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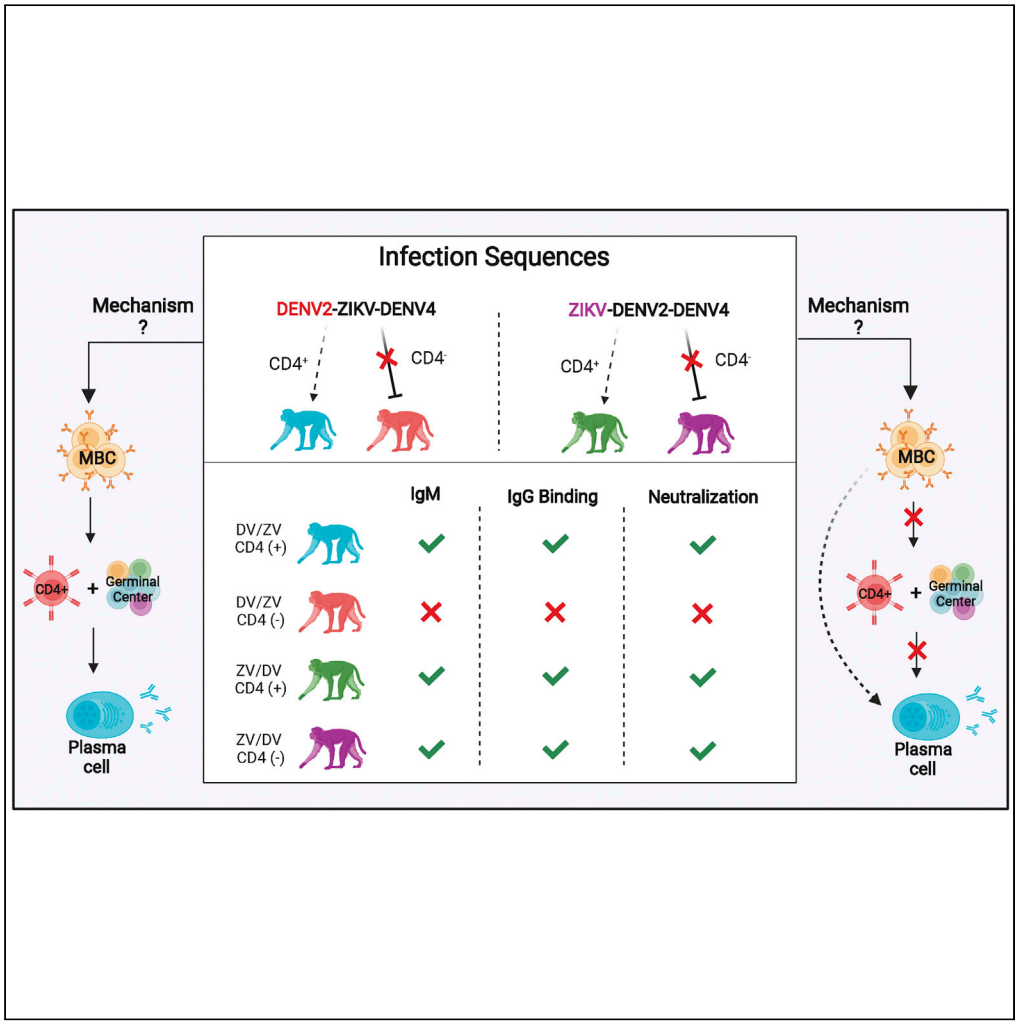

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Highlights

The CD4$^+$T-B cells' response to sequential flavivirus infection is not universal.

Sequence of infections shapes antibody quality in third flavivirus exposure.

The order of infections impacts the magnitude of the recall memory response.

NHP is an excellent model for dissecting the cross-interactions among flaviviruses.

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Infection order outweighs the role of CD4$^+$ T cells in tertiary flavivirus exposure

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SUMMARY

The link between CD4$^+$ T and B cells during immune responses to DENV and ZIKV and their roles in cross-protection during heterologous infection is an active area of research. Here we used CD4$^+$ lymphocyte depletions to dissect the impact of cellular immunity on humoral responses during a tertiary flavivirus infection in macaques. We show that CD4$^+$ depletion in DENV/ZIKV-primed animals followed by DENV resulted in dysregulated adaptive immune responses. We show a delay in DENV-specific IgM/IgG antibody titers and binding and neutralization in the DENV/ZIKV-primed CD4-depleted animals but not in ZIKV/DENV-primed CD4-depleted animals. This study confirms the critical role of CD4$^+$ cells in priming an early effective humoral response during sequential flavivirus infections. Our work here suggests that the order of flavivirus exposure affects the outcome of a tertiary infection. Our findings have implications for understanding the complex flavivirus immune responses and for the development of effective flavivirus vaccines.

INTRODUCTION

Flaviviruses, including Dengue virus (DENV) and Zika virus (ZIKV), are principally arthropod-borne viruses that cause mild to severe diseases in humans worldwide. These members of the Flaviviridae family are transmitted primarily through the bite of *Aedes* spp. mosquitoes, imposing an enormous public health burden in tropical and subtropical areas (Pierson and Diamond, 2020; Suwanmanee and Luplertlop, 2017). Whereas ZIKV transmission has decreased in recent years, its initial emergence into the DENV-endemic regions of the western hemisphere raised concerns (Lessler et al., 2016; Rodriguez-Morales et al., 2016), mainly owing to immunological cross-reactivity limiting serological testing and the implications for the development of severe manifestations in populations exposed to sequential infections (Dejnirattisai et al., 2016; Priyamvada et al., 2016b). Although ZIKV consists of a single serotype, there are four different serotypes of DENV based on antigenic differences within the context of the virion, all of which are pathogenic in humans (Allicock et al., 2020; Chen et al., 2021; Katzelnick et al., 2015; Zellweger et al., 2015). Exposure to one infecting serotype should confer lifelong protection against disease upon secondary homotypic infection. However, heterologous DENV infection can lead individuals to develop dengue or severe dengue, described as hemorrhagic fever or shock syndrome (Bhatt et al., 2020; Guzman et al., 2013). On the other hand, ZIKV cases are generally self-limiting febrile illnesses such as dengue fever, but ZIKV has been associated with more severe outcomes such as Guillain-Barré syndrome (GBS) and birth defects (Culshaw et al., 2017).

During the peak of the ZIKV epidemic in 2016, there was little DENV transmission in the Americas. This has been linked to the extensive cross-reactivity between antibodies (Bhaumik et al., 2018; Priyamvada et al., 2016b; Rogers et al., 2017) and T cells (Grifoni et al., 2017b; Lim et al., 2018) generated against DENV and ZIKV (Katzelnick et al., 2020). The role of cellular immune responses in mediating clearance of subsequent DENV or ZIKV infections has been extensively studied in immunodeficient mice and human infections, highlighting the importance of DENV and ZIKV-specific CD8$^+$ T cells against homotypic infections (Elong Ngono et al., 2016; Regla-Nava et al., 2018; Weiskopf et al., 2013; Wen et al., 2017a, 2017b; Zellweger et al., 2015). Similarly, CD4$^+$ T cells have been shown to be important in flavivirus infections,

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displaying functional plasticity exerting cytotoxic characteristics as a function of previous infections (Weiskopf et al., 2015) and contributing to protection (Elong Ngono et al., 2019; Hassert et al., 2018; Wen et al., 2020). Recently, *Rouers* et al. provided a detailed dissection of the balance between protection or harm depending on T cells' phenotype in response to primary or secondary dengue infections (Rouers et al., 2021). Previously, our group determined that cross-protection is associated with the interval of time between DENV and ZIKV infections and mediated by cellular immune responses, particularly $CD4^+$ T cells (Serrano-Collazo et al., 2020). An area of active discussion is their role in the context of primary and secondary flavivirus infections where it ranges between being polarized to a T helper one cell (St John and Rathore, 2019) and aiding B cells in the germinal center (GC) (Saron et al., 2018) to CD4-restricted HLAs associated with less severe infection outcome, expansion of T follicular cells promoting DENV-specific antibodies and cytotoxic subpopulations as a result of re-exposures (Simon-Lorière et al., 2017; Weiskopf et al., 2015, 2016). Detailed characterization of the maturation of the humoral immune response during secondary infections or vaccinations indicates that during secondary flavivirus exposure, the GC reaction, where the $CD4^+$ T cells play a crucial role in naive B cells activation and immunoglobulin switching, may not be critical for an optimal secondary immune response (Wong et al., 2020). Other groups also have confirmed the involvement of memory B cells (MBC) and MBC-derived plasmablasts in the humoral immune response more than the activation of naive B cells during secondary flavivirus infections (Friberg et al., 2011; Mathew et al., 2011; Priyamvada et al., 2016a; Rogers et al., 2017; Saron et al., 2018; Tsai et al., 2013; Xu et al., 2012) and emphasizing the role of cross-reactive $CD4^+$ T cells (Saron et al., 2018).

Despite these contributions, the changes in the functional quality of flavivirus-induced antibodies during immune recall responses remain less well characterized. More importantly, the role of $CD4^+$ T cells in controlling flaviviral replication by generating polyfunctional responses and the quality of the antibodies produced by tertiary infections in flavivirus experienced humans or non-human primates (NHPs) is largely unknown. NHP models provide advantages such as human-like immune responses and control of external factors, such as injection method, amount of administered viral inoculum, and infection timing (Sariol and White, 2014). Moreover, their competent immune system resembles those of humans, which is essential for understanding the processes driving disease development and has been broadly used to study DENV and ZIKV responses (Borges et al., 2018; Breitbach et al., 2019; McCracken et al., 2017; Pantoja et al., 2017; Sariol et al., 2011; Sariol and White, 2014).

To address the gaps in knowledge regarding the role of $CD4^+$ T cells and the impact of flavivirus priming in sequential infections, in this report we performed a longitudinal study focused on the adaptive immune responses. We assessed the contribution of $CD4^+$ T cells in viral clearance and aid in producing a robust humoral response in rhesus macaques. To test this, DENV/ZIKV or ZIKV/DENV-primed $CD4^+$ T cell-depleted, undepleted, and flavivirus-naïve animals were exposed to DENV-4 (n = 20). We found that the absence of $CD4^+$ T cells in DENV/ZIKV-primed animals with a secondary ZIKV infection before a tertiary heterologous DENV-4 challenge resulted in a significant drawback for the immune responses, including a delay in IgM and IgG responses, as well as a decrease in the overall magnitude of the antibody response, in addition to a reduction in the antibodies binding and neutralization capacity. However, before a third challenge, the lack of $CD4^+$ T cells had no evident impact in ZIKV-primed animals, followed by a sequence of two consecutive heterologous DENV infections (DENV2-DENV4).

Our findings suggest that $CD4^+$ T cells play a crucial role in shaping the quantity, quality, and magnitude of the humoral immune response during tertiary infections. However, that contribution is modulated by multiple variables, including the relatedness among the infecting viruses and the sequence and timing of infections, among others. To the best of our knowledge, this is the first work addressing the cross-immune response scenario in tertiary flavivirus infection combining DENV and ZIKV. This study furthers our understanding of the importance of cellular immune responses in flavivirus endemic areas having significant implications for vaccine development.

RESULTS

Rhesus macaque cohorts, CD4 T cell depletion, and sample collection

The experimental design includes five cohorts of macaques, divided by flaviviral immunological background, time of infections, and immune depletion status were challenged with DENV-4 Dominique (Figure 1). The depletion treatment was efficacious as a 99.8% depletion for cohort A-1 (Figure S1A), and 99.9% depletion for cohort A-3 (Figure S1C) was achieved (Figure S2 for gating strategy). After depletion

Cohort	n	1° Infection	Time between infections	2° Infection	Time between infections	Depletion	3° Infection
A-1	3	DENV2 September 2016	12 months	ZIKVPR September 2017	25 months	CD4	DENV4 October 2019
A-2	3	DENV2 September 2016	12 months	ZIKVPR September 2017	25 months	PBS	DENV4 October 2019
A-3	3	ZIKVPR September 2017	2 months	DENV2 November 2017	23 months	CD4	DENV4 October 2019
A-4	3	ZIKVPR September 2017	2 months	DENV2 November 2017	23 months	PBS	DENV4 October 2019
A-5	8	DENV4 October 2019	N/A	N/A	N/A	N/A	N/A

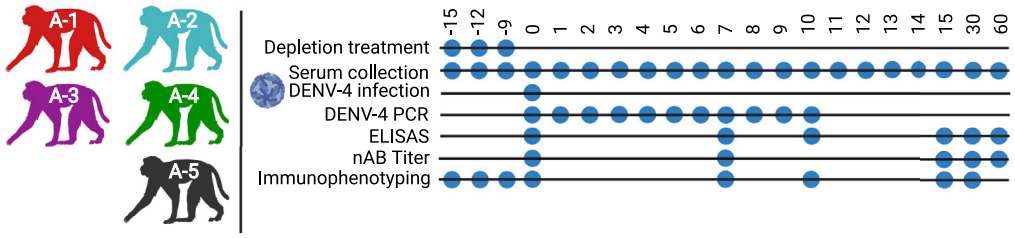

Figure 1. Experimental design for CD4⁺ T cell depletion and heterologous DENV-4 challenge in flavivirus experienced and naive macaques

Five cohorts of rhesus macaques (*Macaca mulatta*) were exposed to DENV and ZIKV virus at different time points. Cohort A-1 (n = 3) and A-2 (n = 3) were exposed to DENV-2 (5×10^5 pfu s.c.) in September 2016 and to ZIKV PRABCV59 (1×10^6 pfu s.c.) in September 2017. Cohort A-3 (n = 3) and A-4 (n = 3) were exposed to ZIKV PRABCV59 (1×10^6 pfu s.c.) in September 2017 and to DENV-2 (5×10^5 pfu s.c.) in November 2017, along with a fifth cohort (A-5) of flavivirus-naïve macaques (n = 8). Depletion of CD4⁺ T cells was performed on experimental groups (A-1 n = 3, denoted DENV/ZIKV CD4 (–) and A-3 n = 3 denoted ZIKV/DENV CD4 (–)) by the initial subcutaneous administration of 50 mg/kg (anti-CD4⁺) at 15 days pre-challenge followed by two intravenous administrations of 7.5 mg/kg (anti-CD4⁺) at 12- and 9-day pre-challenge. Depletion control groups (A-2 (n = 3) denoted DENV/ZIKV CD4 (+) and A-4 (n = 3) denoted ZIKV/DENV CD4 (+) were treated with PBS. All cohorts were challenged subcutaneously (deltoid area) with 5×10^5 pfu/500 μL of DENV-4 Dominique in October 2019. Sample collection was performed at various time points up to 60 days post-infection (dpi) for serum, whole heparinized blood, and PBMC isolation. Figured generated using BioRender. See also Figures S1–S3.

procedures and DENV-4 challenge, neither symptomatic manifestations nor significant differences in weight or temperature were observed in any of the twenty animals belonging to this study. All animals were in range in terms of age, weight, and no variations in rectal temperature were detected (Figures S3A–S3F). We noted differences in liver enzymes alanine transaminase (ALT) levels in the DENV2-ZIKV-DENV4 cohorts on days 2 and 7 post-infection (p.i.) ALT values were significantly higher on both days for DENV/ZIKV CD4-depleted animals when compared to the DENV/ZIKV undepleted animals (Figure S3H, Day 2 and 7, p = 0.0246 and 0.0020, respectively). Interestingly, ZIKV-DENV2-DENV4 cohorts (A-3 and A-4) had a similar ALT value with no major variations in the enzyme profile between the CD4⁺ T cells-depleted and the undepleted control group noted (Figure S3K). On the other hand, no differences in aspartate transaminase (AST) and alkaline phosphatase were detected in either of the cohorts (Figures S3G, S3I, S3J, S3L).

CD4⁺ T cells hindrance has a limited impact on DENV-4 RNAemia in the presence of prior flavivirus immunity

To determine if the depletion of CD4⁺ T cells alters DENV replication kinetics, DENV RNAemia levels were measured in serum using qRT-PCR. Of note, DENV-4 replication in NHP is not as robust and consistent as other DENV serotypes (Hickey et al., 2013; Osorio et al., 2011; White et al., 2013), presenting an intermittent viremia pattern in all cohorts. An early peak viremia was present in the naive cohort when compared to all flavivirus-experienced animals in both sequences of infection. (Figures 2A, 2D, Table 1, and Figure S4) Moreover, by days 7 and 8 p.i. there was no viral RNA detection in any flavivirus-experienced animals while two of the naive animals still had detectable viral RNA. Although the onset of viremia was similar in the depleted and undepleted animals from the DENV/ZIKV infection sequence; for the ZIKV/DENV cohorts,

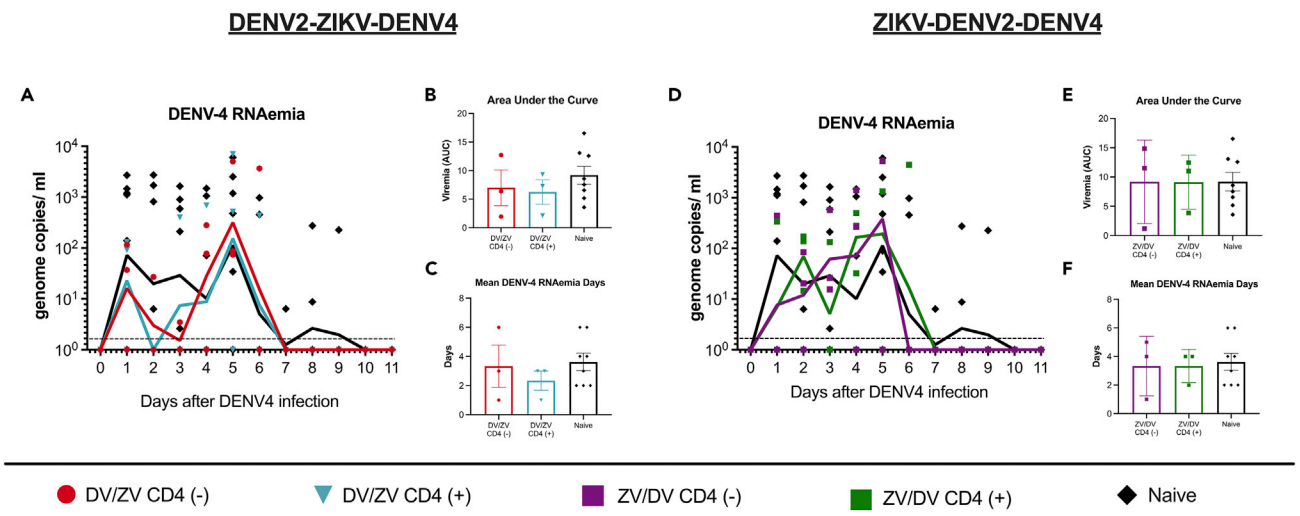

Figure 2. DENV-4 RNA kinetics of depleted and undepleted rhesus macaques

RNAemia is negatively affected by the depletion of CD4$^+$ T cells depending on the infection sequence. Animals depleted of CD4$^+$ T cells are shown in red (DENV-primed) and purple (ZIKV-primed), DENV and ZIKV undepleted animals are shown in blue and green, and flavivirus-naïve animals are shown in black in all panels.

(A and D) DENV-4 genome copies/mL were measured in the serum to monitor viral replication during the first 11 days after infection. Genome copies per mL are shown logarithmically.

(B and E) The area under the curve (AUC) was calculated for individual values.

(C and F) Average RNAemia days were calculated using the following formula: total viremia days divided by the total number of days during which viremia was assessed. The obtained values were placed in a contingency table. Dotted lines indicate the limit of detection of the assay (20 genome copies/mL). See also Figure S4.

we noted an early delay in DENV-4 viremia more evident in the CD4-depleted animals compared to the undepleted group (Figure 2D). However, the ZIKV/DENV CD4-depleted cohort showed the highest viremia peak during the early (Days 0–3) and mid (Days 4–7) periods when compared to the rest of the flavi-experimented animals, raising the question of its link to the CD4-depletion (Figures 2D, S4A and S4B). Interestingly, this cohort had a faster viremia resolution by day 6 p.i. when compared to the rest of the cohorts. When evaluating the area under the curve (AUC), we found that the animals from the DENV/ZIKV with prior flavivirus immunity, although not statistically significant, had on average lower values than the naive group (Figure 2B). Next, we evaluated the average RNAemia days defined as the days with detectable viremia divided by the total number of days during which viremia was assessed, considering the number of animals per group. We noted a tendency where all three DENV/ZIKV undepleted animals grouped in the lower values while the three animals from the DENV/ZIKV CD4-depleted group have a distribution more similar to the naive group (Figure 2C). Nevertheless, the average AUC values (Figure 2E) and the mean viremia days (Figure 2F) were similar among ZIKV/DENV groups. Taken together with the limited number of animals in our cohorts, these results suggest that the role of CD4$^+$ T cells in controlling the viremia is limited, if any, and may have different weights depending on the prior sequence and timing of infections during tertiary flavivirus infection.

The impact of CD4$^+$ T cells depletion in the serological profile depends on the sequence of the infections

To further explore the role of CD4$^+$ T cells, we assessed their impact on the quantity and quality of the humoral response against a tertiary DENV infection. All twenty animals were tested for seroreactivity against DENV-4.

As expected, all flavivirus-experienced animals had a limited level of anti-DENV IgM in comparison to the induction of a primary IgM response in the naive groups (Figure 3A). The AUC of anti-DENV IgM levels was significantly lower in the DENV/ZIKV CD4-depleted groups compared to the DENV/ZIKV undepleted group (Figure 3B, $p = 0.0099$). Moreover, the AUC anti-DENV IgM levels were slightly lower (non-significant) in the ZIKV/DENV CD4-depleted group than in the ZIKV/DENV undepleted group (Figure 3B) but not as

Table 1. DENV-4 viremia of CD4⁺ T depleted, undepleted, and naive rhesus macaques

		RNA emia(Log10 genome copies/mL) Dys Post-DENV-4 Infection											Days	
ID	Immune history	1	2	3	4	5	6	7	8	9	10	11	Total	Mean
0P1	DV/ZV CD4(−)	2.1	ND	ND	2.4	1.9	ND	ND	ND	ND	ND	ND	10	3.3
1O1		1.6	1.4	0.54	1.9	3.7	3.6	ND	ND	ND	ND	ND		
4P0		ND	ND	ND	ND	1.9	ND	ND	ND	ND	ND	ND		
MA023	DV/ZV CD4(+)	2.1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	7	2.3
MA029		ND	ND	ND	2.8	3.8	2.6	ND	ND	ND	ND	ND		
MA062		2.0	ND	2.6	ND	2.7	ND	ND	ND	ND	ND	ND		
BZ34	ZV/DV CD4(−)	2.6	1.3	1.4	2.4	3.7	ND	ND	ND	ND	ND	ND	10	3.3
MA151		ND	ND	1.2	ND	ND	ND	ND	ND	ND	ND	ND		
MA085		ND	1.9	2.8	3.1	4	ND	ND	ND	ND	ND	ND		
MA067	ZV/DV CD4(+)	2.5	2.2	2.1	2.4	3.1	ND	ND	ND	ND	ND	ND	12	4.0
MA068		ND	2.1	ND	1.5	3.7	3.7	ND	ND	ND	ND	ND		
MA143		ND	1.2	ND	2.7	ND	1.9	ND	ND	ND	ND	ND		
0N0	Naive	3.1	3.2	0.42	ND	ND	ND	ND	ND	ND	ND	ND	28	3.5
6K3		3.4	3.4	3.4	ND	2.7	ND	ND	ND	ND	ND	ND		
MA050		ND	ND	0.80	2.8	ND	ND	ND	ND	ND	ND	ND		
MA013		3.2	ND	ND	ND	3.4	ND	ND	ND	ND	ND	ND		
MA019		2.1	ND	2.3	ND	3.1	ND	ND	ND	ND	ND	ND		
MA114		3.0	2.9	ND	2.2	3.8	2.7	ND	1.0	ND	ND	ND		
MA059		ND	ND	3.0	ND	1.5	3.0	0.80	2.4	2.4	ND	ND		
MA063		ND	ND	3.2	ND	2.0	ND	ND	ND	ND	ND	ND		

ND, Not detected.

DENV-4 RNA detection was intermittent in all groups. Mean viremia days per group were calculated using days with detectable RNAemia divided by the number of animals in each cohort. See also Figure S4.

prominent as in the DENV/ZIKV-immune animals. Compared to the depleted group, the presence of CD4⁺ T cells resulted in an increased *de novo* IgM response by a factor of 25 and 15-fold on days 10 and 15 after the infection, respectively, in the DENV/ZIKV sequence of infection. However, the impact of CD4⁺ T depletion was only 4 times, on days 10 and 15, in the ZIKV/DENV sequence of infection (Figure 3C). Interestingly, on average, the induction of a tertiary IgM response in the DENV/ZIKV undepleted animals seems stronger when compared to the ZIKV/DENV counterpart. These results strongly support the concept that the prior sequence of infections alters the IgM humoral response during tertiary infection. DENV/ZIKV-experienced animals all had detectable levels of anti-DENV IgG consistent with a heterotypic DENV infection (Figure 3D). Of note, there was no significant difference in the AUC anti-DENV IgG levels between CD4-depleted animals and their undepleted control cohorts (Figure 3E), suggesting that CD4⁺ T cell depletion has a limited impact on the quantity of the IgG response during tertiary flavivirus infection after sequential flavivirus infections. Next, using an endpoint dilution approach, we assessed the quality of the antibodies from depleted and non-depleted cohorts at 0, 15, and 30 days after DENV-4 infection against the whole DENV-4 viral particle by measuring their binding ability. At baseline, all flavivirus-experienced animals showed cross-reactivity with titers up to a dilution of 1×10^4 with no differences in their AUC values (Figures 4A and 4D). On day 15 p.i., the DENV/ZIKV CD4-depleted cohort showed a statistically significant decrease in the IgG-binding capabilities when compared to the DENV/ZIKV undepleted animals (Figures 4B and 4E, $p = 0.0373$). In contrast, no differences were observed between ZIKV/DENV cohorts. By day 30 p.i., the differences in the binding abilities of the antibodies were still lower in DENV/ZIKV CD4-depleted animals when compared to the rest of the cohorts (Figures 4C and 4F). These results further suggest the significant impact of CD4⁺ T cells have in shaping the antibodies' function early on during tertiary infections. However, as shown for the IgM response, our results demonstrate that the humoral immune response during tertiary flavivirus infection is conditioned by the prior priming of the immune system.

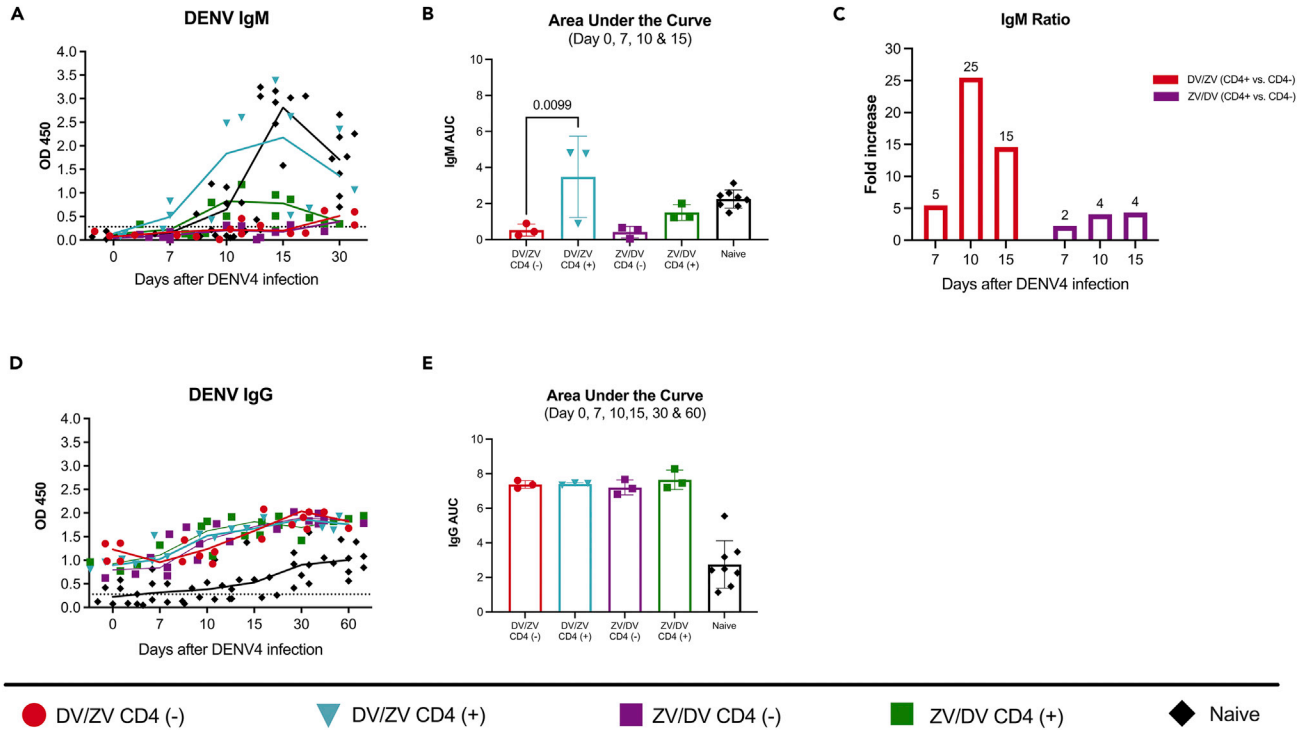

Figure 3. Depletion of CD4$^+$ T cells modifies the serological profile during heterologous infections in DENV/ZIKV-primed individuals

The quantity of the humoral response was assessed using different commercial ELISA tests (A–E). Animals depleted of CD4$^+$ T cells are shown in red (DENV-primed) and purple (ZIKV-primed), DENV and ZIKV undepleted animals are shown in blue and green, and flavivirus-naïve animals are shown in black in all panels.

(B) The area under the curve (AUC) for IgM was calculated for individual values from animals depleted and undepleted with different infecting sequences shown.

(C) IgM fold increase for days 7, 10, and 15 p.i was calculated by dividing the average IgM production for depleted and undepleted groups.

(E) The area under the curve (AUC) for IgM was calculated for individual values from animals depleted and undepleted with different infecting sequences shown. Dotted lines indicate the limit of detection for each test. Statistically significant differences among and within groups were calculated by one-way ANOVA using Tukey's multiple comparisons tests and unpaired t-tests.

Absence of CD4$^+$ T cells negatively impacts the neutralization profile against the infecting DENV serotype in the DENV/ZIKV/DENV sequence

To address if depleting CD4$^+$ T cells further hinders the antibody neutralizing capabilities in a tertiary infection, serum samples were tested using focus reduction neutralization tests (FRNTs) and plaque reduction neutralization tests (PRNTs) against DENV serotypes and ZIKV, respectively. Neutralization assays against the infecting serotype were completed for acute and convalescent periods (Figure S5). The concentration of antibodies required to neutralize 50% of infection ($FRNT_{50}$) for the DENV/ZIKV cohort is shown (Figure 5). The DENV/ZIKV CD4-depleted animals show a significant delay in the neutralization magnitude by day 15 p.i. compared to their respective DENV/ZIKV undepleted group (Figure 5B, $p = 0.0118$). Interestingly, the delay in the expansion of the neutralization in the depleted group is supported by the statistically significant increase in magnitude on day 60 p.i. (Figure 5A, $p = 0.0049$), which was apparent only in this group. Also, the delay was supported by the significant difference in the average increase from day 0–15 ($p = 0.018$) between the undepleted and the depleted groups (Figure 5B). That late increase resulted in a significant difference, with higher values in the depleted group compared to the DENV/ZIKV undepleted group (Figure 5A, $p = 0.0487$). Highly relevant for this work, the CD4$^+$ T cells depletion has no impact on the neutralization profile in the ZIKV/DENV sequence of infection (Figures 5C, 5D, and S5B). Collectively, these results show that CD4$^+$ T cell depletion differentially alters the magnitude of the neutralizing antibody response during a tertiary flavivirus infection depending on the sequence of infection. From here, it can be proposed that different immune mechanisms mediate the induction or recruitment of different antigen-specific B cell populations with optimal function based upon the sequence of flavivirus infection.

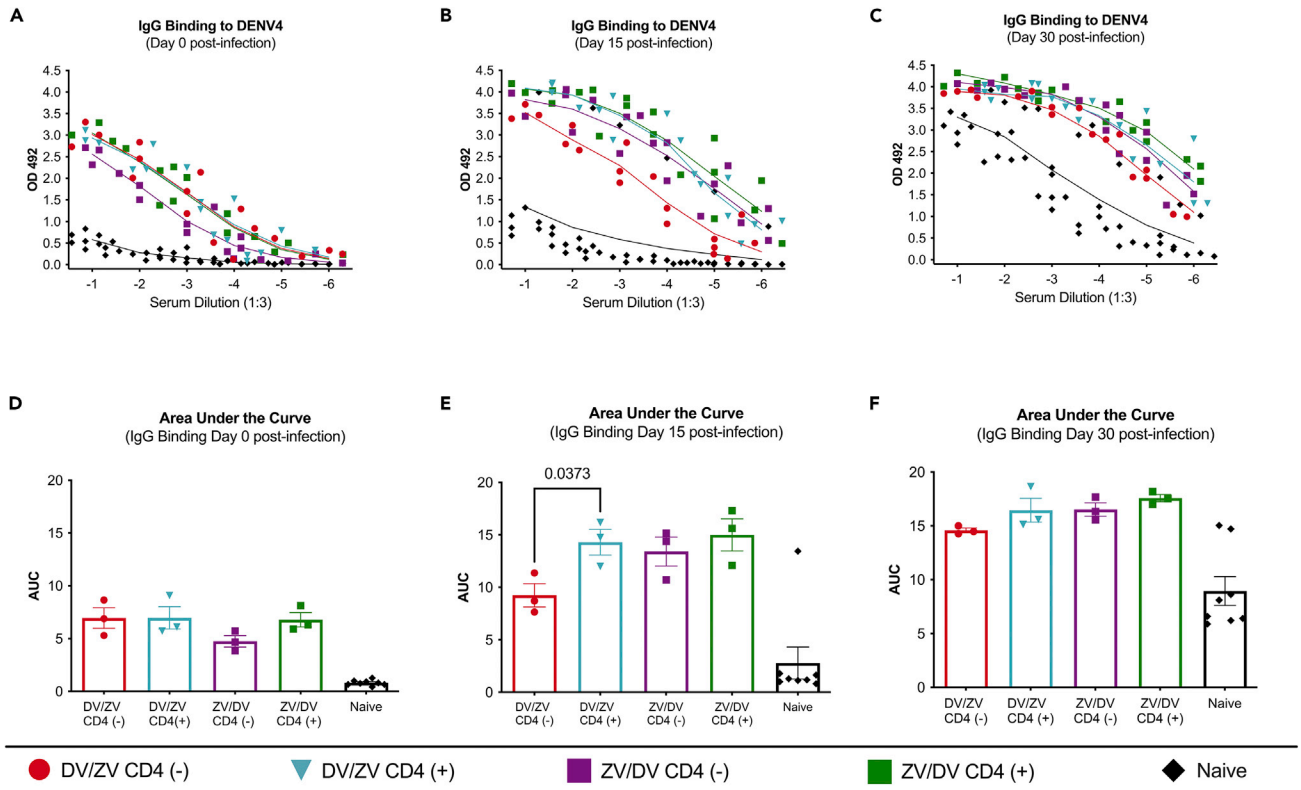

Figure 4. Depletion of CD4$^+$ T cells modifies the IgG-binding capabilities of antibodies against DENV-4 depending on the infection sequence

The quality of the humoral response was assessed using an endpoint dilution binding ELISA (A-F). Animals depleted of CD4$^+$ T cells are shown in red (DENV-primed) and purple (ZIKV-primed), DENV and ZIKV undepleted animals are shown in blue and green, and flavivirus-naïve animals are shown in black in all panels.

(A–C) Binding results against DENV-4 for baseline, day 15, and day 30 p.i. are shown.

(D–F) AUC values from binding results against DENV-4 for baseline, day 15, and day 30 p.i. are shown. Statistically significant differences among groups were observed using one-way ANOVA to compare the AUC values of the depleted and undepleted groups.

Lack of CD4$^+$ T cells hampering the recall immune response is related to the sequence of infection

Next, we reviewed if depleting CD4$^+$ T cells changed the functional quality of the antibodies during the recall memory response to previous infecting serotypes. We measured the binding ability of the antibodies in the sera from depleted and undepleted cohorts before and 15 days after DENV-4 infection against the whole DENV-2 and ZIKV viral particles by an endpoint dilution approach (Figure S6). We observed an expected cross-reactive response against DENV-2, the prior infecting DENV serotype, at day 0 for both infection sequences (Figures S6A and S6C). Nevertheless, on day 15 p.i. the binding against DENV-2 in the DENV/ZIKV CD4 depleted animals showed the lowest AUC values in comparison to all other flavivirus-immune groups with a statistically significant decrease compared to its undepleted control group (Figures S6B and S6D, $p = 0.0001$). A similar profile was seen in the binding against ZIKV with a strong trend to lower, while not significant, AUC values compare to the undepleted control group (Figures S6F and S6H). However, the lack of CD4$^+$ T cells did not affect the binding capabilities of the animals of the sequence ZIKV/DENV/DENV to either DENV or ZIKV. In summary, these results from the binding experiment demonstrate that CD4$^+$ T cells may have a different effect on the recall memory response depending on the prior priming of the immune system during tertiary flavivirus infections.

To further explore how the role of CD4$^+$ T cells can be modulated by order of infections, neutralizing activity against previous infecting flaviviruses (DENV-2, and ZIKV) was tested (Figure S7). The $FRNT_{50}$ of nAbs are shown (Figure 6). As expected, the neutralization of the DENV2/ZIKV/DENV4 sequence was

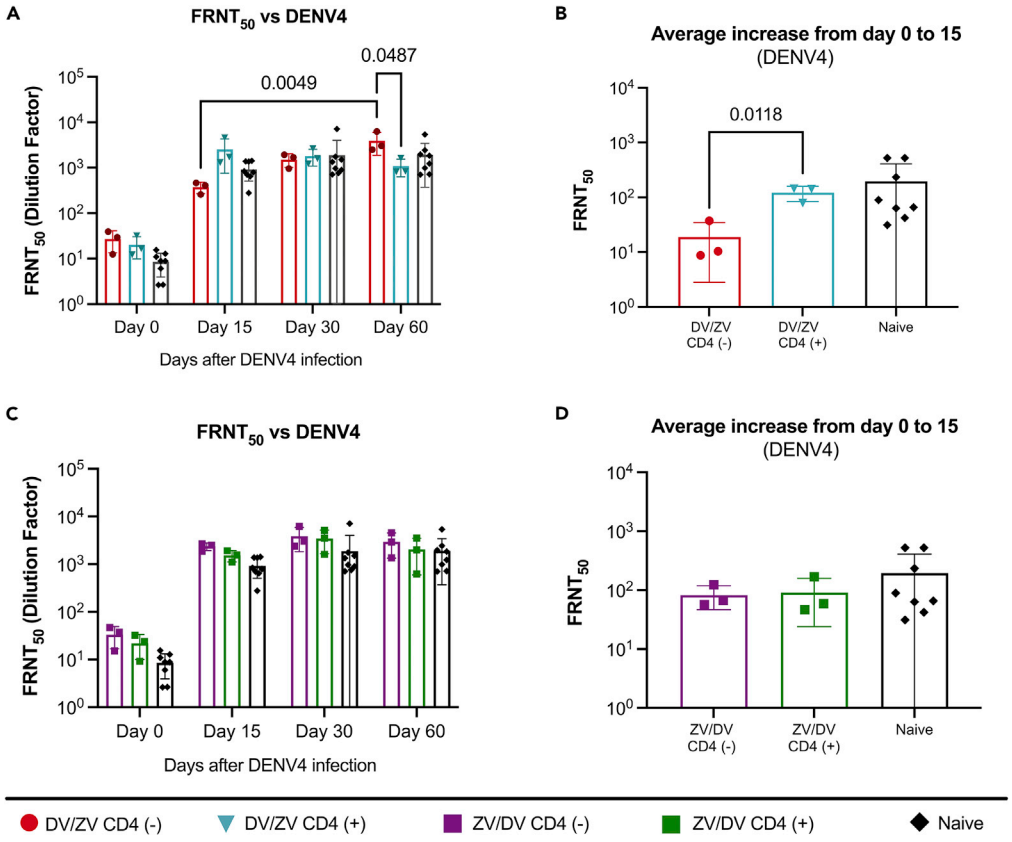

Figure 5. Geometric mean titers of DENV-4 neutralizing antibodies

The concentration of antibodies required to neutralize 50% of infection was determined ($FRNT_{50}$). Animals depleted of $CD4^+$ T cells are shown in red (DENV-primed) and purple (ZIKV-primed), DENV and ZIKV undepleted animals are shown in blue and green, and flavivirus-naïve animals are shown in black in all panels. Non-neutralizing sera were assigned a value of one-half of the detection limit for the visualization and calculation of the geometric means and confidence intervals.

(A) $FRNT_{50}$ values of neutralizing antibodies against DENV-4 after DENV-4 infection for DENV/ZIKV cohorts are shown.

(B) The average $FRNT_{50}$ increase from day 0–15 for DENV-4 was calculated using the average time increase divided by the *n*.

(C) $FRNT_{50}$ values of neutralizing antibodies against DENV-4 after DENV-4 infection for ZIKV/DENV cohorts are shown.

(D) The average $FRNT_{50}$ increase from day 0–15 for DENV-4 was calculated using the average time increase divided by the *n*. Statistically significant differences among groups were calculated by one-way and two-way ANOVA using the Tukey's multiple comparisons test. See also Figure S5.

significantly higher against the primary infecting DENV serotype (DENV-2) than to the heterologous current infecting (DENV-4) serotype (Figure S8A, p = 0.0001). However, the depleted animals showed a noticeable tendency to lower neutralizing titers against all the serotypes compared to all the flavivirus-immune animals (Figures 6 and S8). Interestingly, the animals exposed to ZIKV/DENV2/DENV4 showed a significantly higher neutralization magnitude against the primary infecting DENV serotype and not against ZIKV, the primary infecting flavivirus in this sequence of infection. (Figure S8C, p = 0.0053). Furthermore, the lack of $CD4^+$ T cells did not change the profile of the recall memory response. The naive group had predominantly a typical type-specific neutralizing profile having dominance against DENV-4 the infecting serotype, with a significantly lower cross-neutralizing response to DENV-2 and more limited against ZIKV. The hierarchy of neutralization for this group was DENV4-DENV2-ZIKV (Figure S8E). Interestingly, neutralization magnitude against ZIKV was significantly lower in the DENV/ZIKV CD4-depleted group compared to the ZIKV/DENV CD4-depleted group (Figure 6B, p = <0.0001), and its undepleted counterpart. In summary, these binding, and neutralization results strongly suggest that different populations of antibodies are induced by different molecular mechanisms, and it is depending on the way the immune system is sequentially primed.

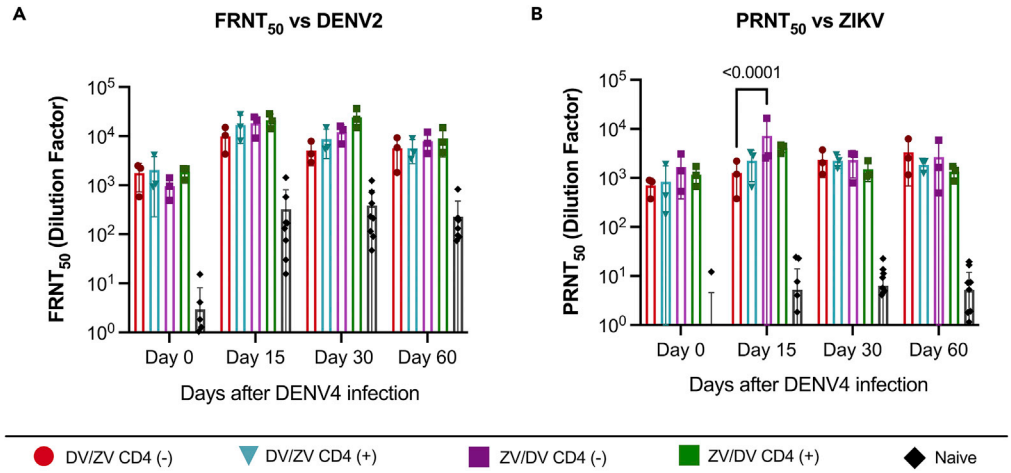

Figure 6. Recall humoral immune response: Geometric mean titers of DENV-4, DENV-2, and ZIKV neutralizing antibodies

The concentration of antibodies required to neutralize 50% of infection was determined ($FRNT_{50}$ and $PRNT_{50}$). DENV/ZIKV CD4-depleted animals are depicted in red, DENV/ZIKV undepleted animals are depicted in blue, ZIKV/DENV CD4-depleted animals are depicted in purple, ZIKV/DENV undepleted animals are depicted in green and flavivirus-naïve animals are depicted in black in all panels. Non-neutralizing sera were assigned a value of one-half of the detection limit for the visualization and calculation of the geometric means and confidence intervals.

(A and B) $FRNT_{50}$ and $PRNT_{50}$ values of neutralizing antibodies against DENV-2 and ZIKV after tertiary DENV-4 infection. Statistically significant differences among groups are reported as multiplicity adjusted p values calculated by two-way ANOVA using Tukey's multiple comparisons test. See also Figures S6–S8.

Infection sequence regardless of $CD4^+$ T cells depletion shapes the activation of a specific ASC subset in Flavi-immune animals

To further explore the role of $CD4^+$ T cells and the hierarchy of infections in shaping the frequency, activation, and proliferation of adaptive immune cell subsets such as B ($CD20^+$ $CD3^-$) and T ($CD3^+$ $CD20^-$) cells, an analysis by flow cytometry was performed. (Figure S10 for gating strategy). No differences were detected in the total B cells following DENV-4 infection compared with baseline levels (Figures S9A and S9B). We found no differences in the memory B cells ($CD20^+CD3^-CD27^+$) and activated memory B cells ($CD20^+CD3^-CD27^+CD69^+$) between both infecting sequences (Figures 7A–7D). Although our gating strategy was not designed to characterize the rhesus plasmablast population as described before (Silveira et al., 2015; Zhang et al., 2019) we detected a significant expansion, $CD4^+$ T cells-independent, in a particular antibody-secreting cells (ASC) population ($CD20^{dim}CD3^-CD27^+$) at seven- and 10 days p.i. only in the ZIKV/DENV cohorts (Figures 7E and 7F $p = 0.0003$, $p = 0.0001$). This last result agrees with our prior findings on the antibodies profile and strengthens the concept that different infection sequences, prior to a tertiary flavivirus infection, may trigger different immune mechanisms driving the humoral immune response.

Lastly, to assess the role of $CD4^+$ T cells in the $CD8^+$ T recall immune response, we measured their effector responses. $CD8^+$ T cells expressing CD107a or producing IFNγ or TNFα in response to previous infecting serotypes were assessed for day 7 p.i. No significant differences were observed between depleted groups or undepleted cohorts (Figures S11 and S12 for gating strategy). Only a trend to the lower number of IFNγ or TNFα -producing cells in response to DENV2 and DENV4 stimulation, but not to ZIKV, was observed in the animals exposed to the ZIKV/DENV/DENV sequence of infection.

DISCUSSION

The role of $CD4^+$ T cells in response to a flavivirus infection has been widely studied (Grifoni et al., 2017a, 2017b; Manh et al., 2020). However, there is still a debate in the field on their crucial contribution to protection (Elong Ngono et al., 2019; Hassert et al., 2018; Weiskopf et al., 2015; Wen et al., 2020) vs. harm (Duangchinda et al., 2010; Mongkolsapaya et al., 2003; Rothman, 2011), making it an area of active discussion in the context of primary and secondary flavivirus infections. Moreover, most of those studies focus on the $CD4^+$ T cell contribution to the recall cellular immune response more than on their role in coordinating

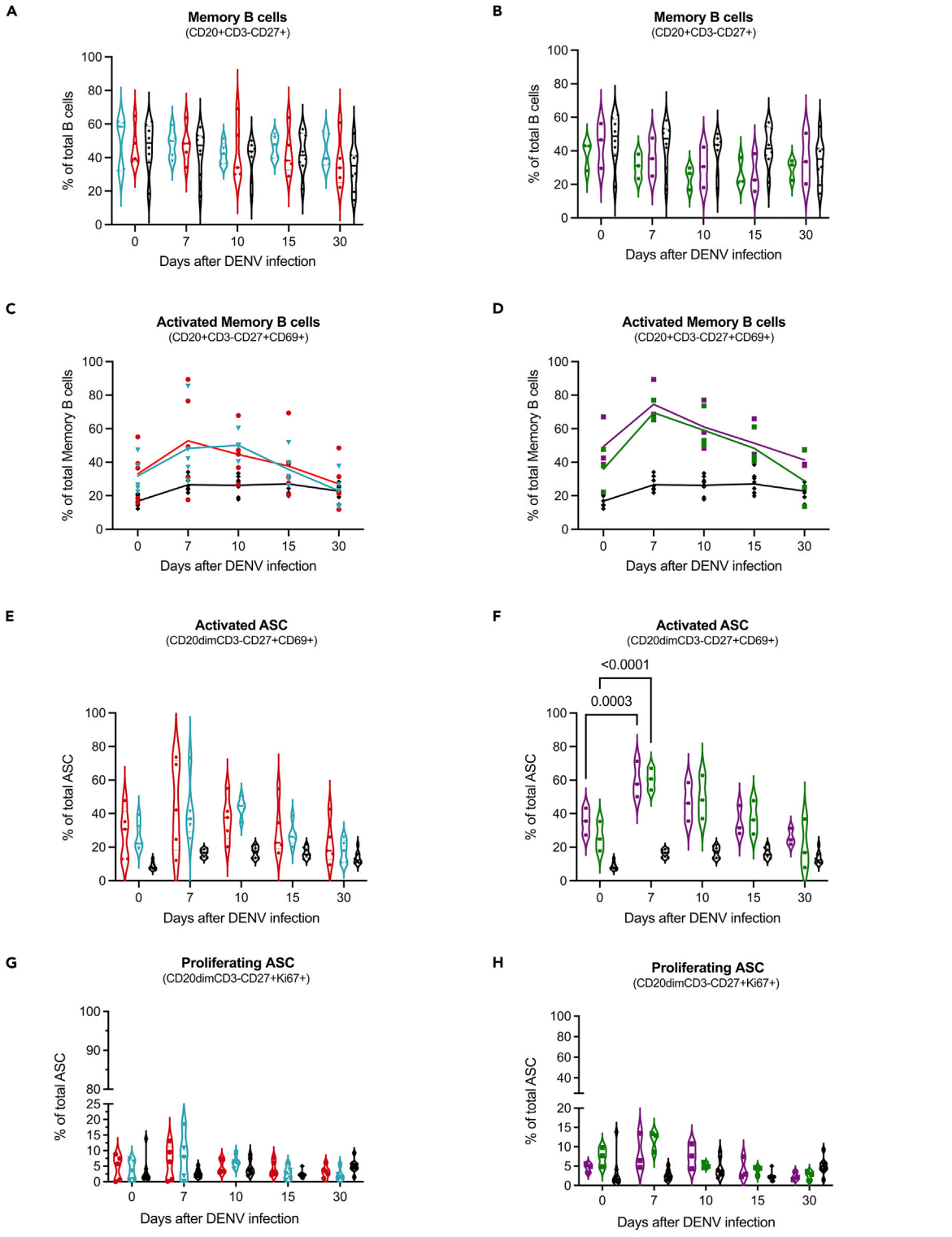
A
Memory B cells
(CD20+CD3-CD27+)
% of total B cells
Days after DENV infection
B
Memory B cells
(CD20+CD3-CD27+)
% of total B cells
Days after DENV infection
C
Activated Memory B cells
(CD20+CD3-CD27+CD69+)
% of total Memory B cells
Days after DENV infection
D
Activated Memory B cells
(CD20+CD3-CD27+CD69+)
% of total Memory B cells
Days after DENV infection
E
Activated ASC
(CD20dimCD3-CD27+CD69+)
% of total ASC
Days after DENV infection
F
Activated ASC
(CD20dimCD3-CD27+CD69+)
<0.0001
0.0003
% of total ASC
Days after DENV infection
G
Proliferating ASC
(CD20dimCD3-CD27+Ki67+)
% of total ASC
Days after DENV infection
H
Proliferating ASC
(CD20dimCD3-CD27+Ki67+)
% of total ASC
Days after DENV infection
DENV CD4 (-)
DENV Flavi-POS
ZIKV CD4 (-)
ZIKV Flavi-POS
Naive

Figure 7. Memory B cells profile before and after DENV-4 infection for DENV/ZIKV & ZIKV/DENV infection sequences

Frequency of B cells and memory B cells were assessed by immunophenotyping using flow cytometry S12 Fig for gating strategy) during baseline and days 7, 10, 15, and 30 post-infection. Animals depleted of $CD4^+$ T cells are shown in red (DENV-primed) and purple (ZIKV-primed), DENV and undepleted animals are shown in blue and green, and flavivirus-naïve animals are shown in black in all panels (n = 20).

(A and B) Percentage of total memory B cells ($CD20^+CD3^-CD27^+$).

(C and D) Frequency of activated memory B cells ($CD20^+$ $CD3^-CD27^+CD69^+$).

(E and F) Percentage of activated antibody-secreting cells (ACS) ($CD20^{dim}$ $CD3^-CD27^+CD69^+$).

(G and H) Frequency of proliferating antibody-secreting cells (ACS) ($CD20^{dim}CD3^-CD27^+Ki67^+$). Comparisons between groups are reported as multiplicity adjusted p values performed by two-way ANOVA using Tukey's multiple comparisons tests and unpaired t-test. Statistically significant differences from the t-test are shown. See also Figures S9–S12.

the humoral immune response during sequential flavivirus infection. Our current report presents the results of depleting $CD4^+$ T cells, the resulting impact on viral replication, the quantity and quality of the humoral immune response, and the $CD8^+$ T cell effector response during tertiary flavivirus infection in NHPs. We provide results from two different flavivirus infection sequences: DENV-ZIKV-DENV vs. ZIKV-DENV-DENV. These scenarios resemble the reality of large geographic endemic areas with more than 390 million people at risk of acquiring multiple flavivirus infections (Bhatt et al., 2013).

In this report, we identified an early delay in DENV-4 viremia in the DENV2/ZIKV/DENV4 infecting sequences in all flavivirus-positive animals, including CD4-depleted animals. This result confirms that antibodies and $CD8^+$ T cells may control early DENV viral replication in subjects with prior flavivirus immunity (Elong Ngono et al., 2016; Lam et al., 2017; Patel et al., 2017; Wahala and Silva, 2011; Weiskopf et al., 2013). Also, the viremia results suggest that $CD4^+$ T cells are needed for sustained viremia control during tertiary flavivirus infection. This agrees with our previous work, where DENV-specific $CD4^+$ T cells induced during primary DENV-2 infection have a role in resolving viral replication during a secondary ZIKV infection (Serrano-Collazo et al., 2020). Moreover, *Weiskopf* et al. and others have shown that DENV $CD4^+$ T cells are readily detectable early following DENV infection, and DENV-specific $CD107a^+$ $CD4^+$ T cells are associated with protection against DENV disease (Weiskopf et al., 2015) and play a crucial role in controlling secondary flavivirus infections (Grifoni et al., 2017b).

Prior results indicate that sequential immunizations for flaviviruses sharing $CD4^+$ epitopes should promote protection during a subsequent heterologous infection. Nonetheless, as supported by our work, sequential infections with different Yellow Fever serocomplex viruses (DENV and JEV) result in different immune profiles (Saron et al., 2018). Additionally, similar amounts of circulating ASC in primary and secondary viral infections helped postulate that pre-existing $CD4^+$ T helper cells may not be required for optimal responses in some viral infections (Balakrishnan et al., 2011; Fink et al., 2007; Querec et al., 2009; Wrammert et al., 2008), and may depend on the nature of the antigen (Fink, 2012). Correspondingly, using a mouse model, *Yauch* et al. showed that during a primary DENV infection without prior flavivirus immunity, $CD4^+$ T cell depletion did not impact the DENV-specific IgM/IgG Ab titers and their neutralizing activity (Yauch et al., 2010). Our data add to the role of the $CD4^+$ T cells in a more complex immune background. We show that $CD4^+$ T cells depleted animals from the DENV2/ZIKV/DENV4 infection sequence produce significantly less total DENV-specific IgM. However, despite the limited binding activity and lower neutralization magnitude in the DENV/ZIKV $CD4^+$ T cells depleted group, we did not detect an impact on the quantity of existing cross-reactive responses (as measured by IgG) in any group. The lack of *de novo* IgM response suggests a role of $CD4^+$ T cells in supporting naive B cells to become IgM-secreting plasmablast or an impact on IgM^+ memory B cell (MBC) reactivation responses in a tertiary DENV-4 with a prior DENV2/ZIKV priming scenario. Nevertheless, our results support that the $CD4^+$ T cells are not essential for an IgM induction in a similar tertiary infection when the immune system was initially primed with ZIKV followed by a secondary DENV-2 challenge.

There is still a considerable gap in our understanding of the intrinsic mechanisms regulating the quantity and quality of the humoral immune response to flavivirus during secondary and more, during tertiary exposure. Our report does not directly explore those mechanisms. However, our results indirectly expose those differences in the context of initial priming and secondary flavivirus infection it is critical to define the underlying immune interactions during tertiary flavivirus infection. We also report that the DENV2/ZIKV/DENV4 CD4-depleted group, but not the ZIKV/DENV2/DENV4 groups, has total DENV-specific antibodies with limited functional quality and activity, defined by significantly lower binding and neutralizing capabilities, respectively, than the $CD4^+$ undepleted group. These differing results confirm a multifactorial setting

controlling the contribution of $CD4^+$ T cells in consecutive flavivirus infections. It is known that the cross-reactive humoral response is broader in secondary DENV infections derived from MBC clonal expansion compared to predominant ZIKV-specific antibodies in primary ZIKV or DENV-ZIKV scenarios (Andrade et al., 2019; Xu et al., 2012). For our results, we hypothesize that sequential secondary and tertiary DENV infections induce a $CD4^+$ T cell-independent clonal expansion of DENV-specific MBC-producing antibodies that, at a functional level, keep their high affinity contributing most strongly to DENV binding and neutralization. On the other hand, during a secondary ZIKV infection, a virus outside the DENV serocomplex and group (Calisher et al., 1989; ICTV, 2020) and with different immunodominant epitopes (Grifoni et al., 2017b; Reynolds et al., 2018; Rivino and Lim, 2017), more DENV-specific MBC generated during the primary DENV-2 infection, may undergo somatic hypermutation and lose cross-reactivity lowering the affinity, determined by the magnitude and specificity of the neutralization to the tertiary infecting DENV-4. This effect is reinforced by the limited binding and neutralization of the anti-DENV and ZIKV antibodies in the absence of $CD4^+$ T cells in the sequence of infection DENV2/ZKV/DENV4; with a pattern supporting a delay in antibody kinetics rather than an impact on overall titers. As shown before, the immune responses induced by ZIKV and DENV as secondary infecting viruses are different. ZIKV infection in DENV-naïve subjects or with prior DENV immunity induces both DENV-MBC and naive B cells with the production of ZIKV type-specific antibodies in both cases. In contrast, cross-reactive MBC predominates after DENV infection (Andrade et al., 2019; Rogers et al., 2017). Other works reported that cross-reactive plasmablast cells more reactive toward the previous infecting DENV serotype (Appanna et al., 2016; Priyamvada et al., 2016a; Zompi et al., 2012). Because of our original staining panel, we were unable to characterize the rhesus plasmablast population as previously described (Silveira et al., 2015; Zhang et al., 2019). Nevertheless, we found a similar neutralization hierarchy profile in both sequences of infections during the tertiary DENV-4 challenge (DENV2>ZIKV > DENV4) resembling the Original Antigen Sin (OAS) postulate. Both sequences of infections were broadly cross-reactive but of significantly higher magnitude against DENV-2, which was the primary infecting DENV serotype. It has been documented that the plasmablast response during secondary DENV infection is mainly derived from MBC (Andrade et al., 2019; Fink, 2012; Wong et al., 2020). Still, there is no information on MBC or plasmablasts dynamic during tertiary flavivirus infection. In our report, the neutralizing magnitude was higher against the priming DENV serotypes (DENV-2) in the ZIKV/DENV2/DENV4 sequence, with two consecutive DENV infections, and not against the priming flavivirus being ZIKV or the current infecting DENV serotype (DENV-4). This may indicate that the antibodies can be originated from the reactivation of DENV-specific MBC plasmablasts after the tertiary infection (Priyamvada et al., 2016a). Also, the finding that the neutralization against the infecting serotype (DENV-4) was independent of $CD4^+$ T cells in the ZIKV/DENV2/DENV4 but not in the DENV2/ZIKV/DENV4 sequence of infection strongly supports that the neutralization in the former sequence may be supported by selecting pre-existing MBC clones without further affinity maturation in GC (Rogers et al., 2017; Wong et al., 2020). Previously *Wong* et al. suggested that for flavivirus challenges, secondary GCs minimally contribute to recall responses, regardless of the serological relatedness among the viruses or the nature of the antigen (Wong et al., 2020). Nonetheless, the serological profile we characterized suggests that in a tertiary flavivirus exposition, the infection's order may play a crucial role in defining the participation of $CD4^+$ T cells in the affinity maturation process in the GC. Although we are not aware of any groups implementing our approach for tertiary infections, our findings on the $CD4^+$ T cell depletions reveal that antigen-specific B cell recruitment and polyclonal antibody response are dependent on the order of flavivirus infections.

Furthermore, the hierarchy of neutralization was unaffected by the position of ZIKV infection related to the two DENV infections. However, it is in line with prior observations showing that DENV/ZIKV cross-reactive MBC response decreased over time post-ZIKV infection (Andrade et al., 2019). In our case, the ZIKV infection occurs precisely 25 months before the tertiary DENV infection in both sequences of infections.

We evaluated the $CD8^+$ T cell response on day 7 p.i. in both sequences of infections by assessing the frequency of virus-specific $CD8^+$ T producing IFN-γ^+ or TNF-α^+ or expressing $CD107a^+$, a marker associated with cytotoxicity. We were unable to identify statistically significant differences when the cells were stimulated with the whole DENV-2, DENV-4 or ZIKV. Still, in animals with the sequence ZIKV/DENV2/DENV4, there was a trend toward lower IFN-γ^+ and TNF-α^+ producing cells compared to the DENV2/ZIKV/DENV4 sequence. Previous studies showed that expanded activated $CD4^+$ T cells located near $CD8^+$ T cells in the spleen after a primary DENV-2 infection did not affect the induction of DENV-2-specific $CD8^+$ T cells (Yauch et al., 2010). Others have highlighted the role of prior heterologous flavivirus immunity

showing that T cell responses to DENV-1 infection are modulated by prior immunity to JEV and YFV to differing extents (Saron et al., 2018). From those results and our limited data, it can be suggested that the specific contribution of the $CD4^+$ T cells in the immune response to flavivirus is impacted by the order of flavivirus infection, more than the priming itself and that the order of infections plays a critical role in defining the recall cellular memory to a tertiary infection. Nevertheless, minimal studies address the interplay between those two key players of the cellular immune response ($CD4^+$ and $CD8^+$ T cells) in the complex immunological scenario of sequential flavivirus interactions.

Previously we confirmed that the length of time between infections during secondary infection with ZIKV or DENV in DENV or ZIKV-primed animals, respectively, does impact the quality of the subsequent response (Perez-Guzman et al., 2019; Serrano-Collazo et al., 2020). Nevertheless, in the current work, it is unclear if the time between subsequent infections prior to a tertiary flavivirus infection plays a critical role in the results presented here. The secondary DENV-2 challenge in the sequence ZIKV/DENV2/DENV4 was performed only three months after a ZIKV priming (Serrano-Collazo et al., 2020), limiting the full expansion of the humoral and cellular components of the ZIKV-specific immune response. However, as mentioned above the ZIKV infection coincided with the tertiary DENV-4 challenge.

Beyond the drawbacks, our work adds new insight into the role of the $CD4^+$ T cells in shaping the humoral immune response by the order and timing of infections during a tertiary infection. Also, it sheds new light on the dynamic of the neutralization hierarchy process, the potential mechanisms, and the multifactorial nature of the immune response to flavivirus during sequential infections. Our contribution to the role of the order of primary and secondary flavivirus infection shaping the immune response during a tertiary exposition provides new insights for an effective flavivirus vaccine development.

Limitations of study

Our report has some limitations, including the number of animals per group, typical in works involving NHPs. Considering the quality of the animals and the multiple contributions in prior works using this model (Kanthaswamy et al., 2016, 2017, 2018; Widdig et al., 2017), we believe in the accuracy and translational potential of the results presented here. Also, more in-depth studies characterizing the plasmablast and MBC profiles, in addition to B cells receptor repertoire and antibody populations at the single-cell level warrant a better understanding in the context of tertiary flavivirus infections. In addition, an extensive characterization of virus-specific $CD8^+$ T cell responses is needed in the context of tertiary flavivirus infections. Our group is currently addressing these gaps in the molecular mechanism behind our findings.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104764.

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AUTHOR CONTRIBUTIONS

C.A.S. and N.M.-R. developed the experimental design. C.R., M.I.M. A.G.B. supervised and performed sample collection and animal monitoring. N.M.-R., C.S.-C., P.P., A.O.-R., L.C., E.G., T.A., and L.B.M. performed the experiments. N.M.-R, C.A.S., C.S.-C, E.G., J.D.B., and A.K.P. analyzed the data. N.M.-R. and C. A.S. drafted the article. C.A.S., N.M.-R., C.S.-C., L.C., P.P., A.O.-R., T.A., M.I.M., A.G.B., C.R., E.G., J.D.B., A.K.P., and L.B.M. reviewed and corrected the last version.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-CD4 Ab CD4R1	NHP Reagent Resource	AB_2716322
CD20 (clone 2H7) PacBlue	Biolegend	Cat# 302238; RRID: AB_2562097
CD3 (clone SP34) PerCP	BD biosciences	Cat# 552851; RRID: AB_394492
CD4 (clone L200) APC	BD biosciences	Cat# 551980, RRID: AB_398521
CD8 (clone DK25) FITC	Sigma Aldrich	FCMAB176F
CD95 (clone DX2) PE	BD biosciences	556674
CD28 (clone 15E8) APC Vio770	Miltenyi	Cat# 130-121-049; RRID: AB_2752213
CD69 (clone FN50) PE-Cy7	BD biosciences	Cat# 561928; RRID: AB_10895389
Ki67 (clone B56) VioGreen	BD biosciences	Cat# 563462; RRID: AB_2738221
CD27 (clone M-T466) PE	BD biosciences	Cat# 555441; RRID: AB_395834
CD8b (clone 2ST8.5H7) Texas Red	BD biosciences	Cat# 641057; RRID: AB_164574
CD3 (clone SP34) PacBlue	BD biosciences	Cat# 558124; RRID: AB_397044
CD20 (clone 2H7) BV605	Biolegend	Cat# 302334;RRID: AB_2563398
CD95 (clone DX2) V510	Biolegend	Cat# 305640; RRID: AB_2629738
CD28 (clone .2) PE-Cy5	BD biosciences	Cat# 561791; RRID: AB_10898345
IFN-g (clone B27) APC	Biolegend	Cat# 506510; RRID: AB_315443
TNF-a (clone MAB11) PE-Cy7	Biolegend	Cat# 502930; RRID: AB_2204079
anti-E mAb 4G2	Dr. Aravinda de Silva and Ralph Baric, University of North Carolina	N/A
anti-prM mAb 2H2	Dr. Aravinda de Silva and Ralph Baric, University of North Carolina	N/A
Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody	Sigma Aldrich	32160702
Bacterial and virus strains		
DENV-4 Dominique strains	Steve Whitehead, NIH/NIAID	N/A
DENV-2 New Guinea 44 (NGC)	Steve Whitehead, NIH/NIAID	N/A
ZIKV PRVABC59	ATCC	VR-1843
Chemicals, peptides, and recombinant proteins		
Dengue Type 4 Protein	Fitzgerald	30-1327
Dengue Type 2 Protein	Fitzgerald	30-1325
Zika Virus Antigen	MyBiosource	MBS569403
Carbonate Bicarbonate Buffer with Azide	Sigma Aldrich	08058-50TAB-F
Bovine Serum Albumin (BSA)	Fisher Scientific	BP9700100
OPD Substrate Tablets	Sigma	34006
HEPES	Fisher Scientific	15630056
NEAA	Fisher Scientific	11140050
Hyclone	Fisher Scientific	SV30079.01
Penicillin/Streptomycin	Fisher Scientific	15140122
L-Glutamine	Fisher Scientific	25030081
Trypsin 0.5%	Fisher Scientific	15400054
DMEM	Fisher Scientific	11960085

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
OptiMEM	Fisher Scientific	31985070
RPMI	Fisher Scientific	A10491-01
FBS	Fisher Scientific	10437-028
PBS	Fisher Scientific	21300-058
DMSO	Sigma Aldrich	D8418-500
Sodium carboxymethyl cellulose	Sigma Aldrich	419273-100G
Non-fat Milk	Denia	N/A
Methanol	Fisher Scientific	A452SK-4
KPL TrueBlue™ Substrate	SeraCare	5510-0030
PFA	Sigma Aldrich	F8775
CytoFix/Cytoperm	BD biosciences	554714
Permeabilizing Solution	BD biosciences	340973
Lysing Solution	BD biosciences	349202
Crystal Violet	Sigma Aldrich	C6158-50G
Critical commercial assays		
Dengue Virus IgM Capture	Focus Diagnostics	EL1500M
Dengue Virus IgG Capture	Focus Diagnostics	EL1500G
Zika Virus IgG	XpressBio	SP856C
QIAmp Viral RNA mini kit	Qiagen	52904
Experimental models: Cell lines		
Vero 81	ATCC	CCL-81
Aedes albopictus clone C6/36	ATCC	CRL-1660
Experimental models: Organisms/strains		
Rhesus macaque (*Macaca mulatta*)	Caribbean Primate Research Center (CPRC)	N/A
Software and algorithms		
FlowJo v10 BD	BD biosciences	https://www.flowjo.com/solutions/flowjo/download; RRID:SCR_008520
Prism 9	Graphpad	https://www.graphpad.com/; RRID:SCR_002798

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Carlos A. Sariol (carlos.sariol1@upr.edu).

Materials availability

All non-commercially available reagents generated in this study are available upon request.

Data and code availability

- All raw data reported in this paper will be shared by the lead contact upon request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
- This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Young male adult rhesus macaques (*Macaca mulatta*) seropositive and seronegative for DENV and ZIKV were housed in the CPRC facilities at the University of Puerto Rico, San Juan, Puerto Rico. All procedures were reviewed and approved by the Institute's Animal Care and Use Committee at Medical Sciences Campus, University of Puerto Rico (IACUC-UPR-MSC), and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (Animal Welfare Assurance number A3421; protocol number, 7890116). Procedures involving all study animals were approved by the Medical Sciences Campus, UPR IACUC. They were conducted following the USDA Animal Welfare Regulations, the Guide for the Care and use of Laboratory Animals, and institutional policies.

METHOD DETAILS

Viral stock

DENV-4 Dominique (kindly provided by Steve Whitehead, NIH/NIAID, Bethesda, MD) was used to obtain comparative results to our previously published data on DENV and ZIKV. Virus was expanded and titered by plaque assay and qRT-PCR using protocols standardized in our laboratories. DENV-2 New Guinea 44 (NGC) strain (provided by Steve Whitehead, NIH/NIAID, Bethesda, MD), and ZIKV PRVABC59 (ATCC VR-1843) were used for neutralization assays. DENV-2 New Guinea 44 (NGC) strain was used to infect macaques in September 2016 and ZIKV PRVABC59 in September 2017.

Immunization, depletion, and viral challenge of macaques

Young adult macaques (6–9 years of age, male) seronegative for DENV and ZIKV were housed in the CPRC facilities, University of Puerto Rico, San Juan, Puerto Rico. Five cohorts of rhesus macaques (*Macaca mulatta*) were exposed to DENV and ZIKV virus at different time points. Cohort A-1 (n = 3) and A-2 (n = 3) were exposed to DENV-2 (5×10^5 pfu s.c.) in September 2016 and to ZIKV PRABCV59 (1×10^6 pfu s.c.) in September 2017. Cohort A-3 (*n* = 3) and A-4 (*n* = 3) were exposed to ZIKV PRABCV59 (1×10^6 pfu s.c.) in September 2017 and to DENV-2 (5×10^5 pfu s.c.) in November 2017, along with a fifth cohort (A-5) of flavivirus-naïve macaques (*n* = 8). All cohorts were challenged subcutaneously (deltoid area) with 5×10^5 pfu/500 μL of DENV-4 Dominique in October 2019. Macaques in experimental cohorts were depleted of $CD4^+$ lymphocytes by administering the anti-CD4 Ab CD4R1 (NHP Reagent Resource; https://www.nhpreagents.org). The initial subcutaneous administration (s.c.) of 50 mg/kg ($CD4^+$) at 15 days pre-challenge was followed by two intravenous administrations (i.v.) of 7.5 mg/kg ($CD4^+$) at 12- and 9-days pre-challenge. The DENV and ZIKV Flavi-POS controls were treated with PBS. For the DENV4 challenge, macaques previously exposed to DENV2 in September 2016 and to ZIKV PRABCV59 in September 2017 (CD4 depleted *n* = 3, DENV Flavi-POS control *n* = 3), to ZIKV PRABCV59 and DENV-2 in September 2017, respectively (CD4 depleted n = 3, ZIKV Flavi-POS control *n* = 3), and DENV/ZIKV naïve animals (*n* = 8) were infected subcutaneously in the deltoid area with 500uL of virus diluted in PBS, using a dose of 5×10^5 pfu. Macaques were monitored after treatments and infections by trained veterinarians for evidence of disease and clinical status. Weights were taken on day 0 and every other day pre-treatment (days −15, −12, −9) and post-infection (days 1–15, 30 and 60). Rectal and external temperatures were taken daily during pre-treatment and post-infection periods. For consistency through the text, the groups are referred to as DENV/ZIKV or ZIKV/DENV CD4-depleted or immune-competent or naïve animals. In the figures, they are referred to as DV/ZV CD4(+) or CD4(−) and ZV/DV CD4(+) or CD4(−) and naïve.

qRT-PCR

DENV viral RNA for real-time PCR assay was extracted from 140 μL virus isolate (previously tittered by plaque assay) and from acute serum samples using QIAmp Viral RNA mini kit (Qiagen, Valencia, CA) as per the manufacturer's instructions as described by our group before (Pantoja et al., 2017; Perez-Guzman et al., 2019; Serrano-Collazo et al., 2020).

ELISA for DENV and ZIKV

Seronegative status for DENV and ZIKV of naïve animals was assessed before DENV-4 challenge using DENV IgG/IgM (Focus Diagnostics, CA) and ZIKV IgG (XpressBio, Frederick, MD) commercial kits. After DENV-4 infection, seroreactivity to DENV was quantified using commercial IgM and IgG ELISA kits (Focus Diagnostics, CA) at baseline, 7-, 10-, 15-, 30-, and 60-days post-infection. All assays were performed per the manufacturer's instructions and as described by our group before (Pantoja et al., 2017; Perez-Guzman et al., 2019; Serrano-Collazo et al., 2020).

Endpoint dilution binding assay

Capture ELISA assay was performed by coating a 96 well plate with DENV-4, DENV-2 (Fitzgerald), or ZIKV (MyBiosource) antigen 2.5 ug/mL coating buffer (Sigma, 08058) overnight at 4°C. Unbound antigen was washed with PBS containing 0.05% Tween 20 and further blocked with 5% BSA (Fisher). Serum samples were serially diluted (1:100, 1:3) in blocking buffer and incubated for 1 h at 37°C. Unbound antibodies were removed by washing and incubated for 1 h at 37 °C with goat-anti-human secondary Ab conjugated with horseradish peroxidase (HRP) (Bio-Rad, CA). Unbound secondary Ab was washed off, and signals were developed with o-phenylenediamine dihydrochloride substrate tablets (Sigma, 34006). OD was read at 492 nm.

DENV and ZIKV titration and neutralization assays

DENV titrations by plaque assay were performed by seeding Vero-81 cells (ATCC-CCL81) at approx. 2.0×10^5 in 96 well plates with supplemented growth medium (DMEM (Dulbecco's Modified Eagle's medium, Thermo Fisher Scientific) for approx. 18 hours. Viral dilutions (ten-fold) were prepared in diluent medium (Opti-MEM (Invitrogen) with 2% FBS (Gibco) and 1% antibiotic/antimycotic (Hyclone). Before inoculation, growth medium was removed, and cells were inoculated with 50 μL per well of each dilution in triplicates; plates were incubated for 1 hour at 37C/5%CO2/rocking. After incubation, 125 μL per well of overlay (Opti-MEM 1% carboxymethylcellulose (Sigma), 2% FBS, 1% non-essential amino acids (Gibco) and 1% antibiotic/antimycotic (HyClone)) was added to the plates containing virus dilutions, followed by an incubation period of 48 hours at 37C/5%CO2. After two days, the overlay was removed with phosphate-buffered saline (PBS 1X) and fixed with 4% paraformaldehyde for 30 minutes. Plates were blocked with 5% nonfat dairy milk in 1X perm buffer (BD Cytofix/Cytoperm™) for 10 min and incubated for 1hr/37C/5%CO2/rocking with anti-E mAb 4G2 and anti-prM mAb 2H2 (kindly provided by Dr. Aravinda de Silva and Ralph Baric, University of North Carolina Chapel Hill, NC, USA), both diluted 1:100 in blocking buffer. Plates were washed 3X with PBS and incubated 1hr/37C/5%CO2/rocking with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma), diluted 1:1,500 in blocking buffer. Foci were developed with TrueBlue HRP substrate (KPL) and counted using an Elispot reader. For the Microneutralization (MN) Assay, sera were diluted two-fold and mixed with approx. Fifty foci per plaque-forming units (FFU per p.f.u. per mL) of virus and then incubated for 1hr/37C/5%/CO2/rocking. Virus-serum dilutions were added to 96-well plates containing Vero-81 cells as mentioned above and incubated with the same conditions. After incubation, overlay was added and processed as previously described. Results were reported as the FRNT with a 60% or greater reduction in DENV foci (FRNT60). A positive neutralization titer was designated as 1:20 or greater, while <1:20 was considered a negative neutralization titer.

ZIKV titration was performed by seeding Vero81 cells (ATCC CCL-81) at approx. 2.0×10^5 per well in 24 well-plates with DMEM (Dulbecco's Modified Eagle's medium, Thermo Fisher Scientific) for approx. 18 hours. Viral dilutions (ten-fold) were prepared in diluent medium (Opti-MEM (Invitrogen) with 2% FBS (Gibco) and 1% antibiotic/antimycotic (Hyclone). Before inoculation, growth medium was removed, and cells were inoculated with 100 μL per well of each dilution in triplicates; plates were incubated for 1 hour at 37C/5%CO2/rocking. After incubation, 1mL per well of overlay (Opti-MEM 1% carboxymethylcellulose (Sigma), 2% FBS, 1% non-essential amino acids (Gibco), and 1% antibiotic/antimycotic (HyClone)) was added to the plates containing virus dilutions, followed by an incubation period of 4 days at 37C/5% CO2. After four days of incubation at 37°C/5%CO_2, overlay was removed; the cells were washed twice with phosphate-buffered saline (PBS), fixed in 80% methanol, and stained with crystal violet. For the Plaque Reduction Neutralization Test (PRNT), sera were diluted two-fold and mixed with approx. Thirty-five foci per plaque-forming units (FFU per p.f.u. per mL) of virus and then incubated for 1hr/37C/5%CO2/rocking. Virus-serum dilutions were added to 24 well-plates containing Vero-81 as mentioned above and incubated with the same conditions. After incubation, overlay was added and processed as previously described. The mean focus diameter was calculated from approx. twenty foci per clone were measured at ×5 magnification. Results were reported as the PRNT with a 60% or greater reduction in ZIKV plaques (PRNT60). A positive neutralization titer was designated as 1:20 or greater, while <1:20 was considered a negative neutralization titer.

Immunophenotyping

Phenotypic characterization of rhesus macaques' adaptive immune response was performed by 8-multicolor flow cytometry using fluorochrome-conjugated Abs at several time points (baseline, 7, 10, 15, and 30 days post-infection). Aliquots of 150uL of whole heparinized blood were incubated with a mix

of antibodies for 30 min. in the dark and at room temperature. After incubation, red blood cells are fixed and lysed with BD FACS fix and lyse solution, and cells are washed twice with BSA 0.05%. Samples were analyzed using a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec, CA). Antibodies used in this study were: CD3-PerCP (SP34), Ki67-Viogreen (B56), CD27-PE (M-T466), and CD69-PeCy7 (FN50) from BD-Biosciences; CD20-PacBlue (2H7) from Biolegend. For analysis, lymphocytes (LYM) were gated based on their characteristic forward and side scatter pattern. B cells were defined as $CD20^{+}CD3^{-}$, Memory ($CD20^{+}CD3^{-}CD27^{+}$) and antibody-secreting cells (ASC = $CD20^{dim}CD3^{-}CD27^{+}$) B cell subpopulations were determined within $CD20^{+}$ B cells. Activation marker CD69 and proliferation marker Ki67 were determined in each different lymphoid cell subpopulation. As we did not label cells with $CD38^{+}$ or $CD19^{+}$ markers, we could not differentiate from plasmablast ($CD20^{-}CD27^{+}CD19^{+}CD38^{+}Ki67^{+}$) or plasma cells ($CD20^{-}CD27^{+}CD19^{-}CD38^{++}Ki67^{-}$). From here, throughout the text, the $CD20^{dim}CD3^{-}CD27^{+}$ are referred to as Antibodies Secreting Cells (ASC). Data analysis was performed using Flowjo (FlowJo LLC Ashland, OR).

Cellular immune response analysis

Intracellular cytokine staining of PBMCs from animals was performed by multicolor flow cytometry using methods similar to those described by Meyer et al. (2015). A baseline line sample collected one week before the depletion and another sample taken seven days after the challenge were assessed. PBMC samples were thawed 1 day before stimulation. Approx. 1.5×10^{6} PBMCs were infected overnight with DENV-4 (Dominique) at a MOI of 0.1, DENV-2 (NGC44) at a MOI of 0.1, or ZIKV at a MOI of 0.5 in RPMI medium with 5% FBS. After stimulation, the cells were stained for the following markers: $CD4^{-}$ PerCP Cy5.5 (Leu-3A (SK3), CD8b-Texas Red (2ST8.5H7), CD3-PacBlue (SP34), CD20-BV605 (2H7), CD95-V510 (DX2), CD28.2-PE-Cy5, IFN-g-APC (B27) and TNF-a-PE-Cy7 (MAB11). The samples were run on an LSRII (BD) and analyzed using Flowjo (Treesar). Lymphocytes were gated based on their characteristic forward and side scatter pattern, T cells are selected with a second gate on the $CD3^{+}$ population, and $CD8^{+}$ T cells are defined as $CD3^{+}$ $CD20^{-}$ $CD8^{+}$ Cytokine expression was determined by the number of $CD8^{+}$ positive cells and then stained positive for IFN-g and TNF-a. CD107a production was also measured in these populations to determine functional cytotoxicity.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). For viral burden analysis, the log titers and levels of vRNA were analyzed by multiple unpaired t-tests and two-way ANOVA. Also, a Chi-squared test was used to analyze a contingency table created from obtained viremia data. The statistical significance between or within groups evaluated at different time points was determined using one-way and two-way analysis of variance (ANOVA) (Tukey's, Sidak's, or Dunnett's multiple comparisons test) or unpaired t-test to compare the means. Significant multiplicity adjusted *p*-values (*< 0.05, **< 0.01, ***< 0.001, ****< 0.0001) show statistically significant differences between groups (Tukey test) or time-points within a group (Dunnett test).