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 10^{11} 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 10^6 viral particles (VP) [10^3 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 10^5 VP (10^2 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 10^6 VP (10^3 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. Log_{10} 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. Log_{10} 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 2×10^5 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 PBS-Tween 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 10^6 cells were calculated. The medium background levels were typically <15 SFC per 10^6 cells.

IgG purification and adoptive transfer. Polyclonal IgG was purified from plasma from PIV vaccinated monkeys at week 8 using protein G purification kits and pooled (Thermo Fisher Scientific, MA, USA). The purified IgG preparation had a log ELISA titer of 3.30 and a log MN50 titer of 3.30. Purified IgG was infused into groups of naïve recipient Balb/c mice or rhesus monkeys by 5-fold serial dilutions prior to ZIKV-BR challenge. Mice received 200, 40, 8, 1.5, or 0 μl of the IgG preparation. Monkeys received 10, 2, or 0 ml of the IgG preparation.

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.

Author Contributions

P.A., R.A.L., N.L.M., S.J.T., and D.H.B. designed the studies. P.A., M.B., M.K., Z.L., D.N., O.N., R.N., and N.B.M. produced the DNA and Ad vaccines and conducted the virologic assays. R.A.D., K.M., R.G.J., K.H.E., N.L.M., and S.J.T. produced the PIV vaccines and conducted the virus neutralization assays. J.P.S.P. and P.M.A.Z. developed the challenge virus. J.M., B.F., and M.G.L. led the clinical care of the rhesus monkeys. P.A., R.A.L., C.A.B., E.T.M., E.N.B., A.A., A.L.B., C.C., A.C., P.B.G., D.J., J.J., B.C.L., S.M., K.M., M.S., G.H.N., K.E.S., and G.A. conducted the monkey and mouse studies and performed the immunologic assays. D.H.B. wrote the paper with all co-authors.

Supplementary Figure Legends

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.