LETTER
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Vaccine protection against Zika virus from Brazil

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Zika virus (ZIKV) is a flavivirus that is responsible for an unprecedented current epidemic in Brazil and the Americas1,2. ZIKV has been causally associated with fetal microcephaly, intrauterine growth restriction, and other birth defects in both humans3-4 and mice5-11. The rapid development of a safe and effective ZIKV vaccine is a global health priority1,2, but very little is currently known about ZIKV immunology and mechanisms of immune protection. Here we show that a single immunization of a plasmid DNA vaccine or a purified inactivated virus vaccine provides complete protection in susceptible mice against challenge with a ZIKV outbreak strain from northeast Brazil. This ZIKV strain has recently been shown to cross the placenta and to induce fetal microcephaly and other congenital malformations in mice11. We produced DNA vaccines expressing full-length ZIKV pre-membrane and envelope (prM-Env) as well as a series of deletion mutants. The full-length prM-Env DNA vaccine, but not the deletion mutants, afforded complete protection against ZIKV as measured by absence of detectable viremia following challenge, and protective efficacy correlated with Env-specific antibody titers. Adoptive transfer of purified IgG from vaccinated mice conferred passive protection, and CD4 and CD8 T lymphocyte depletion in vaccinated mice did not abrogate protective efficacy. These data demonstrate that protection against ZIKV challenge can be achieved by single-shot subcutaneous and intradermal ZIKV vaccines allowed for an analysis of immune correlates of protection. 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 have determined ZIKV isolates from northeast Brazil (Brazil/ZIKV2015; University of São Paulo)11 and Puerto Rico (PRV ABC59; U.S. Centers for Disease Control and Prevention) (Extended Data Fig. 1) and optimized them for increased antigen expression. We also designed deletion mutants lacking prM and/or lacking the transmembrane region (dTM) or the full stem (dStem) 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/group) received a single immunization of 50 μg of each DNA vaccine by the i.m. route at week 0. Env-specific antibody responses were evaluated at week 3 by ELISA. The full-length prM-Env DNA vaccine elicited higher Env-specific antibody titers than did the Env DNA vaccine and all the dTM and dStem 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 full-length prM-Env DNA vaccine also induced ZIKV-specific neutralizing antibodies after a single immunization (Table 1), as measured by a virus-specific microneutralization assay37. In addition, the prM-Env DNA vaccine induced Env-specific CD8+ and CD4+ T lymphocyte responses, as assessed by IFN-γ ELISPOT and multiparameter intracellular cytokine staining (ICS) assays (Fig. 1d-e). To assess the protective efficacy of these DNA vaccines against ZIKV challenge, we injected vaccinated or sham control Balb/c mice at week 4 by the i.v. route with 105 viral particles (VP) [102 plaque-forming units (PFU)] of ZIKV-BR or ZIKV-PR. Viral loads following ZIKV challenge were quantitated by RT-PCR38. Sham vaccinated mice inoculated with ZIKV-BR developed approximately 6 days of detectable viremia with a mean peak viral load of 5.42 log copies/ml (range 4.55-6.57 log copies/ml; N = 10) on day 3 following challenge (Fig. 2a). In contrast, a single immunization with the prM-Env DNA vaccine provided complete protection against ZIKV-BR challenge with no detectable viremia (<100 copies/ml) at any timepoint (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/ml; range 4.80-5.33 log copies/ml; N = 5) than did ZIKV-BR in sham controls. In contrast with the full-length prM-Env DNA vaccine, the DNA vaccines lacking prM as well as the dTM and dStem 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.

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Protective efficacy correlated with Env-specific binding antibody titers (P = 0.0005 comparing protected vs 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 = 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. 5–6). ZIKV-BR replicated efficiently in SJL mice, consistent with a prior study, although at slightly lower levels (mean peak viral load 4.70 log copies/ml; range 3.50-5.92 log copies/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 prior reports, potentially as a result of robust IFN-α-mediated innate immune restriction in this strain of mice.

To investigate the immunologic mechanism of protection against ZIKV-BR challenge, we purified IgG from serum from prM-Env DNA vaccinated Balb/c mice. 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 challenge (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 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 prior to 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/group) received a single immunization of 1 μg of the PIV vaccine with alum or alum alone by the i.m. or s.q. routes. Antibody titers were higher in the group that received the PIV vaccine by the i.m. route as compared with the s.q. route 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 challenged with ZIKV-BR by the i.v. route 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.q. route showed brief low levels of viremia (Fig. 4c), 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 challenges 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 (i) statistical analyses of immune correlates of protection (Figs. 2c–d, 4b), (ii) adoptive transfer studies with purified IgG from vaccinated mice (Fig. 3a–c), and (iii) 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 correlate 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. Moreover, the ZIKV-BR challenge isolate used in the present study has been shown in wildtype SJL mice to recapitulate certain key clinical findings of ZIKV infection in humans, including fetal microcephaly and intrauterine growth retardation. ZIKV-BR did not lead to a fatal outcome in wildtype Balb/c and SJL mice, as has been observed in Ifnar-/- C57BL/6 mice, but the magnitude and duration of viremia in Balb/c and SJL mice appear comparable with that in humans, suggesting the potential relevance of this model. It is notable that ZIKV-BR replicated efficiently in wildtype Balb/c and SJL mice (Fig. 2a, Extended Data Fig. 4), but replicated poorly in wildtype C57BL/6 mice (Extended Data Fig. 5), which is consistent with prior observations. The findings of ZIKV infection in humans, including fetal microcephaly, may offer safety advantages over live attenuated antiviral interventions.

The explosive epidemiology of the current ZIKV outbreak and the devastating clinical consequences for fetuses in pregnant women who become infected demand 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 utilized at comparable doses to develop vaccines for other flaviviruses, including West Nile virus, dengue viruses, tick-borne encephalitis virus, and Japanese encephalitis virus, 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 likely be feasible.

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Figure 1 | Production 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.dTM, (3) prM-Env.dStem, (4) Env, (5) Env.dTM, (6) Env.dStem, and (7) sham DNA vaccines transfected in 293T cells. Balb/c mice (N = 5/group) received a single immunization with 50μg 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. Cellular immune responses were assessed by (d) IFN-γ ELISPOT assays and (e) multiparameter intracellular cytokine staining assays. Error bars reflect s.e.m.
Figure 2 | Protective efficacy of DNA vaccines. (a) Balb/c mice (N = 5 or 10/group) received a single immunization by the i.m. route with 50 μg full-length prM-Env DNA vaccine or a sham vaccine and were challenged at week 4 by the i.v. route with 10^5 VP (10^2 PFU) ZIKV-BR or ZIKV-PR. Serum viral loads are shown. (b) Mice (N = 5/group) received a single immunization with 50 μg of various DNA vaccines and were challenged with ZIKV-BR. Correlates of (c) protective efficacy and (d) day 3 viral loads are shown. Red bars reflect medians. P-values reflect t-tests and Spearman rank-correlation tests.
Figure 3 | Mechanistic studies. (a) Env-specific serum antibody titers in recipient Balb/c mice (N = 5/group) following adoptive transfer of varying amounts (high, mid, low) of IgG purified from serum from prM-Env DNA vaccinated mice or naïve 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 prior to challenge with ZIKV-BR. Red bars reflect medians. P-values reflect t-tests.
Figure 4 | Immunogenicity and protective efficacy of PIV vaccine. Balb/c mice (N = 5/group) received a single immunization by the i.m. or s.q. 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 VP (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.
Table 1 | ZIKV-specific neutralizing antibody titers

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Balb/c mice received a single immunization with 50 μg of various DNA vaccines (Fig. 1–2) or 1 μg purified inactivated virus (PIV) vaccines with alum (Fig. 4), and pooled serum was assessed for ZIKV-specific neutralizing antibodies at week 4. 50% microneutralization (MN50) titers are shown. Also shown are MN50 titers in serum from mice following two immunizations with DNA-prM-Env (boost) and an anti-flavivirus human polyclonal antibody.
METHODS

Animals. Balb/c, SJL, and C57BL/6 female mice at 6-8 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were vaccinated with 50μg DNA vaccine in saline without adjuvant by the i.m. route or with 1μg PIV vaccine with 100μg alum (Alhydrogel; Brenntag Biosector, Denmark) adjuvant by the i.m. or s.q. routes in a 100μl volume and were then challenged at week 4 by the i.v. route with 10^4 viral particles (VP) [10^6 plaque-forming units (PFU)] ZIKV-PR or ZIKV-PR. Animals were randomly allocated to groups. Immunological and virological assays were performed blinded. All animal studies were approved by the BIDMC Institutional Animal Care and Use Committee (IACUC).

DNA vaccines. ZIKV strain BeH15744 (accession number KU365780) was used to design transgenes, which were produced synthetically. Sequences were optimized for enhanced transgene expression. Full-length 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, CA, USA). Deletion mutants lacked the transmembrane (dTM) or stem (dStem) regions and 30 nucleotides were inserted upstream of the start codon to design transgenes, which were produced synthetically. Sequences were optimized for 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 viral particles (VP) per ml. Assay sensitivity was 100 copies/ml. The infection titer was defined as 10% PFUs at 37°C. Cells were then washed, stained, permeabilized with 50% methanol, and incubated with 2μg/ml of each peptide and 5×10^5 murine splenocytes in triplicate in 100μl medium at 37°C. Following an 18h incubation, the plates were washed six times with PBS and stained with 1 μM of 100μl of ZIKV-PR containing 100 PFU were added to 100 μl of each serum dilution and incubated at 35°C for 2h. Supernatants were then transferred to microtiter plates containing confluent Vero cell monolayers (World Health Organization, NIAID-01103001024). After incubation for 2-4 days, cells were fixed with absolute ethanol for 1h at -20°C and washed three times with PBS. The pan-batavirus 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 2h, 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 450nm. For a valid assay, the average absorbance at 450nm of the 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 MNS0 titer was determined by a log-mid point linear regression model. The MNS0 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 interferon-γ (IFN-γ) ELISPOT assays using pools of overlapping 15-aminoo-acid peptides covering the prM or Env proteins (IPT, Berlin, Germany). 96-well multiscreen plates (Millipore, MA, USA) were coated overnight with 100μl/well of 10μg/ml anti-mouse 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 2h with D-PBS containing 5% FBS at 37°C. The plates were then washed three times with D-PBS-Tween, rinsed with RPMI 1640 containing 10% FBS to remove non-specific binding, and incubated with 2μg/ml of each peptide and 5×10^5 murine splenocytes in triplicate in 100μl medium at 37°C. Following an 18h incubation, the plates were washed nine times with PBS-Tween and once with distilled water. The plates were then incubated with 2μg/ml biotinylated anti-mouse IFN-γ (BD Biosciences, CA, USA) for 2h at room temperature, washed six times with PBS-Tween, and incubated for 2h with a 1:50 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 stained with Cytospot (Treestar, OR, USA). The numbers of spot-forming cells (SFC) per 10^6 cells were calculated. The medium background levels were typically ~5 SFC per 10^6 cells.

Intracellular cytokine staining. ZIKV-specific CD4+ and CD8+ T lymphocyte responses were assessed using splenocytes and analyzed by flow cytometry. Cells were stimulated for 1h at 37°C with 2μg/ml of overlapping 15-aminoo-acid peptides covering the prM or Env proteins (IPT, Berlin, Germany). Following incubation, brefeldin-A and monomycin (BioLegend, CA, USA) were added. Cells were then washed, stained, permeabilized with Cytofix/Cytoperm (BD Biosciences, CA, USA), and analyzed using FlowJo v.9.8.3 (TreeStar, OR, USA). Monoclonal antibodies included: CD4 (RM4-5), CD8α (53-6.7), CD4 (IM7), and IFN-γ (XMG1.2). Antibodies were purchased from BD Biosciences, eBioscience, or BioLegend, CA, USA. Viable cell exclusion (LIVE/DEAD) was purchased from Life Technologies, CA, USA.
IgG purification and adoptive transfer. Serum was collected from prM-Env DNA vaccinated mice or naïve mice, and polyclonal IgG was purified using protein G purification kits (Thermo Fisher Scientific, MA, USA). Varying amounts of purified IgG was infused by the i.v. route into naïve recipient mice prior to ZIKV challenge.

CD4+ and CD8+ T lymphocyte depletion. Anti-CD4 (GK1.5) and/or anti-CD8 (2.43) (Bio X Cell, NH, USA) mAbs were administered at doses of 500 μg/mouse to prM-Env DNA vaccinated mice by the i.p. route on day -2 and day -1 prior to 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 v6.03 (GraphPad Software, CA, USA). Comparisons of groups was performed using t-tests and Wilcoxon rank-sum tests. Correlations were assessed by Spearman rank-correlation tests.
Extended Data Figure 1 | ZIKV maximum likelihood phylogenetic tree. The ZIKV-BR and ZIKV-PR challenge isolates are depicted with red arrows.
Extended Data Figure 2 | ZIKV amino acid sequence comparisons. Number of and percentage 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).

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Extended Data Figure 3 | prM-specific antibody responses in DNA vaccinated mice. In the experiment described in Figure 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/group) received a single immunization by the i.m. route with 50 μg full-length prM-Env DNA vaccine or a sham vaccine and were challenged at week 4 by the i.v. route with 10^5 VP (10^2 PFU) ZIKV-BR. Humoral immune responses were assessed at week 3 following 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/group) received a single immunization by the i.m. route with 50 μg full-length prM-Env DNA vaccine or a sham vaccine and were challenged at week 4 by the i.v. route with 10^5 VP (10^6 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/group) received a single immunization by the i.m. route with 50 μg various DNA vaccines and were challenged at week 4 by the i.v. route with $10^5$ VP ($10^2$ 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 Figure 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 mAb treatment of prM-Env DNA vaccinated Balb/c mice.