Long-term protection of rhesus macaques from Zika virus reinfection

Short title: Long-term Zika virus immunity

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Abstract

By the end of the 2016 Zika virus (ZIKV) outbreak, it is estimated that there were up to 100 million infections in the Americas. In approximately one in seven infants born to mothers infected during pregnancy, ZIKV has been linked to microcephaly, developmental delays, or other congenital disorders collectively known as congenital Zika syndrome (CZS). Guillain-Barré syndrome (GBS) in ZIKV infected adults. It is a global health priority to develop a vaccine against ZIKV that elicits long-lasting immunity, however, the durability of immunity to ZIKV is unknown. Previous studies in mice and nonhuman primates have been crucial in vaccine development but have not defined the duration of immunity generated by ZIKV infection. In this study, we rechallenged five rhesus macaques with ZIKV two years after a primary ZIKV infection. We show that primary ZIKV infection generates high titers of neutralizing antibodies (nAbs) that protect from detectable plasma viremia following rechallenge and persist for at least 27 months. While additional longitudinal studies are necessary with longer time frames, this study establishes a new experimentally defined minimal length of protective ZIKV immunity.

Author Summary

ZIKV emerged as a vector-borne pathogen capable of causing illness in infected adults and congenital birth defects in infants born to mothers infected during pregnancy. Despite the drop in ZIKV cases since the 2015-16 epidemic, questions concerning the prevalence and longevity of protective immunity have left vulnerable communities fearful

that they may become the center of next ZIKV outbreak. While pre-existing herd immunity in regions of past outbreaks may dampen the potential for future outbreaks to occur, we currently do not know the longevity of protective immunity to ZIKV after a person becomes infected. Here, we establish a new experimentally defined minimal length of protective ZIKV immunity. We show that five rhesus macaques initially infected with ZIKV two years prior to rechallenge elicit a durable immune response that protected from detectable plasma viremia. While this work establishes a new minimal length of protective immunity, additional studies are necessary to define the maximum length of protective immunity following ZIKV infection.

Introduction

Zika virus (ZIKV) is a mosquito-borne flavivirus first isolated in 1947 in the Zika forest of Uganda [1]. ZIKV is contracted through a mosquito bite or sexual contact and can be passed from mother to fetus during pregnancy [2]. ZIKV infections are typically mild: characterized by low-grade fever, maculopapular rash, arthralgia, and conjunctivitis [3]. The recent outbreak of ZIKV in the Americas was associated with more severe disease. Guillain-Barré syndrome (GBS) has found in a small proportion of ZIKV-positive adults in the French Polynesian and American outbreaks [4, 5]. In addition, infants born to mothers infected with ZIKV during pregnancy have an approximately one in seven chance of having congenital Zika syndrome (CZS), a collection of fetal abnormalities and developmental disorders [6, 7]. A retrospective analysis of infants, now aged 7 - 32 months, born to mothers infected with ZIKV during pregnancy, has shown that one-third

of those infants have below average neurodevelopment [8]. Currently, it is unknown if ZIKV infection in humans confers lifelong immunity, however it is suggested that waning immunity over time leads to more severe ZIKV in the future [9, 10]. It is thought that a single infection or vaccination that generates neutralizing antibody (nAb) titers above 1:10 PRNT₅₀ (50% plaque reduction neutralization test) is enough to confer lifelong immunity in other flaviviruses, such as yellow fever virus (YFV) or dengue virus (DENV) [11, 12]. Antibodies to YFV and DENV have been detected at least 60 years and 35 years after infection, respectively [13–15]. Despite long-term immunity to YFV and DENV, challenges remain in eradication of these viruses due to evidence of circulation in sylvatic (forest) cycles in Asia and Africa, and urban human-mosquito cycles in South America [16–19]. ZIKV circulates as one serotype while DENV circulates as four antigenically distinct serotypes (DENV-1 through DENV-4) [20, 21]. No single serotype of DENV has been eradicated after an outbreak even though there is long-term population-level immunity [15]. Similarly, there is a highly effective YFV vaccine that induces lifelong immunity but hasn't led to the eradication of YFV [14]. Little is known about ZIKV transmission cycles in the Americas, but looking at past flavivirus outbreaks, herd immunity and vector control alone will likely not be enough to eradicate the virus. Determining the longevity of immunity elicited from previous natural ZIKV infection will be important for assessing the risk of reinfection in future outbreaks, as well as for estimating the number of people who are at-risk of future ZIKV infections.

The current understanding of ZIKV immunity in humans is derived primarily from vaccine clinical trials that are in progress [22]. Recipients of a prM+E consensus DNA vaccine (GLS-5700) developed nAb titers ranging from 1:18 to 1:317 fourteen weeks

after the first vaccination. Adoptive transfer of this week fourteen serum provided protection in 92% of mice challenged with a Puerto Rican isolate of ZIKV [23]. Similar work with three purified inactivated virus (PIV) vaccines found that 92% of recipients generated nAb titers greater than 1:100 which corresponds to protection from viremia in mice that were challenged after adoptive antibody transfer of day 57 purified IgG [24]. ZIKV vaccine studies have shown that a range of nAb titers can protect from viremia. However, these studies did not look past 14 weeks to examine the duration of protection of vaccine-derived nAbs.

Several animal models, including mice and rhesus macaques, have been used to explore vaccine protection against experimental ZIKV exposure [25]. Immunocompromised mouse models have been used to advance the knowledge of ZIKV due to their cost effectiveness and availability. Published ZIKV challenge intervals in mice have varied from 3 to 30 weeks after vaccine administration and shown that nAb titers of EC50 (half maximal effective concentration) ranging from 1:75 to 1:100,000 were protective from viremia after ZIKV challenge [26–32]. While immunodeficient mouse models are readily accessible, ZIKV infection is typically lethal because these mice lack necessary components of the antiviral response. Immunodeficient mice provide information on efficacy and safety of therapeutic interventions but fail to provide information on natural virus pathogenicity [25, 33]. Non-human primates (NHP) have also been used in ZIKV studies because they more closely mimic human ZIKV infections [25]. For this reason, NHP studies have been used to assess the durability of immunity from 4 weeks to 1 year following vaccine administration [31, 32, 34, 35]. Looking at the longest duration between vaccination and challenge, Abbink et al. [32]

conducted a study using rhesus macaques to demonstrate the protective efficacy of a ZIKV purified inactivated virus (PIV), DNA-M-Env, and RhAd52-M-Env vaccine candidates. At 52 weeks after administration, they challenged the macaques with Zika Virus/H.sapiens-tc/BRA/2015/Brazil-ZKV2015 (ZIKV-BR). The PIV and RhAd52-M-Env vaccines generated $\log_{10}$ MN50 (50% reduction of microneutralization, comparable to EC50 and PRNT50 values [36]) titers of 1.88 and 2.38 by week 4, but by week 8 after boost immunization $\log_{10}$ MN50 titers increased to and fluctuated between 3.71 and 2.42 through week 52, respectively. The DNA-M-Env vaccine generated $\log_{10}$ MN50 titers of 2.23 at week 8 after boost immunization and rapidly decline to 1.43 by week 14 but remained stable until week 52. These titers of neutralizing antibodies showed that the PIV, RhAd52-M-Env, and DNA-M-Env vaccines protected 75% (6 of 8), 100% (4 of 4), and 29% (2 of 7), respectively, of macaques after ZIKV-BR challenge [32].

Relying on vaccine studies is not sufficient to determine the durability of immunity because vaccine studies rely on booster vaccinations to increase the level of immunity [37]. Natural infections elicit a higher level of immunity that plateaus above the protective threshold thereby maintaining longer protection [38]. For this reason, rechallenge experiments are useful to understand the durability of immunity elicited by natural infection.

Rechallenge experiments using animal models have been used to examine immune responses following natural ZIKV infection, and to confirm that these infections protect from subsequent exposures [25]. Mouse models have been used to rechallenge 3 weeks after primary infection [39–41]. In rhesus macaques, challenge periods have varied from 6 weeks to 10 weeks after primary challenge [42–45]. We previously

demonstrated that rhesus macaques were protected from homologous or heterologous
 rechallenge up to 10 weeks after primary infection [44, 45]. These studies have shown
 that following ZIKV rechallenge, CD8 T cell and CD4 T cell responses are weak, but
 there is an increase in natural killer (NK) cells and nAbs, which correspond to protection
 from disease but not sterilizing immunity [41, 42, 45]. While this is useful for establishing
 the potency of naturally elicited immunity, rapid rechallenge does not mimic the situation
 where someone infected in the 2015/2016 American outbreak is re-exposed years later
 in a subsequent outbreak.

Here we demonstrate a new minimum length of protection resulting from primary ZIKV
 infection using Indian-origin rhesus macaques from previously published studies [44,
 46, 47].

Results

No detectable ZIKV in plasma following rechallenge

Five animals were infected with ZIKV as part of previously published studies [44–47].
 Animal demographics, infection history, and naming scheme used for each animal are
 described in **Table 1**. Following these initial ZIKV infections, peak plasma vRNA load
 occurred between 2 and 6 days post infection (DPI) and ranged from 7.0×10^5 to 2.8
 $\times 10^4$ vRNA copies per ml [44–47]. In non-pregnant animals, vRNA load became
 undetectable by 10–14 DPI. In animals initially infected during pregnancy, vRNA load
 remained detectable for a longer period of time, ranging from 9–70 DPI [46]. To
 determine the durability of immunity following primary ZIKV infection, we

subcutaneously rechallenged these 5 animals with 1×10^4 PFU Zika virus/H.sapiens-
tc/PUR/2015/PRVABC59 (ZIKV-PR) 19-27 months following initial ZIKV infection (**Table**
1). After rechallenge, ZIKV was measured in plasma by qRT-PCR daily on days 3-7
post rechallenge, and on days 10, 14, and 28 days post rechallenge (**Fig 1**). On all days
throughout the study, plasma vRNA loads remained negative, indicating protection from
plasma viremia after ZIKV rechallenge.

Table 1: Animal demographics.

Animal	Public Identifier	Sex	Initial ZIKV Inoculation strain	Initial inoculation dose and route	Rechallenge ZIKV Inoculation strain	Rechallenge inoculation dose, route, and days post initial ZIKV infection (dpi)	Other infections and days post initial ZIKV infection (dpi)
C1	295022	Female	Zika virus/R.macaqu e-tc/UGA/1947/M R766- 3329	10E4 PFU, subcutaneo us	Zika virus/H.sapiens-tc/ FRA/2013/ FrenchPolynesia- 01_v1c	10E4 PFU, subcutaneous, 70 dpi	SHIV, 347 dpi
				10E4 PFU, subcutaneo us	Zika virus/H.sapiens-tc/ PUR/2015/PRVABC5 9	10E4 PFU, subcutaneous, 858 dpi	
C2	321142	Female	Zika virus/H.sapiens -tc/ FRA/2013/ FrenchPolynesi a- 01_v1c1	10E6 PFU, subcutaneo us	Zika virus/H.sapiens-tc/ PUR/2015/PRVABC5 9	10E4 PFU, subcutaneous, 675 dpi	DENV3, 566 dpi
C3	598248	Female	Zika virus/H.sapiens -tc/ FRA/2013/ FrenchPolynesi a- 01_v1c1	10E4 PFU, subcutaneo us	Zika virus/H.sapiens-tc/ PUR/2015/PRVABC5 9	10E4 PFU, subcutaneous, 809 dpi	None
C4	610107	Female	Zika virus/H.sapiens -tc/ FRA/2013/ FrenchPolynesi a- 01_v1c1	10E6 PFU, subcutaneo us	Zika virus/H.sapiens-tc/ FRA/2013/ FrenchPolynesia- 01_v1c1	10E6 PFU, subcutaneous, 70 dpi	SHIV, 326 dpi
				10E4 PFU, subcutaneo us	Zika virus/H.sapiens-tc/ PUR/2015/PRVABC5 9	10E4 PFU, subcutaneous, 837 dpi	

C5	827577	Female	Zika virus/H.sapiens-tc/ FRA/2013/ FrenchPolynesia- 01_v1c1	10E4 PFU, subcutaneous	Zika virus/H.sapiens-tc/ PUR/2015/PRVABC59	10E4 PFU, subcutaneous, 858 dpi	None
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Public animal identifiers are used in studies on the Zika Open Research Portal

(<https://zika.labkey.com/project/OConnor/begin.view?>).

Immune parameters do not change significantly following ZIKV rechallenge

In order to characterize the immune responses following a long-term ZIKV rechallenge, we assessed immune activation by measuring Ki-67 expression, as a marker of activation, in natural killer (NK) cells, CD4, CD8, and CD8 effector memory (T_{EM}) T cells by flow cytometry (**S1 Fig**). The percentage of activated cells per microliter of blood are presented as fold-change from the number of activated cells found 3 days before rechallenge (baseline) to determine fold-change in activation from baseline (**S1 Fig**). Repeated measures ANOVA showed that there were no significant changes in the number of activated CD4, CD8, and CD8 T_{EM} T cell populations over time. In contrast, there was significant variation from baseline measurements in activated (Ki67+) NK cell populations ($F(2.263, 9.051) = 10.36$, $p = 0.0039$), but further analysis by Dunnett's multiple comparisons test showed no significant change in NK cell populations when comparing number of activated NK cells each day after rechallenge to the number 3 days before rechallenge.

Serum nAb responses were measured using a flow cytometry-based ZIKV neutralization assay. Prior to rechallenge, all animals had high EC₅₀ (50% effective concentration) values (**Fig 2A**), that were greater than the 1:10 PRNT₅₀ values

recommended for other flavivirus vaccines [11, 12]. Using a one-way paired t-test, we compared EC50 values on day 0 to EC50 values on day 14 and day 28 (**Fig 2B**). All animals showed increased nAb titers following rechallenge as noted by a greater EC50 value on day 14 compared to day 0 ($p = 0.0025$, $t=5.604$, $df=4$) and on day 28 ($p = 0.0015$, $t=6.446$, $df=4$). The increase in nAbs shows that initial infection may fail to provide sterilizing immunity. Alternatively, it is possible that the antigen present in the virus preparation was sufficient to boost pre-existing antibody responses. However, the combination of immunophenotyping data, neutralization assay data, and persistent negative plasma ZIKV vRNA loads following rechallenge suggest that primary ZIKV infection is sufficient to protect from detectable viremia in the blood.

Clinical evaluation of ZIKV rechallenged macaques

After initial ZIKV challenge and rechallenge, all five animals were monitored daily for signs of disease (for example, inappetence, dehydration, diarrhea, injury, or psychological abnormalities) and had complete blood counts (CBCs) evaluated.

Reference intervals (RIs) developed for Wisconsin National Primate Research Center (WNPRC) animals for species, sex, and age were used to evaluate results [44].

After primary ZIKV infection, animal C1 presented a rash at the injection site. No other clinical abnormalities were noted. Complete blood counts (CBCs) were evaluated for all five animals three days prior to challenge, daily on days 0 through 10, 14, and again on days 21 and 28 DPI. Mild monocytosis with levels exceeding the RI (0-3% of white blood cells (WBC)) was reported for all animals before initial infection and persisted

throughout the duration of the study [44–47]. Animal C1 had lymphocyte levels dip below the RI (20-80% WBC) on days 4 and 7 but rebounded back to within the normal range by day 8. Animal C5 had high platelet counts exceeding the RI (229-524 thousands of platelets/uL) present at day -3 and persisted to be above the RI throughout the study period [44–47].

After rechallenge, animals C1 and C4 developed inappetence 6 days post rechallenge. Animal C1 also developed diarrhea at 6 days post rechallenge. All animals were relocated to new housing 1 day prior to rechallenge which may have contributed to inappetence and diarrhea. No other clinical abnormalities were noted (**S1 Table**). CBC tests were evaluated for all five animals 3 days prior to, on the day of rechallenge, and on days 3-7, 10, 14, and 28 post rechallenge (**S2 Fig**). After rechallenge, all animals displayed persistent mild monocytosis with levels exceeding the RI across all days that CBCs were evaluated. This persistence of monocytosis is consistent with that observed following primary ZIKV infection [44–47]. The stress of frequent blood draws, sedation, experimental infections, and living in captivity have all been associated with an increased risk of monocytosis and lymphopenia potentially explaining these findings in rechallenged animals [48–50].

Discussion

Since ZIKV could re-emerge in areas affected by the 2015 ZIKV outbreak, it is important to evaluate the durability of ZIKV immunity following initial infection in order to understand the risk of re-infection, particularly among women. This study shows that

initial ZIKV infection provides apparently protective immunity at least 27 months after initial ZIKV infection. Primary ZIKV infection generated nAb titers above the 1:10 threshold deemed protective against re-infection for other flaviviruses (**Fig 2**) [11, 12].

These results showing protection from long term rechallenge have important implications for vaccine design and testing. Protection from ZIKV infection following vaccination is thought to require lasting MN50 log₁₀ titers of 2.0 - 2.1, equivalent to log₁₀ EC50 titers. [32]. In our study, prior to rechallenge, EC50 nAb titers were well above the log₁₀ 2.0 threshold that Abbink et al., determined to be protective from ZIKV infection [32]. Does the immunity observed in these animals provide sterilizing protection from reinfection? While we did not detect any ZIKV RNA in the plasma following rechallenge, nAb titers increased modestly. This suggests that ZIKV may replicate at levels below the limit of detection in blood or in sanctuary tissues that are sequestered from the blood. Conversely, it is possible that antigen present in the challenge virus itself was sufficient to stimulate an expansion of pre-existing nAb responses. Others who have encountered similar results have defined empirical thresholds for sterilizing immunity. For example, Kirkpatrick et al. similarly observed a boost in nAb titers in DENV-vaccinated animals following DENV challenge [51]. They defined an increase of less than four-fold nAb titer as sterilizing immunity and a ≥ four-fold increase in nAb titer as non-sterilizing immunity. In our study, two animals, C1 and C3, exhibited 2.5x increases in nAb titer by 14 days post exposure. However, animals C2, C4, and C5 exhibited a 5.7x, 10.0x, and 7.6x increase, respectively, in nAb titer by 14 days post exposure.

Two animals used in our rechallenge study, C3 and C5, were previously part of a published study that assessed fetal outcomes following ZIKV infection in utero [46]. This

study concluded that similar to human pregnancy, maternal-fetal transmission is highly efficient with variable vRNA levels detected in all fetuses assessed. Despite a small sample size in our rechallenge study (n = 2 animals initially infected during pregnancy), we show that these animals develop immune responses similar to non-pregnant animals. Following primary ZIKV infection during pregnancy in 2016, animal C5 has gone on to give birth to two apparently healthy infants (in 2017 and 2018) and C3 has gone on to give birth to one healthy infant (2019). While this study did not look at the impacts of a ZIKV rechallenge during a second pregnancy, we show that subsequent infants born to mothers who were previously infected with ZIKV are in qualitatively good health. Future studies will need to determine if immunity elicited by a primary infection is sufficient to protect a subsequent pregnancy from the effects of a ZIKV rechallenge during pregnancy. Additionally, it will be important to characterize the extent of ZIKV-specific antibody responses in infants exposed to ZIKV *in utero*.

In summary, our study establishes a new experimentally defined minimal length of protective immunity against ZIKV of up to 27 months. Future longitudinal studies will need to assess ZIKV immunity of previously infected individuals at longer time frames than we have in order to continue to define the maximum length of immunity elicited by natural ZIKV infection. Establishing the true maximum length of ZIKV protective immunity will be critical for understanding outbreak dynamics, the future transmission potential of ZIKV, and influence future vaccination strategies.

Materials and Methods

Ethics Statement

All rhesus macaques in this study were cared for by the staff at the Wisconsin National Primate Research Center (WNPRC) in accordance with the regulations, guidelines, and recommendations outlined in the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Weatherall report [52–54]. The University of Wisconsin - Madison, College of Letters and Science and Vice Chancellor for Research and Graduate Education Centers Institutional Animal Care and Use Committee approved the nonhuman primate research covered under protocol number G005401-R01. The University of Wisconsin - Madison Institutional Biosafety Committee approved this work under protocol number B00000117.

All animals were housed in enclosures with required floor space and fed using a nutritional plan based on recommendations published by the National Research Council. Animals were fed a fixed formula, extruded dry diet with adequate carbohydrate, energy, fat, fiber, mineral, protein, and vitamin content. Macaque dry diets were supplemented with fruits, vegetables, and other edible objects (e.g., nuts, cereals, seed mixtures, yogurt, peanut butter, popcorn, marshmallows, etc.) to provide variety to the diet and to inspire species-specific behaviors such as foraging. To further promote psychological well-being, animals were provided with food enrichment, structural enrichment, and/or manipulanda. Environmental enrichment objects were selected to minimize chances of pathogen transmission from one animal to another and from animals to care staff. While on study, all animals were evaluated by trained animal care staff at least twice each day for signs of pain, distress, and illness by observing appetite, stool quality, activity level, physical condition. Animals exhibiting abnormal

presentation for any of these clinical parameters were provided appropriate care by attending veterinarians. Prior to all minor/brief experimental procedures, macaques were anesthetized with an intramuscular dose of ketamine (10 ml kg⁻¹) prior to virus inoculation and blood collection and monitored regularly until fully recovered from anesthesia. Per WNPRC standard operating procedures for animals assigned to protocols involving the experimental inoculation of an infectious pathogen, environmental enhancement included constant visual, auditory, and olfactory contact with conspecifics, the provision of feeding devices which inspire foraging behavior, the provision and rotation of novel manipulanda (e.g., Kong toys, nylabones, etc.), and enclosure furniture (i.e., perches, shelves).

Virus Stocks

Zika virus/H.sapiens-tc/FRA/2013/FrenchPolynesia-01_v1c1 (ZIKV-FP) (GenBank accession: KJ776791) was obtained from Xavier de Lamballerie (European Virus Archive, Marseille, France) and passage history is described in Dudley et al. [44]. African lineage Zika virus (ZIKV-MR766), and Zika virus/H.sapiens-tc/PUR/2015/PRVABC59 (GenBank accession: KU501215) were provided by Brandy Russell (CDC, Ft. Collins, CO). ZIKV-MR766 passage history has been described in Aliota et al [45]. Prior to rechallenge, animal C1 was previously infected with 10⁴ PFU of ZIKV-MR766 as described by Aliota et al. [45]. Animal C4 was previously infected with 10⁶ PFU of ZIKV-FP as described by Dudley et al [44]. Animals C5 and C3 were previously infected with 10⁴ PFU of ZIKV-FP during their first and late second/early third trimesters of pregnancy, respectively, as described by Nguyen et al. [46]. Animal C2

was previously infected with 10^6 PFU ZIKV-FP as previously described by Breitbach et al. [47]. Full infection histories are described in **Table 1**.

Inoculations

Virus stocks were thawed and diluted in PBS to 1×10^4 PFU Zika virus/H.sapiens-tc/PUR/2015/PRVABC59 (ZIKV-PR) for each challenge. Prepared virus stocks were then loaded into a 1 ml syringe that was kept on ice until administration. Animals were anesthetized as described above, and 1 ml of inocula was administered subcutaneously over the cranial dorsum [44]. Animals were monitored for adverse reactions and signs of disease by the veterinary and animal care staff of WNPRC.

Quantification of ZIKV RNA

Plasma and PBMC were isolated from EDTA-anticoagulated whole blood by Ficoll density centrifugation at $1,860 \times g$ for 30 min. Plasma was collected and centrifuged at $670 \times g$ for an additional 8 min to remove remaining cells. Viral RNA was extracted from 300 μ L of plasma using the Viral Total Nucleic Acid Purification kit (Promega, Madison, WI, USA) on a Maxwell 48 RSC instrument (Promega, Madison, WI). The vRNA was then quantified using quantitative RT-PCR previously described in Dudley et al. [44]. Briefly, the qRT-PCR was performed using SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, USA) on a LightCycler 480 or LightCyc instrument (Roche Diagnostics, Indianapolis, IN, USA). Primers and probes

were used at final concentrations of 600 and 100 nM, respectively, with the addition of 150 ng random primers (Promega, Madison, WI). Cycling conditions were as follows: 37°C for 15 min, 50°C for 30 min, and 95°C for 2 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. Viral concentration was calculated by interpolation onto an internal standard curve composed of seven 10-fold serial dilutions of a ZIKV RNA fragment based on ZIKV-FP.

The limit of quantification for this assay was determined using methods adapted from Cline et al [55]. Briefly, half-dilutions of ZIKV RNA were prepared to generate standard curves from 3 to 1,000,000 copies. The overall positivity for each half dilution was assessed by qRT-PCR. The limit of quantification for this assay was established at 100 copies/ml.

Immunophenotyping

The number of activated/proliferating NK cells, CD4, CD8, and CD8 T_{EM} T cells were quantified as described previously [56]. Briefly, 100µL of EDTA-anticoagulated whole blood samples were incubated at room temperature for 15 minutes with an antibody master mix described by Dudley et al. [44]. Red blood cells were lysed using BD Pharm Lyse (BD BioSciences, San Jose, CA), washed twice in media, and fixed with 125µL of 2% paraformaldehyde for 15 minutes. After fixation, cells were washed and permeabilized using Bulk Permeabilization Reagent (Life Technologies, Madison, WI). While the permeabilizer was present, the cells were stained for 15 minutes with Ki-67 (clone B56, Alexa Fluor 647 conjugate). The cells were then washed twice in media and

resuspended in 2% paraformaldehyde until they were run on a BD LSRII Flow Cytometer (BD BioSciences, San Jose, CA). Flow data were analyzed using Flowjo version 10.5.3.

CBC tests

CBC tests were performed on EDTA-anticoagulated whole-blood samples using a SYsmex XS-1000i automated haematology analyzer (Sysmex Corporation, Kobe, Japan). CBC results were reported with species, age, and sex-specific reference ranges. When samples are outside the laboratory-defined criteria (increased total WBC counts, increased monocyte, eosinophil, and basophil percentages, decreased hemoglobin, hematocrit, and platelet values, and unreported differential values) manual slide evaluation was performed.

Flow cytometry-based neutralization assay

Macaque serum samples were screened for ZIKV nAbs by a flow-based ZIKV neutralization assay adapted from Dowd et al [20]. Briefly, Vero cells (ATCC, CLR-1587) were plated at a concentration of 2.5×10^4 cells (100 μ l/well) in a 96 well plate one and incubated at 37°C for 24 hours prior to ZIKV infection. Equal volumes of 1.9×10^2 PFU/mL ZIKV-PR and 1:4 serial serum dilutions were incubated for 30 minutes at 37°C before infecting duplicate wells. The plate was incubated for 72 hours at 37°C. Following incubation, the plates were washed 5 times with $1 \times$ PBS and treated with

trypsin. After the cells separated from the plate, they were transferred to a cluster tube and stained with a LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies, Madison, W). The plate was incubated for 15 minutes at room temperature, 1 mL of R10 media was added, and centrifuged for 5 minutes at 530 x g. Media was aspirated and 125µL of 2% PFA was added to fix the cells. The plates were incubated for 15 minutes at room temperature, 1 mL of R10 media was added, and then they were centrifuged for 5 minutes at 530 x g. Media was aspirated and 100µL of Fix and Permeabilization Medium B (Life Technologies, Madison, W) was added to permeabilize the cell membrane. 2µL of a 1:10 dilution of the flavivirus-specific antibody, 4G2-A647 (Novus Biologicals, Centennial, CO, USA) stock was added to each well. The plate was incubated at room temperature for 15 minutes. 1 mL of R10 media was added, and the plates were centrifuged for 5 minutes at 530 x g. Media was aspirated and 125µL of 2% PFA was added to fix the cells. Infection was monitored by flow cytometry on the BD LSRII Flow Cytometer. Flow cytometry data was analyzed using Flowjo version 10.5.3. Neutralization curves were generated using GraphPad Prism v. 8.0.1 (San Diego, CA). The dilutions required to inhibit 50% and 90% of infection (EC50 and EC90 values) were calculated using a non-linear regression model.

Statistical Analysis

Changes in immunophenotyping and CBC parameters from baseline were analyzed with repeated measures ANOVA, for the populations where ANOVA proved significant, we followed with Dunnett's multiple comparisons test comparing each day after rechallenge back to 3 days before rechallenge.

The log₁₀ serum dilutions corresponding to EC50 nAb titers are plotted for days 0, 14, and 28. Increases in titers were measured by one-way paired t-test. Significance is indicated by * (p-value < 0.05) and ** (p-value <0.005). All statistical analysis was performed in GraphPad Prism v. 8.0.1 (San Diego, CA).

Data management

Complete datasets for these studies have been made publicly available in a manuscript-specific folder on the Zika Open Research Portal (<https://go.wisc.edu/bn71k1>). Authors declare that all other data for these study findings are available via this portal or through supplementary information files from this article.

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References

1. DICK GWA, KITCHEN SF, HADDOW AJ. Zika virus. I. Isolations and serological specificity. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1952;46:509-520. [https://doi.org/10.1016/0035-9203\(52\)90042-4](https://doi.org/10.1016/0035-9203(52)90042-4) PMID: 12995440

2. Petersen LR, Jamieson DJ, Powers AM, Honein MA. Zika Virus. The New England journal of medicine. 2016;374:1552-1563. <https://doi.org/10.1056/NEJMra1602113> PMID: 27028561
3. Musso D, Nilles EJ, Cao-Lormeau V-M. Rapid spread of emerging Zika virus in the Pacific area. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2014;20:O595-6. <https://doi.org/10.1111/1469-0691.12707> PMID: 24909208
4. Parra B, Lizarazo J, Jiménez-Arango JA et al. Guillain-Barré Syndrome Associated with Zika Virus Infection in Colombia. The New England journal of medicine. 2016;375:1513-1523. <https://doi.org/10.1056/NEJMoa1605564> PMID: 27705091
5. Cao-Lormeau VM, Blake A, Mons S et al. Guillain-Barré Syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet. 2016;387:1531-1539. [https://doi.org/10.1016/S0140-6736\(16\)00562-6](https://doi.org/10.1016/S0140-6736(16)00562-6) PMID: 26948433
6. Moore CA, Staples JE, Dobyns WB et al. Characterizing the Pattern of Anomalies in Congenital Zika Syndrome for Pediatric Clinicians. JAMA pediatrics. 2017;171:288-295. <https://doi.org/10.1001/jamapediatrics.2016.3982> PMID: 27812690
7. Rice ME, Galang RR, Roth NM et al. Vital Signs: Zika-Associated Birth Defects and Neurodevelopmental Abnormalities Possibly Associated with Congenital Zika Virus Infection - U.S. Territories and Freely Associated States, 2018. MMWR Morbidity and mortality weekly report. 2018;67:858-867. <https://doi.org/10.15585/mmwr.mm6731e1> PMID: 30091967

8. Nielsen-Saines K, Brasil P, Kerin T et al. Delayed childhood neurodevelopment and neurosensory alterations in the second year of life in a prospective cohort of ZIKV-exposed children. *Nature Medicine*. 2019<https://doi.org/10.1038/s41591-019-0496-1>
9. Baud D, Gubler DJ, Schaub B, Lanteri MC, Musso D. An update on Zika virus infection. *Lancet* (London, England). 2017;390:2099-2109. [https://doi.org/10.1016/S0140-6736\(17\)31450-2](https://doi.org/10.1016/S0140-6736(17)31450-2) PMID: 28647173
10. Shim BS, Kwon YC, Ricciardi MJ et al. Zika Virus-Immune Plasmas from Symptomatic and Asymptomatic Individuals Enhance Zika Pathogenesis in Adult and Pregnant Mice. *MBio*. 2019;10<https://doi.org/10.1128/mBio.00758-19> PMID: 31266863
11. Tauber E, Kollaritsch H, Korinek M et al. Safety and immunogenicity of a Vero-cell-derived, inactivated Japanese encephalitis vaccine: a non-inferiority, phase III, randomised controlled trial. *Lancet* (London, England). 2007;370:1847-1853. [https://doi.org/10.1016/S0140-6736\(07\)61780-2](https://doi.org/10.1016/S0140-6736(07)61780-2) PMID: 18061060
12. Lindquist L, Vapalahti O. Tick-borne encephalitis. *Lancet* (London, England). 2008;371:1861-1871. [https://doi.org/10.1016/S0140-6736\(08\)60800-4](https://doi.org/10.1016/S0140-6736(08)60800-4) PMID: 18514730
13. Amanna IJ, Slifka MK. Questions regarding the safety and duration of immunity following live yellow fever vaccination. *Expert review of vaccines*. 2016;15:1519-1533. <https://doi.org/10.1080/14760584.2016.1198259> PMID: 27267203
14. Vaccines and vaccination against yellow fever. WHO position paper -- June 2013. *Releve epidemiologique hebdomadaire*. 2013;88:269-283. PMID: 23909008

15. Imrie A, Meeks J, Gurary A et al. Antibody to dengue 1 detected more than 60 years after infection. *Viral immunology*. 2007;20:672-675.
<https://doi.org/10.1089/vim.2007.0050> PMID: 18158740
16. Althouse BM, Lessler J, Sall AA et al. Synchrony of sylvatic dengue isolations: a multi-host, multi-vector SIR model of dengue virus transmission in Senegal. *PLoS neglected tropical diseases*. 2012;6:e1928.
<https://doi.org/10.1371/journal.pntd.0001928> PMID: 23209867
17. Moreira-Soto A, Torres MC, Lima de Mendonça MC et al. Evidence for multiple sylvatic transmission cycles during the 2016-2017 yellow fever virus outbreak, Brazil. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*. 2018;24:1019.e1-1019.e4. <https://doi.org/10.1016/j.cmi.2018.01.026> PMID: 29427798
18. Vasilakis N, Cardoso J, Hanley KA, Holmes EC, Weaver SC. Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health. *Nat Rev Microbiol*. 2011;9:532-541.
<https://doi.org/10.1038/nrmicro2595> PMID: 21666708
19. Gardner CL, Ryman KD. Yellow fever: a reemerging threat. *Clin Lab Med*. 2010;30:237-260. <https://doi.org/10.1016/j.cll.2010.01.001> PMID: 20513550
20. Dowd KA, DeMaso CR, Pelc RS et al. Broadly Neutralizing Activity of Zika Virus-Immune Sera Identifies a Single Viral Serotype. *Cell reports*. 2016;16:1485-1491.
<https://doi.org/10.1016/j.celrep.2016.07.049> PMID: 27481466
21. Calisher CH, Karabatsos N, Dalrymple JM et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J*

- Gen Virol. 1989;70:37-43. <https://doi.org/10.1099/0022-1317-70-1-37> PMID: 2543738
22. Barrett ADT. Current status of Zika vaccine development: Zika vaccines advance into clinical evaluation. NPJ vaccines. 2018;3:24. <https://doi.org/10.1038/s41541-018-0061-9> PMID: 29900012
23. Tebas P, Roberts CC, Muthumani K et al. Safety and Immunogenicity of an Anti-Zika Virus DNA Vaccine - Preliminary Report. The New England journal of medicine. 2017<https://doi.org/10.1056/NEJMoa1708120> PMID: 28976850
24. Modjarrad K, Lin L, George SL et al. Preliminary aggregate safety and immunogenicity results from three trials of a purified inactivated Zika virus vaccine candidate: phase 1, randomised, double-blind, placebo-controlled clinical trials. Lancet (London, England). 2018;391:563-571. [https://doi.org/10.1016/S0140-6736\(17\)33106-9](https://doi.org/10.1016/S0140-6736(17)33106-9) PMID: 29217375
25. Dong S, Liang Q. Recent Advances in Animal Models of Zika Virus Infection. Virologica Sinica. 2018;33:125-130. <https://doi.org/10.1007/s12250-018-0007-4> PMID: 29541942
26. Richner JM, Himansu S, Dowd KA et al. Modified mRNA Vaccines Protect against Zika Virus Infection. Cell. 2017;168:1114-1125.e10. <https://doi.org/10.1016/j.cell.2017.02.017> PMID: 28222903
27. Shan C, Muruato AE, Nunes BT et al. A live-attenuated Zika virus vaccine candidate induces sterilizing immunity in mouse models. Nature medicine. 2017;23:763-767. <https://doi.org/10.1038/nm.4322> PMID: 28394328

28. Xu K, Song Y, Dai L et al. Recombinant Chimpanzee Adenovirus Vaccine AdC7-M/E Protects against Zika Virus Infection and Testis Damage. *Journal of virology*. 2018;92<https://doi.org/10.1128/JVI.01722-17> PMID: 29298885
29. Brault AC, Domi A, McDonald EM et al. A Zika Vaccine Targeting NS1 Protein Protects Immunocompetent Adult Mice in a Lethal Challenge Model. *Scientific reports*. 2017;7:14769. <https://doi.org/10.1038/s41598-017-15039-8> PMID: 29116169
30. Kwek SS, Watanabe S, Chan KR et al. A systematic approach to the development of a safe live attenuated Zika vaccine. *Nature communications*. 2018;9:1031. <https://doi.org/10.1038/s41467-018-03337-2> PMID: 29531213
31. Li X-F, Dong H-L, Wang H-J et al. Development of a chimeric Zika vaccine using a licensed live-attenuated flavivirus vaccine as backbone. *Nature communications*. 2018;9:673. <https://doi.org/10.1038/s41467-018-02975-w> PMID: 29445153
32. Abbink P, Larocca RA, Visitsunthorn K et al. Durability and correlates of vaccine protection against Zika virus in rhesus monkeys. *Science translational medicine*. 2017;9<https://doi.org/10.1126/scitranslmed.aao4163> PMID: 29237759
33. Marzi A, Emanuel J, Callison J et al. Lethal Zika Virus Disease Models in Young and Older Interferon α/β Receptor Knock Out Mice. *Front Cell Infect Microbiol*. 2018;8:117. <https://doi.org/10.3389/fcimb.2018.00117> PMID: 29696134
34. Medina LO, To A, Lieberman MM et al. A Recombinant Subunit Based Zika Virus Vaccine Is Efficacious in Non-human Primates. *Frontiers in immunology*. 2018;9:2464. <https://doi.org/10.3389/fimmu.2018.02464> PMID: 30467501

35. Abbink P, Larocca RA, De La Barrera RA et al. Protective efficacy of multiple vaccine platforms against Zika virus challenge in rhesus monkeys. *Science* (New York, NY). 2016;353:1129-1132. <https://doi.org/10.1126/science.aah6157> PMID: 27492477
36. Liu L, Wen K, Li J et al. Comparison of plaque- and enzyme-linked immunospot-based assays to measure the neutralizing activities of monoclonal antibodies specific to domain III of dengue virus envelope protein. *Clin Vaccine Immunol*. 2012;19:73-78. <https://doi.org/10.1128/CI.05388-11> PMID: 22116689
37. cdc.gov [Internet]. Understanding How Vaccines Work c2018 [cited 2019 July 12]. Available from: <https://www.cdc.gov/vaccines/hcp/conversations/downloads/vacsafe-understand-color-office.pdf>
38. Slifka MK, Amanna I. How advances in immunology provide insight into improving vaccine efficacy. *Vaccine*. 2014;32:2948-2957. <https://doi.org/10.1016/j.vaccine.2014.03.078> PMID: 24709587
39. Scott JM, Lebratti TJ, Richner JM et al. Cellular and Humoral Immunity Protect against Vaginal Zika Virus Infection in Mice. *Journal of virology*. 2018;92<https://doi.org/10.1128/JVI.00038-18> PMID: 29343577
40. Turner LH, Kinder JM, Wilburn A et al. Preconceptual Zika virus asymptomatic infection protects against secondary prenatal infection. *PLoS pathogens*. 2017;13:e1006684. <https://doi.org/10.1371/journal.ppat.1006684> PMID: 29145516

41. Huang H, Li S, Zhang Y et al. CD8+ T Cell Immune Response in Immunocompetent Mice during Zika Virus Infection. *Journal of virology*. 2017;91<https://doi.org/10.1128/JVI.00900-17> PMID: 28835502
42. Osuna CE, Lim S-Y, Deleage C et al. Zika viral dynamics and shedding in rhesus and cynomolgus macaques. *Nature medicine*. 2016;22:1448-1455. <https://doi.org/10.1038/nm.4206> PMID: 27694931
43. Rayner JO, Kalkeri R, Goebel S et al. Comparative Pathogenesis of Asian and African-Lineage Zika Virus in Indian Rhesus Macaque's and Development of a Non-Human Primate Model Suitable for the Evaluation of New Drugs and Vaccines. *Viruses*. 2018;10<https://doi.org/10.3390/v10050229> PMID: 29723973
44. Dudley DM, Aliota MT, Mohr EL et al. A rhesus macaque model of Asian-lineage Zika virus infection. *Nature communications*. 2016;7:12204. <https://doi.org/10.1038/ncomms12204> PMID: 27352279
45. Aliota MT, Dudley DM, Newman CM et al. Heterologous Protection against Asian Zika Virus Challenge in Rhesus Macaques. *PLoS neglected tropical diseases*. 2016;10:e0005168. <https://doi.org/10.1371/journal.pntd.0005168> PMID: 27911897
46. Nguyen SM, Antony KM, Dudley DM et al. Highly efficient maternal-fetal Zika virus transmission in pregnant rhesus macaques. *PLoS pathogens*. 2017;13:e1006378. <https://doi.org/10.1371/journal.ppat.1006378> PMID: 28542585
47. Breitbach ME, Newman CM, Dudley DM et al. Primary infection with dengue or Zika virus does not affect the severity of heterologous secondary infection in macaques. *bioRxiv*. 2019613802. <https://doi.org/10.1101/613802>

48. Balcombe JP, Barnard ND, Sandusky C. Laboratory routines cause animal stress. Contemporary topics in laboratory animal science. 2004;43:42-51. PMID: 15669134
49. Dutta P, Nahrendorf M. Regulation and consequences of monocytosis. Immunological reviews. 2014;262:167-178. <https://doi.org/10.1111/imr.12219> PMID: 25319334
50. Ramaekers LH, Theunissen PM, Went K. Acute lymphopenia, stress, and plasma cortisol. Archives of disease in childhood. 1975;50:555-558. <https://doi.org/10.1136/adc.50.7.555> PMID: 1167069
51. Kirkpatrick BD, Whitehead SS, Pierce KK et al. The live attenuated dengue vaccine TV003 elicits complete protection against dengue in a human challenge model. Science translational medicine. 2016;8:330ra36. <https://doi.org/10.1126/scitranslmed.aaf1517> PMID: 27089205
52. Animal Welfare Act of 1970. PL 91-579. Approved 24 December 1970
53. National RCUSCFTUOTGFTCAUOLA. Guide for the Care and Use of Laboratory Animals. 2011<https://doi.org/10.17226/12910> PMID: 21595115
54. Academy of Medical Sciences (Great Britain), Weatherall DJ. The use of non-human primates in research: A working group report. 2006.
55. Cline AN, Bess JW, Piatak M, Lifson JD. Highly sensitive SIV plasma viral load assay: practical considerations, realistic performance expectations, and application to reverse engineering of vaccines for AIDS. Journal of medical primatology. 2005;34:303-312. <https://doi.org/10.1111/j.1600-0684.2005.00128.x> PMID: 16128925

56. Pomplun N, Weisgrau KL, Evans DT, Rakasz EG. OMIP-028: activation panel for Rhesus macaque NK cell subsets. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2015;87:890-893. <https://doi.org/10.1002/cyto.a.22727> PMID: 26218174

Figure Legends

Fig 1: ZIKV viral loads of animals rechallenged with ZIKV-PR from day 0 of primary infection. Schematic of animals included in this study next to their plasma vRNA loads measured by qRT-PCR before and after rechallenge with ZIKV-PR as indicated by an arrow. The limit of detection of ZIKV RNA is 100 copies/mL as indicated by the solid line.

Fig 2: Neutralizing antibody curves. ZIKV neutralizing antibody titers were measured on days 0, 14, and 28. (A) The % viral inhibition corresponding to that $\log_{10}$ reciprocal serum dilution are plotted and fit with a non-linear regression line in order to estimate EC50 and EC90 values. (B) The $\log_{10}$ serum dilutions corresponding to EC50 nAb titers are plotted for days 0, 14, and 28. Increases in titers were measured by one-way paired t-test. Significance is indicated by ** (p-value <0.005).

Supporting information captions

626

627 **S1 Fig: Measurement of immune activation of animals rechallenged with ZIKV-PR.**

628 (A) Schematic of animals included in this study. (B) Spread of Ki-67+ NK cells, (C) CD4
629 T cells, (D) CD8 T cells, and (E) CD8 effector memory (T_{EM}) T cells were measured on -
630 3, 0, 3-7, 10, 14, and 28 days post rechallenge. Percentage of activated cells per μ l of
631 blood are presented as relative to the baseline value set to 100% on -3 days post
632 rechallenge.

633

634 **S2 Fig: Complete blood count graphs of animals rechallenged with ZIKV-PR. (A)**

635 Schematic of animals included in this study. (B) Clinical hematology was performed by
636 WNPRC on whole blood collected daily from macaques on days 3-7, as well as on days
637 -3, 0, 10, 14, and 28 post-ZIKV rechallenge. Horizontal dotted lines indicate the range of
638 normal reference intervals (RI). Four of five animals had lymphocyte levels dip below
639 the RI (20-80% of WBC) at 3 days post exposure but rebounded to the levels within the
640 normal range by 4 days post exposure. Mild monocytosis exceeding the RI (0-3% of
641 WBC) was reported in animal C2 7 days prior to rechallenge. Animal C5 had platelet
642 values exceeding the RI (229-524 ths/uL) ranging from 575-747 ths/uL present at day -3
643 and continuing until day 28. Thrombocytosis was also noted following the initial ZIKV
644 challenge for this animal. No other abnormal CBC data were reported.

645

646 **S1 Table: Clinical remarks for each animal after rechallenge.** BARH = Bright, Alert,

647 Responsive, and Hydrated. BCS = Body Condition Score.

Figure 1

A **Figure 2**