ZIKV PIV vaccine and the DNA-prM-Env vaccine in mice (*15*). The present data confirm and extend these prior studies by demonstrating robust protection with these vaccines

against ZIKV challenge in nonhuman primates, and specifically utilizing the dose, route, and schedule of these vaccines that are typically evaluated in clinical trials. Although the ZIKV PIV vaccine and the DNA-prM-Env vaccine appeared comparably immunogenic in mice (*15*), the PIV vaccine proved more potent than the DNA vaccine in rhesus monkeys under the conditions tested (Figs. 1 and 5). To generalize these observations to a vector-based vaccine, we also evaluated the RhAd52-prM-Env vaccine, which proved highly immunogenic and afforded complete protection after a single immunization in monkeys (Fig. 5). Rhesus adenovirus vectors have the potential advantage of minimal baseline vector-specific neutralizing antibodies in human populations (*16*).

The adoptive transfer studies demonstrate that vaccine-elicited antibodies are sufficient for protection against ZIKV challenge. Moreover, passive protection in mice and rhesus monkeys was observed at relatively low antibody titers (Figs. 3 and 4). Such antibody titers are likely achievable by these vaccine platforms in humans, thus raising optimism for the development of a ZIKV vaccine for humans. Future preclinical and clinical studies will need to address the potential impact of cross-reactive antibodies against dengue virus and other flaviviruses. Secondary infection with a heterologous dengue serotype can be clinically more severe than initial infection, which may or may not reflect antibody-dependent enhancement (*18*, *19*). Cross-reactive antibodies between ZIKV and dengue virus have also been described (*20*, *21*), and dengue-specific antibodies have been reported to increase ZIKV replication in vitro (*22*). The relevance and implications of these findings for ZIKV vaccine development remain to be determined.

The consistent and robust antibody-based correlates of vaccine protection against ZIKV challenge in both rodents and primates suggest the generalizability of these findings. Similar correlates of protection, and specifically neutralizing antibody titers >10, have been reported for other flavivirus vaccines in humans (*23*–*25*). Taken together, these data suggest a path forward for clinical development of ZIKV vaccines in humans. PIV vaccines have been evaluated previously in clinical trials for other flaviviruses, including dengue virus, tick-borne encephalitis virus, and Japanese encephalitis virus (*26*–*30*). Phase 1 clinical trials with the ZIKV PIV

vaccine, as well as other candidate ZIKV vaccines, are expected to begin later this year.

MATERIALS AND METHODS

Animals, vaccines, and challenges

34 outbred, Indian-origin male and female rhesus monkeys (Macaca mulatta) were housed at Bioqual, Rockville, MD. In the first vaccine study, monkeys were immunized by the s.c. route with 5 μg ZIKV purified inactivated virus (PIV) vaccine derived from the PRVABC59 isolate (15) with alum (Alhydrogel; Brenntag Biosector, Denmark) or alum alone at weeks 0 and 4 (N=8/group). In the second vaccine study, monkeys were immunized by the i.m. route with 5 mg DNA vaccines expressing prM-Env (amino acids 216–794 of the polyprotein derived from the BeH815744 isolate and optimized for high expression) (15) at weeks 0 and 4, a single immunization of 1011 VP RhAd52 (16) expressing prM-Env at week 0, or sham controls (N=4/group). Rhesus monkeys were challenged four weeks after the final immunization by the s.c route with 106 viral particles (VP) [103 plaque-forming units (PFU)] ZIKV-BR (Brazil ZKV2015) or ZIKV-PR (PRVABC59) (15). For adoptive transfer studies, Balb/c mice were infused i.v. with IgG purified from PIV vaccinated monkeys at week 8 and were challenged by the i.v. route with 105 VP (102 PFU) ZIKV-BR. Rhesus monkeys were infused i.v. with IgG purified from PIV vaccinated monkeys at week 8 and were challenged by the s.c. route with 106 VP (103 PFU) ZIKV-BR. Animals were randomly allocated to groups. Immunologic and virologic assays were performed blinded. All animal studies were approved by the appropriate Institutional Animal Care and Use Committee (IACUC).

RT-PCR

RT-PCR assays were utilized to monitor viral loads, essentially as previously described (15). RNA was extracted from plasma or other samples with a QIAcube HT (Qiagen, Germany). The wildtype ZIKV BeH815744 Cap gene was utilized as a standard. RNA was purified (Zymo Research, CA, USA), and RNA quality and concentration was assessed by the BIDMC Molecular Core Facility. Log dilutions of the RNA standard were reverse transcribed and included with each RT-PCR assay. Viral loads were calculated as virus

particles (VP) per ml and were confirmed by PFU assays. Assay sensitivity was 100 copies/ml.

PFU assay

Vero WHO cells were seeded in a MW6 plate to reach confluency at day 3. Cells were infected with log dilutions of ZIKV for 1 h and overlayed with agar. Cells were stained after 6 days of infection by neutral red staining. Plaques were counted, and titers were calculated by multiplying the number of plaques by the dilution and divided by the infection volume.

ELISA

Monkey ZIKV Env ELISA kits (Alpha Diagnostic International, TX, USA) were used to determine endpoint binding antibody titers using a modified protocol. 96-well plates coated with ZIKV Env protein were first equilibrated at room temperature with 300 µl of kit working wash buffer for 5 min. 6 µl of monkey serum was added to the top row, and 3-fold serial dilutions were tested in the remaining rows. Samples were incubated at room temperature for 1 h, and plates washed 4 times. 100 µl of anti-human IgG HRP-conjugate working solution was then added to each well and incubated for 30 min at room temperature. Plates were washed 5 times, developed for 15 min at room temperature with 100 µl of TMB substrate, and stopped by the addition of 100 µl of stop solution. Plates were analyzed at 450nm/550nm on a VersaMax microplate reader using Softmax Pro 6.0 software (Molecular Devices, CA, USA). ELISA endpoint titers were defined as the highest reciprocal serum dilution that yielded an absorbance >2-fold over background values. Log10 endpoint titers are reported.

Neutralization assay

A high-throughput ZIKV microneutralization (MN) assay was utilized for measuring ZIKV-specific neutralizing antibodies, essentially as previously described (15). Briefly, serum samples were serially diluted three-fold in 96-well micro-plates, and 100 µl of ZIKV-PR containing 100 PFU were added to 100 µl of each serum dilution and incubated at 35ºC for 2 h. Supernatants were then transferred to microtiter plates containing confluent Vero cell monolayers (World Health Organization, NICSC-011038011038). After

incubation for 4 d, cells were fixed with absolute ethanol: methanol for 1 h at –20°C and washed three times with PBS. The pan-flavivirus monoclonal antibody 6B6-C1 conjugated to HRP (6B6-C1 was a gift from JT Roehrig, CDC) was then added to each well, incubated at 35°C for 2 h, and washed with PBS. Plates were washed, developed with 3,3',5,5'–tetramethylbenzidine (TMB) for 50 min at room temperature, stopped with 1:25 phosphoric acid, and absorbance was read at 450 nm. For a valid assay, the average absorbance at 450 nm of three non-infected control wells had to be ≤ 0.5, and virus-only control wells had to be ≥ 0.9. Normalized absorbance values were calculated, the MN50 titer was determined by a log mid-point linear regression model. The MN50 titer was calculated as the reciprocal of the serum dilution that neutralized ≥ 50% of ZIKV, and seropositivity was defined as a titer ≥ 10, with the maximum measurable titer 7,290. Log10 MN50 titers are reported.

Antibody peptide microarrays

IgG binding to linear peptides spanning ZIKV Env was measured with peptide microarrays (JPT Peptide Technologies, Berlin, Germany), essentially as previously described (17). Briefly, microarrays consisted of 3 identical subarrays containing 153 overlapping 15 amino acid ZIKV Env peptides, which covered 98.2% of available ZIKV Env sequences. Serum was incubated with the microarrays and Alexa Fluor 647-conjugated anti-human IgG. The readout and image processing was performed with Genepix 4300A scanner/software. Mean fluorescent intensity (MFI) equaled the mean of triplicate peptides and was corrected by subtracting values from matched peptides on control microarrays incubated with secondary antibody alone. The threshold for positivity was >5x noise distribution of the sample size.

ELISPOT

ZIKV-specific cellular immune responses were assessed by interferon-γ (IFN-γ) ELISPOT assays using pools of overlapping 15-amino-acid peptides covering the prM, Env, Cap, and NS1 proteins (JPT, Berlin, Germany), essentially as we previously described (15). 96-well multiscreen plates (Millipore, MA, USA) were coated overnight with 100 µl/well of 10 µg/ml anti-human IFN-γ (BD Biosciences, CA, USA) in endotoxin-free Dulbecco's PBS (D-PBS). The plates were then washed three times with D-PBS

containing 0.25% Tween 20 (D-PBS-Tween), blocked for 2 h with D-PBS containing 5% FBS at 37°C, washed three times with D-PBS-Tween, rinsed with RPMI 1640 containing 10% FBS to remove the Tween 20, and incubated with 2 μg/ml of each peptide and 2 × 105 monkey PBMC in triplicate in 100 μl reaction mixture volumes. Following an 18 h incubation at 37°C, the plates were washed nine times with PBSTween and once with distilled water. The plates were then incubated with 2 μg/ml biotinylated anti-human IFN-γ (BD Biosciences, CA, USA) for 2 h at room temperature, washed six times with PBS-Tween, and incubated for 2 h with a 1:500 dilution of streptavidin-alkaline phosphatase (Southern Biotechnology Associates, AL, USA). Following five washes with PBS-Tween and one with PBS, the plates were developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate chromogen (Pierce, IL, USA), stopped by washing with tap water, air dried, and read using an ELISPOT reader (Cellular Technology Ltd., OH, USA). The numbers of spot-forming cells (SFC) per 106 cells were calculated. The medium background levels were typically

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SUPPLEMENTARY MATERIALS

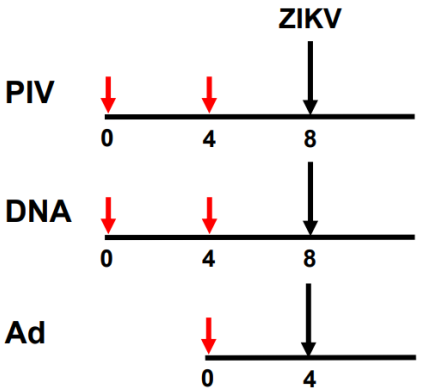

Figure S1. **Vaccine schedules**. Immunization and challenge schedules for the ZIKV purified inactivated virus (PIV) vaccine, plasmid DNA vaccine, and rhesus adenovirus serotype 52 (RhAd52) vaccine studies. Red arrows indicate vaccinations, and black arrows indicate ZIKV challenges. The numbers reflect study weeks

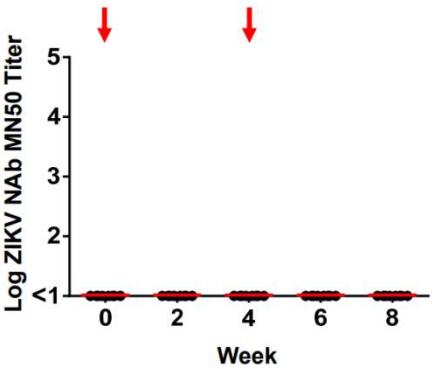

Figure S2. MN50 titers in the sham controls in the ZIKV PIV vaccine study. ZIKV specific microneutralization (MN50) titers following immunization of rhesus monkeys with sham (alum only) at weeks 0 and 4 (red arrows). Red bars reflect medians

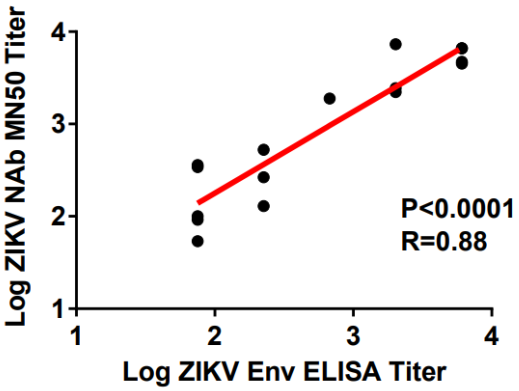

Figure S3. **Correlation of binding and neutralizing antibody titers in the ZIKV PIV vaccine study**. Correlations of binding ELISA titers and microneutralization (MN50) titers at weeks 2 and 6 combined from the ZIKV PIV vaccine study. P-value reflects a Spearman rank-correlation test.

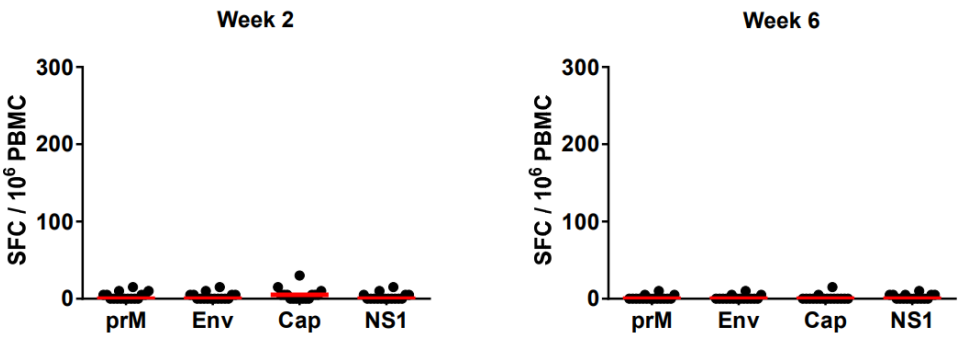

Figure S4. **IFN-γ ELISPOT assays in the sham controls in the ZIKV PIV vaccine study**. Cellular immune responses by IFN-γ ELISPOT assays to prM, Env, Cap, and NS1 at week 2 and week 6 following immunization of rhesus monkeys with sham (alum only) at weeks 0 and 4. Red bars reflect medians.

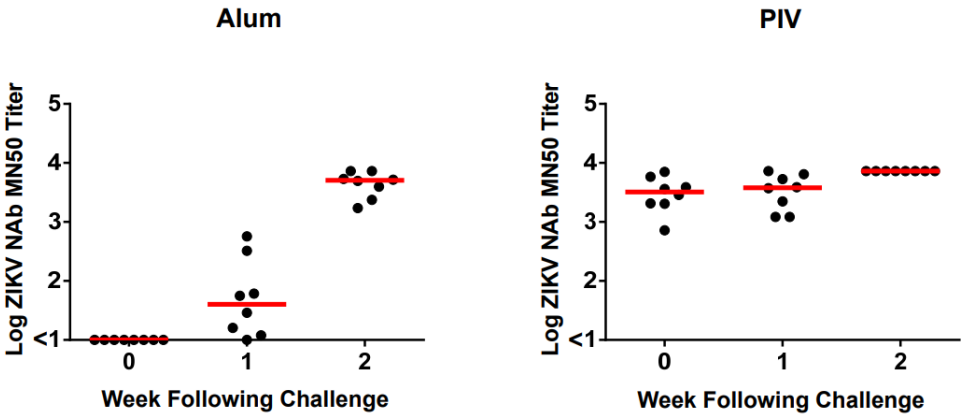

Figure S5. **MN50 titers following ZIKV challenge in the ZIKV PIV vaccine study**. ZIKV-specific microneutralization (MN50) titers following ZIKV-BR challenge in rhesus monkeys that received the ZIKV PIV vaccine or sham (alum only). The maximum measurable log MN50 titer in this assay was 3.86. Red bars reflect medians.

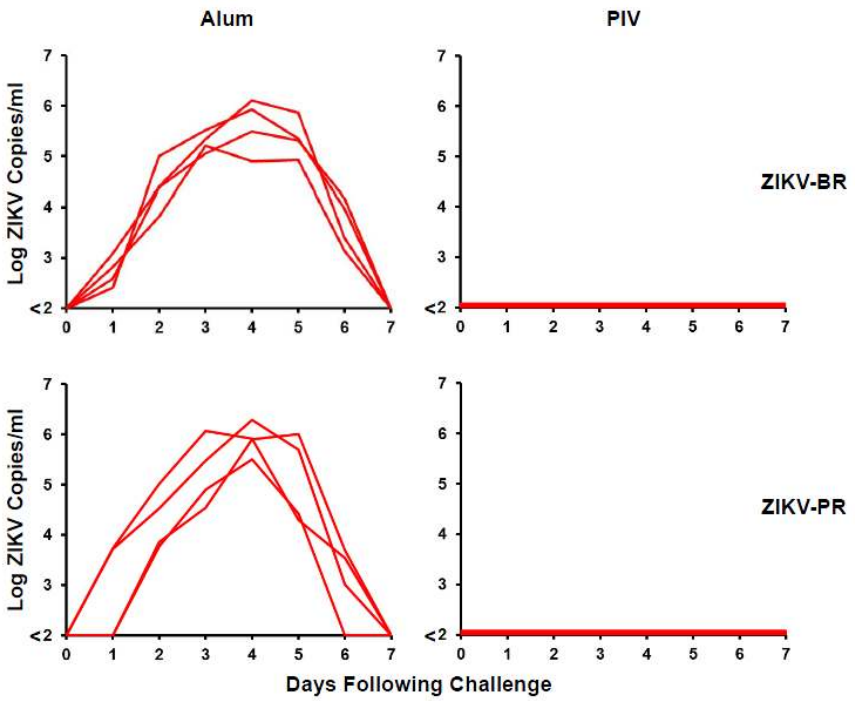

Figure S6. **Viral loads in the ZIKV PIV vaccine study**. Plasma viral loads in PIV vaccinated monkeys and sham controls following challenge with ZIKV-BR or ZIKV-PR (N=4/group).

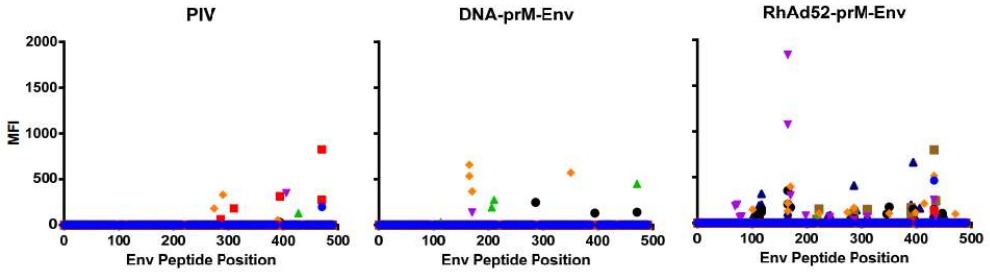

Figure S7. **ZIKV Env peptide microarrays**. Serum samples from week 6 from rhesus monkeys immunized with ZIKV PIV, DNA-prM-Env, and RhAd52-prM-Env vaccines were assayed for linear antibody reactivity using ZIKV Env peptide microarrays. Colors indicate individual monkeys.

4

DURABILITY AND CORRELATES OF VACCINE PROTECTION AGAINST ZIKA VIRUS IN RHESUS MONKEYS

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PATIENCE PAYS OFF

As an individual may not encounter the pathogen for years after they have been vaccinated, efficacious vaccines typically require induction of long-lasting immunity. Abbink and colleagues vaccinated nonhuman primates with one of several candidate Zika virus vaccines and then waited an entire year before conducting a viral challenge. These vaccines had all shown promising results in previous experiments with a more immediate challenge, but here, one vaccine faltered, likely due to waning antibodies. The researchers performed more experiments to suggest that circulating antibodies are mediating protection for these vaccines. These results are useful for Zika virus vaccine development and instructive for vaccine development in general.

ABSTRACT

An effective Zika virus (ZIKV) vaccine will require long-term durable protection. Several ZIKV vaccine candidates have demonstrated protective efficacy in nonhuman primates, but these studies have typically involved ZIKV challenge shortly after vaccination at peak immunity. We show that a single immunization with an adenovirus vector–based vaccine, as well as two immunizations with a purified inactivated virus vaccine, afforded robust protection against ZIKV challenge in rhesus monkeys at 1 year after vaccination. In contrast, two immunizations with an optimized DNA vaccine, which provided complete protection at peak immunity, resulted in reduced protective efficacy at 1 year that was associated with declining neutralizing antibody titers to subprotective levels. These data define a microneutralization log titer of 2.0 to 2.1 as the threshold required for durable protection against ZIKV challenge in this model. Moreover, our findings demonstrate that protection against ZIKV challenge in rhesus monkeys is possible for at least 1 year with a single-shot vaccine.

INTRODUCTION

The development of a safe and effective Zika virus (ZIKV) vaccine has emerged as a global health priority (*1*–*5*). ZIKV infection has been shown to be associated with fetal microcephaly and other congenital malformations (*6*–*9*), as well as Guillain-Barré syndrome in healthy adults (*10*). Protective

efficacy of DNA vaccines, RNA vaccines, adenovirus (Ad) vector–based vaccines, purified inactivated virus (PIV) vaccines, and live attenuated virus vaccines has been demonstrated against ZIKV challenge in rodents and nonhuman primates (*11–19*), and several vaccine candidates are currently in clinical trials (*3–5*). Nonhuman primate challenge studies reported to date have only assessed protection at peak immunity shortly after vaccination (*11*, *13*, *15*). Here, we report the 1-year protective efficacy of three leading vaccine platforms (PIV, DNA, and Ad) in rhesus monkeys and the immune correlates of protection.

RESULTS

We previously designed a DNA vaccine expressing an engineered form of ZIKV BeH815744 prM-Env containing a deletion of the cleavage peptide (amino acids 216 to 794; also termed M-Env), and we showed that this vaccine protected against ZIKV challenge in both mice and rhesus monkeys (*11*, *12*). We compared antigen expression and immunogenicity of DNA vaccines expressing this engineered M-Env, the corresponding full-length prM-Env, and full-length prM-Env containing the stem region of Japanese encephalitis virus (JEV), which has been shown to increase secretion of soluble subviral particles (Fig. 1A) (*15*). The DNA-M-Env vaccine exhibited the highest Env expression by Western blot (Fig. 1B). Groups of Balb/c mice (n = 5 per group) were then immunized by the intramuscular route with a single 50 μg immunization of DNA vaccines expressing M-Env, prM-Env (full length), or prM-Env (JEV stem). The DNA-M-Env vaccine induced the highest antibody responses by enzyme-linked immunosorbent assay (ELISA) at week 4 [P = 0.003 and P = 0.002 comparing titers induced by DNA-M-Env titers with titers induced by DNA-prM-Env (full length) and DNA-prM-Env (JEV Stem), respectively; Fig. 1B]. After challenge with 10^5 viral particles (VP) [10^2 plaque-forming units (PFU)] of ZIKV-BR by the intravenous route (*12*), only the DNA-M-Env vaccine afforded complete protection (Fig. 1C). Env-specific log ELISA titers >2.0 were associated with protection (P < 0.0001; fig. S1). We speculate that the improved performance of the deleted M-Env immunogen may reflect the inefficiency of natural cleavage in the full-length prM-Env immunogen and the lack of the cleavage peptide in the deleted M-Env immunogen.

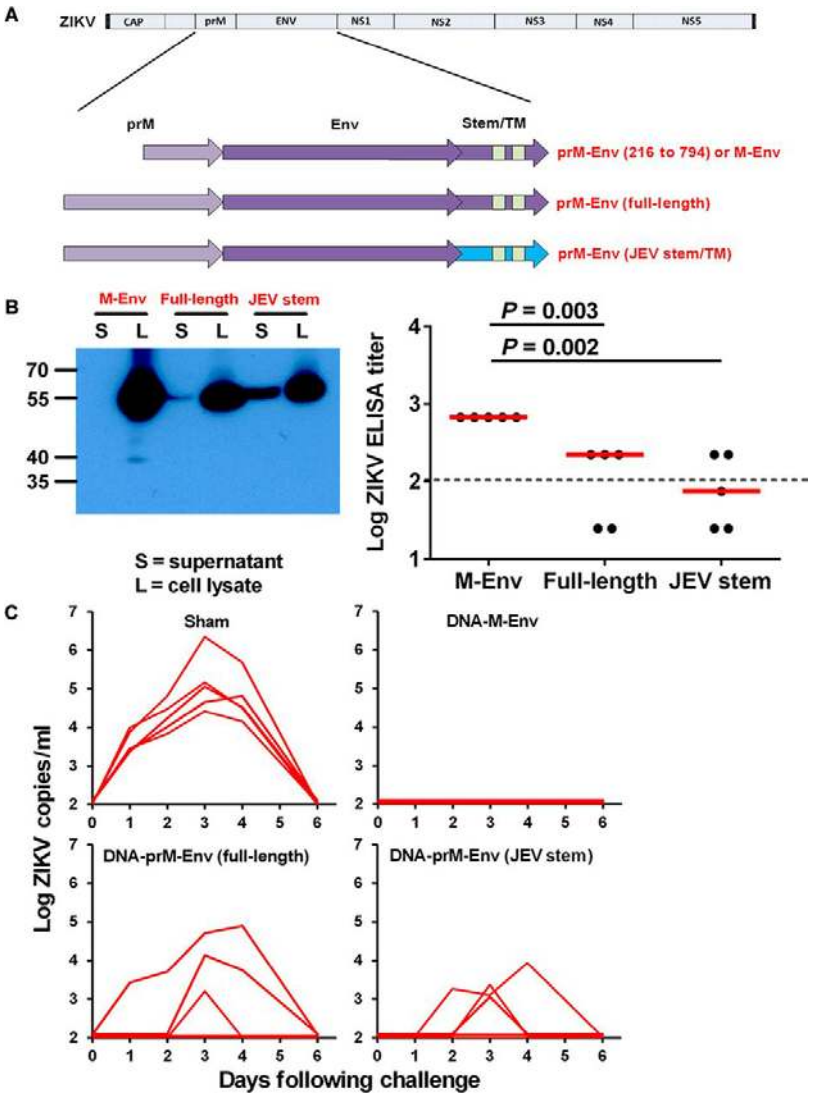

Fig. 1. ZIKV prM-Env antigen development. (**A**) Zika virus (ZIKV) prM-Env antigens tested: cleavage peptide–deleted prM-Env (amino acids 216 to 794; also termed M-Env), full-length prM-Env, and full-length prM-Env with the stem and transmembrane (TM) region of Japanese encephalitis virus (JEV). CAP, capsid. (**B**) Expression from DNA vaccines expressing these three antigens by Western blot and immunogenicity in Balb/c mice (*n* = 5 per group) by Env-specific enzyme-linked immunosorbent assay (ELISA) after a single immunization of 50-µg DNA vaccines expressing M-Env, prM-Env (full-length), or prM-Env (JEV stem). *P* values were determined by *t* test. The dotted line reflects log ELISA titers of 2.0. Red lines reflect medians. (**C**) Mice were challenged by the intravenous route with 10^5 viral particles (VP) [10^2 plaque-forming units (PFU)] ZIKV-BR. Viral loads were determined in serum on days 0, 1, 2, 3, 4, and 6.

We next compared the immunogenicity and protective efficacy of multiple ZIKV vaccine candidates in Balb/c mice. Groups of mice (*n* = 5 per group) were immunized once by the intramuscular route with 10^9 VP Ad26-M-Env, 10^9 VP RhAd52-M-Env, 1-μg PIV with alum, 50-μg DNA-M-Env, 50-μg DNA-prM-Env, or sham vaccine. Env-specific ELISA titers were higher in the Ad26-M-Env, RhAd52-M-Env, and PIV groups as compared with the DNA-M-Env and DNA-prM-Env groups over 20 weeks of follow-up (Fig. 2A). At week 20, all mice were challenged with ZIKV-BR, as described above. Complete protection was observed in the groups of mice that received the Ad26-M-Env, RhAd52-M-Env, and PIV vaccines (Fig. 2B). In contrast, protection was observed in only 80% (four of five) of mice that received the DNA-M-Env vaccine and in only 20% (one of five) of mice that received the DNA-prM-Env vaccine (Fig. 2C), which elicited the lowest Env-specific antibody responses (Fig. 2A), consistent with the previous experiment.

To evaluate the durability of ZIKV vaccine efficacy in nonhuman primates, we immunized 16 rhesus monkeys by the subcutaneous route with 5-μg ZIKV PIV vaccine with alum (*n* = 8) or sham vaccine (alum only) (*n* = 8) twice at weeks 0 and 4 (*11*). We followed ZIKV-specific neutralizing antibodies by microneutralization (MN50) assays (*11*, *12*) for over 52 weeks (Fig. 3A). Median log MN50 titers in the PIV-vaccinated monkeys were 1.88 at week 4 after the initial immunization and increased to 3.71 at week 8 after the boost immunization. Neutralizing antibody titers then declined by 1.33 logs over the next 10 weeks to median log MN50 titers of 2.38 at week 18, and titers then remained largely stable until week 52. Low Env-specific cellular immune responses were also observed by interferon-γ enzyme-linked immunospot assays (fig. S2).

At week 52, all monkeys were challenged with 10^6 VP (10^3 PFU) of ZIKV-BR by the subcutaneous route, as previously described (*11*, *20*). Viral loads were quantitated by real-time polymerase chain reaction (RT-PCR). Sham control monkeys exhibited about 7 days of viremia with median peak log viral loads of 6.47 on days 4 to 5 after challenge (Fig. 3B). Virus was detected for a longer period of time in certain tissue compartments of the sham controls, including cerebrospinal fluid (CSF) and lymph nodes (LN) (Fig. 3, C and D), consistent with previous findings from our laboratory and others (*20*–*23*). In contrast, PIV-vaccinated monkeys showed no detectable viremia (<2 log copies/ml) in 75% (six of eight) of animals (*P* = 0.007 compared with

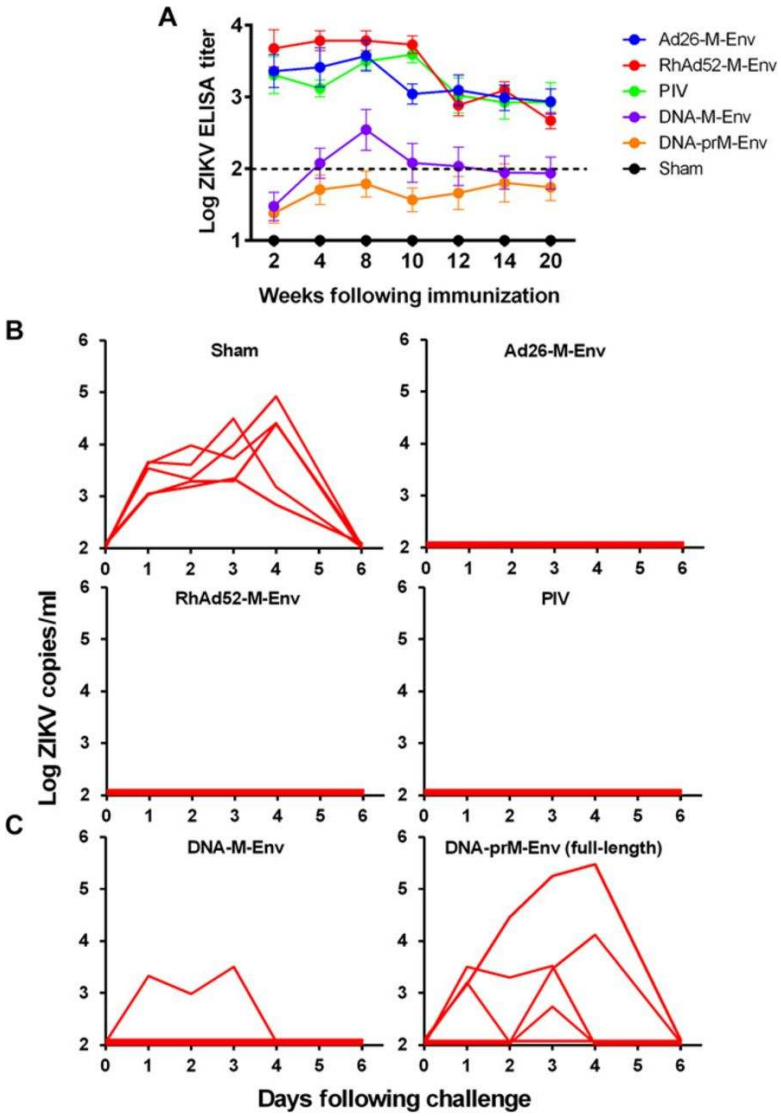

Fig. 2. Long-term immunogenicity and protective efficacy of ZIKV vaccines in Balb/c mice. (A) Balb/c mice (*n* = 5 per group) were immunized once by the intramuscular route with 10^9 VP Ad26-M-Env, 10^9 VP RhAd52-M-Env, 1-µg purified inactivated virus (PIV) with alum, 50-µg DNA-M-Env, 50-µg DNA-prM-Env, or sham vaccine. Median Env-specific ELISA titers are shown. Error bars reflect SEM. The dotted line reflects log ELISA titers of 2.0. (**B** and **C**) Mice were challenged 20 weeks later by the intravenous route with 10^5 VP (10^2 PFU) ZIKV-BR. Viral loads were determined in serum on days 0, 1, 2, 3, 4, and 6.

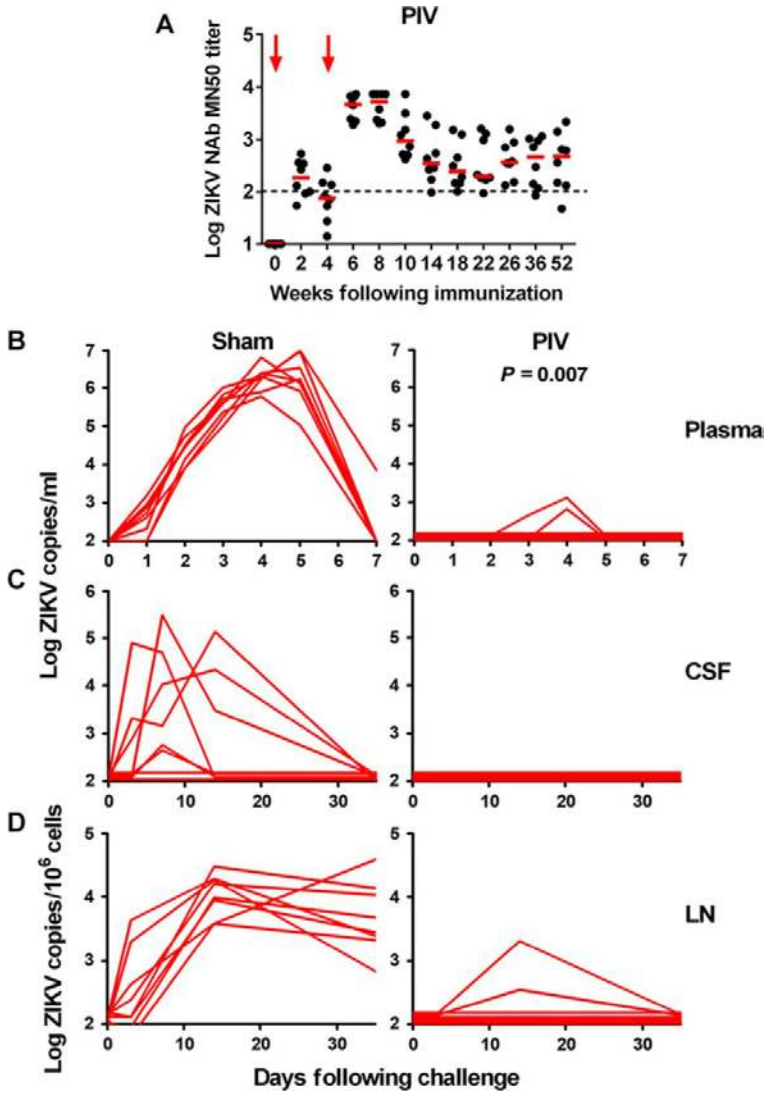

Fig. 3. Long-term immunogenicity and protective efficacy of the ZIKV PIV vaccine in rhesus monkeys. (**A**) Log ZIKV-specific microneutralization (MN50) titers after immunization of rhesus monkeys by the subcutaneous route with 5-µg ZIKV PIV vaccine (*n* = 8) at weeks 0 and 4 (red arrows). The dotted line reflects log MN50 titers of 2.0. Red bars reflect medians. PIV-vaccinated and sham control rhesus monkeys (*n* = 8 per group) were challenged by the subcutaneous route with 10^6 VP (10^3 PFU) ZIKV-BR. Viral loads are shown in (**B**) plasma, (**C**) cerebrospinal fluid (CSF), and (**D**) lymph nodes (LN). Viral loads were determined on days 0, 1, 2, 3, 4, 5, and 7 for the plasma samples and on days 0, 3, 14, and 35 for the other samples. *P*-value determined by Fisher's exact test. NAb, neutralizing antibody.

sham controls) and low and transient viral blips in 25% (two of eight) of animals. These two PIV-vaccinated monkeys also showed low levels of virus in LN.

We next evaluated the durability of protection afforded by the DNA-M-Env and the RhAd52-M-Env vaccines in nonhuman primates. We immunized 15 rhesus monkeys by the intramuscular route with two immunizations of 5-mg DNA-M-Env at weeks 0 and 4 (n = 7), a single-shot immunization of 10^{11} VP RhAd52-M-Env at week 0 (n = 4), or sham vaccine (n = 4). MN50 titers were low after the first DNA-M-Env vaccination but reached peak median log titers of 2.23 at week 8 after the boost immunization (Fig. 4A). Median log MN50 titers in the DNA-M-Env–vaccinated animals declined rapidly to 1.43 by week 14 but then remained largely stable until week 52. Notably, only two of seven DNA-M-Env–vaccinated animals exhibited log MN50 titers of 2.0 or higher during this follow-up period. In contrast, a single immunization of the RhAd52-M-Env induced median log MN50 titers of 2.26 by week 2 (Fig. 4A). MN50 titers in these animals persisted and proved remarkably stable over a year of follow-up, with median log MN50 titers of 2.42 (range, 2.30 to 2.54) at week 52 (Fig. 4A). Env-specific cellular immune responses were also induced in these animals (fig. S3).

After challenge with 10^6 VP (10^3 PFU) of ZIKV-BR at week 52, only 29% (two of seven) of DNA-M-Env–vaccinated animals were protected, and 71% (five of seven) of animals in this group exhibited viremia (Fig. 4B). Notably, the two DNA-M-Env–vaccinated monkeys that were protected were the animals with the highest log MN50 titers. Because the DNA-M-Env vaccine afforded complete protection when challenged at peak immunity (*11*), we speculate that the abrogation of protection reflects the decline of neutralizing antibody titers over the year before challenge to subprotective levels. In contrast, a single immunization with RhAd52-M-Env provided protection in 100% (four of four) of monkeys at 1 year (P = 0.02 compared with sham controls, Fig. 4, B to D), likely reflecting the persistent MN50 titers in these animals.

We next assessed the capacity of week 52 prechallenge serum from the PIV-, DNA-M-Env-, and RhAd52-M-Env–vaccinated monkeys to neutralize a panel of ZIKV strains, and we observed cross-neutralization of

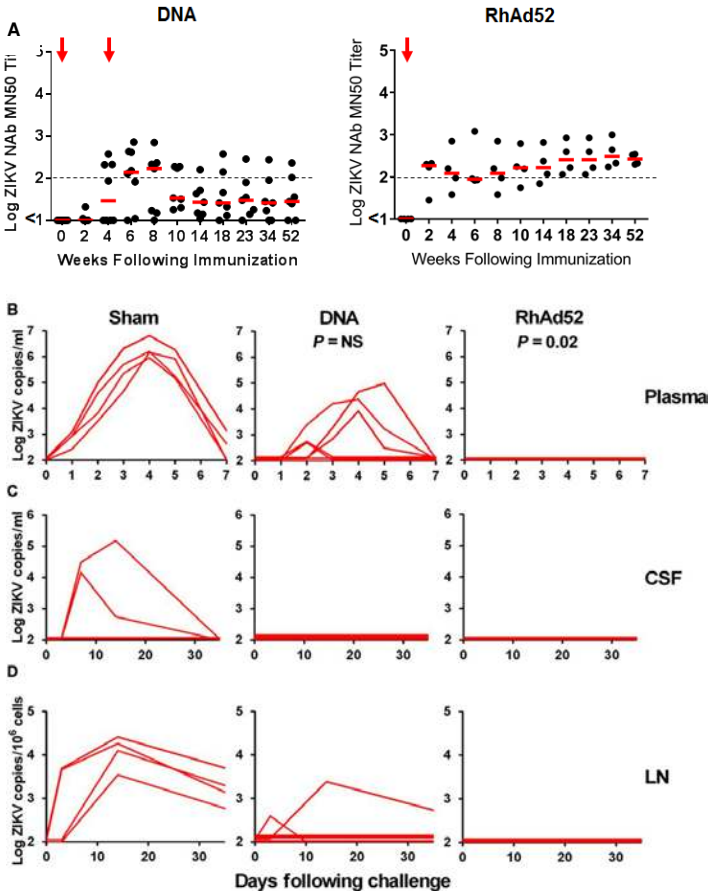

Fig. 4. Long-term immunogenicity and protective efficacy of the ZIKV DNA-M-Env and RhAd52-M-Env vaccines in rhesus monkeys. (**A**) Log ZIKV-specific microneutralization (MN50) titers after immunization of rhesus monkeys by intramuscular with two immunizations of 5-mg DNA-M-Env (*n*= 7) at weeks 0 and 4 (red arrows) or a single-shot immunization of 10^{11} VP RhAd52-M-Env (*n* = 4) at week 0 (red arrow). The dotted lines reflect log MN50 titers of 2.0. Red bars reflect medians. Vaccinated and sham control rhesus monkeys were challenged by the subcutaneous route with 10^6 VP (10^3 PFU) ZIKV-BR. Viral loads are shown in (**B**) plasma, (**C**) CSF, and (**D**) LN. Viral loads were determined on days 0, 1, 2, 3, 4, 5, and 7 for the plasma samples and on days 0, 3, 14, and 35 for the other samples. *P*-values determined by Fisher's exact tests. NS, not significant.

viral strains from Brazil (BR), Uganda (UG), Thailand (TH), Philippines (PH), and Puerto Rico (PR) (fig. S4). We also evaluated the capacity of serum antibodies to enhance ZIKV infection in vitro in K562 cells. As expected, all animals with detectable neutralizing antibodies resulted in enhanced infection in K562 cells at relatively high dilutions of sera (figs. S5 and S6), suggesting that this in vitro assay does not readily distinguish between

protective and enhancing antibodies. No animals demonstrated enhanced ZIKV viremia in this study, including monkeys with subprotective neutralizing antibodies and enhanced infection in K562 cells. We also observed that MN50 titers increased in all the vaccinated animals after challenge (figs. S7 and S8), which may reflect either a lack of complete sterilizing immunity or alternatively an immunologic boost by the 106 VP dose of the challenge virus. Supporting the latter possibility is the lack of observed increased cellular immune responses in the RhAd52-M-Env–vaccinated animals after challenge (fig. S9).

Given the heterogeneous outcome of the challenge studies with the PIV, DNA-M-Env, and RhAd52-M-Env vaccines, we performed an immune correlates analysis to define the threshold MN50 titer required for protection. In the vaccinated animals, the log MN50 titer at the time of challenge (week 52) was inversely correlated with the peak log ZIKV viral load after challenge ($r = -0.81$, $P < 0.0001$; Fig. 5). Moreover, MN50 titers were higher in protected animals than in infected animals ($P < 0.0001$). Specifically, 92% (12 of 13) of animals with MN50 titers >2.0 and 100% (12 of 12) of animals with MN50 titers >2.1 at week 52 were protected. In contrast, 100% (six of six) of animals with MN50 titers <2.0 were infected. Similar results were obtained by an immune correlates analysis that included all animals including the sham controls (fig. S10). Moreover, adoptive transfer studies using purified immunoglobulin G (IgG) from week 52 plasma samples confirmed that the vaccine-induced rhesus monkey antibodies afforded passive protection in Balb/c mice (fig. S11).

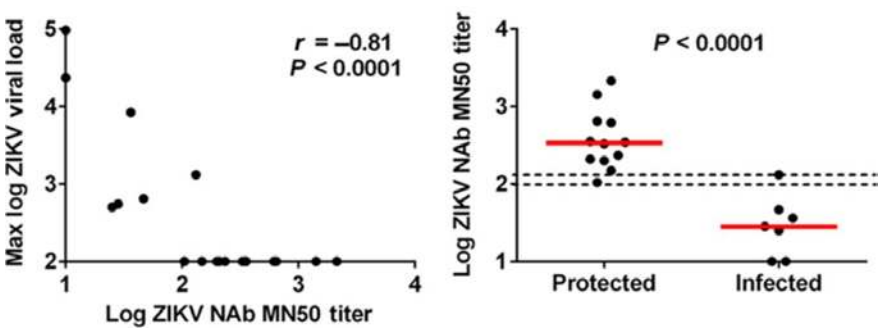

Fig. 5. Immune correlates analysis in vaccinated rhesus monkeys. Correlation of maximum log viral loads after ZIKV-BR challenge with log MN50 titers at week 52 before challenge (left). P value determined by Spearman rank correlation test. Comparison of log MN50 titers at week 52 in protected versus infected animals (right). P value determined by Wilcoxon rank-sum test. The dotted lines reflect log MN50 titers of 2.0 and 2.1. Red lines reflect medians.

DISCUSSION

Here, we demonstrate that a single-shot immunization with RhAd52-M-Env provided complete protection against ZIKV-BR challenge in 100% (four of four) of rhesus monkeys after 1 year. Two immunizations with the ZIKV PIV vaccine also provided robust protection in 75% (six of eight) of animals after 1 year. In contrast, DNA vaccines expressing the same optimized M-Env insert elicited neutralizing antibody titers that declined to subprotective levels during this time period. Protective efficacy strongly correlated with MN50 titers at the time of challenge, which defined the threshold of protection in this model to be log MN50 titers of 2.0 to 2.1 (MN50 titers of 100 to 125).

Previous ZIKV vaccine studies in nonhuman primates from our laboratory and others have challenged animals shortly after vaccination at peak immunity (*11*, *13*, *15*). Although these data provide an important assessment of the theoretical short-term protective efficacy of vaccine candidates, it is critical for a ZIKV vaccine to provide long-term durable protection. Vaccine-elicited antibody responses typically decline with different kinetics depending on the vaccine modality and are likely affected by multiple immunologic and other factors. The PIV vaccine induced high MN50 titers after vaccination that declined over 3 months but still remained above the protective threshold in most of the animals. In contrast, the DNA-M-Env vaccine induced moderate MN50 titers that were sufficient for protection at peak immunity (*11*), but these responses declined to subprotective levels within 2 to 3 months. The RhAd52-M-Env vaccine induced moderate MN50 titers after a single-shot immunization, but these responses remained stable with minimal decline over 52 weeks. The immunologic basis of the persistent neutralizing antibody responses elicited by RhAd52-M-Env remains to be determined.

The strong correlation between ZIKV-specific antibody responses and protective efficacy in both mice and rhesus monkeys, as well as the robustness of this immune correlate across different antigens and different vaccine platforms, suggests the potential generalizability of these observations. Together with previous adoptive transfer studies using polyclonal antibodies from vaccinated animals (*11*, *12*) and monoclonal antibodies (*24*), we suggest that ZIKV-specific neutralizing antibodies

represent the primary mechanistic correlate of protection for ZIKV vaccines. These insights should prove useful in the clinical development of ZIKV vaccines, although the quantitative titer threshold required for ZIKV protection may differ between rhesus monkeys and humans. For other flavivirus vaccines in humans, neutralizing antibody titers of >10 have been reported as correlates of protection (*25*–*27*). Whether or not higher titers will be required for protection against ZIKV in humans remains to be determined. Future studies should also define the Env regions and epitopes that are the target of protective neutralizing antibodies.

The potential for cross-reactive dengue virus (DENV)–specific antibodies to interfere with the immunogenicity and/or protective efficacy of ZIKV vaccines is an important research question. Previous studies have suggested that DENV-specific antibodies can increase ZIKV replication in vitro and in mice (*28*–*30*), but studies in primates have not replicated these findings to date (*31*, *32*). Dedicated studies of ZIKV vaccines in DENV-exposed animals and humans are therefore warranted. It also remains uncertain whether vaccine protection against virus replication in peripheral blood and tissues will translate into prevention of congenital Zika syndrome.

Together, our data demonstrate durable 1-year protection against ZIKV challenge by a recombinant Ad vector–based vaccine and a PIV vaccine in rhesus monkeys. ZIKV Ad, PIV, DNA, and RNA vaccines are currently being evaluated in clinical trials (*33*). Our study also defines the threshold MN50 titers that correlate with long-term protection in this model, although the relationship between the rhesus monkey model and humans remains to be determined. Nevertheless, these findings provide insights that support clinical development of ZIKV vaccines for humans.

MATERIALS AND METHODS

Study design

The objective of these studies was to evaluate the immunogenicity and protective efficacy of ZIKV vaccines in mice and rhesus monkeys. Studies were powered (n = 4 to 8 per group) to detect large differences in protective efficacy. Animals were randomly allocated to groups. Immunologic and

virologic assays were performed blinded. All animal studies were approved by the appropriate Institutional Animal Care and Use Committee.

Animals, vaccines, and challenges

Female Balb/c mice were purchased from commercial vendors and housed at Beth Israel Deaconess Medical Center (BIDMC). Thirty-one outbred, Indian-origin male and female rhesus monkeys (*Macaca mulatta*) were housed at Bioqual Inc., Rockville, MD. Vaccine constructs have been described previously (*11*, *12*). In the first monkey vaccine study, animals were immunized by the subcutaneous route with 5-μg ZIKV PIV vaccine derived from the PRVABC59 isolate with alum (Alhydrogel, Brenntag Biosector) or alum alone at weeks 0 and 4 (n = 8 per group). In the second monkey vaccine study, animals were immunized by the intramuscular route with 5-mg DNA vaccines expressing M-Env (prM-Env amino acids 216 to 794 derived from the BeH815744 isolate with the cleavage peptide deleted) at weeks 0 and 4 (n = 7), a single immunization of 10^{11} VP RhAd52 expressing M-Env at week 0 (n = 4), or sham controls (n = 4). Rhesus monkeys were challenged at week 52 by the subcutaneous route with 10^6 VP (10^3 PFU) ZIKV-BR (Brazil ZKV2015). Studies in Balb/c mice used 1-μg ZIKV PIV, 50-μg DNA vaccines, or 10^9 VP Ad vaccines and were challenged with 10^5 VP (10^2 PFU) ZIKV-BR.

Real-time polymerase chain reaction

RT-PCR assays were used to monitor viral loads, essentially as previously described (*11*, *12*). RNA was extracted from plasma or other samples with a QIAcube HT (Qiagen). The wild-type ZIKV BeH815744 Cap gene was used as a standard. RNA was purified (Zymo Research), and RNA quality and concentration was assessed by the BIDMC Molecular Core Facility. Log dilutions of the RNA standard were reverse-transcribed and included with each RT-PCR assay. Viral loads were calculated as VP per milliliter or per 1×10^6 cells and were confirmed by PFU assays. Assay sensitivity was 100 copies/ml or 1×10^6 cells.

Adoptive transfer studies

Polyclonal IgG was purified with protein G purification kits (Thermo Fisher Scientific) from week 52 plasma samples from rhesus monkeys vaccinated with the PIV, RhAd52-M-Env, DNA-M-Env, and sham vaccines. Total IgG was buffer-exchanged into 1× phosphate-buffered saline (PBS) and pooled for each group. Purified IgG was infused intravenously into groups of naïve recipient Balb/c mice (n = 5 per group) before ZIKV-BR challenge 2 hours after infusion. Mice received 400 µl (high dose) or 25 µl (low dose) of a solution (10 mg/ml) of purified IgG.

Enzyme-linked immunosorbent assay

Mouse and monkey ZIKV Env ELISA kits (Alpha Diagnostic International) were used to determine end point binding antibody titers using a modified protocol. Ninety-six–well plates coated with ZIKV Env protein were first equilibrated at room temperature with 300 µl of kit working wash buffer for 5 min. Six microliters of serum was added to the top row, and threefold serial dilutions were tested in the remaining rows. Samples were incubated at room temperature for 1 hour, and plates were washed four times. One hundred microliters of anti-mouse or anti-human IgG horseradish peroxidase (HRP)–conjugate working solution was then added to each well and incubated for 30 min at room temperature. Plates were washed five times, developed for 15 min at room temperature with 100 µl of 3,5,3′,5′-tetramethylbenzidine (TMB) substrate, and stopped by the addition of 100 µl of stop solution. Plates were analyzed at 450 nm/550 nm on a VersaMax microplate reader using Softmax Pro 6.0 software (Molecular Devices). ELISA end point titers were defined as the highest reciprocal serum dilution that yielded an absorbance >2-fold more than background values. Log_{10} end point titers are reported.

Neutralization assay

A high-throughput ZIKV microneutralization (MN) assay was used for measuring ZIKV-specific neutralizing antibodies, essentially as previously described (*11*, *12*). Briefly, serum samples were serially diluted threefold in 96-well microplates, and 100 µl of ZIKV-PR (PRVABC59) containing 100 PFU was added to 100 µl of each serum dilution and incubated at 35°C for 2

hours. Supernatants were then transferred to microtiter plates containing confluent Vero cell monolayers (World Health Organization, NICSC-011038011038). After incubation for 4 days, cells were fixed with absolute ethanol/methanol for 1 hour at −20°C and washed three times with PBS. The pan-flavivirus monoclonal antibody 6B6-C1 conjugated to HRP (6B6-C1 was a gift from J. T. Roehrig, U.S. Centers for Disease Control and Prevention) was then added to each well, incubated at 35°C for 2 hour, and washed with PBS. Plates were washed, developed with TMB for 50 min at room temperature, and stopped with 1:25 phosphoric acid, and absorbance was read at 450 nm. For a valid assay, the average absorbance at 450 nm of three noninfected control wells had to be ≤0.5, and virus-only control wells had to be ≥0.9. Normalized absorbance values were calculated, and the MN50 titer was determined by a log midpoint linear regression model. The MN50 titer was calculated as the reciprocal of the serum dilution that neutralized ≥50% of ZIKV, and seropositivity was defined as a titer ≥10, with the maximum measurable titer of 7290. Log_{10} MN50 titers are reported. For the cross-strain virus neutralization assays, the following ZIKV strains were used: Brazil (BR; Fortaleza/2015, renamed Paraiba/2015), Uganda (UG; Uganda/1947; MR766), Thailand(TH;SV0127/14), Philippines(PH;CPCC074000Y01U00B001), and Puerto Rico (PR; PRVABC59).

Antibody-dependent enhancement assay

Twofold serial dilutions of heat-inactivated sera were mixed with an equal volume of ZIKV (sufficient to achieve about 15% infection of 5×10^4 K562-DC-SIGN cells) and incubated for 1 hour at 37°C. This mixture was added to a 96-well plate containing medium [RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine (200 mM), and 1% nonessential amino acids (10 mM)] with 5×10^4 K562 cells per well in duplicate and incubated 18 to 20 hours overnight in a 37°C, 5% CO_2, humidified incubator. After overnight incubation, the cells are fixed, permeabilized, and immunostained with flavivirus group–reactive mouse monoclonal antibody 4G2, and secondary polyclonal goat anti-mouse IgG phycoerythrin-conjugated antibody (catalog no. 550589, BD Biosciences). The percent infected cells are quantified on a BD Accuri C6 Plus Flow Cytometer (BD Biosciences).

Statistical analyses

Analysis of virologic and immunologic data was performed using GraphPad Prism version 6.03 (GraphPad Software). Comparisons of groups were performed using *t* tests and Wilcoxon rank sum tests. Correlations were assessed by Spearman rank correlation tests.

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33. NCT02840487, NCT02809443, NCT02887482, NCT02963909, NCT02952833, and NCT02937233.

SUPPLEMENTARY MATERIALS

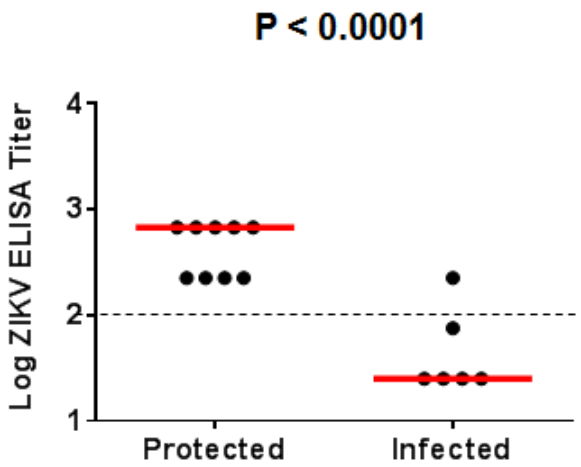

Figure S1. Immune correlates analysis in mice. Comparison of log ELISA titers at week 20 in protected versus infected Balb/c mice in the experiment shown in Fig. 1 (N=15). P-value reflects Wilcoxon rank-sum test. The dotted lines reflect log MN50 titers of 2.0 and 2.1. Red lines reflect medians.

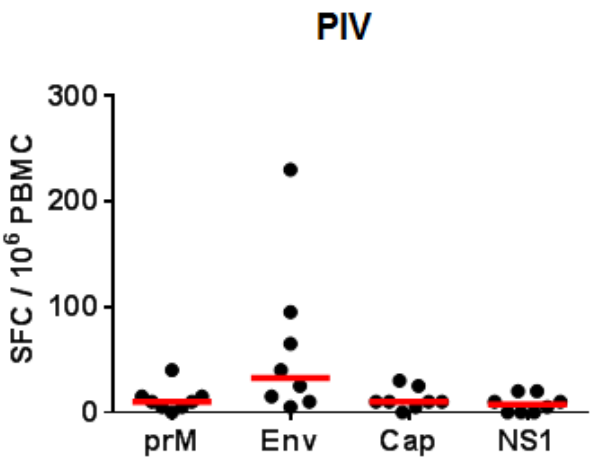

Figure S2. Cellular immune responses in the ZIKV PIV vaccine study. IFN-□ ELISPOT responses are shown to prM, Env, Cap, and NS1 peptide pools at week 6 (N=8/group). Spot-forming cells (SFC) per 106 PBMC are shown. Red bars reflect medians.

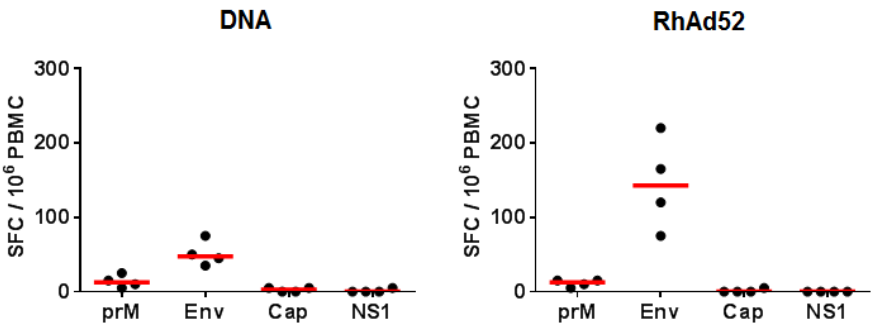

Figure S3. Cellular immune responses in the ZIKV DNA-M-Env and RhAd52-M-Env vaccine study. IFN-ɣ ELISPOT responses are shown to prM, Env, Cap, and NS1 peptide pools at week 6 (N=4/group). Spot forming cells (SFC) per 106 PBMC are shown. Red bars reflect medians.

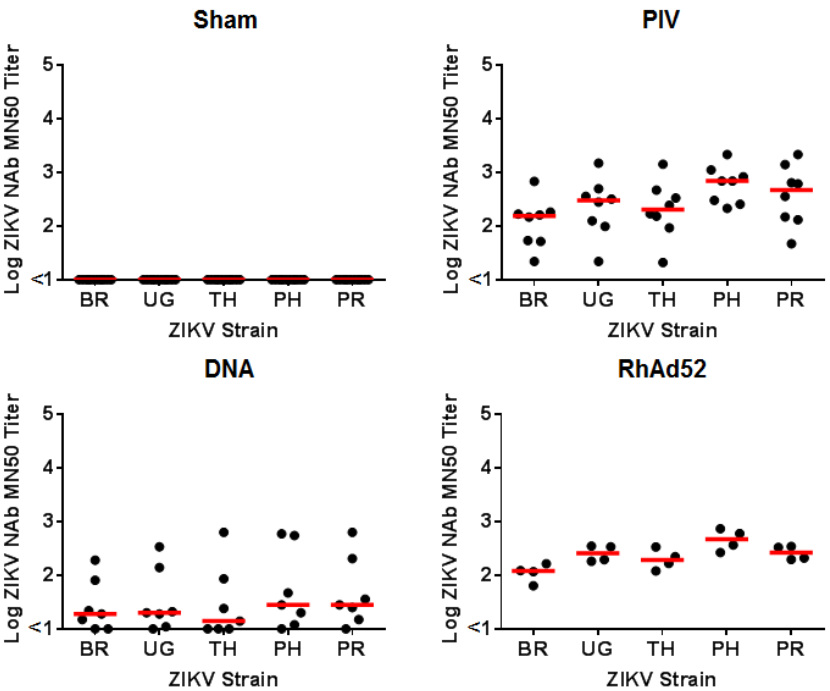

Figure S4. Cross-strain neutralization of a panel of ZIKV strains. Log ZIKV-specific microneutralization (MN50) titers in week 52 pre-challenge serum from the sham, PIV, DNA-M-Env, and RhAd52-M-Env vaccinated monkeys (N=4-8/group), using ZIKV strains from Brazil (BR; Fortaleza/2015, renamed Paraiba/2015), Uganda (UG; Uganda/1947; MR766), Thailand (TH; SV0127/14), Philippines (PH; CPCC074000Y01U00B001), and Puerto Rico (PR; PRVABC59). Red bars reflect medians.

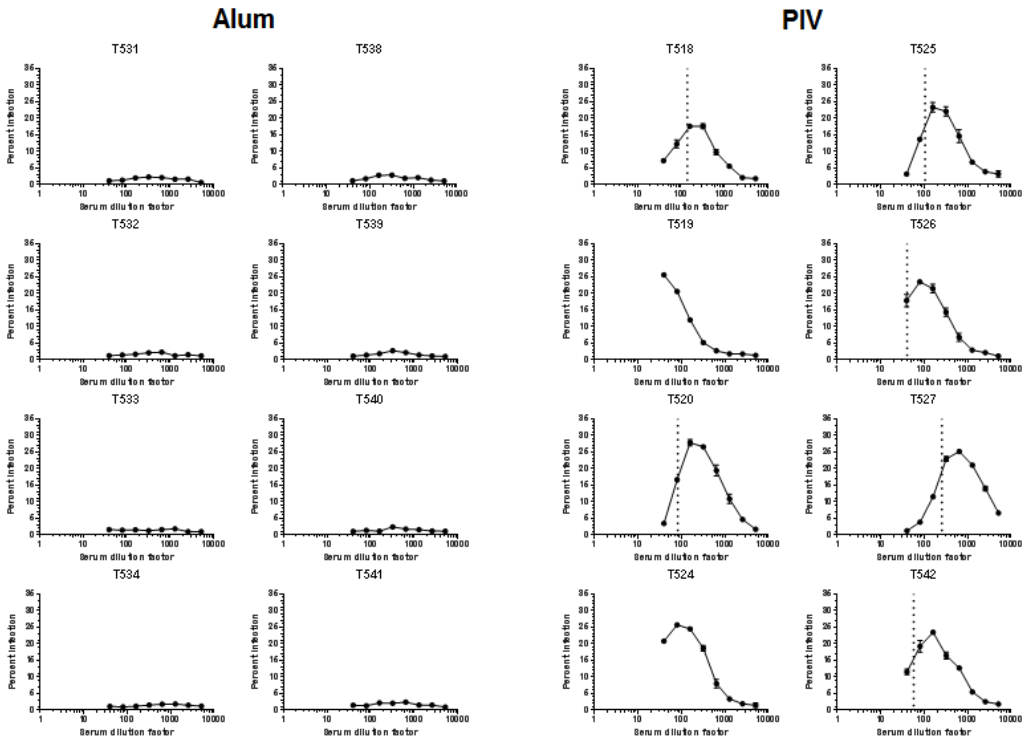

Figure S5. Antibody-dependent enhancement assays in the ZIKV PIV vaccine study. K562 infectivity assays using serum from PIV and sham vaccinated rhesus monkeys in the experiment shown in Fig. 3. The vertical dotted line reflects neutralization titers.

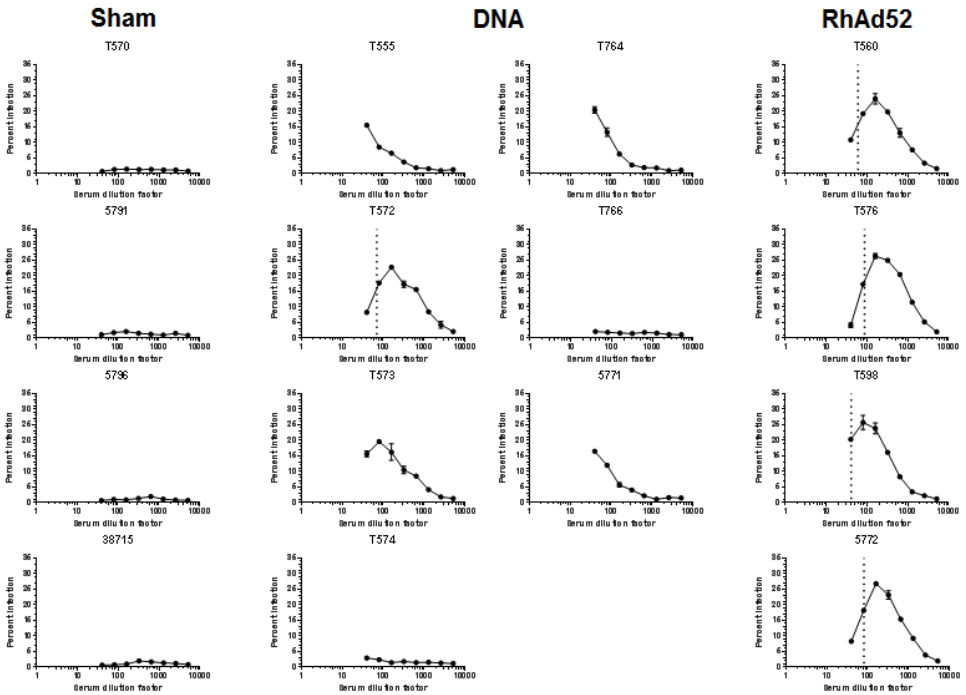

Figure S6. Antibody-dependent enhancement assays in the ZIKV DNA-M-Env and RhAd52-M-Env vaccine study following challenge. K562 infectivity assays using serum from DNA-M-Env, RhAd52-M-Env, and sham vaccinated rhesus monkeys in the experiment shown in Fig. 4. The vertical dotted line reflects 50% neutralization titers in K562-DC-SIGN cells.

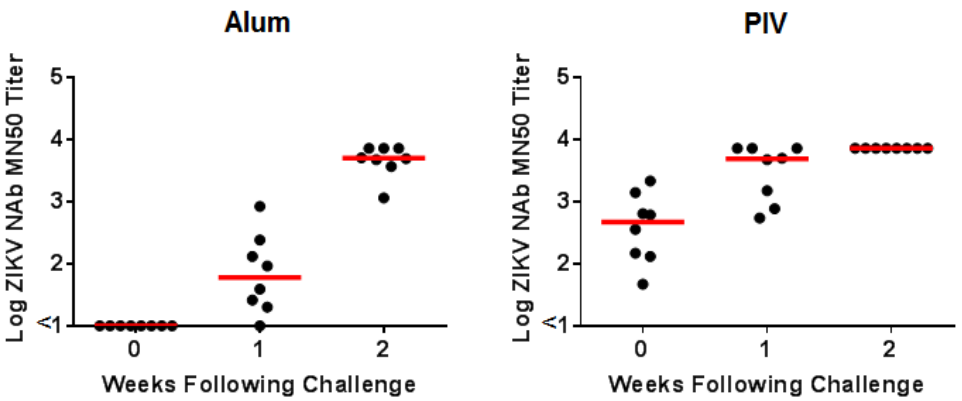

Figure S7. MN50 titers in the ZIKV PIV vaccine study following challenge. ZIKV-specific microneutralization (MN50) titers following ZIKV-BR challenge in PIV and sham vaccinated rhesus monkeys in the experiment shown in Fig. 3 (N=8/group). Red bars reflect medians.

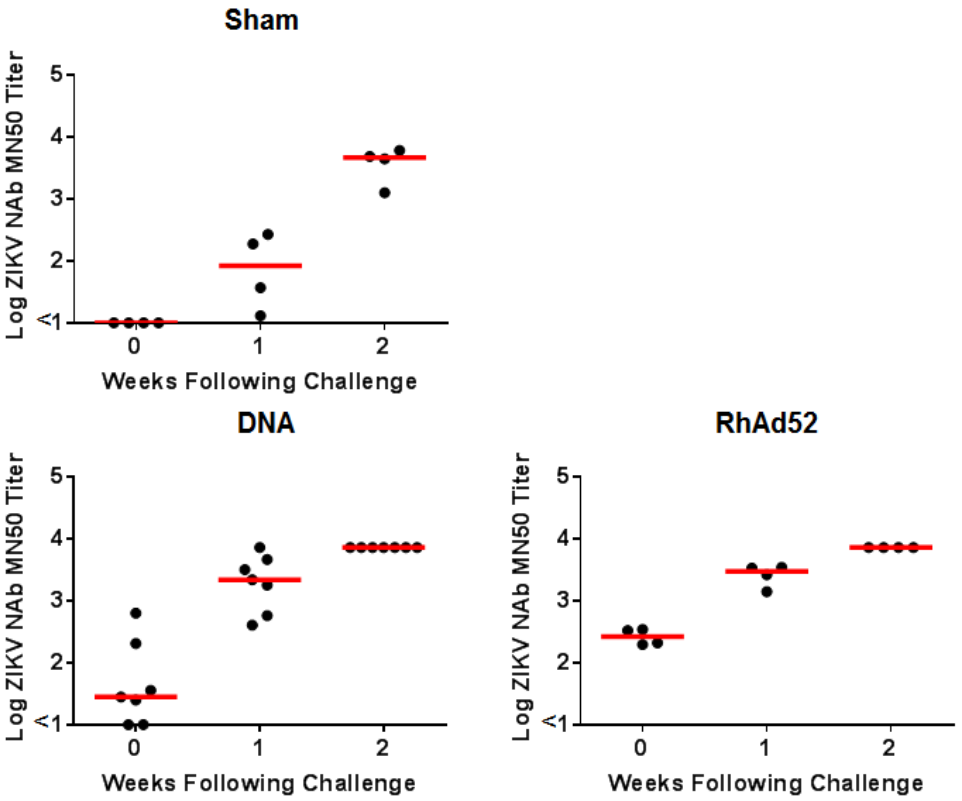

Figure S8. MN50 titers in the ZIKV DNA-M-Env and RhAd52-M-Env vaccine study following challenge. ZIKV-specific microneutralization (MN50) titers following ZIKV-BR challenge in DNA-M-Env, RhAd52-M-Env, and sham vaccinated rhesus monkeys in the experiment shown in Fig. 4 (N=4-7/group). Red bars reflect medians.

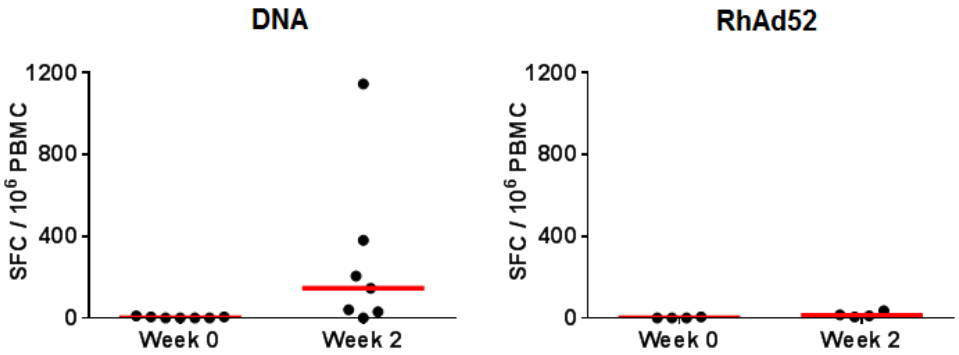

Figure S9. Cellular immune responses in the ZIKV DNA-M-Env and RhAd52-M-Env vaccine study following challenge. Env-specific IFN-□ ELISPOT responses are shown at week 0 prior to challenge and at week 2 following challenge (N=4-7/group). Spot-forming cells (SFC) per 10^6 PBMC are shown. Red bars reflect medians.

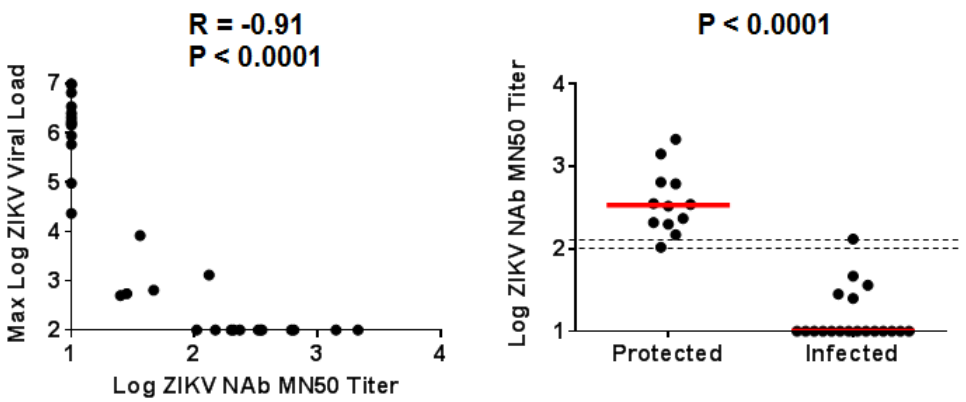

Figure S10. Immune correlates analysis in vaccinated and sham control rhesus monkeys. Correlation of maximum log viral loads following ZIKV-BR challenge with log MN50 titers at week 52 prior to challenge (left). P-value reflects Spearman rank-correlation test. Comparison of log MN50 titers at week 52 in protected versus infected animals (right). P-value reflects Wilcoxon rank-sum test. The dotted lines reflect log MN50 titers of 2.0 and 2.1. Red lines reflect medians.

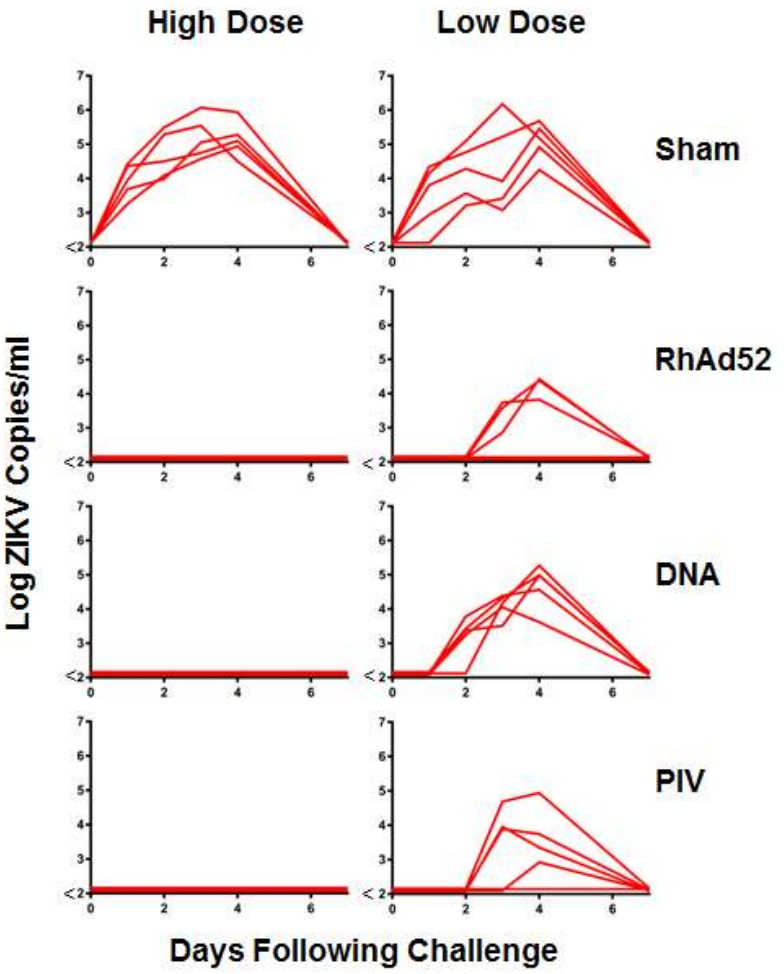

Figure S11. Adoptive transfer studies of rhesus monkey IgG in mice. IgG was purified from week 52 plasma samples from rhesus monkeys vaccinated with the sham, RhAd52-M-Env, DNA-M-Env, and PIV vaccines and infused intravenously into groups of naïve recipient Balb/c mice (N=5/group) prior to ZIKV-BR challenge 2 h after infusion. Mice received 400 µl (High Dose) or 25 µl (Low Dose) doses of a 10 mg/ml solution of purified IgG prior to challenge. Viral loads were determined in serum on days 0, 1, 2, 3, 4, and 7.

5

THERAPEUTIC AND PROTECTIVE EFFICACY OF A DENGUE ANTIBODY AGAINST ZIKA INFECTION IN RHESUS MONKEYS

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ABSTRACT

Strategies to treat Zika virus (ZIKV) infection in dengue virus (DENV) endemic areas are urgently needed. Studies in mice have suggested that ZIKV-specific monoclonal antibodies can treat ZIKV infection[1-4], but the therapeutic efficacy of ZIKV-specific neutralizing antibodies has not previously been determined in nonhuman primates. Here we show that a DENV-specific antibody against the E-dimer epitope (EDE) potently cross-neutralizes ZIKV[1,5-7] and provides robust therapeutic efficacy as well as prophylactic efficacy against ZIKV in rhesus monkeys. Viral escape was not detected *in vitro* or *in vivo*, suggesting a high bar to escape. These data demonstrate the potential for antibody-based therapy for ZIKV.

INTRODUCTION

Zika virus (ZIKV) has been associated with fetal microcephaly and other congenital abnormalities as well as Guillain-Barre syndrome[8-11]. Our laboratory and others have shown that ZIKV-specific neutralizing antibodies correlate with vaccine protection in both mice and monkeys[12-16] as well as with rapid control of viremia following infection in monkeys[17]. Several groups have also demonstrated therapeutic efficacy of ZIKV-specific mAbs in immunosuppressed mice[1-4], and a cocktail of three ZIKV-specific mAbs that targeted domain III was shown to prevent ZIKV infection in nonhuman primates[18]. We therefore assessed the therapeutic efficacy of a potent ZIKV-specific antibody in rhesus monkeys.

Substantial humoral cross-reactivity exists between DENV and ZIKV, and DENV-specific antibodies have been associated with antibody-dependent enhancement of ZIKV infection *in vitro* and in certain murine models[6,19,20]. We previously reported that DENV E-dimer epitope (EDE)-specific mAbs bind a quaternary epitope formed at the interface of head-to-tail E-dimers and efficiently cross-neutralize ZIKV[5-7,21]. EDE-specific mAbs bind poorly to monomeric E-proteins but bind efficiently to stable E-dimers[22] and can be subdivided into two groups, EDE1 and EDE2, by their insensitivity or sensitivity, respectively, to removal of N-linked glycan at position 153, with EDE1 mAbs typically exhibiting greater potency[6,7]. Moreover, the EDE1-specific mAb B10 has been shown to prevent and treat ZIKV infection in mice[1]. We evaluated 33 EDE1-specific antibodies isolated from DENV

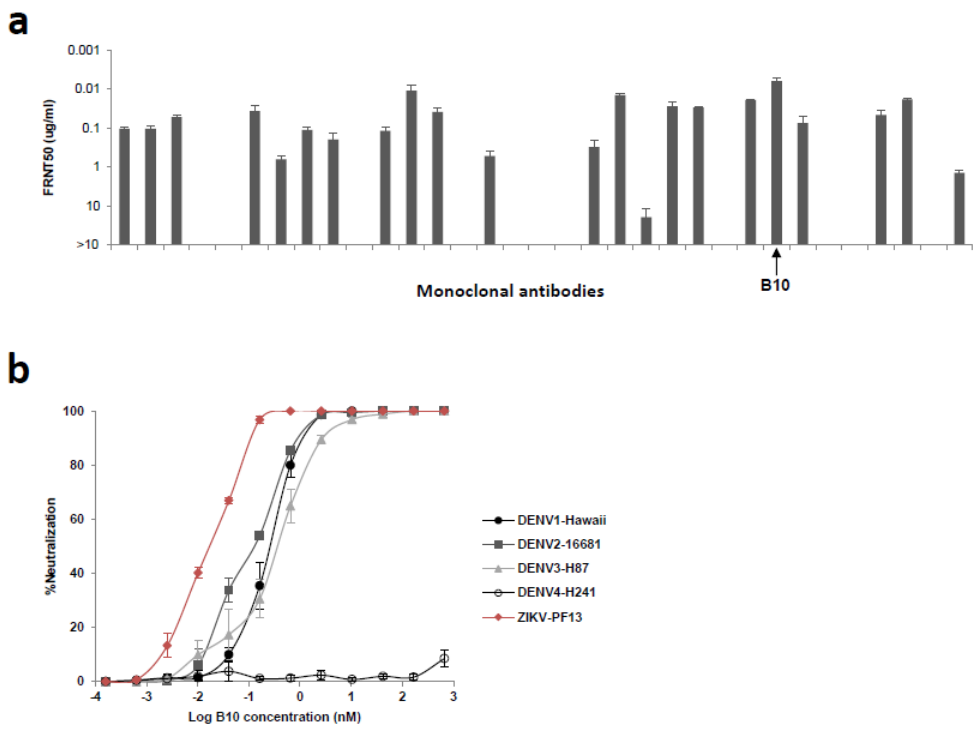


Figure 1. Characterization of human monoclonal antibody B10. (**a**) Neutralization of ZIKV-PF13/251013-18 (PF13), an Asian strain of Zika virus isolated from French Polynesia in 2013, using a panel of 33 EDE1-specific mAbs originally isolated from DENV-infected patients. B10 was the most potent mAb in this panel. (**b**) Neutralization curves of B10 against DENV-1, DENV-2, DENV-3, DENV-4, and ZIKV-PF13. Data are representative of three independent experiments in (**a**), and mean ± s.e.m. are shown in (**b**).

infected patients[7] and found that B10 was the most potent at neutralizing a ZIKV strain from French Polynesia[23] (Fig. 1a). B10 neutralized ZIKV-PF13 (NT50 of 0.016 ± 0.001 nM; NT90 of 0.100 ± 0.009) even more potently than DENV-1/2/3 but showed poor neutralization against DENV4 (Fig. 1b).

To confirm the antiviral activity of B10 against ZIKV in vivo, we performed a titration study in immunocompetent Balb/c mice. Groups of Balb/c mice (N=5/group) received a single infusion of 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.097, 0.048, and 0 µg B10 and were subsequently challenged with 10^5 viral particles (VP) [10^2 plaque-forming units (PFU)] of ZIKV-BR by the intravenous route[13] (Supplementary Fig. S1). In naïve mice, ZIKV-BR infection led to peak viral loads of 5.24-6.18 log RNA copies/ml, similar to

previous findings with this challenge stock[13]. B10 doses as low as 3.12 µg, corresponding to serum levels of 0.5-0.9 µg/ml (data not shown), resulted in complete protection against ZIKV-BR challenge in mice (Supplementary Fig. S1). Sub-protective B10 doses of 0.19-1.56 µg resulted in partial protection of a subset of mice and attenuation of viral loads in infected animals. These data confirm B10 potency against ZIKV challenge in mice.

We next evaluated the therapeutic and prophylactic efficacy of B10 in rhesus monkeys. 16 monkeys received the following antibodies by intravenous infusion either before or after ZIKV-BR challenge (N=4/group): (1) 10 mg/kg B10 on day -1, (2) 10 mg/kg isotype matched control antibody (PGT121)[24,25] on day -1, (3) 10 mg/kg B10 on day +2, or (4) 10 mg/kg isotype matched control antibody (PGT121) on day +2. We selected this antibody dose based on our previous experience with therapeutic HIV-1-specific antibody studies in SHIV-infected rhesus monkeys[24,25]. Antibody pharmacokinetics was monitored by ELISA, and peak B10 levels were 78-306 µg/ml on the day after infusion (Fig. 2).

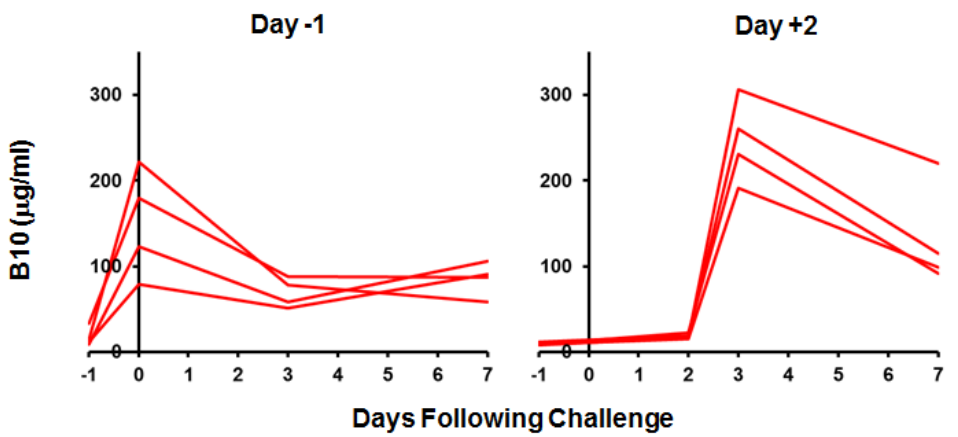


Figure 2. Pharmacokinetics of B10 in rhesus monkeys. Levels of B10 (µg/ml) were determined in monkey serum following B10 infusion by ELISA.

On day 0, all monkeys were challenged by the subcutaneous route with 10^6 VP (10^3 PFU) of ZIKV-BR, and viral loads were quantitated by RT-PCR[12,17]. Animals that received the isotype matched sham control antibody either before or after ZIKV-BR challenge exhibited approximately 7 days of viremia with median peak viral loads of 6.40 (range 5.31-6.60) log RNA

copies/ml on day 3-5 following challenge (Fig. 3a), consistent with our previous studies with this ZIKV-BR challenge stock in rhesus monkeys[12,17]. Administration of B10 on day -1 prior to challenge resulted in complete protection, as evidenced by no detectable plasma viremia at any timepoint (P=0.02 comparing infection of B10 group vs controls, Fisher's exact test). Administration of B10 on day +2 after challenge, which was during the exponential rise of plasma viremia, resulted in an abrupt termination of viral replication and rapid clearance of virus from peripheral blood by day 3 (Fig. 3a; P=0.02 comparing viremia on days 3-7 of B10 group vs controls).

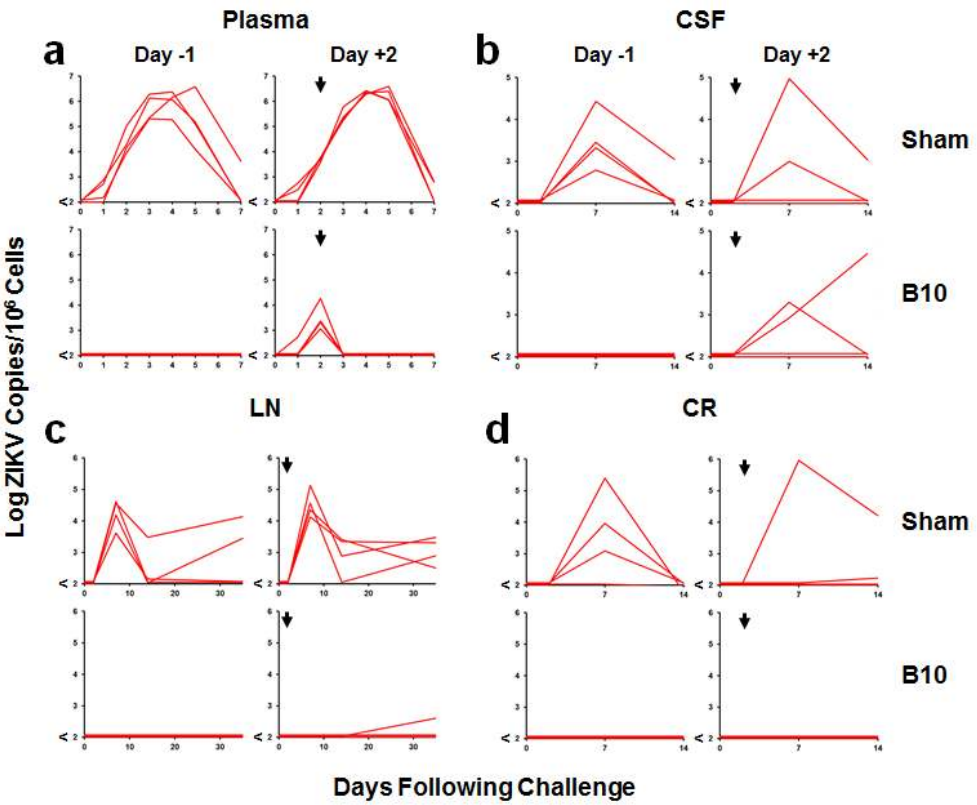


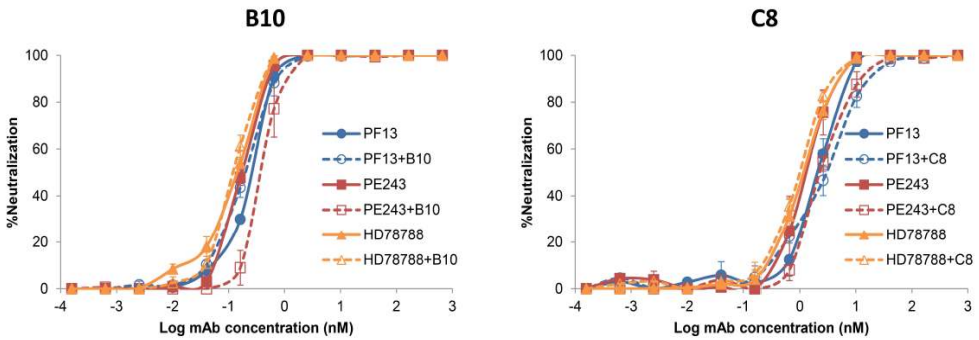
Figure 3. Therapeutic and prophylactic efficacy of B10 in rhesus monkeys. Rhesus monkeys (N=4/group) received 10 mg/kg B10 or the isotype matched sham control antibody PGT121 by the i.v. route on day -1 or day +2. All animals were challenged on day 0 by the s.c route with 10^6 VP (10^3 PFU) ZIKV-BR. Viral loads are shown in (**a**) plasma, (**b**) cerebrospinal fluid (CSF), (**c**) lymph nodes (LN), and (**d**) colorectal (CR) biopsies. Viral loads were determined on days 0, 1, 2, 3, 4, 5, and 7 for the plasma samples and on days 0, 3, 7, 14, and 35 for the other samples. Assay sensitivity 100 copies/ml or $1x10^6$ cells. Arrows designate the day +2 infusions.

We observed prolonged ZIKV-BR shedding in the sham controls in cerebrospinal fluid (CSF), lymph nodes (LN), and colorectal (CR) biopsies (Fig. 3b-d), consistent with our previous observations[17]. Monkeys that received B10 on day -1 prior to challenge had no detectable virus in these tissues, consistent with complete protection against infection. Moreover, these animals had no detectable cellular immune responses following ZIKV-BR challenge, as measured by IFN-γ ELISPOT assays to ZIKV Env, NS1, Cap, and prM peptide pools (Supplementary Fig. S2). Monkeys that received B10 on day +2 after challenge also showed substantial reduction of virus in tissues. However, ZIKV-BR was still detected in 2 of 4 animals in CSF on day 7 and in 1 of 4 animals in CSF on day 14. In this animal, the peak B10 level in CSF was 1 μg/ml (0.5% of plasma levels). The prM-Env sequence from the CSF virus on day 14 was identical to the ZIKV-BR challenge stock (Supplementary Fig. S3), thus suggesting that the virus did not specifically escape from B10. These data demonstrate that therapeutic B10 administration in acutely ZIKV-infected monkeys rapidly controlled virus replication in the periphery within 24 hours but incompletely cleared virus from immunoprivileged sites, likely due to reduced antibody penetration into these anatomic compartments.

To evaluate the capacity of ZIKV to escape EDE1-specific mAbs *in vitro*, we incubated ZIKV with escalating concentrations of the antibodies B10 or C8[5,6] at 0.002, 0.015 and 0.070 ug/ml (corresponding to FRNT50, FRNT90 and FRNT99) for 2, 3, and 5 passages, respectively. After 10 passages, parental and passaged viruses were analyzed for resistance to neutralization by FRNT assays. As shown in Fig. 4, we did not observe viral escape under these conditions, suggesting a relatively high bar to resistance. These findings are consistent with the therapeutic and prophylactic efficacy observed with B10 *in vivo* delivered as monotherapy. In contrast, a cocktail of three domain III-specific mAbs was required to prevent ZIKV infection in nonhuman primates[18].

Our data demonstrate that a DENV EDE1-specific mAb has potent cross-reactive neutralizing activity against ZIKV and provides robust therapeutic as well as prophylactic efficacy against ZIKV infection in rhesus monkeys. Based on the rapid clearance of plasma virus by 24 hours after B10 infusion, we speculate that this antibody functions by opsonization of virus followed by clearance. Previous studies have evaluated ZIKV-specific

mAbs in therapeutic studies in immunosuppressed murine models[1-4]. Our data extend these prior studies by demonstrating for the first time the therapeutic efficacy of a ZIKV-specific antibody in nonhuman primates. These findings encourage clinical development of therapeutic ZIKV-specific mAbs.



	Envelope sequences				
	PF13	PE243		HD78788	
ZIKV-mAb	L/M212M	H401Y	-	I/T156T	-
ZIKV+B10	L/M212L/M	-	K454R	I/T156T	A280V
ZIKV+C8	L/M212M	-	-	I/T156T	-

Figure 4. In vitro selection of ZIKV with B10 and C8. We performed 10 passages of three ZIKV strains (PF13, PE243, HD78788) with escalating concentrations of B10 or C8 antibodies, at 0.002, 0.015 and 0.070 ug/ml (corresponding to FRNT50, FRNT90 and FRNT99) for 2, 3, and 5 passages, respectively. Virus neutralization assays were then performed to determine the resistance of parental vs. B10/C8-selected passaged ZIKV viruses to neutralization. Data are representative of three independent experiments in mean ± s.e.m. ZIKV Env sequences of parental and B10/C8-selected passaged virus were also determined.

The potency of B10 and apparent relatively high bar to escape also raise the possibility of antibody monotherapy, which would be logistically far simpler than the development of antibody cocktails[18] or bi-specific antibodies[2]. The structure of B10 remains to be determined, but the related cross-reactive DENV/ZIKV EDE1-specific mAb C8 binds a conserved quaternary site at the interface between the two Env subunits in the dimer at the interaction site of prM[5], which may explain its high bar to escape and efficacy as monotherapy.

A potential challenge for any antibody-based ZIKV therapeutic strategy involves persistent virus in immunoprivileged sites, since the virus may be seeded in these sites quickly within the first few days of infection. Such sites include the central nervous system, lymph nodes, and placental and fetal tissues. We previously reported that ZIKV persists in CSF, lymph nodes, and colorectal mucosa in monkeys for substantial periods of time after viremia resolves, and viral persistence at these sites correlates with activation of mTOR and proinflammatory signaling pathways[17]. We show here that B10 penetrates poorly into the CSF and thus may not fully clear CSF virus that was seeded prior to antibody administration.

A unique aspect of B10 is that it was derived from a DENV-infected individual prior to the ZIKV epidemic. Certain DENV-specific antibodies have been shown to enhance ZIKV replication in vitro and in mice[6,19,20], although the relevance of these observations for humans remains to be determined. In our experiments, sub-neutralizing doses of B10 did not result in enhanced ZIKV replication in mice (Supplementary Fig. S1), but nevertheless the possibility of antibody-dependent enhancement with a cross-reactive DENV/ZIKV-specific antibody requires further investigation. Our data also raise the possibility of developing antibody therapeutics targeting both flaviviruses in endemic areas.

METHODS

Antibody generation

Activated B cells from dengue infected patients were gated by CD19+, CD3−, CD20lo to CD20−, CD27hi, CD38hi and sorted into 96-well PCR plates containing RNAse. One-step RT-PCR (210212; Qiagen) and nested PCR (203205; Qiagen) were performed to generate PCR products encoding the VH and VLκ/λ genes. The products were cloned into human IgG1 and human Igκ/λ expressing vectors (gifts from H. Wardemann). B10 IgG1 and B10 Igκ containing plasmids were endotoxin-free extracted by using Plasmid Plus Midi Kit (12943; Qiagen) and co-transfected into HEK 293T cells by the polyethylenimine (PEI) method (408727; Sigma). LPS free culture supernatant was collected and purified using Protein G plus/Protein A agarose (IP10; Merck). The endotoxin level was quantitated according to

Pierce LAL Chromogenic Endotoxin Quantitation kit (88282; Thermo Scientific).

Animals, vaccines, and challenges

Female 6-8 week old Balb/c mice were housed at Beth Israel Deaconess Medical Center. 16 outbred, Indian-origin male and female rhesus monkeys (*Macaca mulatta*) were housed at AlphaGenesis, Yemassee, SC. Animals received B10 or isotype matched control antibody (PGT121) infusions by the i.v. route either before or after challenge. Balb/c mice were challenged with 10^5 viral particles (VP) [10^2 plaque-forming units (PFU)] ZIKV-BR (Brazil ZKV2015)[13]. Rhesus monkeys were challenged by the s.c route with 10^6 VP (10^3 PFU) ZIKV-BR[12]. Animals were randomly allocated to groups. Immunologic and virologic assays were performed blinded. All animal studies were approved by the appropriate Institutional Animal Care and Use Committees (IACUCs).

Focus reduction neutralization assay

Virus was incubated with serial dilutions of antibodies at a 1:1 ratio for 1 h at 37 °C. The mAb/virus mixtures were then inoculated onto Vero cells. After 1 h incubation, the cell monolayers were overlaid with 1.5% (w/v) carboxymethyl cellulose and incubated for 2 d (for ZIKV) or 3 d (for DENV). The viral foci were visualized by staining with mAb 4G2 supernatant (mouse anti-DENV fusion loop that cross-reacts to ZIKV) followed by peroxidase-conjugated goat anti-mouse immunoglobulin at a 1:1,000 dilution (P0047; Sigma). The foci (infected cells) were visualized by adding the peroxidase substrate DAB (D5905, Sigma).

RT-PCR

RT-PCR assays were utilized to monitor viral loads, essentially as previously described[12,13]. RNA was extracted from plasma or other samples with a QIAcube HT (Qiagen, Germany). The wildtype ZIKV BeH815744 Cap gene was utilized as a standard. RNA was purified (Zymo Research, CA, USA), and RNA quality and concentration was assessed by the BIDMC Molecular Core Facility. Log dilutions of the RNA standard were reverse transcribed and included with each RT-PCR assay. Viral loads were

calculated as virus particles (VP) per ml or per $1x10^6$ cells and were confirmed by PFU assays. Assay sensitivity was 100 copies/ml or $1x10^6$ cells.

ELISA

Mice and monkey ZIKV Env ELISA kits (Alpha Diagnostic International, TX, USA) were used to assess B10 levels. 96-well plates coated with ZIKV Env protein were first equilibrated at room temperature with 300 µl of kit working wash buffer for 5 min. 6 µl of serum was added to the top row, and 3-fold serial dilutions were tested in the remaining rows. Samples were incubated at room temperature for 1 h, and plates washed 4 times. 100 µl of anti-mouse or anti-human IgG HRP-conjugate working solution was then added to each well and incubated for 30 min at room temperature. Plates were washed 5 times, developed for 15 min at room temperature with 100 µl of TMB substrate, and stopped by the addition of 100 µl of stop solution. Plates were analyzed at 450nm/550nm on a VersaMax microplate reader using Softmax Pro 6.0 software (Molecular Devices, CA, USA). B10 levels were assessed against a standard curve.

***In vitro* selection with B10 and C8**

To try to select ZIKV mutants resistant to neutralization by B10 of C8, ZIKV was incubated with mAb for 1 h at 37 °C. Viruses were then inoculated onto Vero cells and incubated for 2 days. In parallel, mock-neutralized virus was used as wildtype virus control. Viral titers were determined, and virus containing cell suspension was harvested for the next passage. This process was repeated through 10 passages, with 0.002, 0.015, and 0.070 ug/ml of antibody (FRNT50, FRNT90, and FRNT99) for 2, 3, and 5 passages, respectively. After 10 passages, parental and passaged viruses were analyzed for resistance to B10 or C8 neutralization by FRNT assays.

Viral sequencing

Viral RNA was extracted by QIAamp Viral RNA Mini Kit (Qiagen), and RT-PCR was performed to generate cDNA by using SuperScript® III First-Strand Synthesis System (Invitrogen) with a specific Zika 3'UTR primer. The Env region was amplified with Accuprime Taq DNA polymerase High Fidelity

(Invitrogen) using primers specific to the end of capsid and NS1 and sequenced.

Primer	Sequence (5'-3')	Position	Use
R_ZKV_RT_3UTR	CTGGTCTTTCCCACGTCAATATG	10761-10784	RT-PCR
PE243_C17	AGTGTCGGAATTGTTGGCCTCCTGCTG	492-518	PCR
R_PE243_NS1	TGCAGTCACCATTGACCTTAC	3585-3605	PCR
F_ZIK_M963	GAGCCAAAAAGTCATATACTTG	983-1004	Sequencing
F_ZIK_E1347	CATGGMTCCCAGCAYAGYGGGATG	1467-1499	Sequencing
F_ZIK_E1737	GAGGCTGAGATGGATGGTG	1857-1875	Sequencing

Statistical analyses

Analysis of virologic and immunologic data was performed using GraphPad Prism v6.03 (GraphPad Software, CA, USA). Comparisons of groups were performed using Fischer's exact tests and Wilcoxon rank-sum tests.

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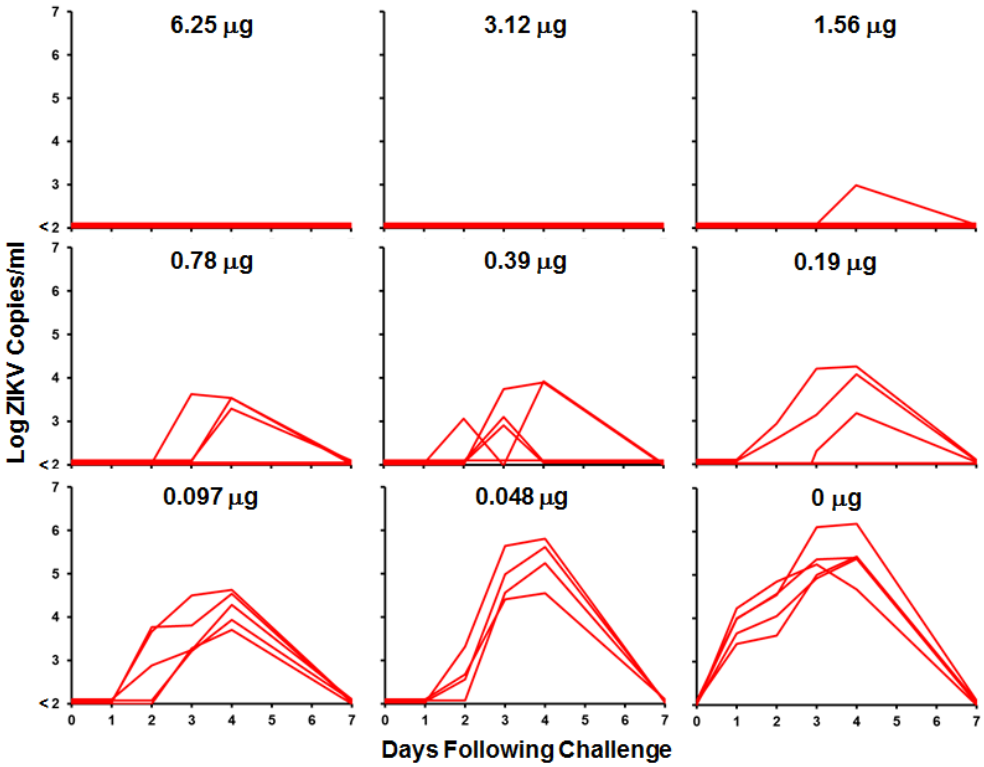
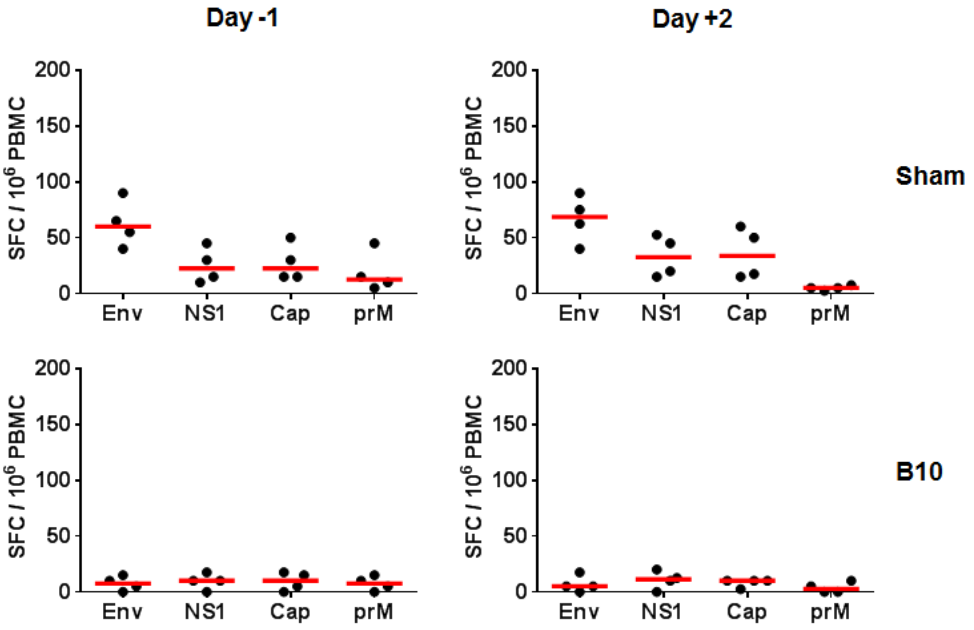
SUPPLEMENTARY MATERIALS



Supplementary Figure S1. Protective efficacy of B10 in mice. Groups of Balb/c mice (N=5/group) received a single infusion of 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.097, 0.048, and 0 µg B10 and were subsequently challenged by the i.v. route with 10^5 VP (10^2 PFU). Plasma viral loads are shown on days 0, 1, 2, 3, 4, and 7. Assay sensitivity 100 copies/ml. Mouse studies were performed twice.



Supplementary Figure S2. Cellular immune responses. IFN-ɣ ELISPOT assays using Env, NS1, Cap, and prM peptide pools were performed in rhesus monkeys at week 2 following ZIKV challenge. Spot-forming cells (SFC) per 10^6 PBMC are shown.

Brazil ZKV2015 prM-Env Sequence (Genbank KU497555.1)

AVTLPSHSTRKLQTRSQTWLESREYTKHLIRVENWIFRNPGFALAAAAIAWLLGSSTSQKVIYLVMI
LLIAPAYSIRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVRSY
CYEASISDMASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSKKMT
GKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPR
TGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTVVVLGT
QEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAETLHGTVT
VEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGEK
KITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAFKSLFGGMS
WFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

ZIKV-BR Challenge Stock prM-Env Sequence

AVTLPSHSTRKLQTRSQTWLESREYTKHLIRVENWIFRNPGFALAAAAIAWLLGSSTSQKVIYLVM
ILLIAPAYSIRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVR
SYCYEASISDMASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSK
KMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLD
CEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTV
VVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAET
LHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYI
VIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAF
KSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

CSF Day 14 (Monkey 12-083) prM-Env Sequence

AVTLPSHSTRKLQTRSQTWLESREYTKHLIRVENWIFRNPGFALAAAAIAWLLGSSTSQKVIYLVM
ILLIAPAYSIRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSNMAEVR
SYCYEASISDMASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGCGLFGKGSLVTCAKFACSK
KMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGHETDENRAKVEITPNSPRAEATLGGFGSLGLD
CEPRTGLDFSDLYYLTMNNKHWLVHKEWFHDIPLPWHAGADTGTPHWNNKEALVEFKDAHAKRQTV
VVLGSQEGAVHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKIPAET
LHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYI
VIGVGEKKITHHWHRSGSTIGKAFEATVRGAKRMAVLGDTAWDFGSVGGALNSLGKGIHQIFGAAF
KSLFGGMSWFSQILIGTLLMWLGLNTKNGSISLMCLALGGVLIFLSTAVSA

Supplementary Figure S3. Sequence of prM-Env in CSF virus from monkey 12-083.
Identical sequences of prM-Env from the ZIKV-BR challenge stock and the day 14 CSF virus (monkey 12-083) are shown. A single amino acid mutation (shown in red) was observed for both sequences compared with the Brazil ZKV2015 sequence (Genbank KU497555.1). Blue indicates region not sequenced.

6.1

ENGLISH SUMMARY

Zika virus (ZIKV) was first isolated in 1947 from a sentinel monkey placed in the Zika forest, Uganda. Analysis verified that it belonged to the *Flaviviridae* family which includes West Nile virus (WNV), dengue virus (DENV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV) and yellow fever virus (YFV). It is an arthropod-borne virus (arbovirus) with the main mosquito vector being *Aedes aegypti.*

The first ZIKV outbreak of public concern was in 2007, located in the Yap Islands, Federate States of Micronesia. From here it quickly spread across the western hemisphere, resulting in an explosive spread across South America with Brazil being particularly hit hard. As ZIKV infections were increasing, so were cases of Guillain-Barré syndrome in infected individuals. Moreover, a spike of microcephaly and other congenital malformations – now collectively called congenital Zika syndrome (CZS)– were also observed in infants born to ZIKV infected mothers, particularly when infected during the first trimester. The link between CZS and ZIKV infection led to the declaration of a Public Health Emergency of International Concern in February 2016, by the World Health Organization (WHO). Additionally, it was the first time in the history of the Center for Disease Control and Prevention (CDC) that it had instated a travel warning within the continental United States. Pregnant women were urged not to travel to areas in Florida with active ZIKV transmission. As the peak of active ZIKV transmission passed, the public health emergency declaration was withdrawn in November, 2016.

Chapter 2

Efforts described in chapter 2 of this thesis have led to the first published vaccine efficacy to ZIKV in pre-clinical models. Experience from vaccine development to related flaviviruses DENV, WNV and YFV provided a stepping stone to critical early development choices. Live-attenuated vaccines have been licensed for DENV and YFV and provided insight that the envelope protein is the primary binding site for neutralizing antibodies. Therefore, we developed subunit DNA vaccines expressing a variety of modified premembrane-envelope antigens. In order to assess protective efficacy of our vaccine candidates we established a wild type Balb/c mouse model. Infection with a Brazilian Zika strain (ZIKV-BR) in naïve mice led to comparable viremia and clinical manifestations as observed in humans.

Immunization of mice with ZIKV PIV or DNA vaccines led to induction of ZIKV neutralizing antibodies (NAbs), as determined by microneutralization 50 (MN50) assay. Complete protection, as evidenced by undetectable viremia in serum by RT-PCR, was observed after ZIKV challenge of mice four weeks post immunization. To determine the mechanism of protection we immunized mice and prior to challenge 4 weeks post immunization, depleted CD4 and CD8 T-cells. Depletion of T-cells did not abrogate protection. To assess if NAbs alone could confer protection we purified serum IgG from immunized mice. Passive transfer of purified IgG in naïve mice blocked infection of ZIKV-BR challenge but was dose dependent. Therefore, NAbs induced by vaccination are sufficient to protect from ZIKV infection in mice, if present at high enough titers.

Chapter 3

In chapter 3, we describe the protective efficacy of our vaccines in rhesus monkeys. In addition to ZIKV PIV and DNA vaccines we also included a viral vectored vaccine based on rhesus adenovirus 52 (RhAd52), expressing the identical antigen expressed by the DNA vaccine that conferred protection in mice. Monkeys were immunized with 2 doses of ZIKV PIV and DNA vaccine or a single dose of RhAd52 vaccine. Neutralizing antibodies were induced by all vaccine candidates as measured by MN50 assay. Four weeks post final immunization all monkeys were infected with ZIKV-BR. All monkeys that received vaccines had undetectable ZIKV viral load in plasma. Furthermore, no virus was detected in other compartments such as cerebrospinal fluid (CSF) or colorectal and cervicovaginal secretions, suggesting sterilizing protection. In addition, vaccine induced antibodies in monkeys were capable to protect both mice and monkeys in passive transfer studies but only if the NAb titer was at least 100 as determined by MN50 assay.

Chapter 4

Protective efficacy against ZIKV infection thus far has been determined at peak immunogenicity, four weeks following vaccination. According to WHO Zika vaccine target product profile (TPP) the preferred duration of protective efficacy is a minimum of 1 year, preferably by a single

dose regimen. In chapter 4 we assessed durability of immune responses and protective efficacy of our vaccine candidates in monkeys. Additionally we showed that of the optimized antigens developed in chapter 2, the M-Env antigen offers better protection than prM-Env.

Mice were immunized with PIV, Ad or DNA vaccines. We monitored binding antibody responses weekly post immunization by ELISA. The PIV, RhAd52 and the human derived Ad26 vector based vaccines all induced higher antibody titers that lasted for 20 weeks. Antibody titers induced by the DNA vaccines declined after the peak at week 4. Mice that received the DNA vaccine expressing M-Env consistently had higher titers of antibody than those that received DNA vaccine expressing prM-Env. Twenty weeks post immunization mice were challenged with ZIKV-BR. All mice immunized with the PIV and Ad based vaccines were completely protected against ZIKV challenge. Four out of five mice in the DNA M-Env group completely protected, whereas only one out of five mice were protected in the DNA prM-Env group.

To validate the durability in monkeys we immunized groups of monkeys with 2 doses of ZIKV PIV, 2 doses of DNA M-Env or a single dose of RhAd52 M-Env. Monitoring of NAbs by MN50 assay over the course of a year showed high titer of NAbs after the PIV boost, that declined rapidly before reaching a setpoint and remaining largely stable after that. The DNA immunized monkeys also reached peak NAb titers following boost with a steady decline in the following weeks. In contrast, RhAd52 induced strong NAb responses after only a single dose, and titers remained remarkably stable over the course of one year. All monkeys were challenged with ZKV-BR at week 52. Monkeys immunized with ZIKV PIV were largely protected. However, there were two monkeys that had low detectable viremia in plasma and LN. DNA immunized monkeys were poorly protected against ZIKV challenge with only 2 monkeys having undetectable virus. In contrast, the RhAd52 immunized monkeys were all completely protected one full year following immunization.

Chapter 5

The promising results obtained with the ZIKV vaccines we developed in this thesis suggest an efficacious vaccine for use in people is possible.

However, vaccines are primarily preventative and measures are also needed in people already exposed to ZIKV. Monoclonal antibodies could potentially be an important tool to treat infection or induce temporary protection.

In chapter 5 we show that neutralizing antibody B10, originally isolated from a DENV infected person, also protects against ZIKV infection in monkeys. Treatment of monkeys with B10 one day prior to infection with ZIKV resulted in complete protection, indicating the NAb can effectively be used as preventative therapy. Treatment two days post ZIKV infection led to rapid and complete control of viremia in plasma. However, in a subset of monkeys, ZIKV was detected in CSF and LN, potentially due to reduced infiltration of these compartments by B10 antibodies.

Conclusion

Research described in this thesis has led to rapid development of multiple vaccines that are protective in pre-clinical animal models. Neutralizing antibodies have been identified as correlation of protection. In mice PIV, Ad and DNA vaccines all led to complete protection against ZIKV challenge four weeks post immunization. However, only PIV and Ad based vaccines completely protected mice against ZIKV challenge twenty weeks post immunization. In monkeys, only the RhAd52 vaccine was able to induce complete durable protection of up to one year against ZIKV infection, with a single dose.

The encouraging data described in this thesis have led to the progression of an Ad26 vaccine expressing M-Env to a phase I clinical trial, which is currently ongoing.

6.2

NEDERLANDSE SAMENVATTING

Het Zika virus (ZIKV) is voor het eerst geïsoleerd in 1947 uit een aap in het Zika bos van Oeganda. Analyse wees uit dat het toebehoorde aan de familie Flaviviridae waar ook het West Nile-virus (WNV), Dengue-virus (DENV), Japanse ecephalitisvirus (JEV) en het door teken overgedragen encephalitisvirus (tick-born encephalitis virus, TBEV) deel van uitmaken. ZIKV wordt hoofdzakelijk door muggen, en voornamelijk door Aedes aegypti, ook wel bekend als de Aziatische tijgermug, overgedragen.

De eerste uitbraak van Zika was in 2007, op de Yap eilanden in de Federale Staten van Micronesië. Vanuit hier verspreidde het virus zich snel over het westelijk halfrond. Voornamelijk Brazilië werd hard getroffen. Met de toename van het aantal Zika infecties werd er ook een toename gezien van het aantal baby's wat geboren werd met neurologische afwijkingen. Deze afwijkingen, waaronder microcefalie, werden samen congenital Zika syndroom (CZS) genoemd. De link tussen CZS en ZIKV-infectie gedurende de zwangerschap leidde tot het uitroepen van een internationale noodsituatie voor de volksgezondheid in februari 2016 door de Wereldgezondheidsorganisatie (WHO). Bovendien werd er voor het eerst in de geschiedenis een reiswaarschuwing ingesteld binnen de continentale Verenigde Staten door het Center for Disease Control and Prevention (CDC). Zwangere vrouwen werd aangeraden om niet naar gebieden in Florida te reizen waar actieve ZIKV-transmissie gaande was. Nadat het aantal nieuwe ZIKV-infecties afnam, werd de noodsituatie in november 2016 opgeheven.
De meest effectieve manier om te beschermen tegen ZIKV is zeer waarschijnlijk door middel van vaccinatie. Vaccins tegen ZIKV zijn echter nog niet beschikbaar. Dit proefschrift beschrijft de ontwikkeling van drie onafhankelijke vaccin kandidaten en een antistof die getest zijn voor hun vermogen om beschermende immuniteit tegen ZIKV infectie te bieden in preklinische modellen.

Hoofdstuk 2

De data zoals gepresenteerd in hoofdstuk 2 van dit proefschrift toonden voor de eerste keer aan dat een vaccin bescherming kan bieden tegen ZIKV infectie in preklinische modellen. Voor de flavivirussen DENV en YFV zijn er live-attenuated virus vaccins beschikbaar. De bescherming van deze DENV en YFV vaccins correleerde met de aanwezigheid van neutraliserende antistoffen die met name gericht zijn tegen het envelop

(Env) eiwit van de virussen. Gebaseerd op de kennis die is opgedaan met deze flavivirusvaccins hebben we geïnactiveerde ZIKV vaccins (PIV) ontwikkeld. Om specifiek antistoffen tegen het Env eiwit van ZIKV op te wekken hebben we ook op DNA gebaseerde subunit vaccins ontwikkeld die verschillende vormen van het natuurlijke premembraan-envelope (prM-Env) complex tot expressie brengen. Om de beschermende werking van de verschillende ZIKV vaccins aan te tonen hebben we een wild type Balb/c muizen model opgezet wat voor wat betreft de distributie van het virus en de ziekteverschijnselen, overeenkomsten vertoont met ZIKV infectie in mensen.

Vaccinatie met PIV en met de verschillende DNA vaccins leidde tot inductie van ZIKV neutraliserende antistoffen die kon worden aangetoond met een microneutralisatie 50 (MN50) assay. Muizen die vier weken na vaccinatie met een enkele dosis vaccin werden blootgesteld aan de Braziliaanse ZIKV stam (ZIKV-BR) ontwikkelden geen viremie of ziekte. Depletie van CD4+ en CD8+ T cellen door middel van toediening van anti-CD4 en anti-CD8 antistoffen aan gevaccineerde muizen, vier weken na vaccinatie, leidde niet tot verminderde bescherming tegen ZIKV challenge. Niet gevaccineerde ZIKV naïeve muizen die gezuiverde ZIKV specifieke antistoffen kregen toegediend afkomstig van ZIKV gevaccineerde muizen waren ook beschermd tegen ZIKV infectie. De bescherming door middel van antistoffen bleek afhankelijk van de antistof concentratie in het bloed. Met deze experimenten is aangetoond dat door vaccins opgewekte antistoffen afdoende zijn om bescherming tegen ZIKV infectie te bieden, mits de concentratie van deze antistoffen hoog genoeg is.

Hoofdstuk 3

In hoofdstuk 3 wordt de werking van verschillende ZIKV vaccins in apen beschreven. Verschillende ZIKV vaccins (PIV, DNA vaccins en een ZIKV vaccin gebaseerd op een virale vector afgeleid van een rhesus adenovirus subtype 52 (RhAd52)) werden vergeleken. De apen werden gevaccineerd met twee doses PIV of DNA vaccin of met een enkele dosis RhAd52. Vier weken na de laatste vaccinatie werden alle apen geïnoculeerd met ZIKV-BR. Alle type vaccins waren in staat om de apen te beschermen tegen ZIKV infectie. In geen enkele aap kon virus gedetecteerd worden in plasma of andere compartimenten zoals hersenvocht of rectale en vaginale

afscheidingen, duidend op een complete bescherming. Gezuiverde, door vaccinatie opgewekte antistoffen in apen, boden ook bescherming als ze getransfereerd werden naar ZIKV naïeve muizen of ZIKV naïeve apen. Volledig bescherming werd echter alleen verkregen als de neutraliserende antistof titer hoger was dan 100, zoals bepaald door middel van een MN50 assay.

Hoofdstuk 4

In de experimenten beschreven in hoofdstukken 2 en 3 werden de gevaccineerde muizen en apen werden kort na vaccinatie, op de piek van de immuunreactie, geïnoculeerd met ZIKV. De WHO heeft aangegeven een voorkeur te hebben voor een enkele dosis ZIKV vaccin welke ten minste 1 jaar beschermende immuniteit zou moeten bieden. In hoofdstuk 4 hebben we onderzocht of de door ons ontwikkelde vaccins langdurige bescherming bieden in het muizen en apen model. Daarnaast hebben we gekeken welke van de twee beste subunit vaccin antigenen, het geoptimaliseerde M-Env en het prM-Env, de meest optimale bescherming biedt.

Muizen werden gevaccineerd met een dosis PIV, DNA of Ad vaccins. Op verschillende tijdspunten na vaccinatie werd de antistof titer bepaald door middel van ELISA. PIV, RhAd52 en een ZIKV vaccin gebaseerd op een virale vector afgeleid van het menselijk adenovirus subtype 26 (Ad26) leidde tot hoge antistof titers die 20 weken lang aanhielden. De antistof titers geïnduceerd door de DNA vaccins echter daalden al 4 weken na immunisatie. Muizen die het M-Env gebaseerde DNA vaccin toegediend hadden gekregen hadden 20 weken na vaccinatie hogere antistof titers dan muizen die het prM-Env vaccin gekregen hadden. Twintig weken na vaccinatie werden de muizen geïnfecteerd met ZIKV. Alle PIV, RhAd52 en Ad26 gevaccineerde muizen waren beschermd tegen ZIKV infectie. Van de vijf muizen gevaccineerd met het prM-Env DNA vaccin was slechts één muis beschermd tegen ZIKV infectie. Van de vijf muizen die gevaccineerd waren met M-Env DNA waren vier muizen beschermd.

In apen konden we aantonen dat met 2 doses PIV beschermende immuniteit kon worden opgewekt die zelfs een jaar na vaccinatie nog gedeeltelijke bescherming bood tegen ZIKV blootstelling. Slechts in twee van de acht gevaccineerde apen kon na blootstelling aan ZIKV virus gedetecteerd

worden in plasma en lymfeklieren. Echter, vijf van de zeven apen die twee doses M-Env DNA vaccin kregen waren een jaar na vaccinatie niet meer beschermd. In tegenstelling, een enkele dosis van het RhAd52 vaccin gaf zelfs een jaar na vaccinatie nog volledige bescherming tegen ZIKV infectie. In geen van de vier apen was virus aantoonbaar na blootstelling aan ZIKV.

Hoofdstuk 5

De vaccins beschreven in dit proefschrift suggereren dat het mogelijk moet zijn om een beschermend ZIKV vaccin voor mensen te maken. Vaccins werken echter voornamelijk preventief en vaak niet meer in mensen die al geïnfecteerd zijn. Monoclonale antistoffen zijn uitermate geschikt voor de behandeling van mensen met een infectie of als zogenaamde post-exposure profylaxe.

In hoofdstuk 5 laten we zien dat neutraliserend antistof B10, die oorspronkelijk geïsoleerd is uit een DENV geïnfecteerd persoon, ook effectieve bescherming geeft tegen ZIKV. Behandeling met B10 een dag voor ZIKV infectie gaf volledige bescherming in apen. Zelfs als B10 twee dagen na infectie toegediend werd, werd de virus replicatie volledig geremd. Hoewel ZIKV in plasma niet meer detecteerbaar was, werd in enkele apen virus aangetroffen in CSF en lymfeklieren, mogelijk door het niet volledig penetreren van de antistoffen in deze anatomische compartimenten.

Conclusie

Het onderzoek verricht in dit proefschrift heeft geleid tot het ontwikkelen van drie onafhankelijke ZIKV vaccin kandidaten. In muizen gaven PIV, DNA en RhAd52 gebaseerde vaccins volledige beschermende kortdurende immuniteit terwijl de immuniteit die werd opgewekt met PIV, RhAd52 en Ad26 langduriger bescherming bood. In apen gaf alleen het vaccin wat gebaseerd was op RhAd52 volledige en langdurige bescherming. Apen gevaccineerd met een enkele dosis RhAd52 waren een jaar later nog steeds volledig beschermd.

Monoclonale antistoffen, zoals B10, kunnen zowel preventief als therapeutisch toegepast worden om respectievelijk ZIKV infectie en ZIKV gemedieerde ziekte, te bestrijden.

De positieve preklinische resultaten beschreven in dit proefschrift hebben uiteindelijk geleid tot het testen van een Ad26 M-Env ZIKV vaccin in een fase I klinische studie, welke momenteel gaande is.

7.1

SUMMARY AND FUTURE PERSPECTIVES

ADAPTED FROM: ZIKA VIRUS VACCINES

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Vaccines in clinical trials

Several vaccine candidates have undergone successful pre-clinical development. Neutralizing antibodies were induced for all vaccines tested in mice (Table 1). All vaccines were able to provide short-term protection in mice against challenge with ZIKV. To date, DNA vaccines, mRNA vaccines, purified inactivated virus vaccines, and adenovirus-based vaccines have also conferred protection in monkeys. There has also been rapid advancement of these candidates into phase 1 clinical trials[1]. To date, there are 13 open clinical trials testing a range of ZIKV vaccine concepts, including DNA vaccines, mRNA vaccines, PIV vaccines and viral vector-based vaccines (Table 2).

DNA vaccines

DNA vaccines are plasmids encoding a transgene of interest under control of a promotor. DNA vaccines can be developed and produced rapidly, and they can induce both humoral and cellular immune responses[2]. The first clinical assessment of safety and immunogenicity of a ZIKV DNA vaccine, expressing the ZIKV precursor membrane and envelope genes (prM-ENV), was led by GeneOne Life Science, Inc and Inovio Pharmaceuticals, Inc (clinical trial NCT02809443)[3]. A total of 40 participants were divided equally between 2 groups and received either a 1 mg or 2 mg dose of the GLS-5700 DNA vaccine by intradermal injection with electroporation at baseline, with boosts at week 4 and week 12 (Table 2). The vaccine was well tolerated with no severe adverse reactions related to the vaccine. ZIKV-specific antibody levels at week 14 were assessed by ELISA and showed 100% seroconversion for binding antibodies in both dose groups, with a geometric mean titer (GMT) of 1,642 (347-7,760) for the 1 mg dose group and a GMT of 2,871 (705-11,688) for the 2 mg dose group. These results indicated that the vaccine-induced antibody responses were dose dependent. Neutralizing antibody titers above the detection limit were detected in 60% and 63% of the 1 mg and 2 mg dose groups, respectively. Passive transfer of week 14 serum into interferon α/β receptor (*Ifnar*) knockout mice followed by a lethal challenge of ZIKV[4] resulted in 92% survival of mice that was independent of neutralizing antibody titer. This phase I clinical trial showed that the DNA vaccine was safe and well tolerated, and that vaccine-induced

Table 1. Zika virus vaccines in pre-clinical and clinical development. NAbs, neutralizing antibodies; ZPIV, ZIKV purified inactivated virus vaccine; Ad, adenovirus-based vaccine; MVA, modified Vaccinia virus Ankara; MV, measles virus-based vaccine; NA, not applicable; NS1, non-structural protein 1; ZIKV-LAV, live-attenuated vaccine; prM-ENV, precursor membrane envelope; ?, not reported or published

Vaccine	Antigen	Induction of NAbs	Short-term protection in mice	Immunocompetence mouse model	Short-term protection in monkeys	Long-term protection in monkeys	Advanced to clinical trial	References
ZPIV	NA	Yes	Yes	Competent	100%	79%	Phase I	Larocca et al. Nature, 2016
								Abbink et al. Science, 2016
								Abbink et al. Sci Trans Med, 2017
DNA	prM-ENV	Yes	Yes	Competent	100%	29%	Phase I/II	Larocca et al. Nature, 2016
								Abbink et al. Science, 2016
								Dowd et al. Science, 2016
								Abbink et al. Sci Trans Med, 2017
Ad	prM-ENV	Yes	Yes	Competent	100%	100%	Phase I	Abbink et al. Science, 2016
								Abbink et al. Sci Trans Med, 2017
								Xu et al. J Virol, 2018
				Competent/Deficient				
mRNA	prM-ENV	Yes	Yes	Competent/Deficient	100%	?	Phase I/II	Richner et al. Cell, 2017
				Competent				Pardi et al. Nature, 2017
MVA	NS1	Yes	Yes	Competent	?	?	Phase I	Brault et al. Sci Rep, 2017
MeV	prM-ENV	Yes	?	?	?	?	Phase I	www.zikavax.eu
ZIKV-LAV	NA	Yes	Yes	Competent	?	?	NA	Shan et al. Nat Med, 2017
				Deficient				Kwek et al. Nat Comm, 2018
				Competent/Deficient				Li et al. Nat Comm, 2018

antibodies were able to protect mice from a lethal challenge of ZIKV. Clinical trials with DNA vaccines have also been conducted by the Vaccine Research Center (VRC) of the National Institute of Allergy and Infectious Diseases (NIAID). The first DNA vaccine was designed to express ZIKV prM-ENV with a JEV envelope stem region; the JEV stem was added to increase sub-virus particle formation (vaccine VRC5288 and study VRC319; clinical trial NCT02840487). The second DNA vaccine expressed wild-type ZIKV prM-ENV (vaccine VRC5283 and study VRC320; clinical trial NCT02996461)[5]. In study VRC319, participants received 4 mg doses at 0 and 8 weeks, 0 and 12 weeks, 0, 4, and 8 weeks, or 0, 4, and 20 weeks by intra-muscular injection (Table 2). In VRC320, participants received 4 mg doses at 0, 4, and 8 weeks through intra-muscular injection or split-dose needle and syringe,or needle-free injection with the Stratis device[6]. Only mild to moderate vaccine-associated adverse events were reported. Neutralizing antibody responses were highest at 4 weeks post final vaccination. In study VRC319, neutralizing antibody GMT titers were 120 (73 to 197) with detectable neutralizing antibodies in 89% of the participants. In study VRC320, neutralizing antibody responses were detected in 100% of participants of the split-dose, needle-free delivery group, with neutralizing antibody GMTs of 304 (215 to 430). Both trials showed the DNA vaccines were well tolerated and immunogenic. The immunogenicity of the wild-type ZIKV prM-ENV DNA vaccine was higher than with the DNA vaccine that included the JEV envelope stem. The VRC5283 vaccine recently advanced into a phase II efficacy trial in regions endemic for ZIKV transmission in South and Central America, the Caribbean and the United States (NCT03110770**).**

Purified inactivated virus vaccines

Three ZIKV purified inactivated virus vaccine (ZPIV) phase I clinical trials (NCT02963909, NCT02952833, and NCT02937233**)** were reported as a combined interim analysis of the preliminary results for the identical group for each individual study[7]. These studies (Table 2) were conducted at Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD, United States; Saint Louis University, Saint Louis, MO, United States; and Beth Israel Deaconess Medical Center (BIDMC), Boston, MA, United States. ZPIV contains a chromatographic-column-purified, formalin-inactivated ZIKV strain (PRVABC59) that was grown in Vero cells. The interim analysis included the group from each study that received the 2 dose regimen of 5

μg aluminum hydroxide adjuvanted ZPIV vaccine, administered intra-muscularly on day 1 and day 29. Adverse events related to the vaccine were mild to moderate with no serious adverse events related to the vaccine reported. Neutralizing antibody titers were determined by microneutralization (MN50) assays at WRAIR for all three trials. A total of 95% of participants had peak neutralizing antibody titers with a GMT of 286 (170–481) after the second dose. Adoptive transfer studies with purified immunoglobulin G resulted in complete protection against ZIKV challenge in 41 out of 50 Balb/c mice, and reduced viremia in the mice that were infected. Results from these trials showed that the inactivated ZIKV vaccine was well tolerated and immunogenic, and that vaccine-induced antibodies were protective in adoptive transfer studies in mice. The impact of different doses and immunization schedules will be determined in follow-up analyses of the completed studies.

Another phase I clinical trial with a ZIKV inactivated vaccine (TAK-426) led by Takeda Pharmaceutical Company Ltd, is ongoing (NCT03343626). A dose escalation study in 240 healthy individual will assess the safety and immunogenicity of this vaccine candidate.

mRNA vaccines

A newer class of vaccines, mRNA vaccines[8], has also been developed against ZIKV. mRNA vaccines encode a gene of interest under control of a promotor. As mRNA is directly translated into protein after entering the cell cytoplasm, mRNA vaccines bypass the need to traverse the nuclear envelope to be expressed. This pathway could potentially lower the doses needed for mRNA vaccines while retaining the immunogenicity observed with DNA vaccines. ZIKV prM-ENV mRNA was encapsulated in a lipid nanoparticle for delivery and stability[9], and immunization of both mice and monkeys with this vaccine induced high levels of neutralizing antibodies that protected against ZIKV infection[10,11]. In mouse pregnancy models, these mRNA vaccines prevented fetal demise, whereas fetal resorption was observed in non-immunized infected pregnant mice. However, levels of ZIKV RNA could still be detected in maternal spleen and brain as well as in placenta and in the fetal head in immunized mice[12].As these results were obtained in the immunocompromised mouse model, which supports increased viral replication, it remains to be determined if viral replication would be

observed in immunocompetent animal models. The first-in-human, phase I/II clinical trial led by Moderna Therapeutics is currently ongoing to assess the safety and immunogenicity of escalating doses of prM-ENV mRNA (NCT03014089) (Table 1). mRNA vaccines could be cost effective as a large number of doses can be produced efficiently. However, it remains to be determined if the promising pre-clinical data translates to humans. Additionally, the stability of mRNA vaccines needs to be taken into consideration.

Viral vector-based vaccines

Viral vector-based vaccines are another promising approach to immunize against various pathogens. These vaccines induce high humoral and cellular immune responses that have been shown to lead to protection against infection in pre-clinical models[13,14]. A MV ZIKV vaccine developed by Themis Bioscience GmbH is currently in a phase I clinical trial. The MV Schwarz vaccine strain[15] was engineered to express ZIKV prM-ENV (MV-ZIKA) and was tested for immunogenicity in mice and monkeys[16]. The ongoing clinical trial is assessing safety and immunogenicity of a high or low dose when given as a single or two dose regimens (NCT02996890). An adenovirus serotype 26 (Ad26) ZIKV based vaccine (Ad26.ZIKV.001), expressing the identical antigen as the rhesus adenovirus serotype 52 (RhAd52) preclinical vaccine candidate, sponsored by Janssen Vaccines and Prevention B.V., is currently also in a phase I clinical trial (NCT03356561). This study aims to test the safety and immunogenicity of two different doses of the vaccine in a double-blind, placebo-controlled clinical trial at two sites in Kansas and Boston, United States. Experience that has already been gained with the Ad26 vaccine vector in clinical trials for other pathogens[17-20] may facilitate the advancement of this vaccine candidate.

Viral vector-based vaccines have shown promising results in pre-clinical models for ZIKV, and certain vectors benefit from prior experience in clinical trials for other pathogens[18,21]. Development of additional vectors with minimal to no pre-existing immunity is also in progress[22].

Protection in pre-clinical models

At the height of the ZIKV epidemic in Brazil, multiple laboratories started to develop vaccine candidates and animal models to assess vaccine efficacy. For example, ZIKV infection in wild type BALB/c mice and rhesus monkeys largely recapitulated the magnitude and duration of ZIKV viremia in humans, exhibiting 7 to 10 days of viremia with minimal clinical symptoms. In contrast, ZIKV infection in immunodeficient mice, such as type I or I/II interferon (A129 or AG129) knockout mice or signal transducer and activator of transcription 2 (*Stat2*) knockout mice, were shown to exhibit prolonged viremia and have been used to study neurological disease in adult and fetal mice. The protective efficacy of a purified inactivated Zika virus vaccine (ZPIV) and a DNA vaccine was first demonstrated in mice[23]. Moreover, ZPIV, a DNA vaccine, and a rhesus adenovirus serotype 52 (RhAd52) vector-based vaccine expressing a modified M-ENV ZIKV antigen were shown to block ZIKV infection in rhesus monkeys[24]. Furthermore, the RhAd52-based vaccine was found to provide durable complete protection in rhesus monkeys against ZIKV a year after immunization with remarkably stable neutralizing antibody titers[25].

Studies from a number of groups have reported the protective efficacy of inactivated virus vaccines, DNA vaccines, viral vector-based vaccines and mRNA vaccines[10-12,24,26-32] (Table 1). DNA vaccines expressing variations of the prM-ENV antigen were quickly developed and tested successfully for efficacy in both mice and monkeys[26]. mRNA vaccines expressing wild-type or modified prM-ENV antigens, leading to the generation of sub-viral particles were able to protect mice with a single dose as low as 10 μg[10] or 50 μg for monkeys[11]. Several live-attenuated vaccines have also been developed, based on the Yellow Fever Virus (YFV) YF17D model, the JEV vaccine SA14-14-2 backbone, or attenuated through systematic deletions in the 3'UTR region in the ZIKV genome[30-32]. All live-attenuated ZIKV vaccines have proved immunogenic and protective in mice and monkeys. Finally, MVA and MV vectors have been engineered to express the NS1 or prM-ENV proteins of ZIKV, respectively. Protection in pre-clinical models with these candidates has also been reported[29].

Table 2. Phase I clinical trial seroconversion rates, neutralizing antibody titers with respective assays and adoptive transfer results in mice. MN50, microneutralization assay; RVP, reporter virus particle assay; NAb, neutralizing antibody; ZPIV, ZIKV purified inactivated virus vaccine; NA, not applicable; N/S, needle and syringe; IM, intramuscular; BIDMC, Beth Israel Deaconess Medical Center; WRAIR, Walter Reed Army Institute of Research; SLU, Saint Louis University; VRC, Vaccine Research Center

Vaccine	Dose	Schedule	NAb seroconversion	MN50 titer	RVP titer	Adoptive transfer in mice
ZPIV (BIDMC)	5 µg	Day 0 and 29, IM	10/10 (100%)	1061·7 (425·8–2489·2)	NA	Yes; 41/50 mice had undetectable viremia
ZPIV (WRAIR)	5 µg	Day 0 and 29, IM	17/20 (85%)	100·8 (39·7–255·7)	NA	No
ZPIV (SLU)	5 µg	Day 0 and 29, IM	25/25 (100%)	345·6 (166·4–718·0)	NA	No
DNA (VRC5288)	4 mg	Weeks 0 and 8 by a single N/S dose	12/20 (60%)	NA	67 (40 to 114)	No
DNA (VRC5288)	4 mg	Weeks 0 and 12 by a single N/S dose	15/20 (75%)	NA	55 (39 to 78)	No
DNA (VRC5288)	4 mg	Weeks 0, 4, and 8 by a single N/S dose	16/20 (80%)	NA	81 (51 to 127)	No
DNA (VRC5288)	4 mg	Weeks 0, 4, and 20 by a single N/S dose	17/19 (89%)	NA	120(73 to 197)	No
DNA (VRC5283)	4 mg	Weeks 0, 4, and 8 by a single N/S dose	10/13 (77%)	NA	48 (28 to 83)	No
DNA (VRC5283)	4 mg	Weeks 0, 4, and 8 by a split N/S dose	14/15 (93%)	NA	150 (99 to 226)	No
DNA (VRC5283)	4 mg	Weeks 0, 4, and 8 by a split needle-free dose	14/14 (100%)	NA	304 (215 to 430)	No
DNA (Inovio Pharmaceuticals Inc)	1 mg	Weeks 0, 4 and 12 by injection and followed by electroporation	12/20 (60%)	1:18 to 1:317	NA	Yes; 92% survival against lethal challenge in *Ifnar* knockout mice
DNA (Inovio Pharmaceuticals Inc)	2 mg	Weeks 0, 4 and 12 by injection and followed by electroporation	12/19 (63%)	1:18 to 1:317	NA	Yes, 92% survival against lethal challenge in *Ifnar* knockout mice

The consistent finding from these studies is that protection against ZIKV infection is predominantly antibody-mediated. Several assays are available to measure vaccine-induced antibodies. The observation that protection is antibody-mediated is concordant with the antibody-based protection observed for WNV, JEV and DENV[33-35]. Data suggest that titers of neutralizing antibodies of ~100, as measured by MN50 assays, are protective against ZIKV[25]. The plaque-reduction neutralization test (PRNT) and a ZIKV reporter viral particle assay (RVP) are other methods that are commonly used to measure neutralizing antibodies[11,26]. Titers between the assays vary, with the RVP reportedly being more sensitive and yielding approximately 10-fold higher titers[26]. CD4+ and CD8+ T cell responses may not be required if levels of neutralizing antibodies exceed this protective threshold[23]. However, CD8+ T cells induced by ZIKV or DENV infection have been shown to be able to reduce ZIKV viral burden in mice[36,37] and further research is needed to determine the impact on short-term and long-term protection.

Protection in pregnancy

ZIKV infection in pregnant women is distinct from infection in non-pregnant women and men[38-40]. For example, more extended periods of viremia have been observed in pregnant women and fetuses[41], and ZIKV RNA was detected throughout the mother and the fetus in animal models[42-44]. Immune responses of pregnant monkeys and mice infected with ZIKV appear similar compared to non-pregnant infected animals[43,45]. The ability of ZIKV to cross the fetal-placental barrier and cause damage to the fetus emphasizes the need for a vaccine and highlights the primary goal of vaccination, that is, to prevent CZS. Therefore, it will be important to measure the efficacy of vaccines to prevent fetal malformations. There are a number of aspects to this research that will need to be considered. For example, is sterilizing immunity required for efficacy or is reducing viral replication sufficient? Additionally, do immune correlates established in non-pregnant animals translate to pregnant animals?

The development of immunodeficient mouse pregnancy models for ZIKV infection has led to important advances due to the increased viral replication and impact on the central nervous system[45,46], and the first prevention of fetal malformations and demise was observed using mRNA and live-attenuated ZIKV vaccines[12,32,47]. However, even though a

statistically significant impact on fetal demise was observed, the protection was not sterilizing. ZIKV RNA was still detected in the maternal brain and spleen as well as in the placenta and fetal heads in the majority of animals. Interestingly, pups born to mothers vaccinated with a LAV were protected against lethal intracranial challenge with ZIKV[32]. Further research is needed to assess the impact of low level viremia in fetal and maternal compartments. In addition, with the increasing knowledge on the long term impact on children born without microcephaly but with confirmed ZIKV infection during pregnancy in humans, it is too early to tell if sterilizing immunity is required to prevent all long term sequela[48]. As a result of differences between rodents and primates[49], a monkey pregnancy model would be preferred[50] and initial progress has been reported[43,51]. In both rhesus and pigtail monkeys, efficient transmission of ZIKV from mother to child during pregnancy has been observed. ZIKV viremia could be detected in various anatomic compartments in the mothers and fetuses, including the brain and placenta[43,51]. In addition, anomalies to the brains of the fetuses were detected, ranging from white matter hypoplasia to pathology to optic nerve and eyes. Similarly as seen in humans, detrimental effects were more evident when infection occurred in early pregnancy[52,53]. It is important to consider that the primary goal of a ZIKV vaccine is to prevent CZS. To realize this goal, vaccines considered for clinical development should ideally be assessed for protective efficacy in preclinical pregnancy models.

Challenges for clinical trials

With the current reduction in ZIKV transmission[54,55], a phase III clinical efficacy trial could prove challenging to execute. Further development of animal pregnancy models that can effectively assess protective efficacy against CZS may therefore be important. In addition, ongoing discussions on alternative paths to licensure are being explored. Human challenge clinical trials have been conducted for other diseases such as typhoid fever[56] and influenza[57]; however, human challenge studies for ZIKV have raised ethical discussions[58]. Invocation of the Food and Drug Administration (FDA) Animal Efficacy Rule (also known as the Animal Rule) could also be considered, if strong correlations of protection in pre-clinical models are deemed likely to translate to humans as outlined below[104]. Recently, a vaccine against anthrax was the first vaccine that was approved based on the Animal Rule[59,60]. According to the FDA, the Animal Rule can be

pursued only if human efficacy studies cannot be performed, for example, because the conduct of such trials is unethical or because field trials after an accidental or deliberate exposure are not feasible[61]. For the Animal Rule to apply, the results of well-controlled animal studies need to demonstrate that clinical benefits in humans would likely be observed with the same study products. For vaccines, a clear immune correlate would facilitate the use of the Animal Rule. For ZIKV, the correlate of protection in nonhuman primates appears to be neutralizing antibody titers[25].

Conclusions and perspectives

In summary, several ZIKV vaccine candidates have been shown to be safe, well-tolerated and immunogenic in humans. In the majority of trial participants, neutralizing antibody titers were induced that were comparable to titers shown to be protective in pre-clinical models. In addition, development of animal models to test vaccine efficacy in the prevention of CZS is underway.

The remarkable speed with which ZIKV vaccines have been developed has led to a rapid increase in our understanding of this virus. Nevertheless, important challenges remain for conducting clinical efficacy trials and vaccine licensure. Because of CZS and the potential lifelong impact on children born from mothers infected with ZIKV, a vaccine is urgently needed. The Rubella vaccine highlights that prevention of congenital defects can be achieved and similar success may be possible for ZIKV.

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7.2

CURRICULUM VITAE

Peter Abbink was born on November 24, 1980 in Lisse, The Netherlands. Upon completion of high school in 1997, Peter enrolled at the ROC Leiden, Middle Laboratory Education (MLO). After 3 years of chemistry and biomedical sciences, he focused on the medical laboratory sciences and entered a one year, full-time internship at the Sylvius Laboratory Leiden (University of Leiden); Department of Radiation Genetics and Chemical Mutagenesis. Here he worked on the mammalian mismatch repair system which ignited his curiosity for molecular biology and biochemistry. To follow his newfound passion, Peter continued his education at the Hogeschool Leiden, majoring in molecular biology and biochemistry. During a full-time internship at Crucell Holland B.V., Peter was exposed to exciting research on adenoviral vaccines. After receiving his Bachelors of Applied Science in 2004, Peter accepted a scientific position at the Center for Virology and Vaccine Research at Beth Israel Deaconess Medical Center, Harvard Medical School in Boston, where he continued his efforts on developing and improving adenoviral vaccines. A highlight of his work is his construction of Ad26, which has recently advanced to a Phase II clinical efficacy trial as a vaccine vector against HIV. Currently the director of the virology group, Peter led the development of the first efficacious Zika vaccines, in 2016. These efforts resulted in the first published vaccine efficacy against Zika virus. In 2017, Peter enrolled in the doctoral program at the University of Amsterdam to further his engagement in vaccine development, with a specific focus on emerging infectious diseases. To date, Peter remains in charge of a group technicians and students and is leading the development of a variety of adenoviral and adeno-associated virus vaccines, assessment of vaccine immunogenicity and efficacy in pre-clinical models and Phase I clinical trials, as well as teach and train students and early career scientists.

7.3

PH.D. PORTFOLIO

AMC Graduate School for Medical Sciences Ph.D. Portfolio

Summary of Ph.D. Training, Teaching and Publications

Ph.D. Candidate: Peter Abbink

Period: 2016-2018

Ph.D. Supervisors: Prof. Dr. J. Schuitemaker and Prof. Dr. D.H. Barouch

Ph.D. Training		Year	Hours	ECTS
Courses				
Vaccine Bioprocessing workshop		2017	32	1.1
Seminars, Meetings, Retreats				
NIH Zika meeting		2017	16	0.6
NIH Zika meeting		2017	16	0.6
CAVD Gates meeting		2016	24	0.9
CAVD Gates meeting		2017	24	0.9
Center for Virology and Vaccine Research		2016-2018	40	1.4
(BIDMC, Zika scientific progress meetings)				
Center for Virology and Vaccine Research		2016-2018	80	2.9
(BIDMC, Departmental and Virology Group team meetings)				
National and International Conferences				
Congress of the Brazilian Society of Immunology		2016	24	0.9
(Campos de Jordao, Brazil)				
International Society of Vaccines		2017	24	0.9
(Paris, France)				
Teaching and Supervision				
Technician (B.Sc.)	Marinela Kirilova	2016-2018	160	5.7
Technician (B.Sc.)	Noe Mercado	2016-2018	160	5.7
Technician (M.Sc.)	Zhenfeng Li	2016-2018	160	5.7
Technician (M.Sc.)	Ramya Nityanandam	2016-2018	160	5.7
Technician (B.Sc.)	Rebecca Peterson	2016-2018	160	5.7
Technician (B.Sc.)	Felix Nampanya	2016-2018	160	5.7
Technician (B.Sc.)	Ovini Nanayakara	2016-2018	160	5.7
Technician (B.Sc.)	David N'gan'ga	2016-2018	160	5.7
Technician (B.Sc.)	Noelix Paulino	2016-2018	160	5.7
Master Student	Michael Boyd	2016	100	3.6
College Student	Hubert Tuyishime	2017-2018	80	2.9
	Tinaye Mutetwa	2016	60	2.1

PUBLICATIONS

**=Authors contributed equally*

Rational Zika vaccine design via the modulation of antigen membrane anchors in chimpanzee adenoviral vectors. Cesar Lopez-Camacho, **Peter Abbink**, Rafael Larocca, Wanwisa Dejnirattisai, Michael Boyd, Alexander Badamchi-Zadeh, Zoe Wallace, Jennifer Doig, Ricardo Sanchez-Velazquez, Roberto Lins, Danilo Coêlho, Young Chan Kim, Claire Donald, Ania Owsianka, Giuditta De Lorenzo, Alain Kohl, Sarah Gilbert, Lucy Dorrell, Juthathip Mongkolsapaya, Arvind Patel, Gavin Screaton, Dan Barouch, Adrian Hill, and Arturo Reyes-Sandoval. *Nat Comm. 2018, Jun*

Zika Virus Vaccines. Peter Abbink*, Kathryn E. Stephenson*, Dan H. Barouch*. *Nat Rev Microbiol. 2018, Jun*

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ACKNOWLEDGEMENTS

"*If you want go fast, go alone; if you want to go far, go together*". This is a quote etched in the wall at the Gates foundation in Seattle which has always stood out to me. The work presented in my thesis reminds me of this, however with slight differences. A passionate team that is set to help others can move mountains in a very short amount of time. We have rapidly developed vaccines that are extremely effective in blocking Zika infection. This resulted in the world's first published vaccine protection against Zika, only 5 months after we started our efforts. New and existing relationships grew stronger which enabled us to start testing our vaccine candidate in clinical trial only a short year after our first proof of protection in mice. Therefore, the statement could potentially read; "*If you want to make a difference, go together*". I truly hope that the work presented here will be able to bring relief to the new challenges experienced by many around the globe in preventing Zika induced congenital syndrome.

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