




Long-Term Protection of Rhesus Macaques from Zika Virus Reinfection

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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, as well as Guillain-Barré syndrome, 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 22 to 28 months after a primary ZIKV infection. We show that primary ZIKV infection generates high titers of neutralizing antibodies that protect from detectable plasma viremia following rechallenge and persist for at least 22 to 28 months. While additional longitudinal studies are necessary with longer time frames, this study establishes a new experimentally defined minimal length of protective ZIKV immunity.

IMPORTANCE 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 decrease in ZIKV cases since the 2015-2016 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. Although preexisting 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 22 to 28 months prior to rechallenge elicit a durable immune response that protected from detectable plasma viremia. This study establishes a new minimal length of protective immunity.

KEYWORDS antibody, immunity, long-term immunity, Zika, Zika virus, arbovirus, flavivirus

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 and are characterized by low-grade fever, maculopapular rash, arthralgia, and conjunctivitis (3). The recent outbreak of ZIKV in the Americas was associated with more severe

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disease. Guillain-Barré syndrome was 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, a collection of fetal abnormalities and developmental disorders (6, 7). A retrospective analysis of infants, now aged 7 to 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 whether ZIKV infection in humans confers lifelong immunity; however, it has been 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 and 35 years after infection, respectively (13–15). Despite long-term immunity to YFV and DENV, challenges remain in the eradication of these viruses due to evidence of circulation in sylvatic (forest) cycles in Asia and Africa and to 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 has not led to the eradication of YFV (14). Little is known about ZIKV transmission cycles in the Americas, but considering 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 14 weeks after the first vaccination. Adoptive transfer of week 14 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 with an 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). Nonhuman primates (NHPs) 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 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-ZIKV2015

(ZIKV-BR). The PIV and RhAd52-M-Env vaccines generated \log_{10} MN₅₀ (50% reduction of microneutralization, comparable to the EC₅₀ and PRNT₅₀ values [36]) titers of 1.88 and 2.38 by week 4, but by week 8 after boost immunization the \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 declined 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 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 corresponds to protection from disease but not sterilizing immunity (41, 42, 45). Although 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 reexposed 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).

(This article was submitted to an online preprint archive [48].)

RESULTS

No detectable ZIKV in plasma after rechallenge. Five animals were infected with ZIKV as part of previously published studies (44–47). Animals C1 and C4 were previously used in rechallenge studies where the animals were rechallenged with 1×10^4 PFU Zika virus/H.sapiens-tc/PUR/2015/PRVABC59 (ZIKV-PR) 70 days after their initial ZIKV infection (44, 45). In addition, animals C1 and C4 were exposed to SHIV on 346 and 326 days after initial ZIKV challenge, respectively. However, neither animal became productively infected, and they have never had a positive viral load, suggesting that there would not be an effect on antigen-specific T cell levels. Animal C2 was previously challenged with dengue virus/H.sapiens-tc/IDN/1978/Sleman/78 (DENV-3) 164 days prior to initial ZIKV challenge. Our group previously showed that previous DENV-3 infection did not affect ZIKV pathogenesis or immunity upon ZIKV challenge (47). Animals C5 and C3 were previously infected with 10^4 PFU of Zika virus/H.sapiens-tc/FRA/2013/FrenchPolynesia-01_v1c1 (ZIKV-FP) during their first and late second/early third trimesters of pregnancy, respectively (46). Full animal demographics, infection history, and naming scheme used for each animal are presented in Table 1. Following these initial ZIKV infections, peak plasma vRNA load occurred between 2 and 6 days postinfection (dpi) and ranged from 2.8×10^4 to 7.0×10^5 vRNA copies per ml (44–47). In nonpregnant animals, vRNA load became undetectable by 10 to 14 dpi. In animals initially infected during pregnancy, vRNA load remained detectable for a longer period of time, ranging from 9 to 70 dpi (46). To determine the durability of immunity following primary ZIKV infection, we subcutaneously (s.c) rechallenged these five animals 22 to 28 months following initial ZIKV infection with 1×10^4 PFU Zika virus/H.sapiens-tc/PUR/2015/PRVABC59 (ZIKV-PR)

TABLE 1 Animal demographics^a

Animal	PAI	Initial			Rechallenge		Other infection, dpi
		Status ^b	ZIKV inoculation strain	Inoculation dose in PFU (s.c.)	ZIKV inoculation strain, dpi	Inoculation dose in PFU (s.c.)	
C1	295022	NP	ZIKV-MR766	10 ⁴	ZIKV-FP, 70 ZIKV-PR, 858	10 ⁴ 10 ⁴	SHIV, 347
C2	321142	NP	ZIKV-FP	10 ⁶	ZIKV-PR, 675	10 ⁴	DENV3, 164
C3	598248	P	ZIKV-FP	10 ⁴	ZIKV-PR, 809	10 ⁴	None
C4	610107	NP	ZIKV-FP	10 ⁶	ZIKV-FP, 70 ZIKV-PR, 837	10 ⁴ 10 ⁴	SHIV, 326
C5	827577	P	ZIKV-FP	10 ⁴	ZIKV-PR, 858	10 ⁴	None

^aPublic animal identifiers (PAI) are used in studies on the Zika Open Research Portal (<https://zika.labkey.com/project/OConnor/begin.view?>). All animals were female. Animal C1 was previously infected with 10⁴ PFU of ZIKV-MR766 and rechallenged with 10⁴ PFU of ZIKV-FP 70 days after initial ZIKV infection (45). Animal C2 was previously infected with 10⁶ PFU ZIKV-FP and Dengue virus 3 164 days prior to initial ZIKV infection (47). 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 (46). Animal C4 was previously infected with 10⁶ PFU of ZIKV-FP and re-challenged 70 days after initial infection with 10⁴ PFU of ZIKV-FP (44). All animals were rechallenged with 10⁴ PFU of ZIKV-PR 675 to 858 days after initial infection. ZIKV-MR766, Zika virus/R.macaque- tc/UGA/1947/MR766-3329; ZIKV-FP, Zika virus/H.sapiens-tc/FRA/2013/FrenchPolynesia-01_v1c1; ZIKV-PR, Zika virus/H.sapiens-tc/PUR/2015/PRVABC59. dpi, days after initial ZIKV infection.

^bThe pregnancy status at the time of initial infection: NP, nonpregnant; P, pregnant.

(Table 1). This stock has been used to productively infect 40 animals from previous studies from our group, confirming infectivity (<https://zika.labkey.com/project/OConnor/begin.view?>). After rechallenge, ZIKV was measured in plasma by quantitative reverse transcription-PCR (qRT-PCR) daily on days 3 to 7 after rechallenge and on days 10, 14, and 28 days after rechallenge (Fig. 1). On all days throughout the study, plasma

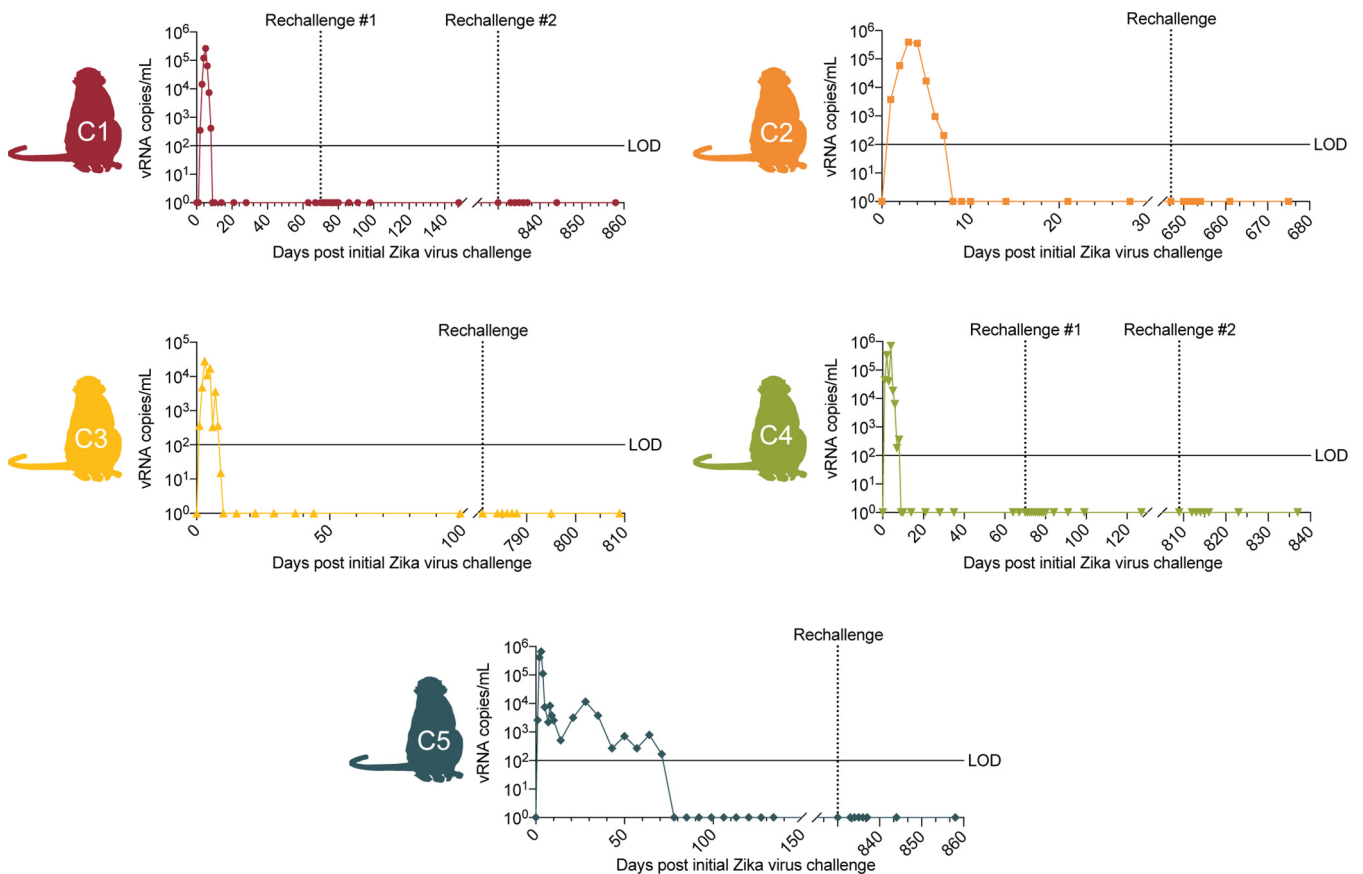


FIG 1 ZIKV viral loads of animals rechallenged with ZIKV-PR from day 0 of primary infection. Schematic of animals included in this study next to their plasma vRNA loads measured by qRT-PCR before and after rechallenge with ZIKV-PR (indicated by an arrow). The limit of detection of ZIKV RNA is 100 copies/mL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

vRNA loads remained negative, indicating protection from plasma viremia after ZIKV rechallenge.

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, CD69, and HLA-DR expression, as markers of activation, in natural killer (NK) cells and CD4, CD8, and CD8 effector memory (T_{EM}) T cells by flow cytometry (Fig. 2 and 3). The percentages of activated cells per microliter of blood are presented as the fold change from the number of activated cells found 3 days before rechallenge (baseline) to determine the fold change in activation from baseline (Fig. 2). Repeated-measures analysis of variance (ANOVA) showed that there were no significant changes in the number of activated Ki-67⁺ CD4, CD8 T_{EM} , CD8, and CD69⁺ CD4 T cell populations over time. In contrast, there was significant variation from baseline measurements in Ki-67⁺ NK cell populations [$F(2.263, 9.051) = 10.36, P = 0.0039$], CD69⁺ NK cell populations [$F(2.381, 9.524) = 9.769, P = 0.0039$], and HLA-DR⁺ NK cell populations [$F(1.524, 6.097) = 11.54, P = 0.0104$]. However, further analysis using Dunnett's multiple-comparison test showed significant changes from baseline in the negative direction in NK cell populations when comparing number of activated NK cells each day after rechallenge to the number 3 days before rechallenge. Similar decreases from baseline were seen in CD69⁺ CD8 T_{EM} and CD8 T cell populations and HLA-DR⁺ CD4, CD8 T_{EM} , and CD8 T cell populations. To further define the cellular immune response following rechallenge, we performed intracellular cytokine staining assays on CD8 and CD4 T cells (Fig. 4 and 5) using whole ZIKV as the stimulating antigen on all animals on 0, 14, and 28 days after rechallenge. As expected, we did not see any ZIKV antigen-specific CD8 or CD4 T cell activation after rechallenge, except for animal C1 on day 14 (Fig. 4B). However, the response was weak and tailed from the original cell population, suggesting that this finding may be a false positive. This further suggests that there was a lack of a cellular immune response following rechallenge.

Serum nAb responses were measured using a flow cytometry-based ZIKV neutralization assay. Prior to rechallenge, all animals had high EC_{50} (50% effective concentration) values (Fig. 6A) 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 the EC_{50} values on day 0 to the EC_{50} values on days 14 and 28 (Fig. 6B). All animals showed increased nAb titers following rechallenge, as noted by a greater EC_{50} 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 preexisting antibody responses. Regardless, 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 complete blood counts (CBCs) were carried out. 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. CBCs were evaluated for all five animals 3 days prior to challenge, daily on days 0 through 10, on day 14, and again on days 21 and 28 dpi. Mild monocytosis with levels exceeding the RI (0 to 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 to 80% WBC) on days 4 and 7 but rebounded to within the normal range by day 8. Animal

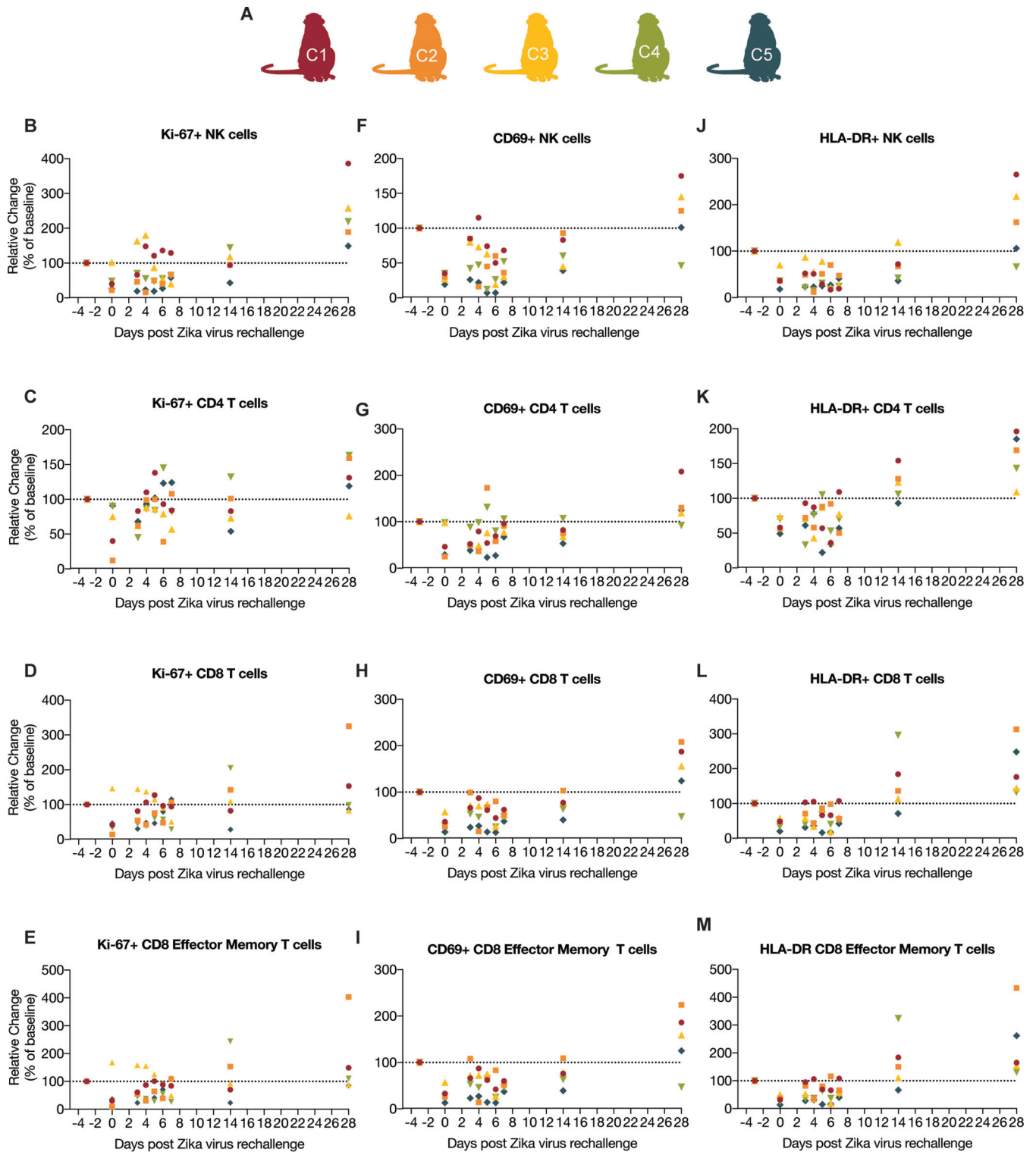


FIG 2 Measurement of immune activation of animals rechallenge with ZIKV-PR. (A) Schematic of animals included in this study. (B to E) Spread of Ki-67⁺ NK cells, CD4 T cells, CD8 T cells, and CD8 effector memory (T_{EM}) T cells. (F to I) Spread of CD69⁺ NK cells, CD4 T cells, CD8 T cells, CD8 effector memory (T_{EM}) T cells. (J to M) Spread of HLA-DR⁺ NK cells, CD4 T cells, CD8 T cells, and CD8 effector memory (T_{EM}) T cells. The percentage of activation in all cell populations was measured on -3, 0, 3, 7, 10, 14, and 28 days after rechallenge. The percentages of activated cells per μ l of blood are presented as relative to the baseline value set to 100% 3 days before rechallenge.

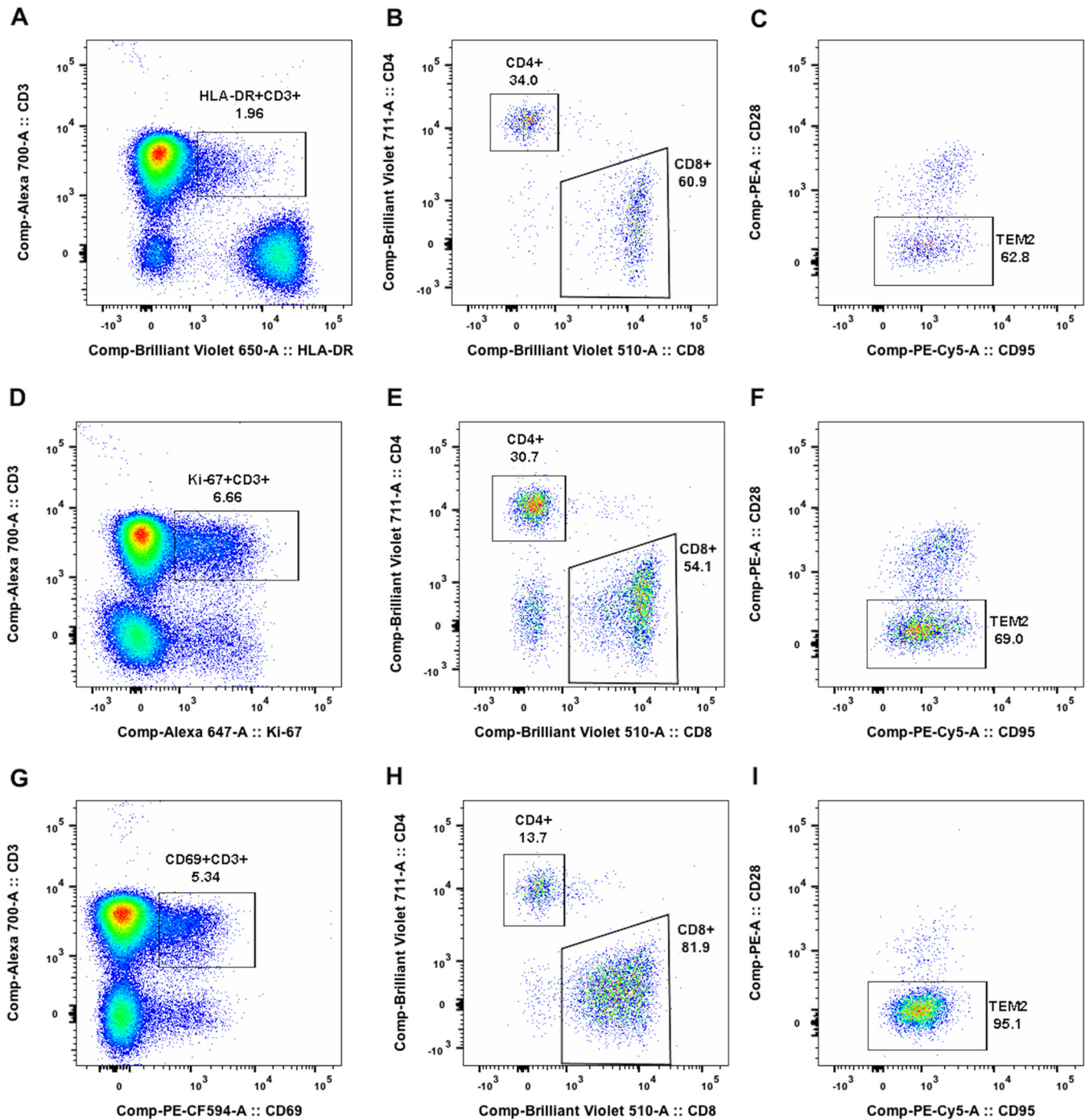


FIG 3 Quantification of activated or proliferating T cells using HLA-DR, CD69 and Ki-67 markers. Panels A to I display the data of animal C2 on day 28 postchallenge. (A, D, and G) Expression of HLA-DR, Ki-67 and CD69 by CD3⁺ T cells within the CD45⁺ lymphocyte gate. (B, E, and H) Frequencies of CD4⁺ and CD8⁺ T cells within the HLA-DR⁺ CD3⁺, Ki-67⁺ CD3⁺, and CD69⁺ CD3⁺ gates, respectively. (C, F, and I) Frequencies of TEM2 (CD95⁺ CD28⁻) cells within the CD8-positive T cell populations.

C5 had high platelet counts exceeding the RI (229,000 to 524,000 platelets/ μ l) present 3 days prior to rechallenge and persisted to be above the RI throughout the study period (44–47).

After rechallenge, animals C1 and C4 developed inappetence 6 days after rechallenge. Animal C1 also developed diarrhea at 6 days after 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 (Table 2). CBC

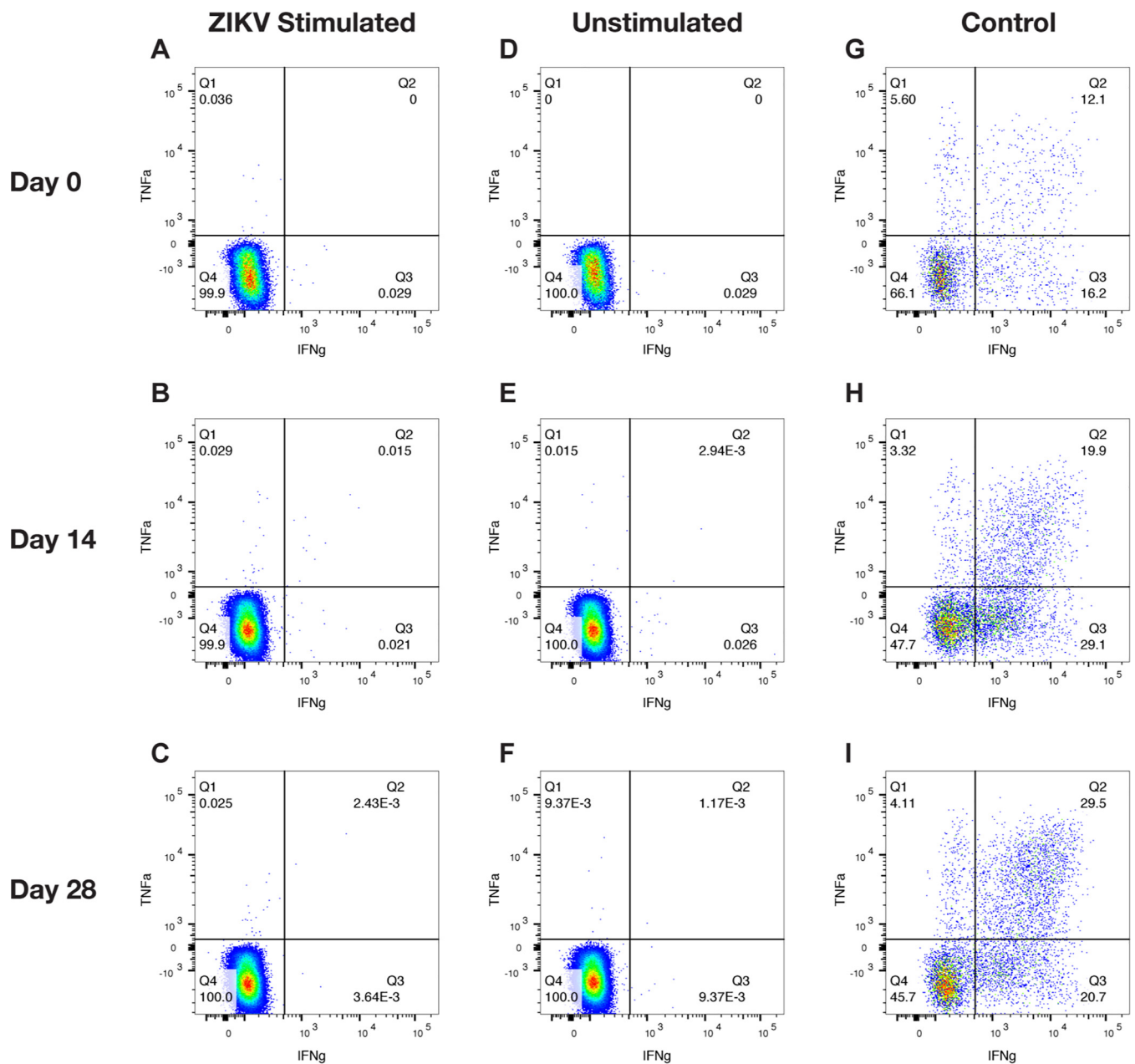


FIG 4 Intracellular cytokine staining for CD8 T cells of ZIKV-rechallenged animals. Representative flow plots for animal C1 on days 0, 14, and 28 after rechallenge show the production of IFN- γ and TNF- α as measures of cellular activation. (A to C) Data for activated CD8 T cells following whole ZIKV stimulation. (D to F) Numbers of activated CD8 T cells when unstimulated. (G to I) Numbers of activated CD8 T cells when stimulated with a leukocyte activation cocktail as a positive control.

tests were evaluated for all five animals 3 days prior to rechallenge, on the day of rechallenge, and on days 3 to 7, 10, 14, and 28 after rechallenge (Fig. 7). 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 (49–51).

DISCUSSION

Since ZIKV could reemerge in areas affected by the 2015 ZIKV outbreak, it is important to evaluate the durability of ZIKV immunity after initial infection in order to

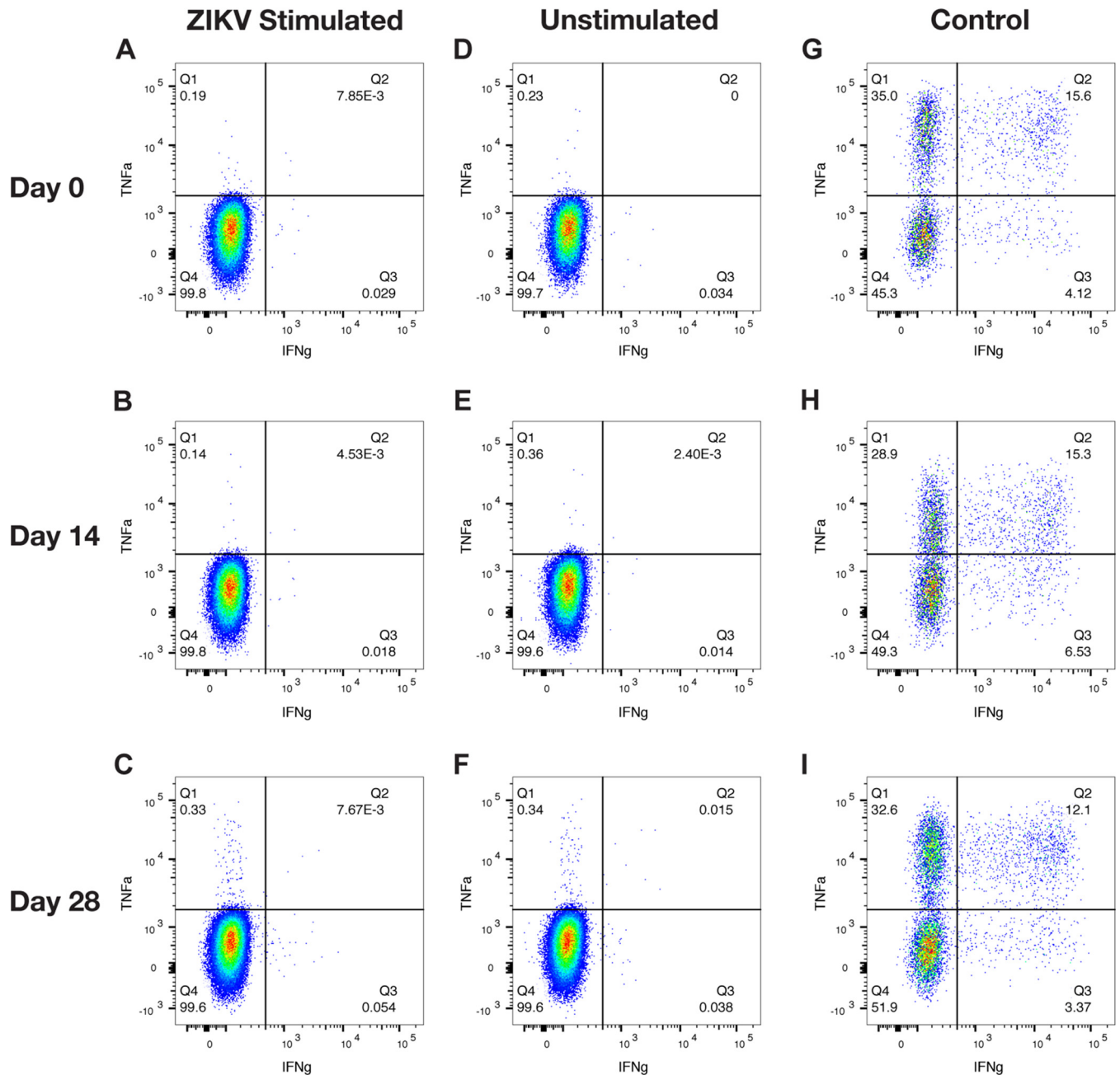


FIG 5 Intracellular cytokine staining for CD4 T cells of ZIKV-rechallenged animals. Representative flow plots for animal C4 on days 0, 14, and 28 after rechallenge the production of IFN- γ and TNF- α as measures of cellular activation. (A to C) Data for activated CD4 T cells following whole ZIKV-PR stimulation. (D to F) Numbers of activated CD4 T cells when unstimulated. (G to I) Numbers of activated CD4 T cells when stimulated with a leukocyte activation cocktail as a positive control.

understand the risk of reinfection, particularly among women. This study shows that initial ZIKV infection provides apparently protective immunity at least 22 to 28 months after initial ZIKV infection. As with many NHP studies, this was an opportunistic rechallenge study using previously ZIKV-infected animals that were available despite having different medical histories. Nonetheless, regardless of previous infection history, we show that primary ZIKV infection generated nAb titers above the 1:10 threshold deemed protective against reinfection for other flaviviruses (Fig. 6) (11, 12). This finding bodes well for those who have had different exposure histories; however, further work will need to corroborate this in humans.

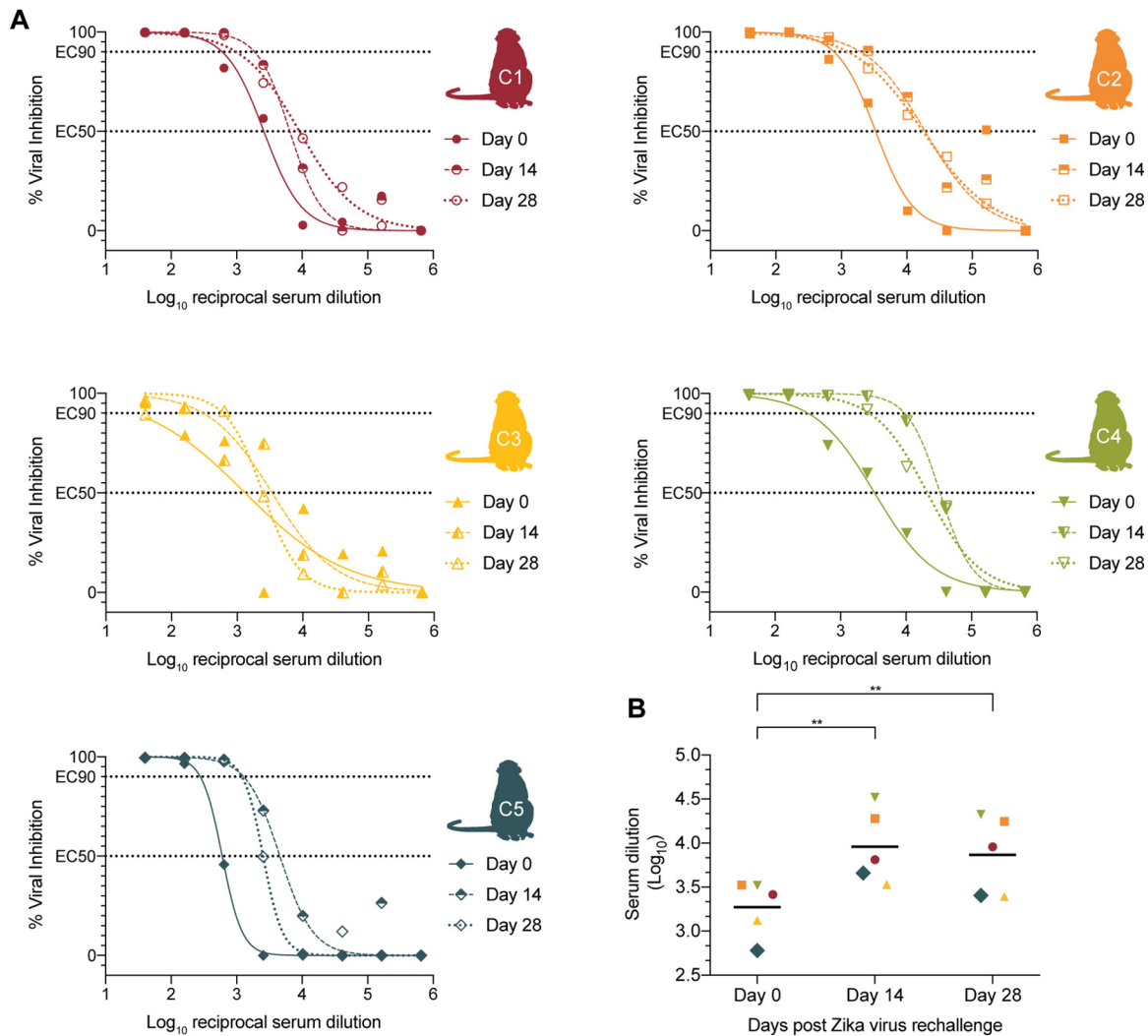


FIG 6 Neutralizing antibody curves. ZIKV neutralizing antibody titers were measured on days 0, 14, and 28. (A) The percent viral inhibitions corresponding to that log₁₀ reciprocal serum dilution are plotted and fit with a nonlinear regression line in order to estimate the EC₅₀ and EC₉₀ values. (B) The log₁₀ serum dilutions corresponding to EC₅₀ nAb titers are plotted for days 0, 14, and 28. Increases in titers were measured by a one-way paired *t* test. Significance is indicated (**, *P* < 0.005).

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 to 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. (32), determined to be protective from ZIKV infection. Does the immunity observed in these animals provide sterilizing protection from reinfection? Although we did not detect any ZIKV RNA in the plasma following rechallenge, we cannot exclude the possibility that ZIKV RNA persisted in sanctuary tissues following initial ZIKV infection. Others have shown that while an increase of ZIKV nAbs rapidly controls blood viremia, sanctuary tissues were found to harbor persistent ZIKV RNA for up to 72 dpi (52, 53). The impact of virus that might be replicating in sanctuaries on subsequent immunity from reinfection would be of interest in future studies.

After rechallenge, we observed a modest increase in ZIKV nAb titers, which suggests that the antigen present in the challenge virus itself was sufficient to stimulate an expansion of preexisting nAb responses. However, it is possible that the inoculum virus was able to replicate below the limit of detection. Future studies could begin to discern

TABLE 2 Clinical remarks for each animal after rechallenge^a

Animal	Days after rechallenge (dpi)	Clinical remark(s)
C1	-1	Relocated to new housing.
	0	Possible chow inappetence.
	3	Third consecutive day of 50% or greater chow remaining; expect appetite to improve with acclimation; normal fecal production suggests adequate intake; weight stable, BCS 5/5 at last physical exam; attitude good.
	6	Chow inappetence; ninth consecutive report of 50% chow left over; diarrhea; overall weight stable; animal appears stable.
	14	Animal reported for redness at right temple over the weekend; did not observe reported erythema; animal appeared BARH and accepted treats.
	17	Animal reported for redness at right temple over the weekend; did not observe reported erythema; animal appeared BARH and accepted treats.
C2	-1	Relocated to new housing.
	28	Animal reported for a scab on the left brow; a 3-mm scab was observed on the lateral L brow; mild erythema was present; no swelling or discharge was seen; animal appeared BARH and accepted treats.
C3	-1	Relocated to new housing.
C4	-2	Possible chow inappetence.
	-1	Relocated to new housing.
	0	During rounds, observed a mild amount of blood in cage; no wounds observed on animal; suspect minor trauma.
	3	Third consecutive day of 50% or greater chow remaining; expect appetite to improve with acclimation; normal fecal production suggests adequate intake; weight stable, BCS 5/5 at last physical exam; attitude good.
	6	Chow inappetence; ninth consecutive report of 50% chow left over; animal appears stable, BARH, and accepted treats.
	10	Confirmed chow inappetence; commonly reported in this animal; has unresolved problem of chow inappetence and is on supplemental diet; weight stable; animal appeared BARH and accepted treats.
	13	Confirmed chow inappetence; commonly reported in this animal; firm stool, first report; has unresolved problem of chow inappetence and is on supplemental diet; weight stable; animal appeared BARH and accepted treats.
	17	Confirmed chow inappetence; commonly reported in this animal; possible persistent firm stool; currently on supplemental diet; weight stable; animal appears stable, BARH, and accepted treats.
	18	Chow inappetence; animal on supplemental diet for chow inappetence; possible firm stool, rarely reported in this animal; animal appears stable, BARH, and accepted treats.
	24	Chow inappetence; animal on supplemental diet for chow inappetence; possible firm stool, rarely reported in this animal; animal appears stable, BARH, and accepted treats.
25	Mild chow inappetence; common report for this animal; most recent weight from one month ago was stable; animal appeared BARH and accepted treats.	
26	Animal reported for a scab above the brow; a 4-mm scab was seen above the brow on midline; mild erythema was observed, no swelling or discharge was seen; animal appeared BARH and accepted treats.	
C5	-1	Relocated to new housing.

^aAbbreviations: BARH, bright, alert, responsive, and hydrated; BCS, body condition score.

if the antigen present in the rechallenge inoculum was sufficient to boost antibody titers or if the virus replicated below the limit of detection. This could be done by rechallenging again with an equivalent low dose of inactivated ZIKV and measuring the effects on neutralizing antibody levels. 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 (54). These researchers defined an increase of <4-fold nAb titer as sterilizing immunity and a ≥ 4 -fold increase in nAb titer as nonsterilizing immunity. In our study, two animals, C1 and C3, exhibited 2.5 \times increases in nAb titer by 14 days after exposure. However, animals C2, C4, and C5 exhibited a 5.7 \times , 10.0 \times , and 7.6 \times increases, respectively, in nAb titer by 14 days postexposure. While we did not look specifically at B cell activation, we showed that ZIKV-specific nAbs became elevated following rechallenge, suggesting that there was ZIKV-specific activation in the humoral arm of the immune response. Additional work will need to be done to determine the effects of ZIKV rechallenge on antibody binding, as well as the isotypes recognizing ZIKV.

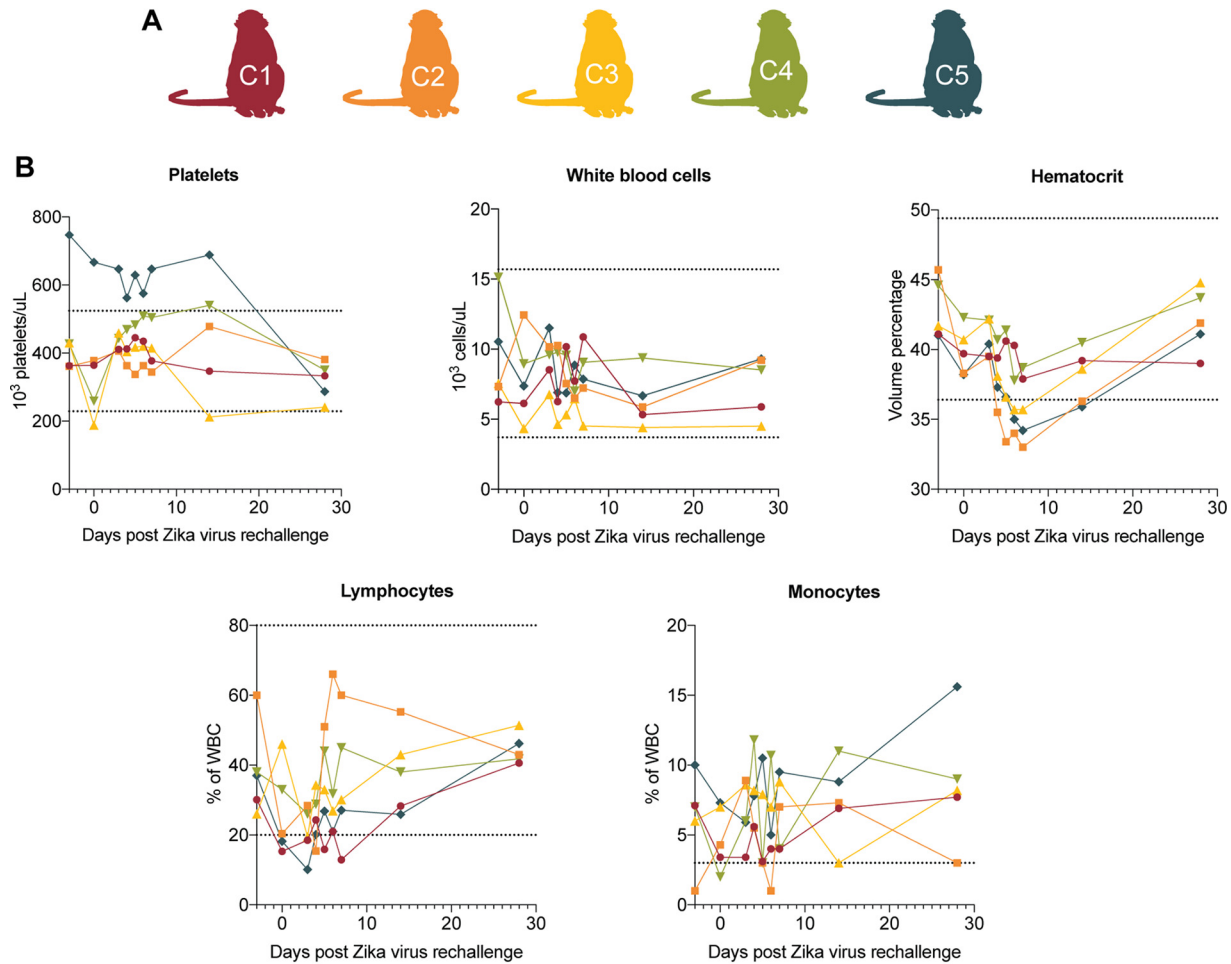


FIG 7 Complete blood count graphs of animals rechallenged with ZIKV-PR. (A) Schematic of animals included in this study. (B) Clinical hematology was performed by WNPRC on whole blood collected daily from macaques on days 3 to 7, as well as on days -3, 0, 10, 14, and 28 after ZIKV rechallenge. Horizontal dotted lines indicate the range of normal reference intervals (RI). Four of five animals had lymphocyte levels dip below the RI (20 to 80% of WBC) at 3 days after exposure but rebounded to the levels within the normal range by 4 days after exposure. Mild monocytosis exceeding the RI (0 to 3% of the WBC) was reported in animal C2 7 days prior to rechallenge. Animal C5 had platelet values exceeding the RI (229,000 to 524,000 platelets ths/ μ l) ranging from 575,000 to 747,000 platelets ths/ μ l present at day -3 and continuing until day 28. Thrombocytosis was also noted following the initial ZIKV challenge for this animal. No other abnormal CBC data were reported.

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 nonpregnant animals. After 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 (in 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 whether immunity elicited by a primary infection is sufficient to protect a subsequent pregnancy from the effects of a ZIKV rechallenge during pregnancy. In addition, 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 22 to 28 months. Future longitudinal studies

will need to assess ZIKV immunity of previously infected individuals with longer time frames than we have used here 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, help elucidate 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 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 (55–57). 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 NHP research covered under protocol G005401-R01. The University of Wisconsin—Madison Institutional Biosafety Committee approved this work under protocol 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 and shelves).

Virus stocks. Zika virus/H.sapiens-tc/FRA/2013/FrenchPolynesia-01_v1c1 (ZIKV-FP) (GenBank accession no. [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 (ZIKV-PR) (GenBank accession no. [KU501215](#)) were provided by Brandy Russell (Centers for Disease Control and Prevention, 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 and rechallenged with 10⁴ PFU of ZIKV-FP 70 days after initial ZIKV infection, as described by Aliota et al. (45). Animal C4 was previously infected with 10⁶ PFU of ZIKV-FP and rechallenged with 10⁴ PFU of ZIKV-FP 70 days after initial ZIKV infection as described by Dudley et al. (44). In addition, animals C1 and C4 were exposed to SHIV on 346 and 326 days after the initial ZIKV challenge, respectively, but never had a positive viral load. 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 dengue virus/H.sapiens-tc/IDN/1978/Sleman/78 (DENV-3) 164 days prior to being infected with 10⁶ PFU ZIKV-FP as previously described by Breitbart et al. (47). Full infection histories are presented in Table 1.

Inoculations. Virus stocks were thawed and diluted in phosphate-buffered saline (PBS) to 1 × 10⁴ 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 s.c. 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 × g for 30 min. Plasma was collected and centrifuged at 670 × g for an additional 8 min to remove remaining cells. Viral RNA was extracted from 300 μl of plasma using a viral total nucleic acid purification kit (Promega, Madison, WI) on a Maxwell 48 RSC instrument (Promega). The vRNA was then quantified using qRT-PCR previously as 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) on a LightCycler 480 or LightCycler instrument (Roche Diagnostics, Indianapolis, IN). Primers and probes were used at final concentrations of 600 and 100 nM, respectively, with the addition of 150 ng of random primers (Promega). 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 s 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. (58). 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 T cells, CD8 T cells, and CD8 T_{EM} cells were quantified as described previously (59). Briefly, 100- μ l portions of EDTA-anticoagulated whole-blood samples were incubated at room temperature for 15 min 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 125 μ l of 2% paraformaldehyde for 15 min. After fixation, the cells were washed and permeabilized using bulk permeabilization reagent (Life Technologies, Madison, WI). While the permeabilizer was present, the cells were stained for 15 min 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). 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 hematology analyzer (Sysmex Corporation, Kobe, Japan). CBC results were reported with species-, age-, and sex-specific reference ranges. When samples were 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 and incubated at 37°C for 24 h 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 min at 37°C before infection of duplicate wells. The plate was incubated for 72 h at 37°C. After incubation, the plates were washed five 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, WI). The plate was incubated for 15 min at room temperature, and 1 ml of R10 medium was added, followed by incubation for 5 min at $530 \times g$. The medium was aspirated, and 125 μ l of 2% paraformaldehyde (PFA) was added to fix the cells. The plates were incubated for 15 min at room temperature, 1 ml of R10 medium was added, and then the cells were centrifuged for 5 min at $530 \times g$. The medium was aspirated, and 100 μ l of fix and permeabilization medium B (Life Technologies) was added to permeabilize the cell membrane. Next, 2 μ l of a 1:10 dilution of the flavivirus-specific antibody (4G2-A647; Novus Biologicals, Centennial, CO) stock was added to each well. The plate was incubated at room temperature for 15 min. Then, 1 ml of R10 medium was added, and the plates were centrifuged for 5 min at $530 \times g$. The medium was aspirated, and 125 μ l of 2% PFA was added to fix the cells. Infection was monitored by flow cytometry on the BD LSRII flow cytometer. Flow cytometry data were 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 nonlinear regression model.

Intracellular cytokine staining. Cryo-stored peripheral blood mononuclear cells from rhesus macaques were screened for activated CD4 and CD8 T cells by intracellular cytokine staining assays using whole-virus ZIKV-PR as a stimulant on days 0, 14, and 28 after rechallenge. Approximately 1.5×10^6 to 2×10^6 cells were plated and stimulated with ZIKV-PR leukocyte activation cocktail (BD Biosciences) or tissue culture medium for 90 min at 37°C in a 5% CO₂ incubator in the presence of 0.5 mg of anti-CD28 (clone L293), 0.5 mg of anti-CD49d (clone 9F10), and CD107a PE (clone H4A3) antibodies. After the 90-min incubation, 0.5 mg of brefeldin A (BioLegend, San Diego, CA) and 0.1 nmol of monensin solution (BioLegend) were added, and the samples were returned to the incubator overnight. The cells were stained for the surface expression of CD3 PE-CF594 (clone SP34-2), CD4 PerCP-Cy5.5 (clone L200), CD8 brilliant violet 711 (clone RPA-T8), and Live/Dead Fixable Near-IR dead cell stain kit (Life Technologies). They were then washed twice with fluorescence-activated cell sorting (FACS) buffer and fixed with 2% PFA. Cells were then permeabilized with 0.1% saponin buffer, intracellularly stained for gamma interferon (IFN- γ)/FITC (clone 4S.B3; BD Biosciences), tumor necrosis factor alpha (TNF- α)/Alexa Fluor 700 (clone MAb11; BD Biosciences), and interleukin-2 (IL-2)/APC (clone MQ1-17H12; BD Biosciences); the samples were then washed twice with saponin buffer and fixed with 2% paraformaldehyde. All antibodies were acquired from BD Biosciences.

Samples were acquired using FACS DIVA (v8.0.1) on a special-order research product BD LSR II apparatus equipped with a 50-mW 405-nm violet, a 100-mW 488-nm blue, and a 50-mW 640-nm red laser. We collected up to 300,000 events in the lymphocyte gate defined by forward- and side-scatter parameters.

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-comparison 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 asterisks (*, *P* < 0.05; **, *P* < 0.005). All statistical analysis was performed using Prism v8.0.1 (GraphPad, San Diego, CA).

Data availability. Complete data sets for these studies have been made publicly available in a manuscript-specific folder on the Zika Open Research Portal (<https://go.wisc.edu/bn71k1>). We declare that all other data for these study findings are available via this portal.

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