CD4+ T Cell Depletion Before Primary Dengue and/or Secondary Zika Infection Reveals Mechanistic Correlates of Antibody Functionality in Rhesus Macaques.

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Running Title: CD4 T cell depletion before dengue or zika of rhesus macaques

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Abstract

Dengue (DENV) virus and Zika virus (ZIKV) are two flaviviruses of major public health concern. One drawback designing effective vaccines is our limited understanding of the mechanisms ruling protection or harm among DENV serotypes, or between DENV and ZIKV. Here, we depleted rhesus macaques of $CD4^+$ T cells *in vivo* before primary DENV infection and/or secondary ZIKV challenge to recreate a sub-optimal priming of the humoral immune response. Our results support that $CD4^+$ T cells are needed to induce a quantitative and type-specific effective humoral immune response against primary DENV, but also against secondary ZIKV in DENV-experimented subjects. Our results also indicate a limited contribution of the DENV-Memory B cells to anti-ZIKV response. Furthermore, our results suggest that a suboptimal B cell priming during a primary DENV infection does differentially impact different antibody (Abs) properties. While binding or neutralization of ZIKV or DENV during a subsequent exposure to ZIKV is not affected by the lack of $CD4^+$ T - B cells interaction during a primary DENV infection, that interaction is critical to guarantee the Abs specificity. Also, we found that depleting $CD4^+$ T cells before DENV primary infection but not before ZIKV challenge significantly increases Abs cross- reactivity against DENV-EDIII domain and DENV-NS1 protein but not against ZIKV-EDIII domain or NS1 protein. Furthermore, there was more cross-reactivity among the DENV-NS1 proteins than against DENV-EDIII domains, suggesting that during a primary DENV infection $CD4^+$ T cells have a different weight in the responses against EDIII domain and NS1 protein. The proper Abs binding and neutralization with increased cross-reactivity profile was associated with limited frequency of circulating peripheral T helper cells (pTfh) with T helper 1 phenotype (CD4+/CXCR5+/CXCR3+) and expressing markers related to B cell activation (CXCR5+/CXCR3+/PD-1+/ICOS+) in the group depleted of $CD4^+$ T cells only before primary DENV infection. However, memory B cells – but not Antibody Secreting Cells (ASC) activation 7 days after the infection – positively correlate with those two populations of pTfh. Finally, when Abs cross-reactivity values were incorporated in a Principal Component Analysis (PCA), the DENV- $CD4^+$ T depleted group separates from the other two groups with similar Abs binding and neutralization profiles. Our result strongly suggests that during a heterologous sequential DENV/ZIKV infections Abs binding, and neutralization, may be regulated by different factors than their specificity. Before, the induction of cross-neutralizing Abs has been described in the context of secondary DENV infection. Here, for the first time, we are reproducing the experimental conditions leading to the generation of such Abs population *in vivo*. In summary, we show that suboptimal immune priming during a primary flavivirus infection has functional consequences during a secondary heterologous infection. These results have huge implications understanding the immune response to DENV vaccines (and maybe ZIKV), including why an optimal vaccine or natural-induced neutralizing response not necessarily protects or enhances pathogenesis during a subsequent natural heterologous exposure.

Introduction

Dengue (DENV) and Zika (ZIKV) viruses are flaviviruses of significant public health concern (Li et al., 2021; Paixao et al., 2022; Qiao et al., 2021; Tian et al., 2022; Wong et al., 2022). DENV is responsible for about 390-500 million infections per year, of which 96 million present different levels of disease severity (Bhatt et al., 2013) including Dengue Hemorrhagic Fever/ Shock Syndrome with up to 20 % mortality rate if untreated (WHO/TDR, 2009). It is estimated that the population at risk will increase in 60% by 2080 for a total of 6.1 billion people (Messina et al., 2019). ZIKV infections are usually mild but have been associated with unique severe adverse outcomes such as fetal loss (Campos et al., 2016), congenital ZIKV syndrome (CZS) (Brasil et al., 2016), Guillain-Barré syndrome (GBS) (Cao-Lomeau et al., 2016), and rare cases of encephalopathy (Roze et al., 2016), meningoencephalitis (Carteaux et al., 2016), myelitis (Mecharles et al., 2016), uveitis (Furtado et al., 2016), and severe thrombocytopenia (Sharp et al., 2016).

DENV and ZIKV circulate in similar geographic areas (Braga et al., 2017; Rodriguez-Morales et al., 2016), and the effect of prior DENV or ZIKV immunity to each other is subject of an ongoing debate. Some evidence from animal models and humas suggest that prior DENV immunity has a protective role in secondary ZIKV infection (Ana Carolina Bernardes Terzian, 2017; Breitbach et al., 2019; McCracken et al., 2017; Pantoja et al., 2017). Other works propose that prior DENV infection may increase ZIKV disease severity (Bardina et al., 2017; Crooks et al., 2021; Katzelnick et al., 2020b; Katzelnick et al., 2021). Intermediate pre-existing levels of naturally acquired anti-DENV or anti-ZIKV antibodies (Abs) were found to be associated with enhancement of symptomatic and severe DENV. However, subjects with high anti-DENV or anti-ZIKV Abs are at much lower risk of symptomatic and severe DENV2, with rates similar to DENV-naïve individuals (Katzelnick et al., 2020a). To add complexity to the field, recently it has been documented that different DENV and ZIKV interactions can be different or asymmetrical depending on the DENV serotype {Bos, 2023 #2166; Katzelnick, 2020 #2029. Nevertheless, the mechanisms supporting a proportionate immune response among all four DENV serotypes or DENV and ZIKV after a natural infection – particularly after vaccination – still remains as a critical knowledge gap.

Immunity to one DENV serotype is believed to confer type specific (TS) protective Abs or immunity to that infecting serotype, but can also induce cross-reacting (CR) Abs able to amplify the pathogenesis during a secondary infection with a different serotype – a phenomenon known as antibody-dependent enhancement (ADE) (Guzman et al., 2013; Halstead, 2003; Halstead et al., 1983; Katzelnick et al., 2017). As a result, DENV vaccines are designed as tetravalent formulations to induce balanced Abs (Abs) response against all four DENV serotypes at the same time. Two of the leading DENV vaccines (Dengvaxia and TAK-003), based on attenuated chimeric viruses (Deauvieau et al., 2007; Guy et al., 2017; Huang et al., 2013; Osorio et al., 2011b) has shown to induce unbalanced immune responses (Biswal et al., 2020; Guirakhoo et al., 2002; Hadinegoro et al., 2015; Halstead, 2018; Sabchareon et al., 2012; White et al., 2021). A third vaccine candidate developed by the Laboratory of

Infectious Diseases at the National Institutes of Health (TV003) based on live attenuated DENV viruses (Durbin et al., 2013; Durbin et al., 2011; Whitehead, 2016), induced TS Abs to all four DENV serotypes and protection against challenge (Kirkpatrick et al., 2016; Nivarthi et al., 2021). Recent results from phase 3 are encouraging, but differences in efficacy were still reported between DENV1 [89.5% (95% CI, 78.7 to 95.0)] and DENV2 [69.6% (95% CI, 50.8 to 81.5)] the two serotypes detected in the three years of follow up period (Kallás et al., 2024). The limitations of Dengvaxia and TAK-003 formulations in inducing a balanced immune response to all four DENV serotypes have been associated to unequal replication of vaccine components or to the lack of/incomplete cellular immune response to the nonstructural (NS) proteins, among others (Grifoni et al., 2020; Guy et al., 2008; Guy et al., 2020; Thomas et al., 2009; Waickman et al., 2019a; Waickman et al., 2019b). For Dengvaxia, the DENV4 component was replicating and immunodominant, compared to the other three serotypes (Guy et al., 2009); a similar situation is documented with DENV2 in the TAK-003 vaccine in non-human primates (NHP) and humans (Osorio et al., 2011a; Rupp et al., 2015; Tricou et al., 2020).

Total Abs to each DENV serotypes have been used as a marker of protection for DENV vaccines, as it has shown to correlate with the efficacy of other flavivirus vaccines like yellow fever vaccine (YFV), Japanese encephalitis vaccine (JEV) (Appaiahgari et al., 2006; Goncalvez et al., 2008; Gupta et al., 2008; Raengsakulrach et al., 1999; Saini and Vrati, 2003), and tick-borne encephalitis (TBEV) (Beran et al., 2019; Leonova and Pavlenko, 2009; Maikova et al., 2016; Venturi et al., 2009; Vorovitch et al., 2019). But recent growing evidences point out that TS neutralizing Abs (nAbs) above a certain threshold may be a better indicator of vaccine efficacy than the total Abs (Henein et al., 2021; Nivarthi et al., 2021). Earlier works also provide explanations as of why some Abs might neutralize DENVs in cell culture, but not protect *in vivo* (Raut et al., 2019). Martinez et al. assembled a comprenhensive review on the multiple factors modifiying the efficacy of the immune response to DENV and DENV vaccines, incluidng the intra and inter serotypic viral antigenic diversity and the genotypic diversity among others {Martinez, 2021 #2068}.

All those observations and findings from natural infections and vaccines are supported on Abs response mainly against the structural proteins, reinforcing the concept that immunogenicity of the structural proteins does not guarantee protection from natural infection or vaccination efficacy. Most humoral immunity epitopes are located within the domains of the DENV envelope (E) protein, while cellular immunity epitopes are located on the NS proteins (Pinheiro et al., 2021; Rothman, 2010). Additionally, the NS1 protein is particularly interesting and unique, as it is a protein produced and secreted during viral replication, which make it highly immunogenic (Zhang et al., 2023) inducing significant levels of anti-NS1 Abs in DENV- and ZIKV-infected individuals (Aquino et al., 2023; Castro-Trujillo et al., 2023; Churdboonchart et al., 1991; Costa et al., 2007; Petphong et al., 2023). It is documented that DENV E and NS1 proteins equally score 4 – out of the 10 proteins – in an immunogenicity score (Weiskopf et al., 2016), and some reports have pointed NS1 as a target antigen for the development of DENV vaccines (Costa et al., 2007; Costa et al., 2006; Henchal et al., 1988; Wan et al., 2014), while others suggest a role

123 for this protein in the pathogenesis (Perera et al., 2024; Shu et al., 2000; Zhang et al., 2023). Thus, it still remains
124 a puzzle due to its ability to elicit both protective and potentially pathogenic immune responses. On this work, in
125 addition to the conventional Abs to the structural proteins, we explore the contribution of NS1 to the immune
126 response during the primary DENV infection and a secondary ZIKV challenge.
127 Beyond the cannonical humoral component, a robust T cell response is needed to confer protection after a natural
128 DENV or ZIKV infection (Elong Ngono and Shresta, 2019; Roth et al., 2018; Tian et al., 2019; Weiskopf and
129 Sette, 2014; Yauch et al., 2009) and most of the cognates epitopes for the T cells ($CD4^+$ and $CD8^+$ T cells) are
130 located in the NS proteins (Grifoni et al., 2017b; Rivino et al., 2013; Rivino and Lim, 2017). Despite this, one
131 common aspect shared among all three leading DENV vaccines is that they do not present NS proteins of all
132 DENV serotypes to the immune system. Dengvaxia is based on a Yellow Fever (YF) 17D backbone and contains
133 no DENV NS proteins (Guirakhoo et al., 2001). The Takeda vaccine uses a DENV-2 backbone and has only
134 DENV-2 NS proteins (Huang et al., 2013; Kinney et al., 1997). The NIH/Butantan/MSD vaccine has full genome
135 DENV-1, -3, and -4 components with a DENV-2/-4 chimera based on a DENV-4 backbone. This vaccine possesses
136 NS proteins from DENV-1, -3, and -4 but not of DENV2 (Blaney et al., 2006; Blaney et al., 2008; Whitehead et
137 al., 2003a; Whitehead et al., 2003b). Further, data on the contribution of T cells and their cognate epitopes on the
138 NS proteins to the vaccine efficacy, and potentially protection, is still restricted (Angelo et al., 2017; Guy et al.,
139 2008; Tricou et al., 2022; Waickman et al., 2019a; Weiskopf et al., 2015a). These data underscore how the
140 contribution of NS proteins and T cells has been understudied in the context of DENV vaccination. Pinheiro et al.
141 completed a comprehensive analysis of B and T cells epitopes shared by all three leading DENV vaccines, and
142 concluded that investing in vaccines that contain most of the epitopes involved in protective immunity (cellular
143 and humoral arms) is an important issue to be considered (Pinheiro et al., 2021). In fact, a few years ago the World
144 Health Organization (WHO) recommended that the cell mediated immunity (CMI) induced by DENV vaccines
145 should be studied in detail (Thomas et al., 2009).
146 Also, it has been shown that $CD4^+$ T cells are essential for inducing a protective immune response against DENV
147 and ZIKV (Elong Ngono et al., 2019; Grifoni et al., 2017a; Grifoni et al., 2017c; Hassert et al., 2018; Manh et al.,
148 2020; Weiskopf et al., 2015b; Wen et al., 2020); these cells play a critical role connecting the cellular and humoral
149 immune responses though unique interactions with B cells (Crotty, 2015; Swain et al., 2012). Moreover,
150 immunological characterization of T cells has identified the presence of follicular $CD4^+$ T helper cells (Tfh) or
151 "Tfh-like" in blood, known as circulating (cTfh) or peripheral (pTfh) cells. These cells have been described, in
152 terms of function, as the equivalent counterpart of germinal center (GC) Tfh cells in peripheral blood (Bentebibel
153 et al., 2013; Crotty, 2014; Morita et al., 2011). However, there is limited literature available on the role of pTfh
154 cells in DENV (Haltaufderhyde et al., 2018; Izmirly et al., 2022; Wijesinghe et al., 2020) and ZIKV vaccination
155 or infections (Liang et al., 2019; Pardy et al., 2022). Despite their critical role helping B cells form an optimal
156 humoral immune response, there are scarce works studying the direct interactions of $CD4^+$ T cells/pTfh cells

157 subsets with B cells and NS proteins, and its effect in Abs functionality against DENV and/or ZIKV (Elong Ngono
158 et al., 2019; Rivino et al., 2013; Rouers et al., 2021). Using a NHP model – rhesus macaques – we asked how the
159 lack of essential cells, such as $CD4^+$ T cells, impacts the quality of the humoral immune response (including the
160 response to NS1 protein) during a primary DENV2 infection and its consequences in the response to a secondary
161 heterologous ZIKV infection. We believe this is the type of mechanistic questions that needs to be addressed in
162 order to identify correlates of protections and ultimately design more efficient flavivirus vaccines.
163 In this work, we depleted rhesus macaques of $CD4^+$ T cells before a primary DENV2 infection and/or before a
164 subsequent ZIKV infection one year apart. A cohort was depleted of $CD8^+$ T cells before the secondary challenge
165 with ZIKV.
166 We found that the lack of $CD4^+$ T cells before infections results in a significant increase in DENV viremia and
167 DENV-NS1 antigenemia and ZIKV viremia. Lack of $CD4^+$ T cells before DENV or ZIKV also results in a delay
168 in total Abs switch documented by limited IgM and IgG production and in a significantly lower anti-DENV and
169 anti-ZIKV NS1 Abs response. We also found that Abs binding or neutralizing DENV or ZIKV after heterologous
170 challenge with ZIKV secondary infection were minimally impacted by the lack of $CD4^+$ T cells during DENV
171 primary infection. Nevertheless, the presence of these cells during the primary DENV infection were critical to
172 preserve the specificity of the Abs against the DENV, but not ZIKV EDIII domain and NS1 protein after ZIKV
173 challenge.
174 This profile of Abs functionality (proper binding and neutralization with increased cross-reactivity) was
175 associated with a significant lower frequency of pTfh TH1 and expression of markers related to B cell activation
176 – PD-I and ICOS – when $CD4^+$ T cells were not present during the primary DENV infection. Furthermore, the
177 expression of these markers correlates with the activation of the MBC, but not ASC, in the same group.
178 Interestingly, the depletion of $CD8^+$ T cells abrogates the direct correlation between those cell subsets and MBC
179 or ASC activation.
180 With our experimental design, we could reproduce *in vivo*, first the time, the mechanism inducing those CR nAbs.
181 This Abs population was described in the context of secondary DENV infections (Patel et al., 2017) and has been
182 at the center of an active debate on their protective or pathogenic contribution to the flavivirus response.
183 Our findings are critical to understand the efficacy limitations of Abs induced by current approved DENV
184 vaccines or protecting from natural infections and thus are essential to guide the new generations of DENV and
185 ZIKV vaccines. We also provide an animal model to reproduce an immunological signature of the response to
186 flaviviruses that can be used to test and challenge the protective or disease-enhancing attributes of current and
187 new vaccine candidates or, as in this work, to better characterize flavivirus interactions.
188 We are unaware of any prior work addressing the complex interactions among $CD4^+$ T cells, B cells and NS
189 proteins in response to flavivirus infection in NHPs.
190

Results

Rhesus macaque cohorts, T cell depletion and sample collection. Five groups of flavivirus-naïve rhesus macaques (*Macaca mulatta*) (SFig 1). Prior to any experimental activity, all animals were put through a quarantine period of 40 days. Depletion of T cells was performed by initial subcutaneous (s.c.) administration of 50 mg/kg of anti-CD4+ treatment at 15 days pre-challenge, followed by two intravenous (i.v.) administrations of 7.5 mg/kg at 12- and 9-days pre-challenge. Animals were depleted of CD4+ or CD8 T+ cells, or administered phosphate buffer saline (PBS), followed by a primary infection with DENV-2 ($5x10^5$ pfu s.c.) and a subsequent ZIKV challenge (1×10^6 pfu s.c.) one year later, after a second round of T cell depletion – or administration of PBS – before infection. CD4+ T cell depletion was performed on groups 1 and 3 prior to primary DENV2 infection and on groups 1 and 2 prior to secondary ZIKV infection (group 1: n=6, denoted CD4-/DV2/CD4-/ZV; group 2: n=6, denoted CD4+/DV2/CD4-/ZV; group 3: n=6, denoted CD4-/DV2/CD4+/ZV). Group 4 is CD4-competent but CD8-depleted prior to secondary ZIKV infection (n=6, denoted CD4+/DV2/CD8-/ZV). Group 5, the control group, was treated with PBS before each infection (n=5, denoted CD4+/DV2/CD4+/ZV).

Depletion of CD4+ T cells elicits a CD8+ T cell overcompensation, and vice-versa. To determine if the depletion treatment was effective, frequency of CD4+ and CD8+ T cells was assessed by immunophenotyping using flow cytometry during pre-treatment (pre-TX), post-treatment (post-TX), and post-primary DENV2 infection. Before depletion, CD4+ T cell frequency was similar in all animals and decreased by day 2 post-TX, resulting in a depletion of 99.8% (SFig 2A). This reduction was consistent until day 7 post infection (p.i.), when they began to slowly recover by day 15 post infection p.i.. Further, the absence of CD4+ T cells in depleted animals promoted an increase in CD8+ T cell frequency (SFig 2B).

CD4+ and CD8+ T cell frequencies were also measured before, during and after depletions prior to and during ZIKV challenge (SFig 2C and D). Similar to the prior scenario, CD4+ T cell frequency was similar in all animals, and decreased by day 2 after treatment start (SFig 2C). Interestingly, animals that underwent CD8+ T cell depletion experimented an increase in CD4+ T cell frequency (SFig 2C). Lastly, CD8+ T cells behaved similarly to CD4+ T cells after depletion, with frequency decreasing by day 2 post-TX and beginning to recover by day 7 post-challenge (p.c.), while CD4-depleted animals showed an increase in CD8+ T cell frequency (SFig 2D).

CD4+ T cell depletion impairs DENV2 viral clearance in flavivirus-naïve macaques. To determine if CD4+ T cell depletion enhances or reduces DENV2 replication, and how it changes depending on various depletion conditions, DENV2 RNAemia was measured in serum during the first 15 days after primary DENV2 infection. During the first 3 days p.i., viral RNA detection increased similarly in all groups (Fig 1A). By day 5 p.i., the control group starts resolution of viremia, while the CD4-depleted group still had high detectable RNAemia levels. By day 10 p.i. there was no viral RNA detection in any animal from the control group, indicating a faster

225 RNAemia resolution in this group compared to the CD4-depleted animals. This suggests a delay in viral clearance
226 in animals that are CD4-deficient. By day 13 p.i. all animals tested negative for DENV2 (Fig 1A and Table S1).
227 When evaluating the area under the curve (AUC), we found that the CD4-competent animals, although not
228 statistically significant, had lower values, on average, than the CD4-depleted group (Fig 1B). Next, we evaluated
229 the average RNAemia days, defined as the days with detectable viremia divided by the total number of days
230 during which viremia was assessed, considering the number of animals per group (Fig 1C). The undepleted
231 animals had significantly less mean RNAemia days compared to the depleted animals ($p= 0.0132$). Taken
232 together, these results suggest that $CD4^+$ T cells have an early role controlling DENV2 RNAemia, but it remains
233 unknown if this is due to a direct action or to an interaction with B cells.
234
235 **CD4+ but not CD8+ T cells are necessary to control ZIKV RNAemia in DENV-immune macaques**. To
236 assess the impact of T cell depletions in ZIKV replication in DENV2-immune animals, and how it changes
237 depending on various depletion conditions, ZIKV RNAemia was measured in serum during the first 15 days and
238 on day 30 after secondary ZIKV challenge using qRT-PCR. The CD4+/DV2/CD8-/ZV group had the lowest
239 ZIKV RNAemia compared to group CD4-/DV2/CD4+/ZV on day 1 ($p< 0.043$), to the control group
240 CD4+/DV2/CD4+/ZV on day 2 ($p< 0.007$) and to the double negative group CD4-/DV2/CD4-/ZV on days 2 and
241 4 p.c. ($p< 0.005$ and $p< 0.02$ respectively). Also, the control double positive group had significantly lower ZIKV
242 RNAemia compared to the double-depleted group on day 4 p.c. ($p< 0.01$) (Fig 1D and Table S2).
243 By day 7 p.c., there was no viral RNA detection in any group. Serum samples from days 15 and 30 p.c. were
244 tested in order to evaluate intermittent viremia. Historical data from our laboratory shows that ZIKV replication
245 in naive undepleted animals is significantly higher than ZIKV replication in DENV-immune animals regardless
246 of CD4+ T cell status.
247 We observed a difference when comparing all five groups based on AUC (Fig 1E). Although not statistically
248 significant, the CD8-depleted group had slightly lower AUC levels, even lower than the undepleted control group.
249 Additionally, the CD4-double depleted group had the highest AUC levels.
250 Lastly, when evaluating the mean RNAemia days, the control undepleted animals had the least viremia days,
251 which was significantly lower compared only to the double-depleted group (Fig 1F).
252 Next, we wanted to evaluate the actual CD4+ T cell contribution controlling the ZIKV RNAemia. For this, we
253 grouped the animals together according to their depletion status. Comparing the depleted (groups 1 and 4) vs.
254 non-depleted animals (groups 2, 3 and 5) before DENV2 infection resulted in a non-significant trend ($p< 0.064$)
255 to lower ZIKV viremia days in the undepleted groups (Table S2) and in very similar AUC values (351.8 and
256 370.2 for the depleted and undepleted groups, respectively).
257 However, when the two groups depleted before ZIKV infection (groups 1 and 2) were compared with the other
258 three undepleted groups (groups 3, 4 and 5), there was a significantly higher viremia peak by day 4 in the $CD4^+$

259 T cells-depleted animals (p= 0.03). Also, the AUC value for the CD4-undepleted groups before ZIKV infection
260 (490.0) almost doubles the value of the depleted groups (272.0) (Fig 1E). Furthermore, together, the depleted
261 groups have significantly higher mean viremia days than the CD4+ T cells undepleted groups (p= 0.006) (Fig
262 1F).
263 Taken together, these results confirm the requirement of CD4+ T cells for an optimal control of DENV2
264 replication during a primary infection. In addition, our findings propose an important role for $CD4^+$ T cells
265 controlling secondary ZIKV infection and that role being independent of prior DENV experience. Lastly, this
266 indicates that CD8+ T cells may not be required for ZIKV viral replication control in individuals with prior
267 DENV2 immunity.
268
269 **$CD4^+$ T cell depletion results in higher detection of DENV NS1.** To confirm viremia and to explore the role of
270 CD4+ T cells in the generation of NS1 antigen, we measured DENV NS1 levels in serum. Since NS1 antigen is
271 generally detected during days 1 to 9 p.i., we tested samples from baseline and days 3, 5, 7, 10, 15 and 30 post
272 DENV2 infection (SFig 3). As expected, no detectable levels of NS1 were observed at day 0 on any of the groups.
273 On days 3, 5, 7 and 10 p.i., an increase in NS1 levels was observed on all animal groups, with CD4-depleted
274 animals having slightly higher NS1 levels in comparison to the undepleted animals by day 10 p.i.. By day 15 p.i.,
275 NS1 levels were significantly higher in depleted animals ($p< 0.0001$) compared to undepleted animals, where
276 NS1 levels are completely undetectable. This is of particular interest because by this time, detection of DENV-
277 NS1 is not expected. This suggests that the lack of $CD4^+$ T cells enhances NS1 circulation, which correlates with
278 enhanced pathogenesis, further confirming a protective role for $CD4^+$ T cells during primary DENV infection.
279
280 **Lack of CD4+ T cells during a primary DENV2 infection does not hinder antibody binding to ZIKV in a**
281 **secondary exposure.** We evaluated the impact of $CD4^+$ and $CD8^+$ T cell depletion on the quantity and quality of
282 the humoral response against DENV2 and ZIKV infections. Anti-DENV IgM and IgG levels were measured after
283 primary DENV2 infection (Fig 2). CD4-depleted animals had significantly lower levels of anti-DENV IgM in
284 comparison to the undepleted animals by days 10 and 15 p.i. ($p< 0.001$) (Fig 2A). Furthermore, total anti-DENV
285 IgM produced from day 0 to day 15 p.i. was significantly lower in the CD4-depleted group in comparison to the
286 control group (p= 0.007) (Fig 2C). On the other hand, anti-DENV IgG levels were statistically different between
287 groups only on day 15 p.i., where the CD4-depleted group had lower levels compared to the control group ($p<$
288 0.001) (Fig 2B). Similar results were observed with the total anti-DENV IgG produced (from day 0 to day 30),
289 where CD4-depleted animals had significantly lower IgG values in comparison to undepleted animals (p= 0.0153)
290 (Fig 2D). IgG levels by days 60 and 90 p.i. were at similar levels in both groups. These results suggest a delay in
291 B cell activation and in isotype class switching during primary DENV infection related to the lack of $CD4^+$ T
292 cells.

293 Additionally, DENV and ZIKV binding IgM and IgG levels were measured after secondary ZIKV challenge (Fig
294 3). As expected, all animals had no detectable anti-DENV IgM at baseline and remain consistently low throughout
295 days 7,10 and 15 p.c. (Fig 3A). At day 15 p.c. only one animal from the control group and one from the
296 CD4+/DV2/CD8-/ZV group had values over the assay threshold, further indicating the positive role of $CD4^+$ T
297 cells in the magnitude of the humoral immune response during primary DENV infection. In contrast, all animals
298 had similar detectable anti-DENV IgG levels at baseline (Fig 3B). As expected, ZIKV infection transiently
299 boosted cross-reacting binding Abs which declined by day 30 p.c. in all groups (Fig 3B). However, the magnitude
300 of this boost was significantly impacted on day 15 p.c. by the lack of CD4+ T cells. Lower anti-DENV IgG levels
301 were observed in the two groups lacking CD4+ T cells before ZIKV challenge (double CD4-depleted and
302 CD4+/DV2/CD4-/ZV). The significance between group CD4-/DV2/CD4-/ZV with groups
303 CD4+/DV2/CD4+/ZV, CD4+/DV2/CD8-/ZV and CD4-/DV2/CD4+/ZV was statistically significant ($p< 0.019$,
304 $p< 0.008$ and $p< 0.047$ respectively). The differences between the CD4+/DV2/CD4-/ZV with the
305 CD4+/DV2/CD4+/ZV and CD4+/DV2/CD8-/ZV groups were also significant ($p< 0.018$ and $p< 0.040$
306 respectively). There were no differences among the three groups with $CD4^+$ T cells before ZIKV infection.
307 Altogether, these results indicate that DENV-experimented $CD4^+$ T cells do not contribute to an optimal humoral
308 immune response to ZIKV. This also suggests that DENV MBCs have a limited expansion during a secondary
309 ZIKV infection and if they have, the expansion is CD4-independent.
310 Anti-ZIKV IgM levels became detectable by day 7 p.c. with a peak by day 15 and started declining by day 30 p.c.
311 (Fig 3C). By day 10 p.c., the control group had significantly higher levels than at least two groups, the doubled-
312 depleted group ($p< 0.030$) and the CD4-/DV2/CD4+/ZV group ($p< 0.033$). By day 15 p.c., the differences between
313 the control undepleted group and the two other groups undepleted of CD4+ T cells before ZIKV challenge were
314 significant (CD4-/DV2/CD4+/ZV, $p< 0.022$ and CD4+/DV2/CD8-/ZV, $p< 0.028$). However, the two groups
315 depleted of CD4+ T cells before ZIKV challenge have the lowest levels of ZIKV-binding IgM compared to the
316 three undepleted groups (group CD4+/DV2/CD4+/ZV vs. CD4-/DV2/CD4-/ZV and CD4+/DV2/CD4-/ZV $p<$
317 0.0001 and $p< 0.0001$ respectively, group CD4-/DV2/CD4+/ZV vs. CD4-/DV2/CD4-/ZV and CD4+/DV2/CD4-
318 /ZV $p< 0.001$ and $p< 0.053$ respectively and group CD4+/DV2/CD8-/ZV vs. CD4-/DV2/CD4-/ZV and
319 CD4+/DV2/CD4-/ZV $p< 0.001$ and $p< 0.004$ respectively).
320 Of note, some of the CD4-/DV2/CD4-/ZV animals had a late modest boost in ZIKV IgM levels by day 30 p.c.
321 after the initial observed delay; interestingly, all depleted animals before ZIKV challenge induced the lowest IgM
322 levels. Together, these observations suggest a delayed ZIKV-naïve B cell expansion in absence of CD4+ T cells.
323 Results from ZIKV-binding IgG Abs were similar to the IgM results (Fig 3D). By day 10 p.c. the two groups
324 depleted of CD4+ T cells before the challenge showed the lowest values. The values were significantly lower in
325 the group depleted of CD4+ T cells only before ZIKV challenge (CD4+/DV2/CD4-/ZV) compared to the
326 CD4+/DV2/CD4+/ZV ($p< 0.004$) and CD4-/DV2/CD4+/ZV ($p< 0.0001$) groups. By day 15 p.c. this trend was

327 maintained. Results reached statistical significance between the control undepleted group and the double-depleted
328 group ($p< 0.003$) and the CD4+/DV2/CD4-/ZV group ($p< 0.001$) and between the CD4-/DV2/CD4+/ZV group
329 with the CD4-/DV2/CD4-/ZV and the CD4+/DV2/CD4-/ZV groups ($p< 0.001$ and $p< 0.0007$ respectively).
330 An interesting finding was the presence of detectable cross-reacting anti-ZIKV IgG levels at baseline only in the
331 group depleted of CD4+ T cells before primary DENV infection but not before ZIKV challenge (CD4-
332 /DV2/CD4+/ZV) with significant higher values when compared to the CD4+/DV2/CD4-/ZV and the
333 CD4+/DV2/CD4+/ZV groups ($p< 0.031$ and $p< 0.037$ respectively). The double-depleted group also had higher
334 cross-reactive IgG levels compared to the undepleted groups but did not reach statistical significance. This result
335 suggests an increased expansion of DENV-cross-reactive B cell clones in animals without $CD4^+$ T cells during
336 primary DENV infection.
337 To confirm our results on the anti-ZIKV IgG, we analyzed the total ZIKV-specific IgG produced on days 10, 15
338 and 30 p.c. (Fig 3E). Double-depleted animals had significantly less total ZIKV IgG production compared to the
339 CD4-competent animals before ZIKV challenge ($p= 0.0002$ compared to CD4-/DV2/CD4+/ZV group, $p= 0.0200$
340 compared to CD4+/DV2/CD8-/ZV group and $p= 0.0065$ compared to CD4+/DV2/CD4+/ZV). Similarly, the
341 CD4+/DV2/CD4-/ZV group had lower IgG production compared to the CD4-/DV2/CD4+/ZV, CD4+/DV2/CD8-
342 /ZV and CD4+/DV2/CD4+/ZV groups ($p= 0.0003$, $p= 0.0322$ and $p= 0.0108$, respectively). In general, CD4-
343 depleted animals before ZIKV challenge produced significantly less total ZIKV-specific IgG.
344 These results indicate that while $CD4^+$ T cells during a primary DENV infection have limited impact in the binding
345 of ZIKV-specific Abs, their absence may result in a significant induction of flavivirus CR B cell clones (Fig 2D)
346 during a secondary infection. This also indicates that DENV-induced MBC populations generated during a
347 primary DENV infection have limited contribution in the anti-ZIKV-binding Abs after ZIKV infection. In
348 addition, these results suggest that $CD4^+$ T cells are required for a more effective and specific antibody response
349 against ZIKV.
350
351 **Recall memory to DENV NS1 is expanded by lack of CD4+ T cells before DENV2 infection.** Next, we
352 performed an anti-DENV NS1 assay using samples from baseline and days 15, 30 and 90 after primary DENV2
353 infection (SFig 4A). No DENV NS1 anti-IgG levels were detected on day 0 as expected. However, by day 15 p.i.,
354 depleted animals had significantly lower levels in comparison to the undepleted animals, similar to anti- DENV
355 IgG levels previously presented ($p= 0.0003$). By day 30 p.i., both groups had reached similar anti-NS1 IgG levels.
356 Interestingly, by day 90 p.i. depleted animals showed significantly higher anti-NS1 IgG levels compared to
357 undepleted animals ($p= 0.0003$), altogether supporting the delay in Abs switch in this group as seen in the total
358 IgG Abs response.
359 We also measured DENV NS1 anti-IgG levels in samples from baseline and days 15, 30 and 90 after secondary
360 ZIKV challenge (SFig 4B). Of note, on baseline the group depleted of $CD4^+$ T cells before DENV infection but

361 not ZIKV (CD4-/DV2/CD4+/ZV) had higher values when compared to the groups with $CD4^+$ T cells prior to
362 DENV infection, and they were significantly higher particularly in comparison to the CD4+/DV2/CD4-/ZV group
363 (p= 0.002). Although not significant, the double-depleted group had similarly high values in comparison to the
364 groups with CD4+ T cells prior to DENV infection. This implies that the lack of $CD4^+$ T cells before primary
365 DENV infection also results in an increased durability of NS1 Abs. By day 15 p.c., we observed significantly
366 higher values of DENV NS1 anti-IgG in the CD4-/DV2/CD4+/ZV group in comparison to all other groups (CD4-
367 /DV2/CD4-/ZV, p= 0.0124; CD4+/DV2/CD4-/ZV, p= 0.0002; CD4+/DV2/CD4+/ZV, p= 0.0094 and
368 CD4+/DV2/CD8-/ZV, p= 0.0152). This trend is maintained by day 30 p.c. (CD4-/DV2/CD4-/ZV, p= 0.0053;
369 CD4+/DV2/CD4-/ZV, p< 0.0001; CD4+/DV2/CD4+/ZV, p< 0.0001 and CD4+/DV2/CD8-/ZV, p< 0.0001).
370 Intriguingly, similar to what we stated on the previous section, these results seem to imply that deficiency of $CD4^+$
371 T cells during the primary DENV2 infection modifies the recall memory quantitatively resulting in an increased
372 DENV NS1-cross-reactivity induced by ZIKV.
373 Lastly, no significant differences between groups were observed on day 90 p.c.. Of note, some animals belonging
374 to the CD4+/DV2/CD4-/ZV group exhibited a marked separation between IgG values, indicating that antibody
375 repertoire may be different between individuals even sharing similar conditions.
376
377 **Depletion of CD4+ T cells only prior to DENV infection increases ZIKV NS1-binding IgG after ZIKV**
378 **infection.** To further study the impact of CD4+ T cells in the generation of CR and specific Abs, we measured
379 anti-ZIKV NS1 IgG levels in serum at days 0, 15, 30 and 90 p.c. (SFig 5). Contrary to the total IgG, no animals
380 had, regardless of their CD4+ T cell status, detectable anti-ZIKV-NS1 IgG Abs at baseline, indicating that those
381 Abs are very specific. However, by day 15 p.c., only animals with CD4+ T cells before ZIKV challenge produced
382 detectable IgG levels, with the exception of one animal from the CD4+/DV2/CD4-/ZV group (SFig 5). Double-
383 depleted animals had the lowest IgG levels of all groups, significantly different from the control group (p= 0.0003)
384 and the CD4-/DV2/CD4+/ZV group (p= 0.0004) on day 15 p.c. Additionally, NS1 IgG levels from the double-
385 depleted group were significantly lower in comparison to the CD4-/DV2/CD4+/ZV group (p= 0.0071) also on
386 day 30 p.c.. The latter group was consistently the highest IgG-producing group, even higher than the control
387 group. Unsurprisingly, CD8-depleted animals behaved similarly to the control group as they both have intact CD4
388 T cells populations before the primary infection and the challenge. By day 90 p.c. almost all animals, regardless
389 of their depletion status, had no NS1-IgG levels. Collectively, these data suggests that DENV-experimented $CD4^+$
390 T cells have limited or no effect on the production of anti-ZIKV NS1-abs during a secondary ZIKV exposure.
391
392 **Early DENV2 neutralization activity is delayed by lack of CD4+ T cells.** To determine the contribution of Abs
393 to DENV2 and ZIKV neutralization and the impact of CD4+ and CD8+ T cell depletion, all groups were tested
394 using FRNT and PRNT assays against DENV2 and ZIKV respectively (Fig. 2 and 3). Neutralization assays were

395 completed for baseline and days 15, 30 and 90 p.i. for DENV-2 and days 15, 30 and 90 p.c. for ZIKV. The 50%
396 effective concentration (EC) of neutralizing Abs is shown in Figures 3 and 4 as well. As expected, no animals
397 had nAbs against DENV2 at baseline before primary infection (Fig 4A). By day 15 p.i., a significantly lower
398 neutralizing activity is observed in the CD4-depleted animals in comparison to the undepleted group ($p < 0.0001$)
399 (Fig 4B). This trend is not observed on day 30 p.i., when both groups behave similarly (Fig 4C). Endpoint titers
400 dilutions are shown in Figures 3D and 3E in order to facilitate a better interpretation. Similar to the scenario
401 described above, significantly lower EC50 values are observed in the depleted animals ($p = 0.0032$) (Fig 4D).
402 PRNT assays were performed after ZIKV challenge; despite the detection of anti-ZIKV IgG at baseline in some
403 groups, no neutralizing activity was observed at that time point against ZIKV (Fig 5A). However, on day 15 p.c.,
404 the CD4-double depleted group was the lowest at neutralizing ZIKV (Fig 5B). Animals belonging to the CD4-
405 /DV2/CD4+/ZV group and interestingly those from group CD4+/DV2/CD8-/ZV, having a higher frequency of
406 CD4+ T cells, have better neutralizing activity by day 15 p.c. compared to the other three groups – even the
407 undepleted control group. Those differences were statistically significant compared to the double-depleted group
408 ($p = 0.0231$ and $p = 0.0155$ respectively). Taken together, these results lead us to consider that DENV-primed
409 $CD4^+$ T cells have a limited contribution to an effective early neutralization during a secondary ZIKV infection
410 and that the magnitude of neutralization can correlate with the frequency of those cells.
411 By day 30 p.c., a change in neutralization profile is observed in all groups except the double-depleted animals,
412 which still show a delay in neutralization in comparison to the other groups (Fig 5C). By day 90 p.c., all groups
413 behave similarly in terms of neutralization magnitude (Fig 5D). Endpoint titers dilutions are shown in Figures 4F-
414 H in order to facilitate a better interpretation.
415 To further study the impact of CD4+ T cells on the humoral response, neutralization assays were performed
416 against DENV2 using serum samples from 90 days post ZIKV challenge (Fig 5E and 5I). No statistical differences
417 were detected between any of the groups.
418
419 **Lack of CD4+ T cells during a primary DENV2 infection compromises antibody specificity in a secondary**
420 **ZIKV exposure**. To further explore the role of CD4+ T cells in shaping the immune response against DENV and
421 ZIKV, epitope reactivity against EDIII and NS1 was evaluated using a Luminex assay. EDIII was selected as a
422 major neutralizing target and NS1 due to its proposed role in pathogenesis. Type-specific and CR responses were
423 measured at day 90 p.i. after DENV2 and ZIKV infections against EDIII and NS1 antigens from all four DENV
424 serotypes and ZIKV. To normalize the data a cross-reactivity index (CRI) was calculated by subtracting the
425 background from the homologous value (values against ZIKV) and the heterologous values (values against
426 DENV2) followed by dividing the subtracted homologous by the subtracted heterologous values (Fig 6).
427 As expected, after primary DENV2 infection, both undepleted and depleted animals had a high similar type-
428 specific response against EDIII and NS1 to the infecting serotype DENV2, and a similar limited cross-reactivity

429 against EDIII to the other DENV serotypes, with a significant difference observed in DENV4 EDIII (p= 0.0265)
430 (Fig 6A). As reported in the literature, primary DENV infection in flavivirus-naïve individuals triggers minimal
431 or no cross-reactivity to ZIKV (Collins et al., 2017; Duffy et al., 2009). Both groups show a similar high type-
432 specific response against DENV2 NS1 protein. In contrast to the response to EDIII domain, a higher CR response
433 against NS1 to the other DENV serotypes, particularly against DENV serotype 1, was observed (p= 0.0045).
434 Interestingly, there was a limited but detectable cross-reactivity against ZIKV NS1 protein in both groups (Fig
435 6A). This suggests that during a primary DENV infection, CD4+ T cells have a different weight in the responses
436 against EDIII domain and NS1 protein.
437 A more complex scenario is observed after secondary ZIKV infection (Fig 6B). All animals show an increase in
438 the type-specific response against EDIII and NS1 towards the infecting ZIKV, but their reactivity against other
439 DENV serotypes varies. Additionally, all groups show reactivity against DENV2 antigens. Of note, two animals
440 from the undepleted control group NS1 (MA254 and MA264) had a higher type-specific response against DENV2
441 EDIII, and two had a lower response (MA311 and MA350) (Fig 6B), indicating that antibody repertoire can be
442 different between individuals, a point to take into consideration in vaccine design. Lastly, as expected, the
443 CD4+/DV2/CD8-/ZV group behaved very similar to the CD4-competent groups, demonstrating that $CD8^+$ T cell
444 depletion does not have an impact in epitope reactivity (Fig 6B). Taken together, these results indicate that while
445 $CD8^+$ T cell depletion increases $CD4^+$ T cell frequency and results in better ZIKV viremia control, quantity has
446 no effect on the specificity of epitope recognition. Overall, the cross-reactivity to NS1 protein was more
447 significant among DENV serotypes than to EDIII domain suggesting that, like after a primary DENV infection,
448 CD4+ T cells have a different weight in the response against EDIII domain and NS1 protein after a secondary
449 ZIKV infection in DENV-immune individuals. Animals belonging to all groups, regardless of depletion condition,
450 show an increased but similar response against EDIII and NS1 to the infecting ZIKV. These results suggest that
451 pre-existing DENV immunity has a very limited impact in the cross-reactivity to ZIKV EDIII domain and NS1
452 protein, and that $CD4^+$ T cells have a limited role regulating that cross-reactivity to those ZIKV targets.
453
454 **CD4+ T cells modulate early response IgG+ MBC activation and functionality during a heterologous ZIKV**
455 **infection with previous DENV-2 immunity**. To determine the contribution of CD4+ T cells in modulating the
456 surface IgG+ memory B cell (IgG MBC) (CD3-CD20+CD27+sIgG+) response to ZIKV, we measured expression
457 levels of surface IgG in total MBCs via Flow Cytometry. Whole blood for Immunostaining was collected on
458 baseline and days 7, 15, and 30 p.c. with ZIKV. By day 15 p.c., a discrete peak in frequencies was observed in all
459 groups, except the CD4+/DV2/CD8-/ZV group which remain at the baseline levels (Fig 7A). On day 15 p.c., the
460 CD4+/DV2/CD4+/ZV and the CD4-/DV2/CD4+/ZV were the only groups showing a significant increase in IgG
461 expression when compared to their baseline status. By day 30 p.c., all groups showed similar levels of IgG
462 expression to their baseline status. We did not detect a significant difference in IgG expression among groups

463 compared at each timepoint suggesting that sIgG expression is not impacted by depletion treatment status. Taken
464 together, this data suggests that a secondary ZIKV infection in individuals with prior DENV-2 immunity, IgG+
465 MBCs levels suffer limited increase in frequency and if it happens it is CD4+ T cell-dependent.
466 To determine if CD4+ T cells have a role in modulating the activation pathways of IgG MBCs after the ZIKV
467 challenge, we surveyed the expression of CD69 in this cell population. Compared to their baseline, a significant
468 increase in the frequency of IgG MBCs expressing CD69 was observed at day 7 p.c. only on those groups with
469 intact CD4+ T cells before ZIKV challenge (CD4-/DV2/CD4+/ZV, $p< 0.034$; CD4+/DV2/CD4+/ZV, $p< 0.024$;
470 and CD4+/DV2/CD8-/ZV, $p< 0.010$) (Fig 7B and 7C). Taken together, this data suggests that IgG MBCs go
471 through $CD4^+$ T cell-dependent interactions for their activation during a secondary ZIKV infection.
472 To determine if CD4+ T cells have a role in the differentiation of activated B cells into ASC (possibly through
473 GC) formation) during a heterologous ZIKV infection we measured CD69 expression in total ASCs
474 ($CD20^{dim}CD3^-CD27^+$). Despite our gating strategy being designed to characterize rhesus macaque plasmablast
475 populations (Silveira et al., 2015) (Zhang et al., 2019), we were unable to segregate that population. However as
476 previously reported by us (Marzan-Rivera et al., 2022), we idetified the total ASC population and observed a
477 trend in activation of that population during the ZIKV infection. At day 7 p.c., we detected a significant increase
478 in CD69 expression in the CD4+/DV2/CD4+/ZV and the CD4-/DV2/CD4+/ZV groups, compared to the other
479 groups (Fig 7D, $p< 0.005$). This peak in CD69 expression was maintained up to day 15 p.c. in the
480 CD4+/DV2/CD4+/ZV group followed by a significant drop in CD69 expression levels at day 30 p.c.. Levels of
481 expression observed during this timepoint were of similar magnitude as on baseline. On the other hand, groups
482 that lacked CD4+ T cells before the ZIKV infection (CD4+/DV2/CD4-/ZV and CD4-/DV2/CD4-/ZV) showed a
483 trend of lower levels of ASCs expressing CD69 compared to the $CD4^+$ T cell competent groups before the ZIKV
484 infection. Our data points to a *de novo* B cell response pathway that is T cell-dependent to promote the formation
485 of both IgG MBCs and ASC subsets with good neutralizing capacity towards ZIKV.
486
487 **$CD4^+$ Peripheral T helper cells (pTfh) are present in circulating blood of Rhesus macaques during**
488 **heterologous ZIKV infection with prior DENV2 immunity**. To confirm the presence and frequency of pTfh
489 CD4+ T cells, we measured expression levels of surface markers such as CXCR5, CXCR3, PD-1, and ICOS via
490 flow cytometry analysis. Chemokine receptor CXCR3+ expression has been associated with Type 1 T helper
491 immune responses (Th1) (Sánchez-Vargas and Mathew, 2019; Sandberg et al., 2021; White et al., 2021).
492 Moreover, PD-1 and ICOS expression is associated with B cell interaction in order to induce B cell activation
493 (Lei et al., 2021; Shi et al., 2018). This analysis was performed only on groups CD4+/DENV2/CD4+/ZIKV, CD4-
494 /DENV2/CD4+/ZIKV and CD4+/DENV2/CD8-/ZIKV because they have CD4+ T cell populations before ZIKV
495 infection. Frozen PBMCs were used for Immunostaining on day 0 (baseline), day 7, and day 15 p.c. with ZIKV.
496 Surface expression of CD4+/CXCR5+ was detected at similar levels in all three tested groups, confirming

497 circulation of pTfh cells. No significant differences were established among groups after ZIKV challenge on day
498 7 p.c.. By day 15 p.c. the control undepleted group and the group lacking CD4+ T cells before DENV showed a
499 trend to higher frequencies compared to the group depleted of CD8+ T cells before ZIKV challenge, and that
500 difference reached significant values between that group and the CD4-/DENV2/CD4+/ZIKV group on day 15
501 p.c. ($p< 0.039$) (Fig 8A). Despite having similar frequency of pTfh T cells (CD4+/CXCR5+), results demonstrate
502 significance on days 0, 7, and 15 p.c. regarding the frequency of total pTfh TH1 CD4+ T (CXCR5+ and CXCR3+)
503 among groups with the group depleted of CD4+ T cells before DENV infection, having significant lower
504 frequencies of those cells since baseline ($p< 0.000$ and $p= 0.0034$) and on day 7 p.c. ($p< 0.000$ and $p= 0.0306$)
505 compared to groups CD4+/DENV2/CD4+/ZIKV and CD4+/DENV2/CD8-/ZIKV, respectively. On day 15 p.c.
506 there was a marked decrease in the frequency of TH1 cells in the CD4+/DENV2/CD8-/ZIKV group compared to
507 the control undepleted group ($p= 0.0009$). The difference in frequency was also significant between the control
508 group and the CD4-/DENV2/CD4+/ZIKV group ($p= 0.0012$) (Fig 8B). Further, we wanted to assess the co-
509 expression of B cell co-stimulating markers PD-1 and ICOS in order to correlate these results with B cell
510 activation. The frequency of total CD4+ pTfh T cells CD4+/CXCR5+/CXCR3+/PD-1+/ICOS+ showed a trend to
511 increase from baseline to day 7 p.c. in all three groups. We found same profile as with the TH1 pTfh cells were
512 the CD4-/DENV2/CD4+/ZIKV show statistically significantly lower frequency of these co-stimulating markers
513 at baseline and on days 7 and 15 p.c. compared to groups CD4+/DENV2/CD4+/ZIKV and CD4+/DENV2/CD8-
514 /ZIKV ($p<0.0001$ in all comparisons) (Fig 8C). Gating strategy is shown in Figure 6D.
515 When evaluating the total CD4+ T cell memory phenotype subsets (naïve CD28+CD95-, effector memory (EM)
516 CD28-CD95-, and central memory (CM) CD28+CD95+), CM pTfh CD4+ T cells (CD4+/CD28+/CD95+) remain
517 to be the more abundant phenotype of CD4+ memory T cells. (data not shown). To explore the impact of CD4+
518 and CD8+ T cell depletion on the frequencies of CM CD4+ pTfh T cells (CD4+/CD28+/CD95+/CXCR5+) we
519 measured frozen Peripheral Blood Mononuclear Cells (PBMC) collected on day 0 (baseline), day 7, and day 15
520 p.c. with ZIKV via flow cytometry analysis (Fig 9A). Overall, there was a non-significant trend to increased
521 frequency in all groups from day 0 to day 7 p.c. followed by a contraction on day 15 p.c. On baseline, the frequency
522 was significantly lower in the CD4-/DENV2/CD4+/ZIKV group compared to the CD4+/DENV2/CD8-/ZIKV
523 group ($p< 0.002$). However, there were no differences among groups after the challenge. Results also demonstrate
524 a limited expansion of CM CD4+ pTfh T cell subsets co-expressing CD28+/CD95+/CXCR5+/CXCR3+ in all
525 groups on day 7 p.c. with a contraction in the CD4+/DENV2/CD8-/ZIKV group by day 15 p.c.. As similarly seen
526 with prior CD4+ pTfh profiles, the CD4+ T cell-deficient group before primary DENV infection result in the
527 lowest frequency since baseline ($p< 0.0001$ and $p= 0.0024$), on day 7 ($p< 0.0001$ and $p= 0.0209$), and on day 15
528 p.c. ($p< 0.0001$ and $p= 0.0008$) compared to groups CD4+/DENV2/CD4+/ZIKV and CD4+/DENV2/CD8-/ZIKV,
529 respectively (Fig 9B). We also measured the CM subpopulation co-expressing the B cell co-stimulating markers
530 PD-1+ and ICOS+ (CD28+/CD95+/CXCR5+/CXCR3+/PD1+/ICOS+) (Fig 8C). All three groups showed the

531 same trend as in the other CM population with a very limited expansion on day 7 and a trend to contraction on
532 day 15 p.c. in the control undepleted and in the CD4+/DENV2/CD8-/ZIKV groups but not in the CD4-
533 /DENV2/CD4+CD4+/ZIKV, which remains with similar frequency as day 7 p.c.. Interestingly, at baseline the
534 CD4+/DENV2/CD8-/ZIKV group showed a frequency of these cells that is significantly higher compared to the
535 control group ($p< 0.035$). But again, the CD4+ T cell-deficient group before primary DENV infection resulted in
536 the lowest frequency since baseline ($p= 0.0412$ and $p< 0.000$), on day 7 ($p= 0.0046$ and $p< 0.0001$), and on day
537 15 p.c compared to groups CD4+/DENV2/CD4+/ZIKV and CD4+/DENV2/CD8-/ZIKV respectively (Fig 9C).
538 There were no significant differences among groups on day 15 p.c. This data on the pTfh cells subsets show a
539 detectable, but minimal expansion of the pTfh cells, and its CM compartment, during heterologous ZIKV
540 infection in prior DENV2 immune individuals.
541
542 **Total and DENV primed CM pTfh cells modulate both B cell subset activation and serum antibody**
543 **response functionality towards ZIKV.** To determine if the frequencies of peripheral pTfh cells are directly
544 associated with the humoral response to the secondary infection with ZIKV, we performed Pearson correlation
545 and simple linear regression analysis comparing pTfh cell frequencies with the neutralization assays and the IgG^+
546 MBC immunophenotyping data. For the IgG^+ MBC analysis, we compared three pTfh cell phenotypes: $CXCR5^+$,
547 $CXCR5^+CXCR3^+$, and $CXCR5^+CXCR3^+PD1+ICOS^+$, at days 7 and 15 p.c., with frequencies of activated IgG^+
548 MBCs, and activated ASCs, at 7 days p.c.. While we did perform correlation analysis comparing the pTfh cell
549 frequencies with frequencies of total IgG^+ MBCs, at 15 days p.c., we did not observe any significant correlation
550 (data not shown). Interestingly, we observed a very low yet significant positive correlation ($R^2= 0.3237$, $P=$
551 0.0214) when we compared frequencies of $CXCR5^+$ pTfh cells from day 7 p.c.. with total IgG^+ MBC frequencies
552 from day 15 p.c. from all animals in the study (SFig 6A). From these observations, we decided to perform these
553 correlation analyses with the experimental groups separately as we observed that correlations vary significantly
554 depending on both cell phenotype and T-cell depletion treatment. When comparing the $CXCR5^+CXCR3^+$ pTfh
555 cell phenotype, from Day 7 p.c., with the activated IgG^+ MBC phenotype, we observed very high and significant
556 correlations in both the CD4+/DV2/CD4+/ZV and the CD4-/DV2/CD4+/ZV groups ($R^2= 0.8790$, $P= 0.0186$ and
557 $R^2= 0.7424$, $P= 0.0274$, respectively) (SFig 6B and 6C). In addition, a very positive and significant correlation
558 with activated IgG^+ MBC and $PD1^+ICOS^+$ pTfh cell frequencies was observed in the CD4-/DV2/CD4+/ZV group
559 ($R^2= 0.7028$, $P= 0.0371$) (SFig 6D). When comparing the $CXCR5^+$ pTfh cells, from Day 7 p.c., with activated
560 ASCs, we only observed a highly negative and significant correlation in the CD4-/DV2/CD4+/ZV group ($R^2= -$
561 0.7, $P= 0.0378$) (SFig 6E). On the other hand, we did not observe a significant correlation when performing this
562 comparison in the CD4+/DV2/CD4+/ZV group. Furthermore, when we compared the frequencies of $PD1^+ICOS^+$
563 pTfh cell, from Day 7 p.c., with activated ASCs we observed a very high and significant positive correlation in
564 the CD4+/DV2/CD4+/ZV group only ($R^2= 0.8657$, $P= 0.0218$) (SFig 6F) These observations suggest that not

565 only the B cell response is modulated by possible interactions with these pTfh cell subsets, but also these
566 mechanisms are strongly influenced by the nature of the initial priming with DENV. Also, the opposed correlation
567 (MBC vs. ASC) in the CD4-/DV2/CD4+/ZV group strongly suggest that lack of DENV-experimented pTfh
568 modify the interactions with the MBC and ASC in different ways. As part of this analysis, we also measured
569 possible correlations between these B cell subsets and these same pTfh cell subsets but focusing on CM pTfh
570 cells. Here, we only observed very high and significant positive correlations between the $CXCR5^{+}CXCR3^{+}$ and
571 $PD1^{+}ICOS^{+}$ CM pTfh cells, from day 7 p.c., and activated IgG^{+} MBCs and activated ASCs in the
572 CD4+/DV2/CD4+/ZV group (SFig 7). This data adds new insight to the impact of the initial DENV priming in
573 the secondary ZIKV infection response, as CM pTfh cells only show a significant correlation in the expansion of
574 activated B cell subsets in animals with DENV- primed $CD4^{+}$ T cells. Lastly, the CD4+/DV2/CD8-/ZV group
575 showed no significant correlations between any of the pTfh, and B cell subsets analyzed (data not shown). This
576 could suggest a role or possible interactions of CD8+ T cells with either pTfh cells and/or B cell subsets during
577 the ZIKV infection.
578 To characterize the impact of pTfh cells in the functionality of the antibody response towards the ZIKV infection,
579 we performed Pearson correlation and linear regression analysis comparing the previous pTfh cell phenotypes,
580 during day 7 and 15 p.c.., with the EC50 data (from ZIKV and DENV neutralizations) gathered from days 30 and
581 90 p.c.. Similar to what we observed in the correlation analysis with IgG^{+} MBCs and ASCs, only the
582 CD4+/DV2/CD4+/ZV group showed highly positive and significant correlations between pTfh cell phenotypes
583 and the ZIKV EC50 values (SFig 8). When comparing frequencies of $CXCR5^{+}$ pTfh cells, from both days 7 p.c.
584 and 15 p.c., with EC50 values from day 30 p.c., we observed a high and significant positive correlation at both
585 timepoints (R^2= 0.8317, p= 0.0309 and R^2= 0.9115, p= 0.0115, respectively) (SFig 8A and 8B). In the EC50 from
586 day 90 p.c., we only observed significant positive correlations with the frequencies of $PD1^{+}ICOS^{+}$ pTfh cells in
587 both day 7 and 15 p.c. timepoints (R^2= 0.7796, p= 0.0472 and R^2= 0.9182, p= 0.0102, respectively) (SFig 8C and
588 8D). We did not observe any significant correlations with $CXCR5^{+}CXCR3^{+}$ pTfh cells (data not shown). As part
589 of this analysis, correlations were performed with CM pTfh cell subsets as well. Similar to previous results, only
590 the CD4+/DV2/CD4+/ZV group showed significant positive correlations between CM pTfh cell frequencies and
591 ZIKV EC50 values from days 30 and 90 p.c.. Interestingly, for both the $CXCR5^{+}$ from day 15 p.c. and the
592 $CXCR5^{+}CXCR3^{+}$ pTfh cells from days 7 and 15 p.c., a strong positive correlation was observed when compared
593 with EC50 values from day 30 p.c. when comparing all animals in the study (R^2= 0.3282, p= 0.0204, R^2= 0.2863,
594 p= 0.0269, R^2= 0.5100, p= 0.0019 respectively) (SFig 8E, 8F and 8G). Similar to previous observations, there
595 were significant positive correlations for $CXCR5^{+}$ CM pTfh cells from days 7 and 15 p.c., when compared to
596 EC50 values from day 30 p.c. (R^2= 0.9505, p= 0.0470, and R^2= 0.9063, p= 0.0125 respectfully) (SFig 8H and 8I).
597 Lastly, the only positive correlation observed in EC50 values from day 90 p.c. was observed when comparing
598 frequencies of $PD1^{+}ICOS^{+}$ CM pTfh cells from day 15 p.c. (R^2= 0.8214, p= 0.0339) (SFig 8J). Taken together,

599 this data adds new insight to the importance of the nature of the initial priming with DENV and its impact on the
600 ZIKV infection, as we also saw strong correlations between CM pTfh cell phenotypes and EC50 values in the
601 CD4+/DV2/CD4+/ZV group only. This suggests that DENV-primed CM pTfh cells may play an important role
602 in modulating an efficient antibody response to ZIKV. In addition, this data adds more weight to the role of
603 $ICOS^{+}PD1^{+}$ pTfh cells in priming a robust Abs response with efficient neutralizing capacity towards flavivirus.
604 Interestingly, we observed no significant correlations when we compared total and CM pTfh cells with EC50
605 values, from day 90 p.c., that neutralized DENV-2 in any experimental group (data not shown). These results
606 suggest that, while possible DENV-primed CM pTfh cells help to modulate the potency of nAbs to ZIKV, these
607 mechanisms between pTfh cells and the B cell response may be only part of the type-specific humoral response
608 to ZIKV. These results further support a T-cell dependent humoral immune response to ZIKV in DENV-
609 experimented individuals.
610
611 **Principal component analysis recapitulates infection dynamics.** To reduce the dimensionality of our data –
612 which included ELISAs, neutralization assays, flow cytometry, and cross reactivity indexes – we performed
613 principal component analysis (PCA). Interestingly, PCA projections of days 15 and 30 p.c. corroborate our assay-
614 by-assay results. More specifically, both principal component projections separate most of the CD4+/DV2/CD4-
615 /ZV and the double depleted groups (CD4-/DV2/CD4-/ZV) samples – most samples have positive values on PC2
616 on days 15 p.i. (Fig 10A and 10B). Also, most of the samples from the undepleted CD4+/DV2/CD4+/ZV group
617 and from the CD4-/DV2/CD4+/ZV and CD4+/DV2/CD8-/ZV groups having in common the presence of $CD4^{+}$ T
618 cells before ZIKV infection cluster together – having positive values in PC1 and negative value in PC2 on day 15
619 p.c. and negative values in PC1 on day 30 p.c.–, which corroborates our results that demonstrates that these groups
620 behave similarly at day 15 p.i.. Of particular interest is the group with CD4+ T cells depleted before primary
621 DENV infection but not before ZIKV challenge. On day 15 p.c., half of samples (n=3) clustered very close with
622 the double-depleted group with a positive value in PC1 similar to the other three samples from same group, but
623 with a positive value in PC2 as the double depleted group.
624 PCA projections of day 90 post ZIKV challenge shows how groups return to baseline, as there is no clear
625 separation between groups (Fig 10C). This is in line with our individual assay-by-assay results that show that,
626 after 90 days of infection, there is no significant difference between groups. Eighty three percent (83%) of the
627 samples from the CD4-/DV2/CD4+/ZV showed negative PC1 clustering with 100% of samples from the double
628 depleted group and 66% of samples from the group depleted of CD4+ T just before ZIKV infection
629 (CD4+/DV2/CD4-/ZV). Nevertheless, after incorporating the CRI to our PCA matrix there is a dramatic shift
630 with the CD4-/DV2/CD4+/ZV group (83% of samples) clustering together with all samples from the CD4-
631 /DV2/CD4-/ZV double-depleted groups with most having now positive PC1 values of over 0.5 between most of
632 the samples. This way those groups remained separated from the other groups (Fig 10D). These results are in line

633 with our individual assay-by-assay results that show that, in most of the results the double depleted group and the
634 CD4+/DV2/CD4-/ZV trend to have similar results while the CD4-/DV2/CD4+/ZV have similar profile from the
635 two undepleted groups (CD4+/DV2/CD4+/ZV and CD4+/DV2/CD8-/ZV). Such a drastic change in the
636 dimensionality of the data reflects the impact of the CRI and most importantly, this analysis strongly suggest a
637 differential impact of CD4+ T cells on the Abs binding and neutralization properties versus specificity generating
638 immune memory during a primary DENV infection.
639 Overall, PCA projections recapitulated the individual trends in the various analysis performed within our dataset.
640 PCA further provides an excellent resource to corroborate the validity and biological importance of our results.
641

642 **Discussion**

643 Despite many years or research, DENV pathogenies is not completely understood. However, it is recognized that
644 a balanced humoral and T cell immune response is essential to drive protection.

645 The role of $CD4^+$ T cells in DENV pathogenesis evolved over the years. Early data and subsequent works
646 highlighted their role in DENV pathogenesis (Duangchinda et al., 2010; Mangada and Rothman, 2005; Rivino et
647 al., 2013; Rouers et al., 2021; Yu et al., 2022). However, more recent works show a relationship between these
648 cells and DENV protection (Graham et al., 2020; Weiskopf et al., 2015b). Also, a contribution of $CD4^+$ T cells in
649 protection against ZIKV has been documented (Cimini et al., 2017; Elong Ngono et al., 2019; Hassert et al., 2018;
650 Lucas et al., 2018). Nevertheless, as it has been pointed out, there are currently limited data available on the
651 relationship between the generation of cross-reactive T cells following vaccination and protection from infection
652 or severe DENV disease (Guy et al., 2008; Tricou et al., 2022; Waickman et al., 2019a) or ZIKV (Rivino and Lim,
653 2017; Wen and Shresta, 2017). In this work, we found that the presence of $CD4^+$ T cells was essential for optimal
654 control of primary DENV infection and an effective Abs switching, as the depletion of those cells induced a delay
655 in anti-DENV IgM and IgG production. These results indicating a delay in B cells activation and in isotype class
656 switching during primary DENV infection related to the lack of $CD4^+$ T cells. Our finding are in contraposition
657 with prior work in mice showing that depletion of $CD4^+$ T cells did not have a significant effect on viral clearance
658 and were not required for the induction of the DENV2-specific Ab or CD8+ T cell responses, and that the early
659 anti-DENV2 Ab response is $CD4^+$ T cells-independent (Yauch et al., 2010). However, earlier works also
660 performed in mice showed that Abs were not required to protect against DENV; in contrast, depletion of $CD4^+$ T
661 and $CD8^+$ T lymphocytes significantly reduced survival of ACS46-primed mice challenged with the DENV JHA1
662 strain, suggesting that cellular immune response targeting NS proteins may be a promising route in vaccine
663 development against DENV (Amorim et al., 2016). In addition to their role interacting with B and $CD8^+$ T cells,
664 $CD4^+$ T cells have also been shown to be protective against encephalitis induced by West Nile Virus (WNV),
665 another flavivirus, in RAG(-/-) and C57BL/6 (H-2(b)) mice (Brien et al., 2008). Again, differences in animal
666 models may account for the radical differences in our results from prior published work. However, the
667 implications are very relevant to advice on DENV vaccines, and our results, as from others, indicate the need to
668 pay attention to $CD4^+$ T cell contribution in DENV vaccine design and efficacy. Angelo et al. addressed this issue
669 by characterizing CD4+ T cells contribution in donors vaccinated with a tetravalent DLAV vaccine (TV005) with
670 pools of DENV virus-derived predicted major histocompatibility complex (MHC) class II binding peptides. They
671 found that DENV-specific CD4+ T cells were focused predominantly on the capsid and NS3 and NS5 antigens
672 and, more importantly, that responses in vaccinees were similar in magnitude and breadth to those after natural
673 infection (Angelo et al., 2017). Another study characterizing T cell response to the same vaccine found that
674 multifunctional DENV-specific $CD8^+$ and $CD4^+$ T cells developed 8-14 days after vaccination and were
675 maintained for at least six months and identified a phenotypic profile of antiviral $CD8^+$ and $CD4^+$ T cells

676 associated with protective immunity to DENV infection (Graham et al., 2020). In addition, a particular highly
677 polarized CX3CR1+ cytotoxic $CD4^+$ T cell phenotype was associated with protective immunity to
678 DENV(Weiskopf et al., 2015b). Previously we found that $CD4^+$ T cell depletion during a tertiary flavivirus
679 infection results in a delay in DENV-specific IgM/IgG antibody titers, binding, and neutralization in
680 DENV/ZIKV-primed animals but not in ZIKV/DENV-primed animals, highlighting the fact that the contribution
681 of those cells can also be relative to the sequence of flavivirus infection, adding complexity to the interpretation
682 (Marzan-Rivera et al., 2022).
683 We found significant higher ZIKV viremia in the group with higher frequency of DENV-experimented $CD4^+$ T
684 cells prior ZIKV challenge compared to the control undepleted, and the double depleted groups and the group
685 depleted before the primary DENV infection carrying DENV-naïve $CD4^+$ T cells. Also the control undepleted
686 group had significantly less ZIKV viremia days compared to the double depleted group. This result is coherent
687 with prior reports confirming the role of those cells controlling ZIKV viremia even in naïve and DENV-
688 experimented animals (Wen et al., 2020). Mice depleted of $CD4^+$T cells have more severe neurological sequela
689 and significant increases in viral titers in the central nervous system. When those cells were transfered from ZIKV
690 immune mice, CD4+ T cells protected type I interferon receptor-deficient animals from a lethal challenge,
691 showing that the response by these cells is necessary and sufficient for control of ZIKV disease (Hassert et al.,
692 2018). On another work, depletion of CD4+ T cells in mice reduced plasma cell, GC B cells, and IgG responses
693 to ZIKV without affecting CD8+ T cell response, and those cells were required to protect mice from a lethal dose
694 of ZIKV after infection intravaginally, but not intravenously (Elong Ngono et al., 2019).
695 These results are also in line with our finding that depletion of $CD4^+$ T cells before ZIKV challenge in animals
696 with prior DENV immunity significantly dampened the B cells immune response, delaying the early IgM and IgG
697 responses and ZIKV neutralization, supporting a protective role of $CD4^+$ T cells against ZIKV mediated by
698 different mechanisms. Other results from mice showed that reduced viral load in the brain, prevention of rapid
699 disease onset and survival was dependent on $CD4^+$ T cells and IFN gamma signaling, and requires $CD8^+$ T cells
700 and B lymphocytes (Lucas et al., 2018). However, Weng et al. showed that anti-ZIKV IgG production was not
701 necessary to protect naive HLA-DRB1(∗)0101 transgenic mice immunized with $CD4^+$ T specific peptides. These
702 results showed that immunization induces a cell response sufficient to reduce tissue viral burden following ZIKV
703 infection. Furthermore, another recent work in mice revealed that the role of $CD4^+$ T cells may be modulated
704 differentially by various ZIKV strains (Pardy et al., 2021). Pardy et al. showed that $CD4^+$ T cells were
705 differentially polarized to the Th1 subtype after challenging mice with pre-epidemic or contemporary ZIKV
706 strains. The differences were characterized by enhanced GC B cell response and robust production of higher
707 avidity-nAbs following the infection with the contemporary strain, suggesting that ZIKV strains have evolved to
708 differentially induce T cell, B cell, and Ab responses including their avidity and neutralization properties (Pardy
709 et al., 2022). These results are relevant while considering ZIKV vaccine designs.

710 More controversial is our finding that depleting $CD8^+$ T cells before ZIKV challenge resulted in a strong trend to
711 have lower viremia and significant better IgM and IgG response and neutralizing activity than the $CD4^+$ T cell-
712 depleted group, suggesting that $CD8^+$ T cells depletion does not have a relevant role in controlling ZIKV infection
713 and are not required for an optimal humoral immune response in DENV-immune individuals. Several works
714 support the role of $CD8^+$ T cells and CR DENV virus-specific $CD8^+$ T cells controlling ZIKV replication (Regla-
715 Nava et al., 2018; Rivino and Lim, 2017; Wen et al., 2017; Wen and Shresta, 2017; Yauch et al., 2010).
716 Nevertheless, these works have been performed in flavivirus naïve mice – which may account for the difference
717 with our finding.
718 Our results also showed that the $CD4^+$ T cell-depleted animals had a significant compensatory increase in $CD8^+$
719 T cells and vice-versa; animals depleted of $CD4^+$ T cells had a significant compensatory increase in $CD8^+$ T cells,
720 which has been priorly documented in rhesus macaques (Chowdhury et al., 2015; Mueller et al., 2009; Okoye et
721 al., 2009; Schouest et al., 2020). Considering such cellular profile, our model suggests that other components of
722 the immune system, like precisely DENV-experimented $CD4^+$ T cells and the humoral immune response from a
723 prior DENV2 immunity, play a role controlling ZIKV infection and that $CD8^+$ T cells are not critically required
724 in this scenario. It is known that $CD4^+$ T cells can have a dissimilar contribution to the humoral immune response
725 depending on the type of prior flavivirus exposure. A prior T cell receptor repertoire analysis revealed preferential
726 expansion of CR clonotypes between JEV and ZIKV, suggesting that pre-existing immunity against JEV may
727 prime the establishment of stronger $CD4^+$ T cell responses to ZIKV purified-inactivated virus vaccination. These
728 $CD4^+$ T cell responses correlated with titers of ZIKV-nAbs in JEV- pre-vaccinated group, but not in flavivirus-
729 naïve or YFV- pre-vaccinated individuals, suggesting a stronger contribution of $CD4^+$ T cells in the generation of
730 nAbs in the context of JEV-ZIKV cross-reactivity (Lima et al., 2021). This finding, together with our results and
731 from others, emphasize that the efficacy of a DENV or ZIKV vaccine may also depend on the nature of prior or
732 post exposure to flavivirus.
733 Our results on multiple $CD4^+$ T depletions also shed light on the quantity and quality of the immune response in
734 sequential DENV/ZIKV infection in the context of an optimal or suboptimal priming of B cells.
735 The binding or magnitude of neutralization against DENV2 or ZIKV after ZIKV challenge was essentially
736 unaffected by the lack of $CD4^+$ T cells before primary DENV infection. These results indicate that DENV-
737 experimented $CD4^+$ T cells do not contribute to an optimal humoral immune response against ZIKV and support
738 prior reports confirming the specificity of ZIKV-induced humoral immune response in naïve (Collins et al., 2017;
739 Duffy et al., 2009), but also in DENV-pre-exposed, subjects (Andrade et al., 2020). However, depletion before
740 primary infection results in a significant increase in ZIKV-cross reactivity prior ZIKV challenge evidenced by the
741 presence of CR ZIKV-binding IgG. This implies an essential role of $CD4^+$ T cells to guarantee a more specific
742 Abs repertoire after the *primo* infection. That role may contribute to avoid potential disease enhancement during
743 a secondary flavivirus exposure, due to generation of wide CR DENV MBC as consequence of a suboptimal

priming. In contrast to the total IgG, no animals had detectable anti-ZIKV-NS1 IgG Abs at baseline, indicating that those Abs are very specific, supporting the efforts to use this protein as a diagnostic marker (Castro-Trujillo et al., 2023; Guzman et al., 2010; Petphong et al., 2023; Vazquez et al., 1995), but also considering the antigen as a target for vaccination. However, removing $CD4^+$ T cells prior to primary infection resulted in a significant increase in DENV anti-NS1 Abs durability. The implications of this finding need further considerations to determine their probable or not pathogenic role, during a subsequent natural exposure or DENV or ZIKV vaccine administration.

Despite this, NS1 has also been used as a strategy for protection against TBE (Beicht et al., 2023) and against JEV (Li et al., 2020; Nath et al., 2020; Zhou et al., 2020). Gonçalves et al. nicely showed in mice that the cooperation between $CD4^+$ T cells and the humoral response induce a robust protection against DENV mediated by a DNA-NS1 vaccine (Goncalves et al., 2015). Rhesus macaques is a good model to evaluate the resppnse to NS1 protein as prior work using the same species and DENV2 strain we used confirmed that $CD4^+$ T cells from these animals targeted NS1, NS3 and NS5 proteins (Mladinich et al., 2012).

Our results confirmed an enhanced circulation of DENV-NS1 antigen after DENV infection and significant lower DENV or ZIKV-specific anti-NS1 IgG response after the primary infection of challenge respectivelly, associated to the lack of $CD4^+$ T cells, confriming a protective role for these cells.

Consistent with the Abs response, our data on the MBC and ACS after ZIKV challenge points to a *de novo* B cell response pathway that is T cell-dependent to promote the formation of both IgG MBCs and ASC subsets with good neutralizing capacity towards ZIKV. Only the groups with $CD4^+$ T cells before the ZIKV challenge recorded a significant activation of both MBC and ACS, while the depleted groups showed a trend for activation but without reaching significant differences with their baseline frequencies.

Previosly, we showed a similar profile ($CD4^+$ T cells-dependent MBC and ACS activation) during tertiary flavivirus infection, but only in the sequence where the ZIKV challenge was preceded by a DENV infection and not when two consecutive DENV infections were secondary and terciary exposures (Marzan-Rivera et al., 2022). Our results are in agreement with data from humans showing that ZIKV infection in DENV-naïve subjects (or with prior DENV immunity) induces both DENV-MBC and naive B cells with the production of ZIKV TS Abs in both cases (Andrade et al., 2020; Rogers et al., 2017). The recall memory against DENV2 detected in our work after ZIKV challenge was significantly higher in the undepleted groups suggesting to be $CD4^+$ T cells-dependent. However, more specific studies on BCR and Abs repertoir diversity, which are being implemented by our group, are needed. A recent work characterized the plasmablast response in rhesus macaques exposed to primary ZIKV or sequential ZIKV/ZIKV or DENV3/ZIKV infections. Authors reported the highest VH and VL diversity levels upon primary ZIKV infection and lower levels in both homologous and heterologous secondary ZIKV or DENV responses (Singh et al., 2024). In addition to the authors' discussion, those results strongly suggest dissimilar T-B cell interactions in those different sequential flavirus infections.

A different scenario compatible with our prior findings on sequential DENV infections (Marzan-Rivera et al., 2022), showing that the pool of CR policlonal Abs were generated mainly from MBC-derived plasmablasts and not from naïve B cells, was characterized for repeated DENV infections (Xu et al., 2012).

Cells phenotyping results were consitent with the correlation analysis we performed. Only the groups that were CD4+ before ZIKV challenge showed a significant positive correlation between some pTfh phenotypes and MBCs activation. However, only the control undepeleted group showed significant correaltion with the activiation of the ACS. Interestingly, the group depleted of $CD4^+$ T cells only before ZIKV infection showed a significant inverse correlation with the activation of the ACS. This finding and its relationship with the increased cross-reactivity in this group need further considerations. Intringuingly, the group depleted of $CD8^+$ T cells before ZIKV challenge – but with intact $CD4^+$ T cells – did not showed any correlation with the activiation of these cells, suggesting a potential role of $CD8^+$ T cells in an effective activation of the MBC and the ASC as it has been discussed before (Rouers et al., 2021). Finally, we found that pTfh (CXCR5+) and pTfh with TH1 pehonotype (CXCR5+/CXCR3+), but not the the pTfh expressing B cell markers for activation (PD1+/ICOS+), had a strong correlation with EC50 values against ZIKV (but not DENV). However, when we split the groups, only the control double positive group kept the significant correlation with the EC50 values, suggesting a contribution of the DENV pre-exposed T helper population in an effective neutralization against ZIKV. Because pTH cells are essential for the formation of GCs and to regulate GC B cell differentiation into plasma cells and memory B cells (Crotty, 2015), our correlation results support our other finidngs suggesting that the humoral immune response to ZIKV in DENV-experimented subjects is most likelly T-cell dependent; any disruption or suboptimal T-B cell interactions during a primary DENV infection has consequences in the quality of the immune response to a secondary ZIKV exposure. Vaccination of naive HLA-DRB1*0101 transgenic mice with DENV/ZIKV-cross-reactive $CD4^+$ T cell epitopes induced a $CD4^+$ T cell response sufficient to reduce tissue viral burden following ZIKV infection suggesting that those cells expressing a TH1 pehnotype (as we characterized in our model) can suppress ZIKV replication, even in a Abs-independient manner (Wen et al., 2020). A recent work on the response to the TV003 vaccine in phase II clinical trial revealed that sero-positive subjects have increased baseline/pre-vaccination frequencies of circulating cTfh cells and, more importantly and inline with our findings, they found that the baseline/pre-vaccination cTfh profile correlated with the vaccine's ability to launch nAbs response against all four DENV serotypes (Izmirly et al., 2022).

The findings on the correlation between the pTfh cells with the MBC and the magnitude of neutralization from our work have implications for vaccine designs. Lack of inducing an effective and specific $CD4^+$ T cells (and most likeely pTfh cells) responses might thus impair vaccine performance. Our results add on the mechanisms guiding the immune response during heterologous flavivrus infection and may contribute to rational vaccine development efforts.

811 One key finding from our work is the increased cross-reactivity to both the DENV-EDIII domain and NS1 (but
812 not to similar ZIKV regions) in absence of $CD4^+$ T cells before primary DENV infection but not before ZIKV
813 challenge. The fact that the cross-reactivity was identified against both the structural EDIII domain and the NS1
814 protein agrees with the finding that $CD4^+$ T cell epitopes in humans are skewed toward recognition of viral
815 components that are also targeted by B lymphocytes and, from here, guiding the production of Abs mainly against
816 structural proteins (Rivino et al., 2013) or NS1 protein having also T and B cell epitopes (Aquino et al., 2023;
817 Beicht et al., 2023; Perera et al., 2024; Shu et al., 2000). We are not sure why the cross-reactivity was higher
818 against the NS1 protein than to the EDIII domain, but the factors responsible for directing the $CD4^+$ T cell to
819 similar sites at the virus protein surface (or to NS1) in different flaviviruses are unknown (Aberle et al., 2018).
820 However, they are most likely related to the structural context of these epitopes and their accessibility during
821 antigen processing (Koblischke et al., 2017; Koblischke et al., 2018; Landry et al., 2017; Schwaiger et al., 2014).
822 The involvement of NS1 can be explained as 50% or more of the MBC repertoire represented in monoclonal
823 antibodies (mAbs) from DENV patients bind to NS1, NS3, prM, or capsid in addition to the E (Beltramello et al.,
824 2010; de Alwis et al., 2011). A prior work showed that half of anti-NS1 mAbs had limited cross-reactivity among
825 DENV, while most of the structural Abs showed full cross-reactivity against all virus serotypes (Dejnirattisai et
826 al., 2010). Supported in that data, the increased cross-reactivity against NS1 we found, reinforce the critical
827 contribution of $CD4^+$ T cells during a primary infection to the Abs specificity during a subsequent flavivirus
828 exposure.
829 Strikingly, that increase in cross-reactivity was not accompanied by a detriment in Abs binding or DENV
830 neutralization after the challenge, indicating that $CD4^+$ T cells were not essential for those properties, but they
831 were required for Abs specificity. Interestingly, while $CD8^+$ T cell depletion increased $CD4^+$ T cell frequency, it
832 had no effect on the specificity of epitope recognition, supporting that our finding is related to the quality of the
833 priming. This suggests that the specificity of DENV Abs need to be secured during a *primo* infection with an
834 optimal B cell priming and may be regulated through a different mechanism than the binding and neutralization
835 functions. After many years of extensive research, the mechanism regulating the Abs somatic hypermutation and
836 affinity maturation are still not well understood (Elsner and Shlomchik, 2020; Stebegg et al., 2018; Victora and
837 Nussenzweig, 2012). How this process occurs in flaviviruses is also largely unknown. However, we know that
838 high affinity Abs with higher SHM rate are generated in the GCs (Fink, 2012; Fink et al., 2007; Godoy-Lozano
839 et al., 2016; Wong et al., 2020) where the encounter with antigen-presenting cells, including $CD4^+$ T cells, occurs.
840 Our results agree and provide, for the first time, mechanistic evidence to prior findings where a population of
841 broadly cross-neutralizing Abs in addition to the TS nAbs was identified after secondary DENV infections (Patel
842 et al., 2017).
843 Currently, our group is performing Ab depletion to characterize the TS vs. CR Abs and ADE experiments to test
844 the potential enhancement of these CR Abs. We understand that results from these experiments will add

845 instrumental information in support of our results. We also acknowledge that we used samples from day 90 p.i.
846 where CR Abs start to decline but are still present (Katzelnick and Harris, 2017; Patel et al., 2017; Wahala and
847 Silva, 2011). However, it will not change the fact that, in our experimental conditions, the Abs population after
848 ZIKV challenge, in DENV-experimented individuals with suboptimal T-B cell interaction, behave differently in
849 their binding and neutralization properties vs. their specificity.
850 Also, experiments reproducing the sequence of infections ZIKV/DENV2 are underway, and DENV/DENV and
851 ZIKV followed by different DENV serotypes are on the pipeline.
852 Before, it has been highlighted that the risk of immunopathology associated with sub-optimal cross-reactive B-
853 cell and T-cell responses to heterologous serotypes represent critical factors for the development of a fully
854 protective DENV vaccine (Rivino and Lim, 2017). Our work adds on the relevance and uniqueness of the
855 contribution of $CD4^+$ T cells to the specificity of the nAbs to increase vaccine efficacy and to potentially prevent
856 or decrease the risk of pathogenic manifestations mediated by ADE or similar mechanisms.
857 Finally, and not less relevant, our work reinforces the value of rhesus macaques as a reliable model with a strong
858 translational capability and a needed complement to results obtained with other animal models.
859 One limitation to advance our knowledge on the correlates of protection towards DENV is the lack of an adequate
860 animal model to reproduce DENV clinical manifestations. Despite the proven value of small animal models, most
861 of the studies performing a fine dissection of the mechanisms addressing the protection against DENV and ZIKV
862 use transgenic mice deficient in some essential anti-viral pathways (Yauch and Shresta, 2008; Zompi and Harris,
863 2012). These genetic modifications in the murine model prevent to recognize the contribution of all components
864 of the immune response as a whole and from here, the translation to humans may be narrow.
865 In addition, it is known that the T cell immune response to flaviviruses in humans is restricted by the HLA system
866 (Angelo et al., 2017; Sierra et al., 2007; Weiskopf et al., 2016; Weiskopf et al., 2011; Wen et al., 2010; Zivna et
867 al., 2002).
868 The value of rhesus macaques as NHPs to study the pathogenesis and immune response after natural infection, or
869 vaccination to DENV (Mladinich et al., 2012; Onlamoon et al., 2010; Osorio et al., 2014; Sariol et al., 2011;
870 Sariol et al., 2007; Sariol and White, 2014; White et al., 2021; Young et al., 2023) or ZIKV (Abbink et al., 2017;
871 Aliota et al., 2016; Best et al., 2017; Breitbach et al., 2019; Carroll et al., 2017; Coffey et al., 2018; Coffey et al.,
872 2017; Dudley et al., 2019; Dudley et al., 2016; Haddow et al., 2017) is well documented. Also, our group and
873 others have shown the usefulness of this model to dissect many aspects of DENV and ZIKV interactions (Crooks
874 et al., 2021; George et al., 2017; Larocca et al., 2021; Marzan-Rivera et al., 2022; McCracken et al., 2017; Pantoja
875 et al., 2017; Perez-Guzman et al., 2019; Serrano-Collazo et al., 2020; Valiant et al., 2018; Valiant et al., 2019).
876 Rhesus macaques have also shown to be very reliable to anticipate vaccine outcomes (Borges et al., 2019). Early
877 immunization studies of rhesus macaques with Dengvaxia, either with the tetravalent formulation CYD-TDV
878 (CYD1–4) or with two bivalent formulations (CYD1,2 + CYD3,4), resulted in higher responses against the

879 dominant DENV4 and DENV1, and very limited response to DENV2 serotypes in both approaches (Guy et al.,
880 2009). Later, results from humans showed a vaccine efficacy of 55.6%, 75.3% and 100.0% for serotypes 1, 3 and
881 4 respectively. The efficacy for serotype 2 was as low as 9.2 % resulting in an overall efficacy of 30.2% for all
882 serotypes (Sabchareon et al., 2012). Rhesus macaque studies with DENVax (formulation used for TAK-003
883 vaccine) likewise anticipated the findings from the clinical trials. Different DENVax formulations failed to
884 generate adequate responses against DENV4 and stimulate lower levels of nAbs to DENV 1, 3 and 4 but higher
885 levels to DENV2 (Osorio et al., 2011a).
886 Overall, our findings have enormous impact for the planning and evaluation of DENV and ZIKV vaccine
887 schedules, design, and monitoring, strongly suggesting the consideration of CD4+ T cell epitopes for the
888 composition of new generation flavivirus vaccines.
889

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898

899 **Author contributions**

900 C.A.S., A.M.d.S, C.S.-C., A.M.-S. and L.C. developed the experimental design. C.R., M.I.M. and A.G.B.
901 supervised and performed sample collection and animal monitoring. C.S.-C., A.M.-S., L.C., S.H. and L.A.
902 performed the experiments. C.A.S., C.S.-C, A.M.-S., L.C. and M.S.-R. analyzed the data. C.A.S., C.S.-C, A.M.-
903 S. and L.C drafted the article. C.A.S., C.S.-C., A.M.-S, L.C., S.H., M.S.-R., L.A., T.A., M.I.M., C.R., A.G.B. and
904 A.M.d.S. reviewed and corrected the last version.

905 **Declaration of interests**

906 The authors declare no competing interest.
907

908 **Materials and Methods**

909 **Experimental model.** Young adult (4–7 years of age) male rhesus macaques (*Macaca mulatta*),
910 seronegative for DENV and ZIKV, were housed in the CPRC facilities at the University of Puerto Rico,
911 San Juan, Puerto Rico. All procedures were reviewed and approved by the Institute's Animal Care and Use
912 Committee at the Medical Sciences Campus, University of Puerto Rico (IACUC-UPR-MSC) and
913 performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory
914 Animal Care (AAALAC) (Animal Welfare Assurance number A3421; protocol number, 7890116). In
915 addition, the study was conducted following the USDA Animal Welfare Regulations, the Guide for the Care
916 and Use of Laboratory Animals, and institutional policies, to ameliorate animal suffering by the
917 recommendations of the Weatherall Report on the use of nonhuman primates in research. All procedures
918 were conducted under anesthesia by intramuscular injection of ketamine at 10–20 mg/kg of body weight,
919 as approved by the IACUC. Anesthesia was delivered in the caudal thigh using a 23-gauge sterile syringe
920 needle. During the period of the entire study, the macaques were under the environmental enrichment
921 program of the facility, also approved by the IACUC. Animals were continuously monitored by trained
922 veterinarians at the Animal Research Center.

923

924 **Viral Stock.** DENV-2 New Guinea 44 (NGC) strain (provided by Steve Whitehead, NIH/NIAID, Bethesda,
925 MD) and ZIKV PRVABC59 (ATCC VR-1843) were used to infect macaques at different timepoints. Viruses
926 were expanded and tittered by plaque assay and qRT-PCR using protocols standardized in our laboratories.
927 In addition, both viruses were used for neutralization assays.

928

929 **Depletion treatment, viral infection, and sample collection.** A total of 29 rhesus macaques seronegative
930 for DENV and ZIKV were housed in the CPRC facilities, at the University of Puerto Rico, San Juan, Puerto
931 Rico. During the first year, macaques were divided into two cohorts based on their depletion status. Twelve
932 (12) animals were depleted of CD4+ T cells by administration of anti-CD4 Ab CD4R1 (NHP Reagent
933 Resource; https://www.nhpreagents.org). Administration of the depletion treatment consisted of an initial
934 s.c. inoculation of 50 mg/kg at 15 days prior to viral infection, followed by two i.v. injections of 7.5 mg/kg
935 12 days and 9 days before viral infection respectively. The seventeen (17) remaining animals received
936 phosphate-buffered saline (PBS) as a control. Both cohorts were then infected with DENV-2 NGC strain
937 (5.0×10^5 pfu s.c.), which is denoted from here on as the primary infection. Following one year after
938 infection, animals were further divided into five cohorts based on depletion status. Animals were depleted
939 of CD4+ or CD8+ T cells, or treated with PBS, followed by a secondary infection with ZIKV (1×10^6 pfu
940 s.c.). CD8+ T cell depletion was performed by administration of anti-CD8 alpha MT807R1 at the same
941 dosage as CD4+ T cells depletion treatment. CD4+ T cell depletion was performed on groups 1 and 2 (group

942 1: n=6, denoted CD4-/DV2/CD4-/ZV; group 2: n=6, denoted CD4+/DV2/CD4-/ZV). Group 3 was depleted
943 of CD8+ T cells (n=6, denoted CD4+/DV2/CD8-/ZV). Lastly, the remaining two groups were administered
944 PBS (group 4: n=6, denoted CD4-/DV2/CD4+/ZV; group 5: n=5, denoted CD4+/DV2/CD4+/ZV).
945 Blood samples were collected on days $^{-}15$, $^{-}12$ and $^{-}9$ pre-infection, and during the first fifteen consecutive
946 days after infection, followed by a monthly sample collection from there onwards until a year later when
947 sample collection pattern described is repeated for the secondary infection. Collected samples included
948 serum, heparinized whole blood, and citrate whole blood for PBMCs isolation. Macaques were monitored
949 after treatments and infections by trained veterinarians for evidence of disease and clinical status. Rectal
950 and external temperatures and weights were taken on day 0 and every other day pre- and post-infection. A
951 summary for the experimental design and groups is provided (SFig 1).
952
953 **Immunophenotyping.** Phenotypic characterization of Rhesus macaque's adaptive immune response was
954 performed by 16- multicolor flow cytometry using fluorochrome-conjugated Abs at several time points.
955 Depletion efficacy was confirmed by measuring the frequency of $CD4^{+}$, $CD8^{+}$ T cells, and $CD20^{+}$ B cells
956 on the following timepoints: pre-treatment/depletion baseline, and days 2, 5, and 9 post-treatment. In
957 addition, B and T cell frequencies were measured on baseline and days 1, 2, 3, 7, 15, 30, 60 and 90 p.i. and
958 p.c.. Aliquots of 150 uL of heparinized whole blood were incubated with a mix of antibodies for 30 min. in
959 the dark at room temperature. After incubation, red blood cells are fixed and lysed with BD FACS fix and
960 lyse solution, and cells were washed twice with BSA 0.05%. Samples were analyzed using a MACSQuant®
961 Analyzer 16 Flow Cytometer (Miltenyi Biotec, CA). Antibodies used in this study were: CD8-FITC (DK25)
962 from Sigma; CD3- PerCP (SP34), Ki67- Viogreen (B56), CD4- APC (L200), CD69- PeCy7 (FN50), CD95-
963 PE (DX2), IgG- APC- H7 (G18- 145), CD11b- AF 700 (ICRF44), CD80- BV650 (L307.4), CD27- PE (M-
964 T466) from BD Biosciences; CD20- PacBlue (2H7), HLA-DR- BV570 (L243), CD138- FITC (DL- 101),
965 ICOS- PE/Dazzle 594 (C398.4H), CXCR3- PECy7 (GO25H7), PD-1- BV650 (EH12.2H7) from
966 Biolegend; CD28- APC- Vio770 (I5E8) and CCR7- PerCP Vio770 (REA546) from Miltenyi; CXCR5- PE
967 (MU5UBEE) from Thermo eBiosciences; and CD38- APC (AT-1) from Adipogen.
968 For analysis, lymphocytes (LYM) were gated based on their characteristic forward and side scatter patterns.
969 B cells were defined as $CD20^{+}CD3^{-}$, Memory (MBC= $CD20^{+}$, $CD3^{-}$, $CD27^{+}$), class-switched IgG Memory
970 (IgG MBC= $CD20^{+}$, $CD3^{-}$, $CD27^{+}$, surface IgG+ (sIgG), plasmablasts as (PB= $CD20^{-/low}$, $CD3^{-}$, $CD11b^{-}$,
971 $HLA\text{-}DR^{+}$, $CD80^{+}$) as described by Silveira et al., 2015 (Silveira et al., 2015), plasma cells as (PC= $CD20^{-}$
972 $^{/low}$, $CD3^{-}$, $CD138^{+}$) as described by Martinez- Mirullo et al., 2016 (Martinez-Murillo et al., 2016). Gating
973 strategy for B cells and subsets is presented on Supplementary Figure 9 (SFig 9). T cells were defined as
974 $CD3^{+}CD20^{-}$, $CD4^{+}$ T cells ($CD3^{+}CD20^{-}CD4^{+}$), $CD8^{+}$ T cells ($CD3^{+}CD20^{-}CD8^{+}$), and subpopulations were
975 defined inside the $CD3^{+}$ T cells. Naïve ($CD3^{+}CD20^{-}CD28^{+}CD95^{-}$), EM ($CD3^{+}CD20^{-}CD28^{-}CD95^{+}$), and

976 CM ($CD3^+CD20^-CD28^+CD95^+$) T cell subpopulations were determined within $CD4^+$ and $CD8^+$ T cells.
977 Gating strategy for T cells and memory subsets is presented on Supplementary Figure 10 (SFig 10), while
978 gating strategy for the characterization of $CD4^+$ pTfh cells was represented by selecting CM and EM T cells
979 ($CD4^+/CXCR5^+/CXCR3^+$) expressing activation and migration markers such as ($CCR7^+/PD\text{-}1^+/ICOS^+$)
980 (SFig 11). All data from cell population frequencies and immune markers expression were analyzed using
981 Flow/Jo software and GraphPad Prism (FlowJo LLC Ashland, OR).
982
983 **qRT- PCR.** DENV-2 and ZIKV viral RNA for real-time PCR assay was extracted from 200uL of virus
984 isolate (previously tittered by focus reduction neutralization assay) and from acute serum samples using a
985 MagMax Viral/Pathogen Nucleic Acid Isolation Kit (Applied Biosystems, MA) as per the manufacturer's
986 instructions. Viral RNA extraction was performed using a KingFisher Duo Prime Purification System
987 (Thermo Fisher Scientific, MA). In brief, a Viral Pathogen DNA/RNA binding magnetic bead solution is
988 prepared, 200uL of sera, 500uL of Viral Pathogen Nucleic Acid isolation kit wash buffer, 5uL of a protein
989 kinase solution, and 1mL of ethanol solution (80% ethanol in DNAse free water) were added to specified
990 wells in a KF Duo Deepwell 96 plate (Thermo Fisher Scientific, MA) and loaded on the KingFisher Duo
991 Prime Purification System. Purified RNA was collected and stored at -80°C in kit elution buffer solution.
992 Real-time PCR for DENV-2 and ZIKV isolated RNA was carried out using the Gensig Real-Time PCR
993 detection kit for Dengue Virus and the Gensig Real-Time PCR detection kit for Zika Virus (Primer Design,
994 UK), respectively, as per the manufacturer's instructions. Briefly, a kit Positive Control is diluted in 500uL
995 of a kit Template buffer and a series of tenfold dilutions were prepared as follows: 2×10^5/uL, 2×10^4/uL,
996 2×10^3/uL, 2×10^2/uL, 20/uL, 2/uL (copy number/ mL). For the reaction mix, 10uL of oasig 2X Precision
997 one step qRT-PCR was combined with 1uL of kit probe/primer mix and 4uL of sterile water, for a total
998 volume of 20uL once the RNA sample was added. The reaction mix was added to a 96 well plate, alongside
999 the experimental RNA samples, a kit non-template control (NTC), an RNA extraction negative control
1000 (DNAse free water), a positive control (RNA extraction from virus stock), and calibration standards, in
1001 specified wells. The plate was then loaded, and the RT-PCR reaction was performed using a Quant Studio
1002 5 Real-Time PCR Instrument (Applied Biosystems, MA). For quantification, a standard curve was
1003 generated from the ten-fold dilutions of the kit RNA positive control.
1004
1005 **ELISAs for DENV and ZIKV.** Serostatus of animals for DENV and ZIKV was assessed before and after
1006 DENV-2 infection using DENV IgG/IgM commercial kits (Focus Diagnostics, CA). ZIKV serostatus was
1007 also assessed using a commercial kit for ZIKV IgM (InBios, International Inc, Seattle) and ZIKV IgG
1008 (XpressBio, Frederick, MD). Timepoints measured include baseline and days 7, 10, 15, 30, 60, and 90 post-

1009 infection. All assays were performed per the manufacturer's instructions and as described by our group
1010 (Pantoja et al., 2017; Perez-Guzman et al., 2019; Serrano-Collazo et al., 2020).
1011
1012 **ELISAs for detection of DENV NS1 antigen, DENV anti-NS1 IgG and ZIKV anti-NS1 IgG**. Detection
1013 of DENV NS1 antigen after primary DENV-2 infection, ZIKV anti-NS1 IgG after secondary ZIKV
1014 infection, and DENV anti-NS1 IgG after both primary and secondary infections were measured using
1015 commercial kits (InBios, International Inc, Seattle). The assays were performed per the manufacturer's
1016 instructions. Timepoints measured include baseline and days 3, 5, 7, 15 and 30 p.i. for DENV NS1, and
1017 baseline and days 15, 30 and 90 p.c. for ZIKV and DENV anti NS1 IgG.
1018
1019 **DENV neutralization assays.** For the Focus Reduction Neutralization Test (FRNT) for DENV, Vero81
1020 cells (ATCC CCL-81) were seeded at approx. 2.0 x 10^5 per well in 24 well-plates with DMEM (Dulbecco's
1021 Modified Eagle's medium, Thermo Fisher Scientific) for approx. 18 hours. Serum dilutions (ten-fold) were
1022 prepared in diluent medium Opti-MEM (Invitrogen) with 2% FBS (Gibco) and 1% antibiotic/antimycotic
1023 (Hyclone). Virus was added to each dilution and incubated for 1 hour at 37ºC/5%. Before inoculation, the
1024 growth medium was removed, and cells were inoculated with 50 uL per well of serum-virus dilution in
1025 triplicates; plates were incubated for 1 hour at 37ºC/5%CO_2/rocking. After incubation, 125 uL per well of
1026 overlay (Opti-MEM 1% carboxymethylcellulose (Sigma), 2% FBS, 1% non-essential amino acids (Gibco)
1027 and 1% antibiotic/antimycotic (HyClone)) was added to the plates containing virus dilutions, followed by
1028 an incubation period of 48 hours at 37ºC/5%CO_2. After two days, overlay was removed with PBS and fixed
1029 with 4% paraformaldehyde for 30 minutes. Plates were blocked with 5% non-fat dairy milk in 1X perm
1030 buffer (BD Cytofix/Cytoperm) for 10 min and incubated for 1hr/37ºC/5%CO2/rocking with anti-E mAb
1031 4G2 and anti-prM mAb 2H2 (kindly provided by Dr. Aravinda de Silva and Ralph Baric, University of
1032 North Carolina Chapel Hill, NC, USA), both diluted 1:100 in blocking buffer. Plates were washed three
1033 times with PBS and incubated 1hr/37ºC/5%CO_2/rocking with horseradish peroxidase (HRP)-conjugated
1034 goat anti-mouse antibody (Sigma), diluted 1:1,500 in blocking buffer. Foci were developed with TrueBlue
1035 HRP substrate (KPL) and counted using an Elispot reader. Results were reported as FRNT with a 60% or
1036 greater reduction in DENV foci (FRNT60). A positive neutralization titer was designated as 1:20 or greater,
1037 while <1:20 was considered a negative neutralization titer.
1038
1039 **ZIKV neutralization assays.** For the Plaque Reduction Neutralization Test (PRNT) for ZIKV, Vero81 cells
1040 (ATCC CCL-81) were seeded at approx. 2.0 x 10^5 per well in 24 well-plates with DMEM (Dulbecco's
1041 Modified Eagle's medium, Thermo Fisher Scientific) for approx. 18 hours. Serum dilutions (ten-fold) were
1042 prepared in a diluent medium (Opti-MEM (Invitrogen) with 2% FBS (Gibco) and 1% antibiotic/antimycotic

1043 (Hyclone). Virus was added to each dilution and incubated for 1 hour at 37ºC/5%/CO2. Before inoculation,
1044 the growth medium was removed, and cells were inoculated with 100 uL per well of each dilution in
1045 triplicates; plates were incubated for 1 hour at 37ºC/5%CO2/rocking. After incubation, 1mL per well of
1046 overlay (Opti-MEM 1% carboxymethylcellulose (Sigma), 2% FBS, 1% non-essential amino acids (Gibco),
1047 and 1% antibiotic/antimycotic (HyClone)) was added to the plates containing virus dilutions, followed by
1048 an incubation period of 4 days at 37ºC/5% CO2. After four days of incubation at 37ºC/5%CO2, the overlay
1049 was removed; the cells were washed twice with phosphate-buffered saline (PBS), fixed in 80% methanol,
1050 and stained with crystal violet and foci were counted. The mean focus diameter was calculated from approx.
1051 twenty foci per clone were measured at 35 magnifications. Results were reported as the PRNT with a 60%
1052 or greater reduction in ZIKV or DENV plaques (PRNT60). A positive neutralization titer was designated
1053 as 1:20 or greater, while < 1:20 was considered a negative neutralization titer.
1054
1055 **EDIII and NS1 Antigen Production.** ZIKV (H/PF/2013) EDIII antigen used in this study was produced
1056 as previously described (Adams et al., 2021). EDIIIs of DENVs were also cloned and expressed similarly
1057 to ZIKV EDIII. Briefly, codon-optimized genes encoding the EDIIIs of DENV1 (AAB70694·1, E protein
1058 aa 297-394), DENV2 (ADA00411·1, E protein aa 297-394), DENV3 (AAB69126·2, E protein aa 295-392)
1059 and DENV4 (ADA00410·1, E protein aa 297-394) with an N-terminal human serum albumin signal
1060 peptide, a polyhistidine tag, and a HaloTag were cloned into the mammalian expression plasmid pαH.
1061 Recombinant DENV EDIII antigens were expressed in mammalian Expi293 cells and purified from the
1062 culture supernatant using Ni-NTA agarose (Qiagen). Purified EDIII antigens were biotinylated using
1063 HaloTag PEG biotin ligand (Promega), according to the manufacturer's protocol. For NS1 antigen
1064 production, codon-optimized genes encoding the NS1s of DV1 NS1 Region 37, DV2 NS1 Region 39, DV3
1065 NS1 Region22, DV4 NS1 Region 45 and ZKVNS1 Region 47 were used. Mobility-shift analysis was used
1066 to assess the biotinylated EDIII antigens using SDS-PAGE. The mammalian expression plasmids and
1067 sequences will be made available in the plasmid repository and Genbank, respectively.
1068
1069 **Coupling antigens to beads via avidin-biotin interaction:** Site-specifically biotinylated EDIII antigens
1070 were coupled to unique MagPlex®-Avidin Microspheres (DENV1 (region 12), DENV2 (region 26),
1071 DENV3 (region 36), DENV-4 (region 38) and ZIKV (region 30)) at a concentration of 5 µg of antigen per
1072 106 beads in phosphate-buffered saline, pH 7.4 (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and
1073 2 mM KH2PO4) for 1 hour at 37°C with shaking at 700 rpm. Beads were then washed/blocked three times
1074 in block buffer (PBS + 1% BSA) and resuspended in wash buffer at 2x106 beads/mL.
1075

1076 **EDIII and NS1 multiplex Assay:** DENV and ZIKV EDIII coupled beads were combined in equal ratios
1077 by plating 50 µL containing 2,500 beads per antigen into each well of a 96-well assay plate (Cat.
1078 No:655906). Human serum samples were diluted 1:500 in block buffer. Mouse monoclonal antibodies
1079 specific for each EDIII antigen (ZV-67 (ZIKV), E103 (DENV1), 3H5 (DENV2, 8A1 (DENV3), E88
1080 (DENV4)) and NS1 antigen were titrated in each assay as controls for assay consistency across couplings,
1081 plates, and assay days. A magnetic plate separator was used to remove the block buffer from the assay plate,
1082 and 50 µL of diluted serum or mAb was added to the beads. The serum and bead mixture were incubated
1083 for 1 hour at 37°C with shaking at 700 rpm. Beads were washed thrice200 µL block buffer per well using
1084 a magnetic separator. 50 µl of Goat anti-Human IgG Fc Multi-species SP ads-PE (Southern Biotech,
1085 catalog:2014-09) and Goat anti-Mouse IgG Fc Human ads-PE (Southern Biotech, catalog:1030-09) were
1086 added to the beads (6 µg/ml in block buffer) and incubated for 1 hour at 37°C with shaking at 700 rpm to
1087 detect respective antibody species. Beads were washed thrice with 200 µL block buffer per well using a
1088 magnetic separator and resuspended in 100 µL of block buffer before acquiring data. Fluorescence data
1089 were obtained using a Luminex 200 analyzer set to acquire at least 50 beads per bead region.

1090 **Principal Component Analysis (PCA).** All PCA analyses were performed using R statistical software R
1091 version 4.2.3 using the *prcomp* function of the *stats* package (Team et al., 2018; Team, 2010). To examine
1092 global patterns of infection, we carried out a PCA using all the parameters measured during the infection
1093 timeline; these analyses were performed for day 15, day 30 and day 90 of infection. The parameters used
1094 to perform PCA are depicted in Supplementary Table 3 (Table S3).
1095
1096 **Quantification and statistical analysis.** Statistical analyses were performed using GraphPad Prism 9.0
1097 software (GraphPad Software, San Diego, CA, USA). For viral burden analysis, the log titers and levels of
1098 vRNA were analyzed by multiple unpaired t-tests and two-way ANOVA. The statistical significance
1099 between or within groups evaluated at different time points was determined using one-way or two-way
1100 analysis of variance (ANOVA) (Tukey's, Sidak's, or Dunnett's multiple comparisons test) or unpaired t-test
1101 to compare the means. Significant multiplicity-adjusted p-values (*< 0.05, **< 0.01, ***< 0.001, ****<
1102 0.0001) show statistically significant differences between groups (Tukey test) or time points within a group
1103 (Dunnett test).
1104

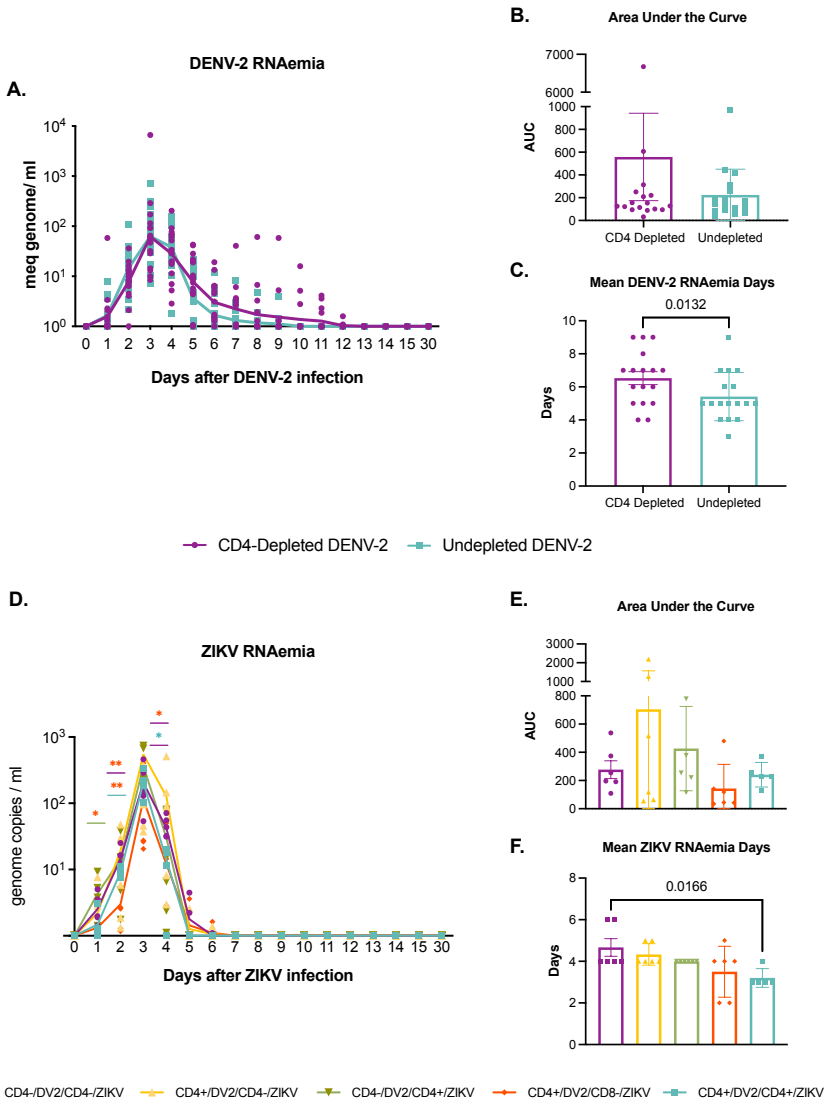


Fig 1. DENV2 and ZIKV RNA kinetics in flavivirus-naïve CD4-depleted and undepleted animals. RNAemia is affected by depletion of CD4+ or CD8+ T cells. **Upper panel**. CD4-depleted animals are depicted in purple and undepleted animals are depicted in turquoise. **(A)** DENV-2 genome copies/mL were measured in serum to monitor viral replication during the first 15 days and day 30 after infection. Genome copies per mL are shown logarithmically. **(B)** The area under the curve (AUC) was calculated for individual values. **(C)** Average RNAemia days were calculated using the following formula: total viremia days divided by the total number of days during which viremia was monitored. **Lower panel**. Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), CD4+/DV2/CD4-/ZV (shown in yellow), CD4-/DV2/CD4-/ZV (shown in purple), and CD4+/DV2/CD8-/ZV (shown in red). **(D)** ZIKV genome copies/mL were measured in serum to monitor viral replication during the first 15 days and day 30 after infection. Genome copies per mL are shown logarithmically. **(E)** The area under the curve (AUC) was calculated for individual values. **(F)** Average RNAemia days were calculated using the following formula: total viremia days divided by the total number of days during which viremia was monitored. Statistically significant differences among and within groups were calculated by two-way ANOVA using Tukey's multiple comparisons test and unpaired t-tests. Colored stars represent a significantly different group, while colored lines represent the group that it is compared to.

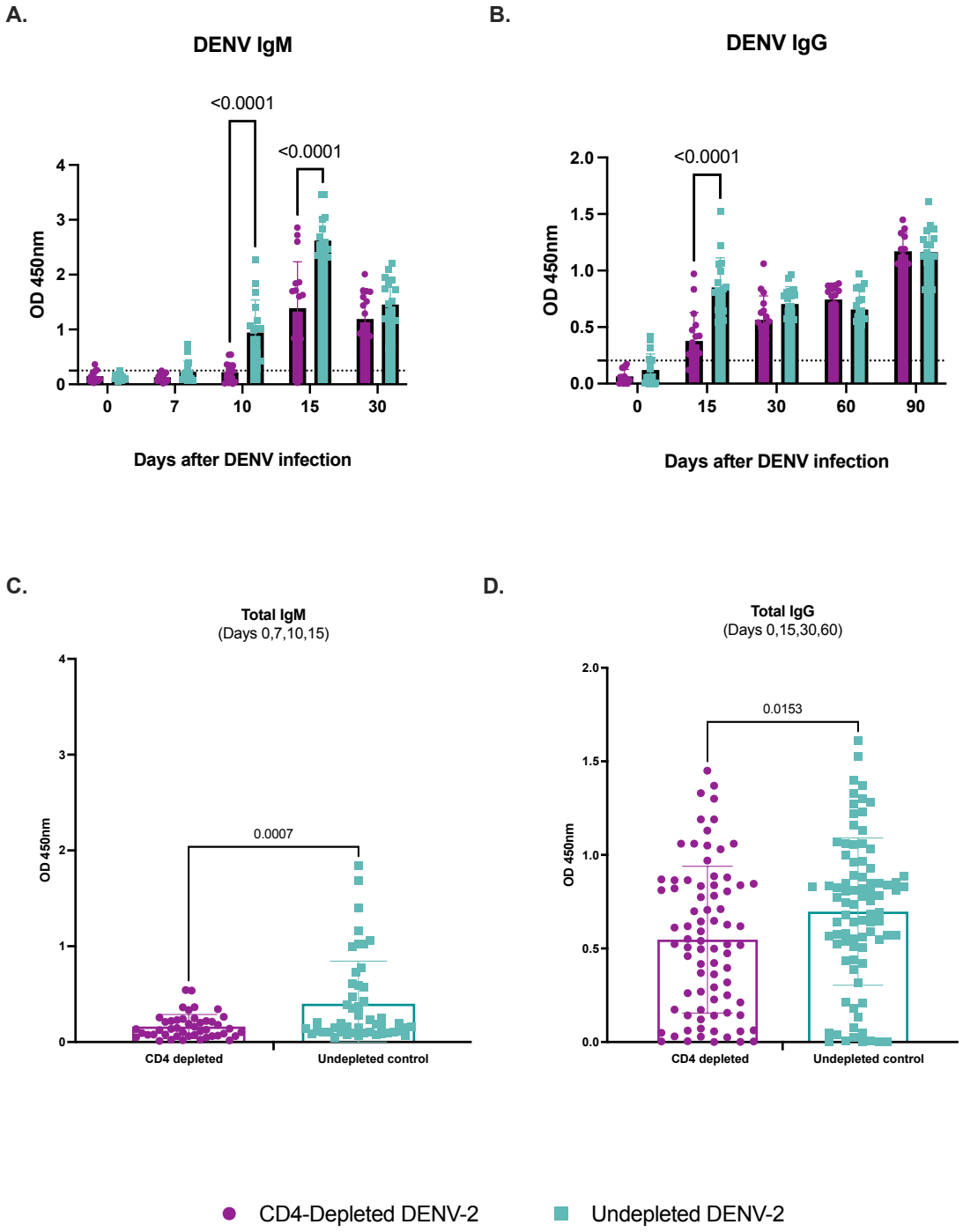


Fig 2. T cell depletion modifies serological profile during primary DENV2 infection. IgM and IgG response after DENV-2 infection was assessed using commercial ELISA tests. CD4-depleted animals are depicted in purple and undepleted animals are depicted in turquoise. Dotted lines indicate the limit of detection for each test. **(A-B)** Binding capacity of DENV IgM and IgG antibodies from CD4-depleted and undepleted animals after DENV-2 infection. **(C-D)** Total DENV IgM and IgG levels throughout different timepoints. Statistically significant differences among and within groups were calculated by two-way ANOVA using Tukey's multiple comparisons test and unpaired t-tests.

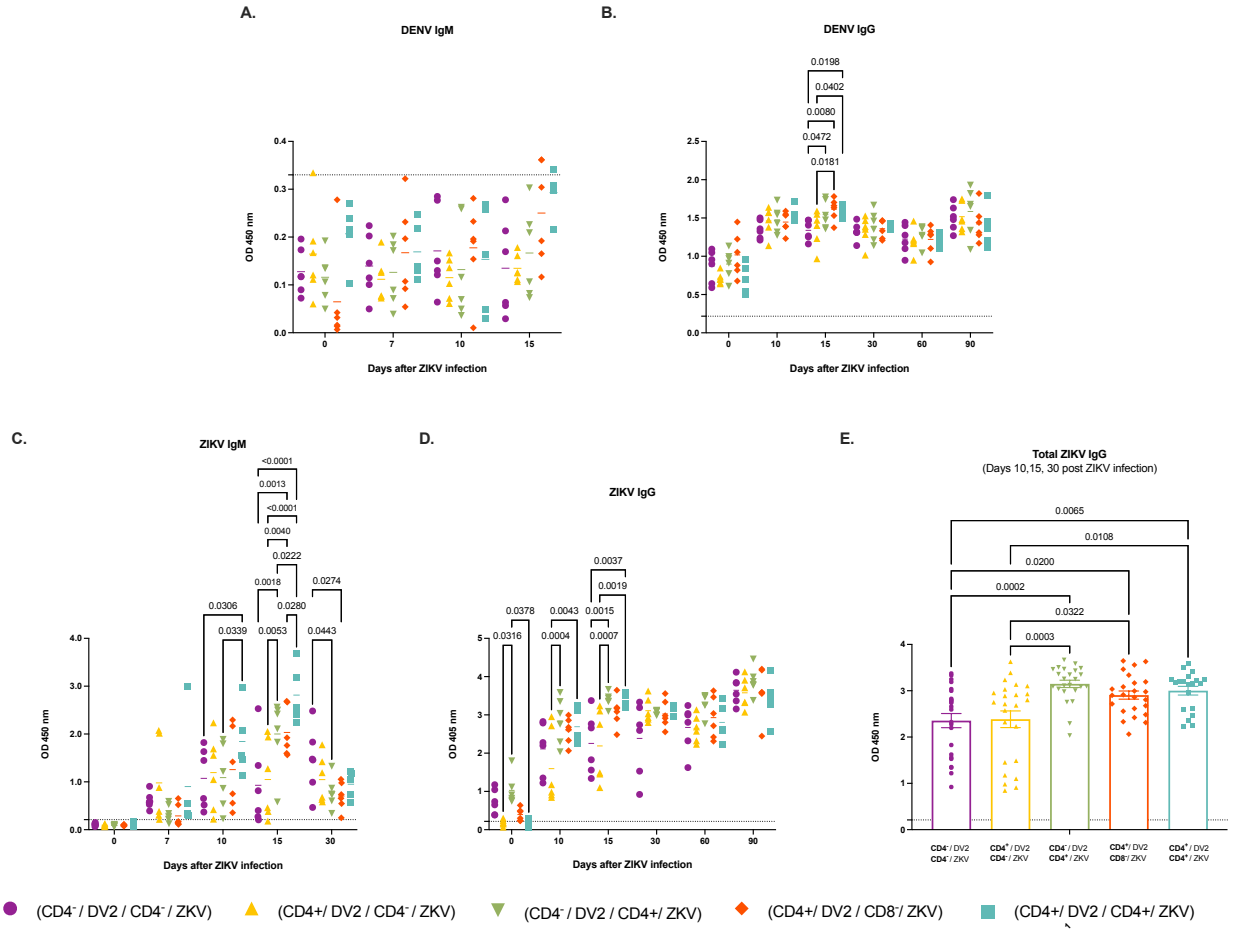


Fig 3. T cell depletion modifies serological profile during secondary heterologous ZIKV infection. IgM and IgG response after ZIKV infection in the presence of DENV immunity was assessed using commercial ELISA tests. Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), CD4+/DV2/CD4-/ZV (shown in yellow), CD4-/DV2/CD4-/ZV (shown in purple), and CD4+/DV2/CD8-/ZV (shown in red). Dotted lines indicate the limit of detection for each test. **(A-B)** Binding capacity of DENV IgM and IgG antibodies from CD4-depleted and undepleted animals after ZIKV infection. **(C-D)** Binding capacity of ZIKV IgM and IgG antibodies from CD4-depleted and undepleted animals after ZIKV infection. **(E)** Total ZIKV IgG levels after ZIKV infection were analyzed. Statistically significant differences among and within groups were calculated by two-way ANOVA using Tukey's multiple comparisons test and unpaired t-tests, and by One-way ANOVA using Tukey's multiple comparisons test.

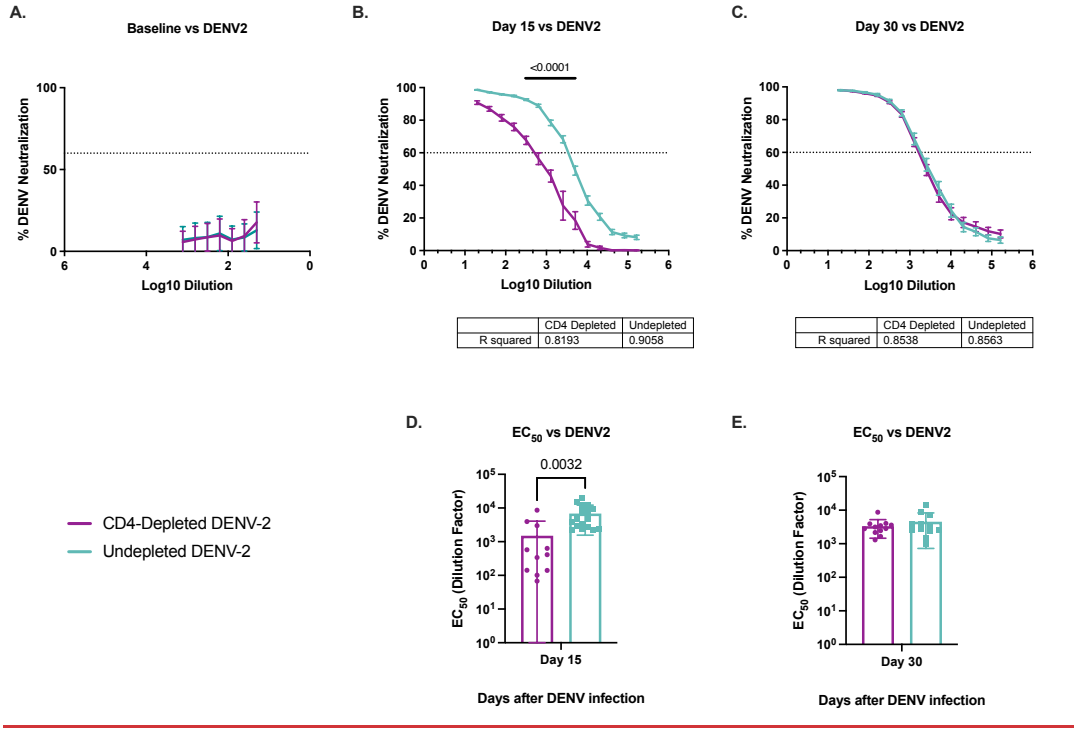
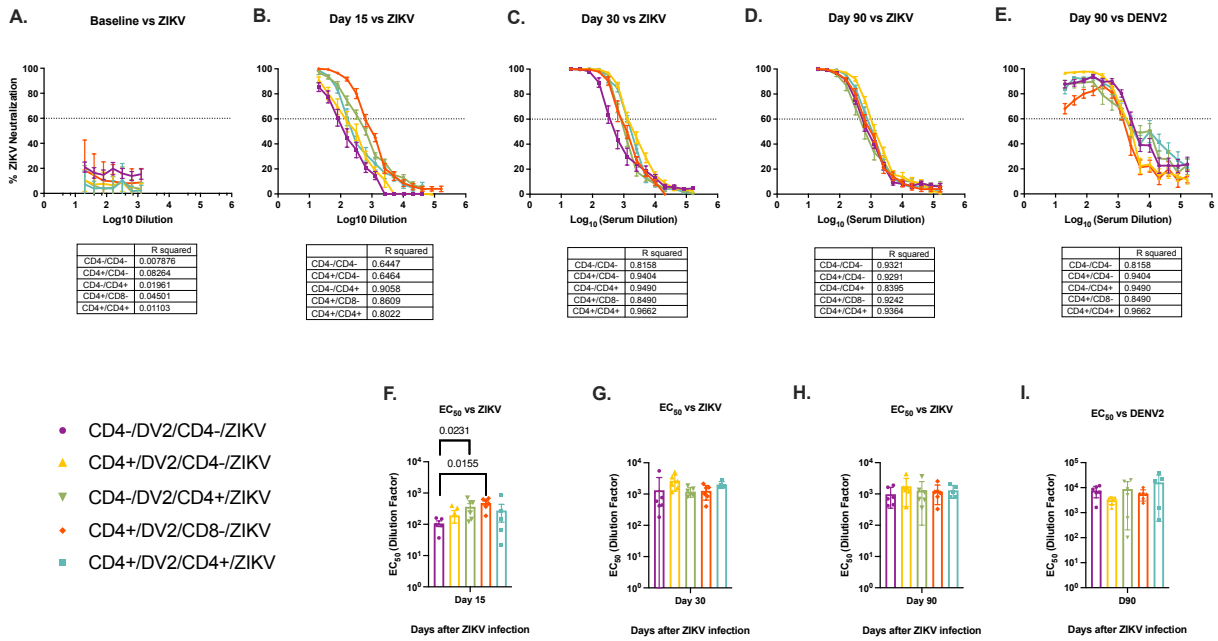


Fig 4. FRNT values of neutralizing antibodies against DENV-2 in depleted or undepleted flavivirus-naïve macaques. Neutralization capacity against DENV-2 is shown. CD4-depleted animals are depicted in purple and undepleted animals are depicted in turquoise. Dotted lines indicate the limit of detection for each test. **(A-C)** FRNT60 values of neutralizing antibodies against DENV-2 after DENV-2 infection are shown. **(D-E)** EC50 values of neutralizing antibodies against ZIKV after ZIKV infection. Statistically significant differences among groups were calculated by one-way and two-way ANOVA using the Tukey's multiple comparisons test and unpaired t-tests.



	R squared
CD4-/CD4-	0.007876
CD4+/CD4-	0.08264
CD4-/CD4+	0.01961
CD4+/CD8-	0.04501
CD4+/CD4+	0.01103

	R squared
CD4-/CD4-	0.6447
CD4+/CD4-	0.6464
CD4-/CD4+	0.9058
CD4+/CD8-	0.8609
CD4+/CD4+	0.8022

	R squared
CD4-/CD4-	0.8158
CD4+/CD4-	0.9404
CD4-/CD4+	0.9490
CD4+/CD8-	0.8490
CD4+/CD4+	0.9662

	R squared
CD4-/CD4-	0.9321
CD4+/CD4-	0.9291
CD4-/CD4+	0.8395
CD4+/CD8-	0.9242
CD4+/CD4+	0.9364

	R squared
CD4-/CD4-	0.8158
CD4+/CD4-	0.9404
CD4-/CD4+	0.9490
CD4+/CD8-	0.8490
CD4+/CD4+	0.9662

Fig 5. PRNT values of neutralizing antibodies against ZIKV in depleted or undepleted DENV-immune macaques. Neutralization capacity against ZIKV is shown. Animal cohorts are depicted as follows based on treatment regime: Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), CD4+/DV2/CD4-/ZV (shown in yellow), CD4-/DV2/CD4-/ZV (shown in purple), and CD4+/DV2/CD8-/ZV (shown in red). Dotted lines indicate the limit of detection for each test. **(A-D)** FRNT60 values of neutralizing antibodies against ZIKV after ZIKV infection are shown. **(E-G)** EC50 values of neutralizing antibodies against ZIKV after ZIKV infection. Significant differences among groups were calculated by one-way and two-way ANOVA using the Tukey's multiple comparisons test and unpaired t-tests.

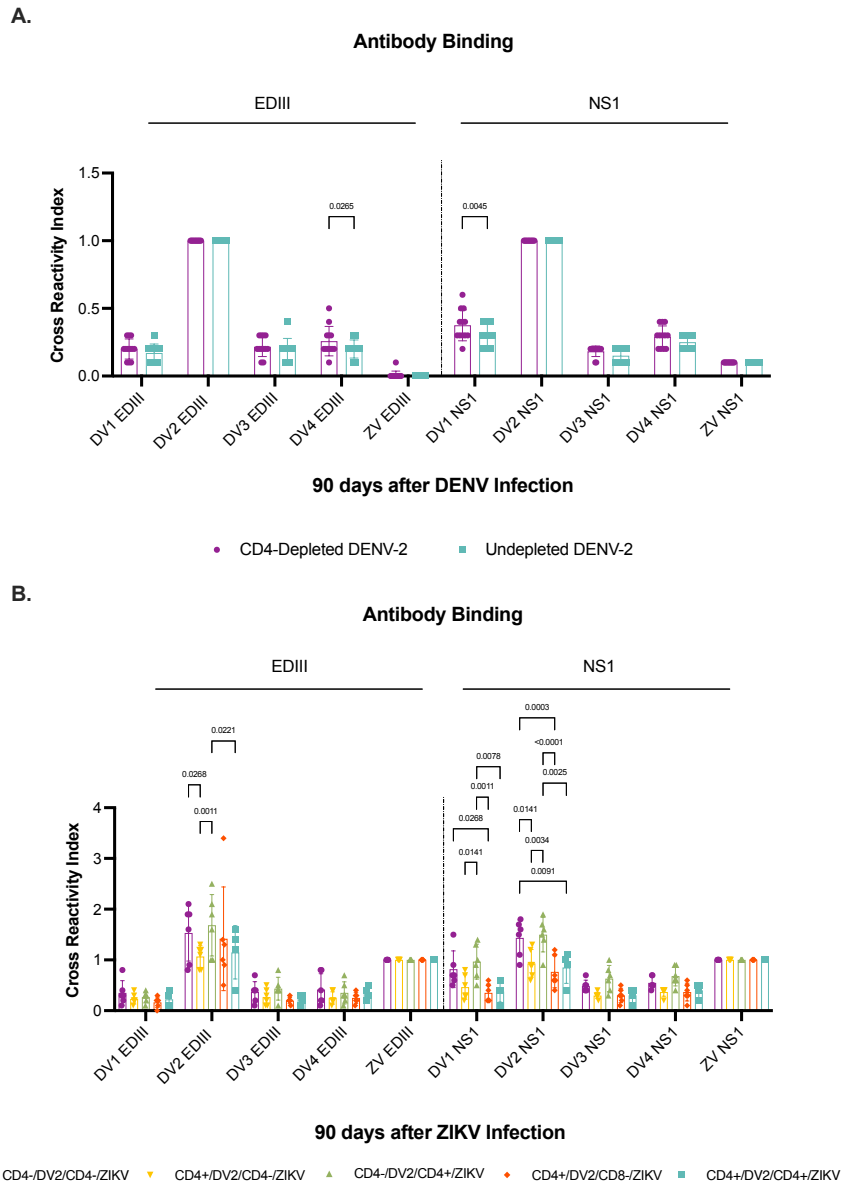


Fig 6. Epitope reactivity against DENV and ZIKV antigens in depleted and undepleted macaques. Antibody type-specific and cross-reactive responses against EDIII and NS1 antigens were assessed. Data generated by Luminex assay was normalized to a cross-reactivity index (CRI) by subtracting background (BSA) from the homologous value (values against infecting virus, HOM) and the heterologous values (values against heterologous virus, HET) followed by dividing the subtracted homologous by the subtracted heterologous values [$\frac{(HOM-BSA)}{(HET-BSA)}$]. **Upper panel.** CD4-depleted animals are depicted in purple and undepleted animals are depicted in turquoise. **(A)** Antibody response to EDIII (left side) and NS1 (right side) antigens from all four DENV serotypes and ZIKV 90 days after DENV2 infection**. Lower panel.** Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), CD4+/DV2/CD4-/ZV (shown in yellow), CD4-/DV2/CD4-/ZV (shown in purple), and CD4+/DV2/CD8-/ZV (shown in red). **(B)** Antibody response to EDIII (left side) and NS1 (right side) antigens from all four DENV serotypes and ZIKV 90 days after ZIKV infection**.** Significant differences among groups were calculated by two-way ANOVA using the Tukey's multiple comparisons test.

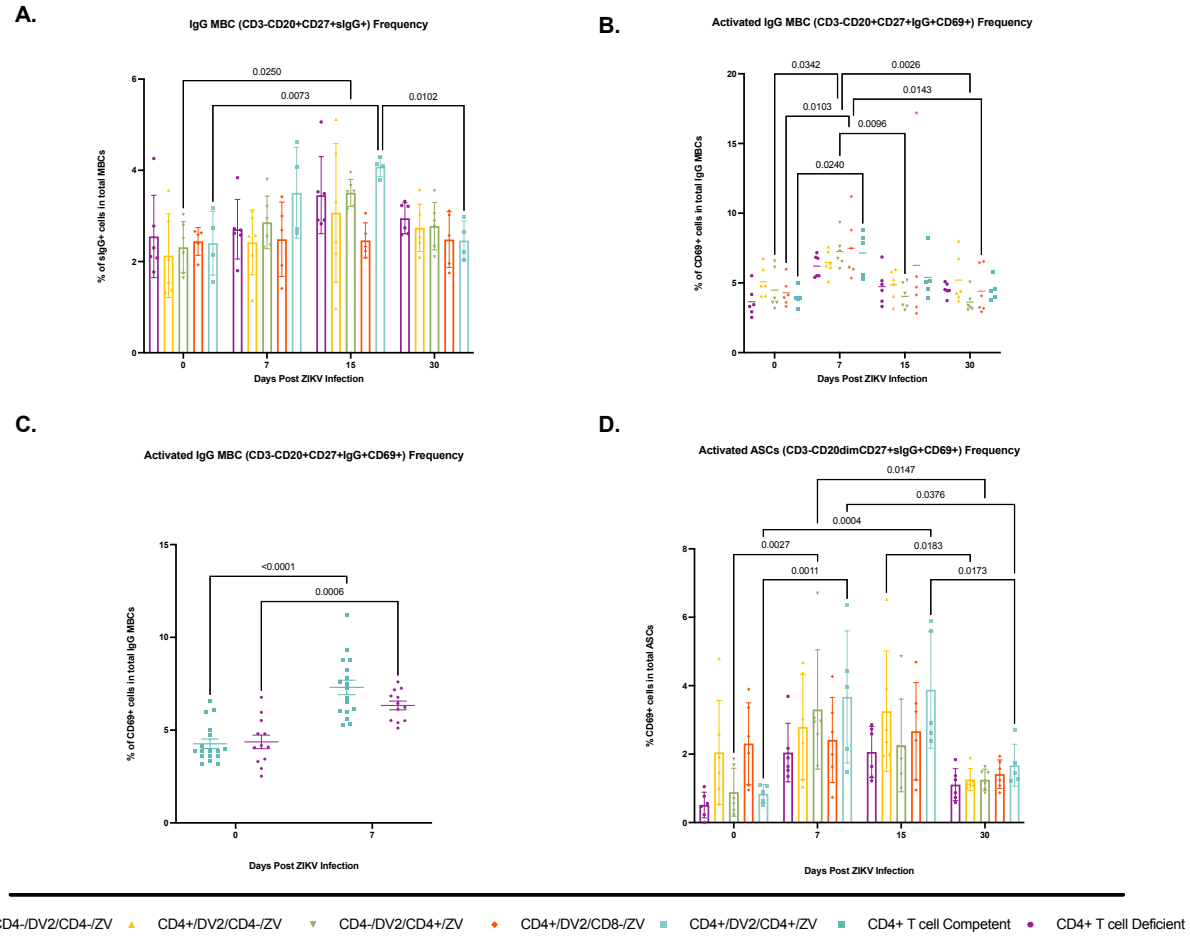


Fig 7. IgG memory B cell and ASC population kinetics after ZIKV infection in CD4+ T cell-competent and -deficient animals with previous DENV-2 immunity. The frequency of IgG MBCs and ASCs was assessed by immunophenotyping using flow cytometry. Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), CD4+/DV2/CD4-/ZV (shown in yellow), CD4-/DV2/CD4-/ZV (shown in purple), and CD4+/DV2/CD8-/ZV (shown in red). **(A)** Percentages of surface IgG expression in total MBCs (CD3-CD20+CD27+sIgG+). **(B)** Frequencies of CD69+ cells in total IgG+ MBCs (CD3-CD20+CD27+sIgG+CD69+). **(C)** Frequencies of CD69+ cells in total IgG MBCs with all cohorts organized into CD4+ T cell competent (shown in turquoise) and CD4+ T cell depleted (shown in purple). **(D)** Frequencies of CD69+ cells in total ASCs (CD3-CD20dimCD27+CD69+). Statistically significant differences among groups were calculated by two-way ANOVA using the Tukey's multiple comparisons test and unpaired t-tests.

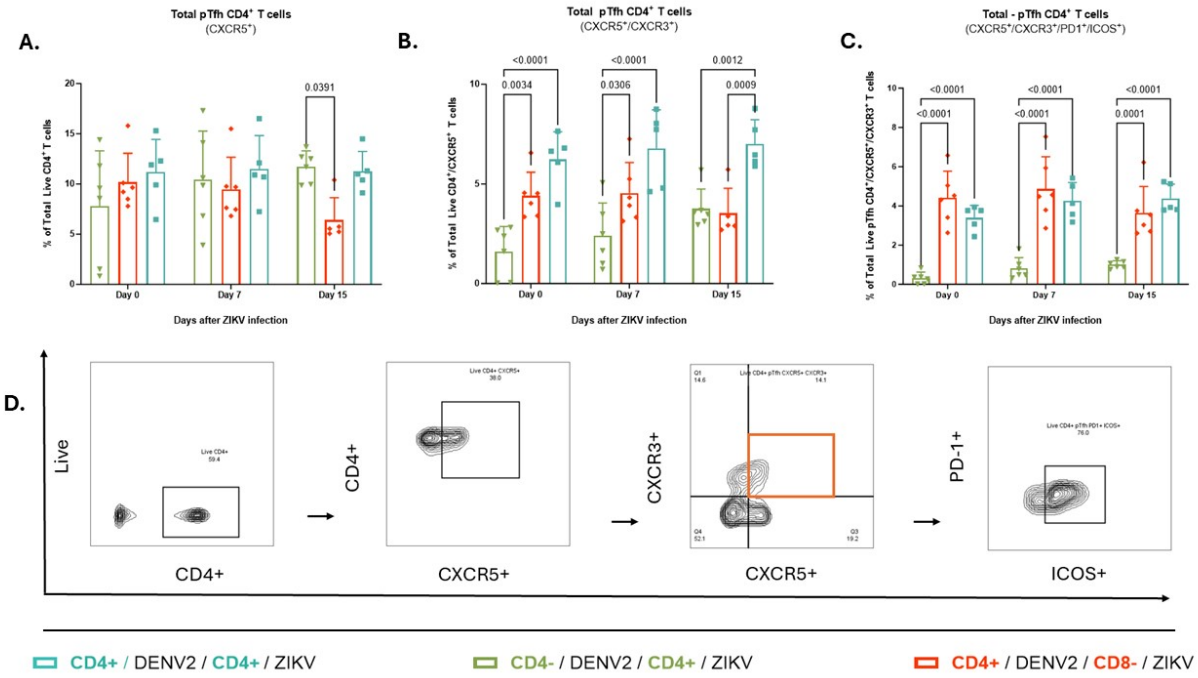


Fig 8. Total pTfh CD4+ T cell characterization. Immunophenotyping of CD4+ pTfh T cells was assessed via flow cytometry using frozen PBMCs collected on baseline, day 7, and day 15 pi with ZIKV. Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), and CD4+/DV2/CD8-/ZV (shown in red). **(A)** Frequency of total CD4+ T cells expressing follicular canonical marker CXCR5. **(B)** Frequency of total CD4-pTfh T cells expressing CXCR5 and CXCR3. **(C)** Frequency of total pTfh CD4 T cells expressing B cell co-stimulating surface makers PD1, and ICOS. **(D)** Gating Strategy for total pTfh CD4+ T cells characterization based on CXCR5, CXCR3, PD1, and ICOS surface markers expression. 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.

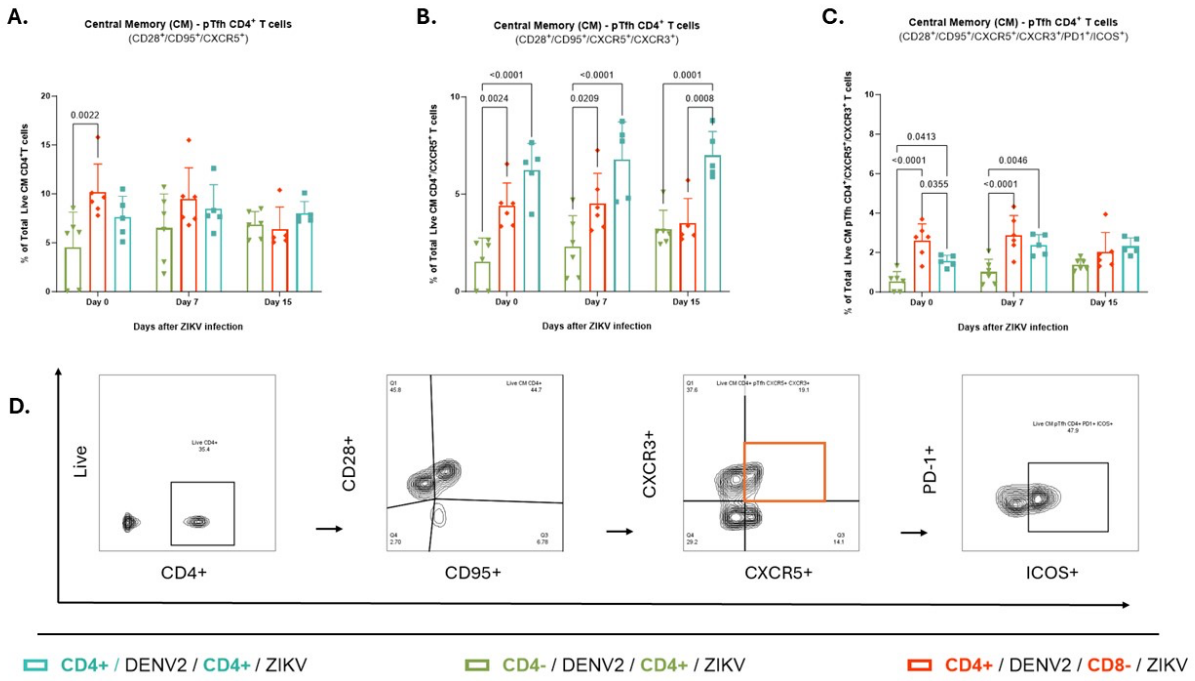
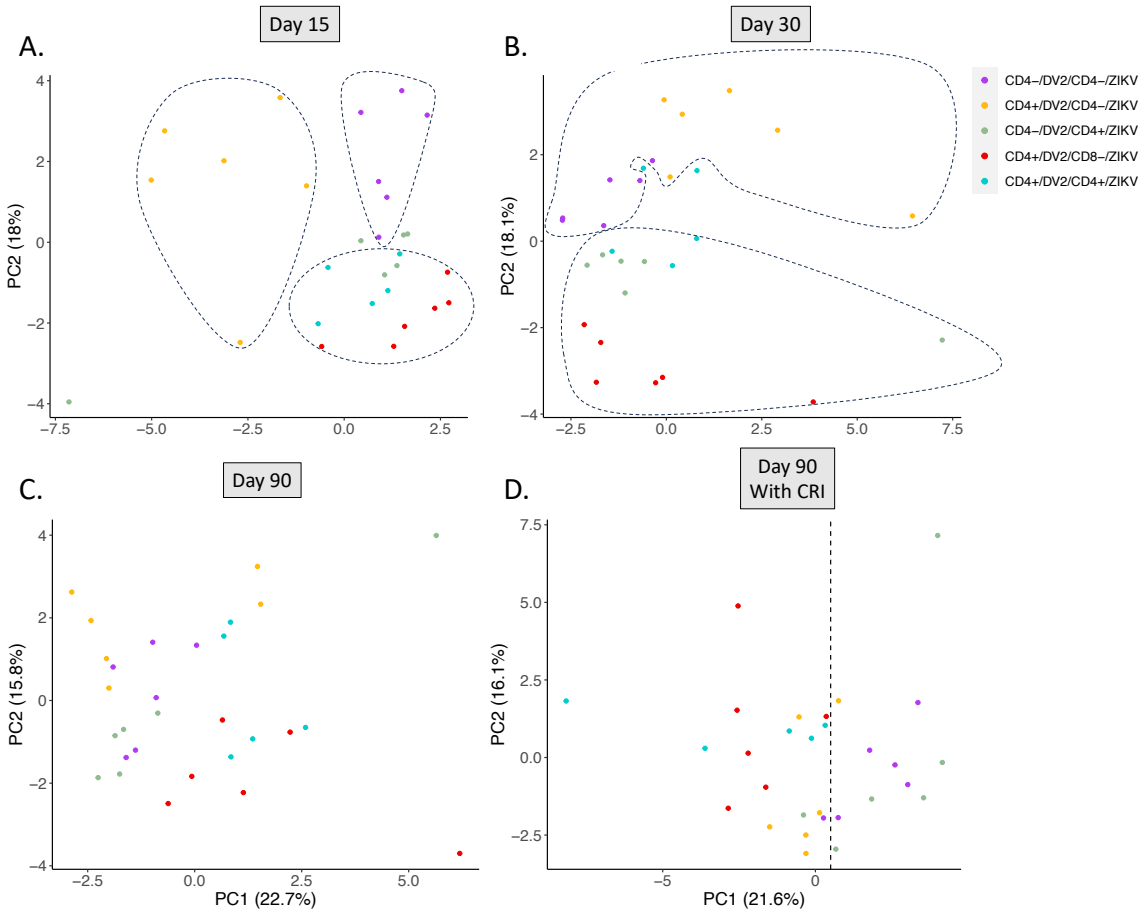


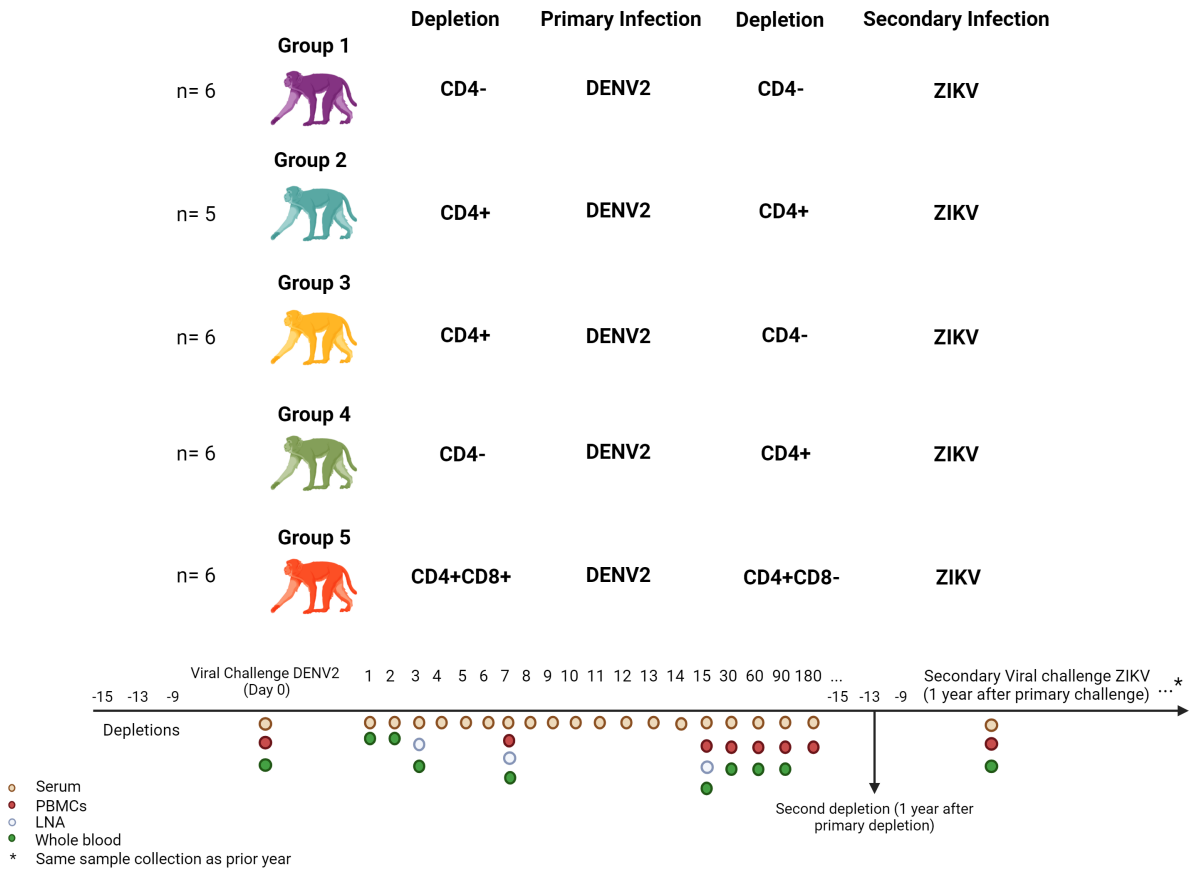
Fig 9. Total CM-pTfh CD4+ T cell characterization. Immunophenotyping of CM-pTfh CD4+ T cells after secondary ZIKV infection was accessed via flow cytometry using frozen PBMCs collected on baseline, day 7, and day 15 pi with ZIKV. Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), and CD4+/DV2/CD8-/ZV (shown in red). **(A)** Frequency of total CM CD4+ T cells expressing CXCR5. **(B)** Frequency of total CM- pTfh CD4+ T cells expressing CXCR5 and CXCR3. **(C)** Frequency of total CM-pTfh CD4+ T cells expressing B cell co-stimulating surface makers PD1, and ICOS. **(D)** Gating Strategy for total CM-pTfh CD4+ T cells characterization based on CXCR5, CXCR3, PD1, and ICOS surface markers expression. 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.

1284

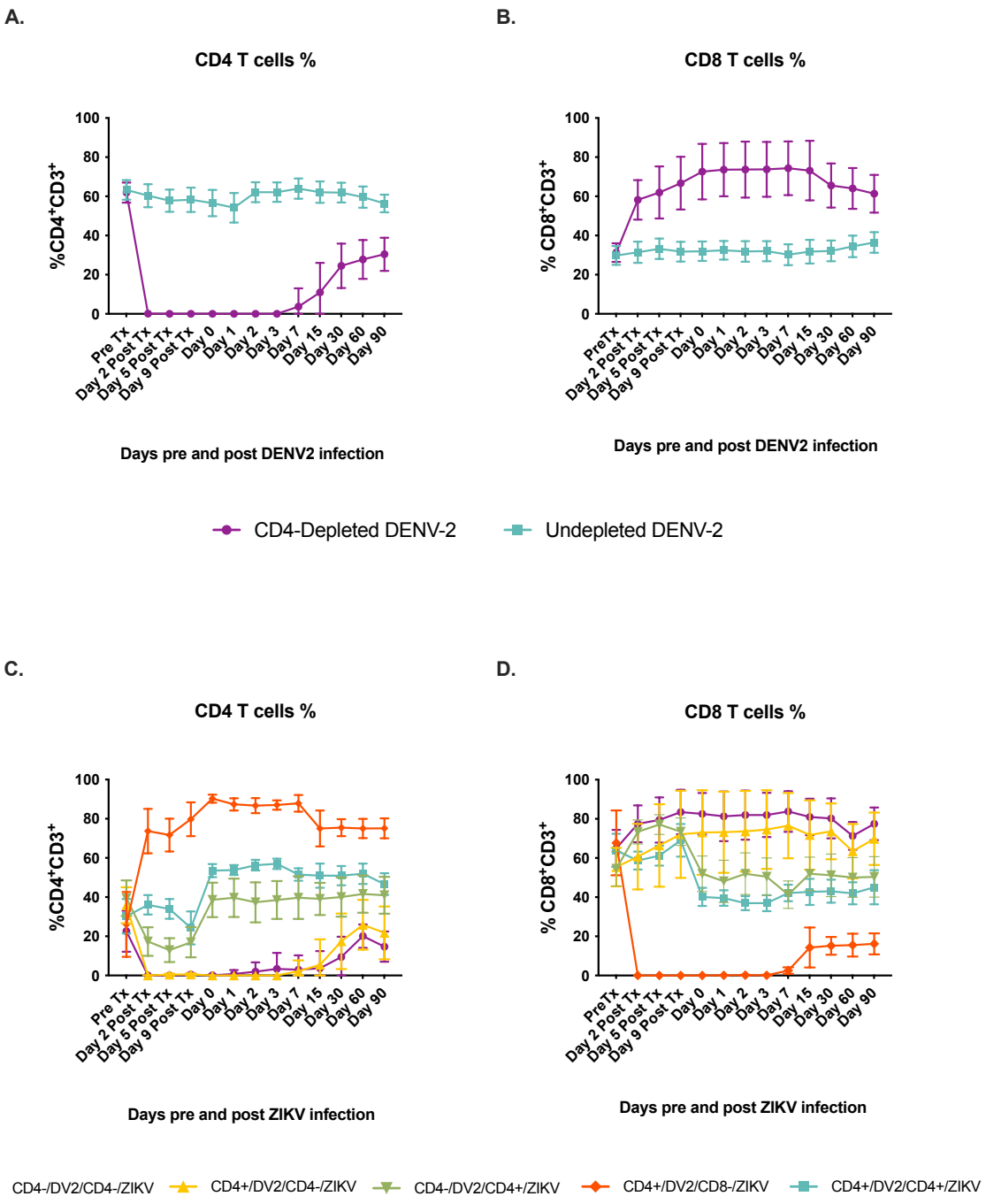


1285
1286
1287 **Fig 10. Principal component analysis of the effect of CD4+ T cell depletion on the ZIKV immune**
1288 **response**. Principal component analysis was conducted in order to evaluate the overall effect of CD4+ T
1289 cells on various elements of the immune response against ZIKV including neutralization activity, IgM and
1290 IgG response and B and T cell response. Animal cohorts are depicted as follows based on treatment regime:
1291 CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), CD4+/DV2/CD4-
1292 /ZV (shown in yellow), CD4-/DV2/CD4-/ZV (shown in purple), and CD4+/DV2/CD8-/ZV (shown in red).
1293 Principal component analysis of day 15 **(A)**, day 30 **(B)** and day 90 **(C)** post ZIKV challenge**.** Principal
1294 component analysis of day 90 p.c. including CRI data **(D)**.
1295

SUPPLEMENTARY FIGURES



SFig 1. Experimental design of T cell depletion and heterologous DENV2 and ZIKV infections in flavivirus-naïve macaques. Five cohorts of rhesus macaques (*Macaca mulatta*) underwent depletion of CD4+ T cells (or PBS in control group) followed by DENV-2 (5 x 10^5 pfu s.c.) infection at different timepoints. One year later, all cohorts were exposed to ZIKV strain PRABCV59 (1 x 10^6 pfu s.c.) after a second round of CD4+ or CD8+ T cell depletion (or PBS in control group). Sample collection was performed at various time points up to 90 days post-infection (p.i.) for serum, whole heparinized blood, and PBMC isolation.



SFig 2. CD4+ and CD8+ T cell frequency following depletion treatments prior to DENV2 and ZIKV infections. Frequency of CD4+ and CD8+ T cells was assessed by immunophenotyping before and after depletion treatments using flow cytometry. **Upper panel**. CD4-depleted animals are depicted in purple and undepleted animals are depicted in turquoise. CD4+ **(A)** and CD8+ **(B)** T cell depletion efficacy for depleted and undepleted animals before, during and after DENV2 infection. **Lower panel**. Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), CD4+/DV2/CD4-/ZV (shown in yellow), CD4-/DV2/CD4-/ZV (shown in purple), and CD4+/DV2/CD8-/ZV (shown in red). CD4+ **(C)** and CD8+ **(D)** T cell depletion efficacy for depleted and undepleted animals before, during and after ZIKV infection.

ID	Immune History	RNAemia (Log10 genome copies/mL) Post-DENV Infection																	Days	
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	30	Total	Mean
0Z6	CD4-/DV2	0.000	0.000	0.924	1.505	1.245	0.875					0.446	0.615	0.039	0.000	0.000	0.000	0.000	50	2.94
AE9		0.000	0.000	1.204	1.759	1.059	0.758	0.164				0.000	0.272	0.202	0.000	0.000	0.000	0.000		
1Z3		0.000	0.120	1.123	1.672	1.528	0.641	0.768		0.828		0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA317		0.000	0.000	1.557	2.235	1.888	1.291	0.176	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA312		0.000	0.000	0.908	2.060	0.718	0.961	0.217	0.425	0.285	0.107	0.713	0.000	0.000	0.000	0.000	0.000	0.000		
MA209		0.000	0.000	0.323	1.003	0.844	0.000	1.283	1.608	1.784	1.765	1.202	0.555	0.000	0.000	0.000	0.000	0.000		
5Z1		0.000	0.357	0.655	1.967	1.298	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
0Z7		0.000	0.326	0.991	3.821	1.282	0.663	0.493	0.490	0.000	0.720	0.000	0.362	0.000	0.000	0.000	0.000	0.000		
MA333		0.000	0.000	1.111	1.812	1.456	0.692	0.147	0.429	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA321		0.000	0.000	0.873	1.753	1.844	1.016	0.658	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA267		0.000	0.030	0.805	1.459	1.467	1.334	0.236	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA316		0.000	0.192	0.928	1.785	1.840	1.632	1.127	0.785	0.515	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA249		0.000	1.766	0.000	2.463	2.308	1.618	0.799	0.040	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA345		0.000	0.529	1.293	1.970	1.961	1.452	0.813	0.545	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
7X5		0.000	0.000	0.608	0.927	1.716	0.936	0.706	0.566	0.107	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
6S1		0.000	0.000	1.047	1.157	1.784	1.279	0.109	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA353		0.000	0.000	0.891	1.117	0.454	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
9X5	CD4+/DV2	0.000	0.076	0.879	1.741	1.379	0.663					0.000	0.000	0.000	0.000	0.000	0.000	0.000	50	2.94
MA341		0.000	0.000	2.037	2.855	2.111	1.006	0.174	0.053	0.115	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
4Z0		0.000	0.000	1.092	1.865	1.364	0.085	0.052	0.474	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA320		0.000	0.566	1.598	1.650	1.641	0.674	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
8X2		0.000	0.598	1.078	1.505	0.255	0.000	0.257	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA258		0.000	0.000	0.330	1.924	1.692	1.249	0.768	0.313	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
9X7		0.000	0.000	1.247	2.148	1.305	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
5Z8		0.000	0.901	1.555	2.487	1.782	0.123	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
9X2		0.000	0.638	1.313	1.662	1.704	1.080	0.457	0.000	0.067	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
9X1		0.000	0.000	0.527	1.242	1.009	0.552	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
2Z4		0.000	0.604	0.827	1.477	1.215	0.089	0.000	0.162	0.000	0.159	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA163		0.000	0.102	0.762	1.026	1.581	1.030	1.078	0.911	0.282	0.605	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA254		0.000	0.000	1.443	2.228	2.012	0.087	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA264		0.000	0.000	1.210	0.857	2.160	1.331	0.097	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA311		0.000	0.243	1.216	1.511	1.387	0.000	0.000	0.000	0.670	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA350		0.000	0.000	1.534	2.385	2.190	0.504	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA364		0.000	0.000	1.229	2.033	2.035	1.128	0.654	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		

Table S1**. DENV-2 RNAemia of CD4-depleted and undepleted animals.** DENV-2 RNA detection during the first 15 days and day 30 p.i. Mean viremia days per group was calculated using days with detectable RNAemia divided by the number of animals in each group.

ID	Immune History	RNAemia (Log10 genome copies/mL) Post-ZIKV Infection																	Days	
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	30	Total	Mean
0Z6	CD4-/DV2/CD4-/ZV	0.000	0.550	1.401	2.114	1.305	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	13	2.17
AE9		0.000	0.277	0.878	2.158	1.496	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
1Z3		0.000	0.296	1.213	2.459	1.744	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA317		0.000	0.118	1.214	2.663	1.639	0.649	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA312		0.000	0.702	1.096	2.188	1.850	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA209		0.000	0.156	0.939	1.730	1.507	0.347	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
9X5	CD4+/DV2/CD4-/ZV	0.000	0.455	1.477	2.582	1.955	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	12	2
MA341		0.000	0.884	1.680	3.208	2.709	0.412	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
4Z0		0.000	-0.038	0.272	1.574	0.473	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA320		0.000	-0.377	0.770	1.662	-0.553	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
8X2		0.000	-0.029	0.122	1.982	0.911	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA258		0.000	-0.467	1.456	3.038	2.164	0.000	0.143	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
5Z1	CD4-/DV2/CD4+/ZV	0.000	0.508	0.686	2.364	0.378	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	9	1.5
0Z7		0.000	0.967	1.585	2.828	1.904	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA333		0.000	0.724	1.097	2.244	1.185	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA321		0.000	0.468	1.183	2.437	1.873	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA267		0.000	0.150	0.243	1.994	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA316		0.000	0.578	1.121	2.871	0.820	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
9X7	CD4+/DV2/CD8-/ZV	0.000	0.006	-1.268	1.311	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	7	1.17
5Z8		0.000	0.637	1.010	2.605	1.701	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
9X2		0.000	0.455	0.429	1.991	0.458	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
9X1		0.000	0.000	0.410	1.420	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
2Z4		0.000	0.041	0.109	1.438	0.420	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA163		0.000	0.000	0.069	2.038	1.161	0.559	0.212	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA254	CD4+/DV2/CD4+/ZV	0.000	0.109	0.989	2.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	6	1
MA264		0.000	0.000	0.874	2.521	1.261	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA311		0.000	0.000	0.964	2.271	1.307	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA350		0.000	0.493	0.901	2.262	1.290	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
MA364		0.000	0.000	1.045	2.340	1.059	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		

Table S2. ZIKV RNAemia days of DENV-immune macaques with different T cell depletion status. ZIKV RNA detection during the first 15 days and day 30 p.i. Mean viremia days per group was calculated using days with detectable RNAemia divided by the number of animals in each group.

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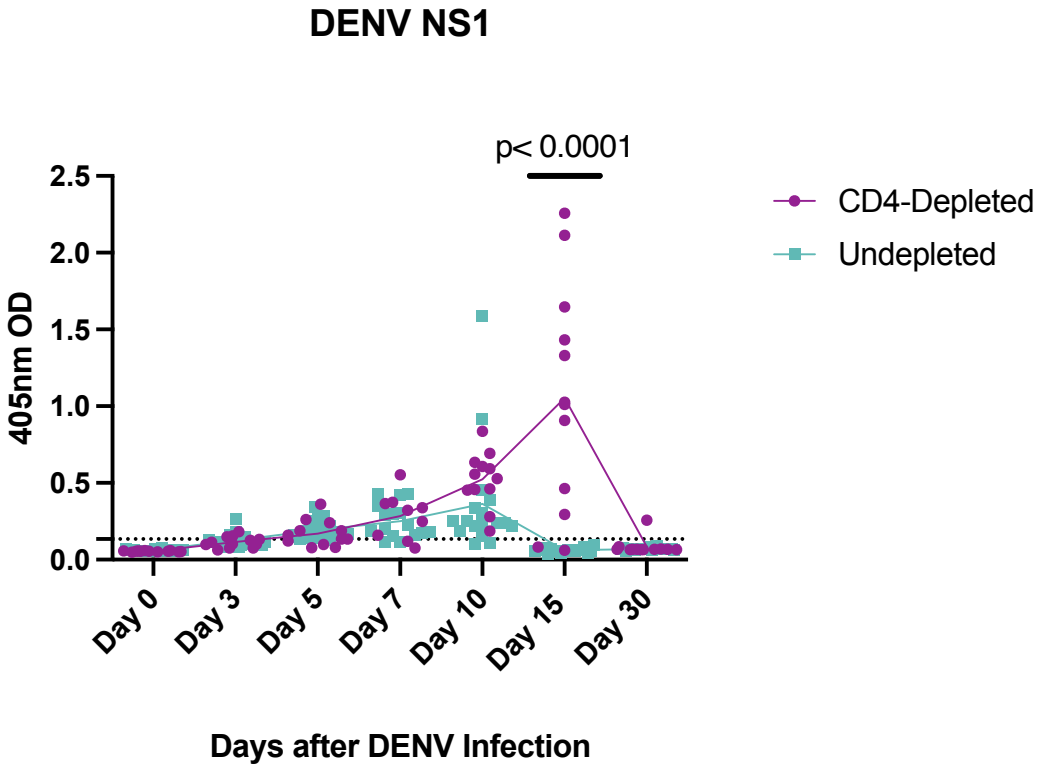

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1341

1342 **SFig 3. DENV NS1 levels are affected by absence of CD4+ T cells after DENV-2 infection.** DENV-NS1
1343 levels after DENV-2 infection were measured using a commercial ELISA test. CD4-depleted animals are
1344 depicted in purple and undepleted animals are depicted in turquoise. Dotted lines indicate the limit of
1345 detection for each test. Statistically significant differences among and within groups were calculated by
1346 two-way ANOVA using Tukey's multiple comparisons test and unpaired t-tests.

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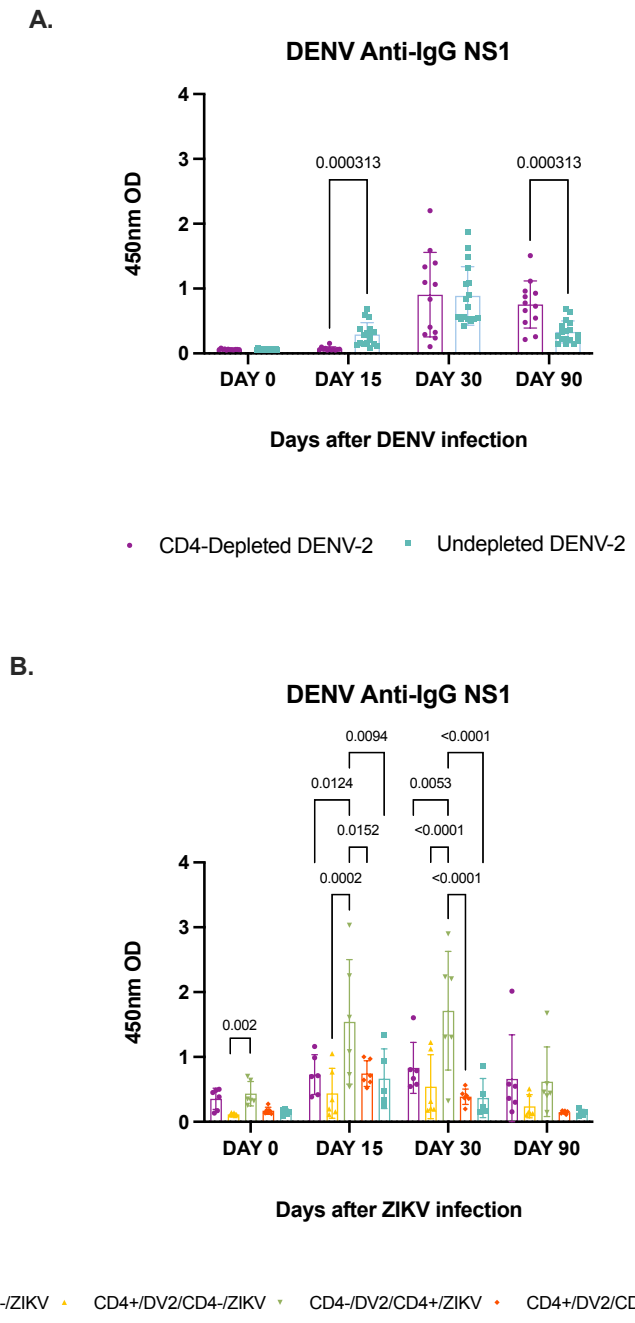
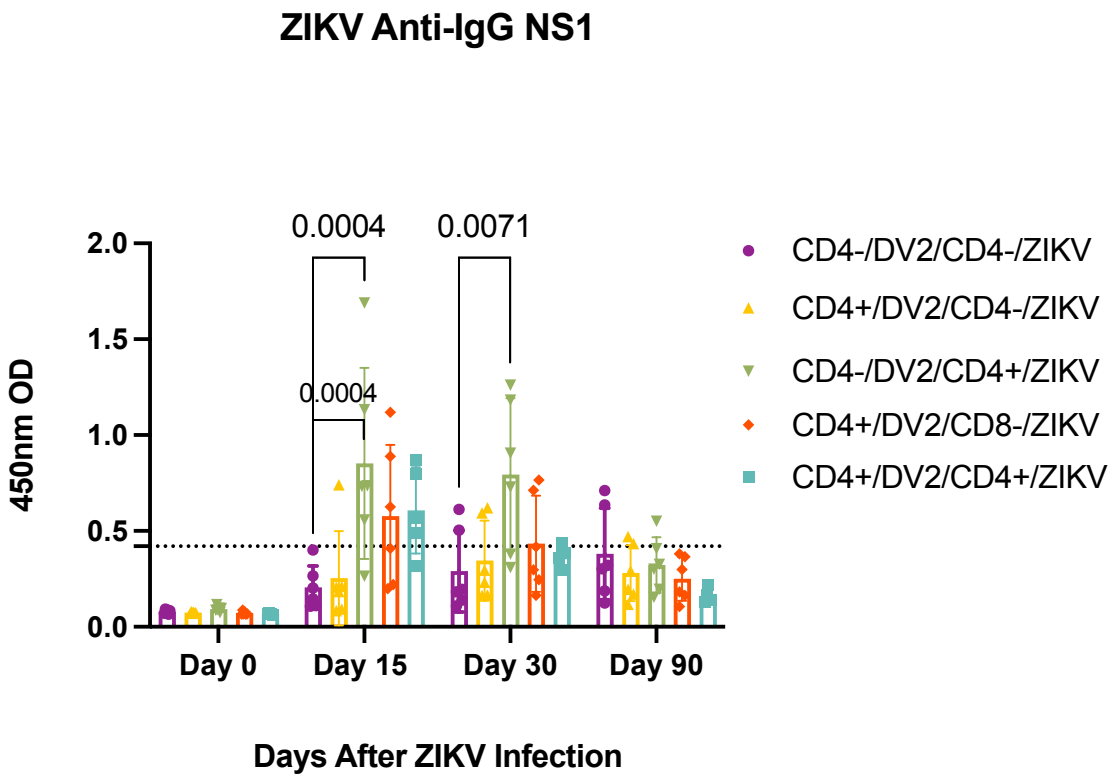
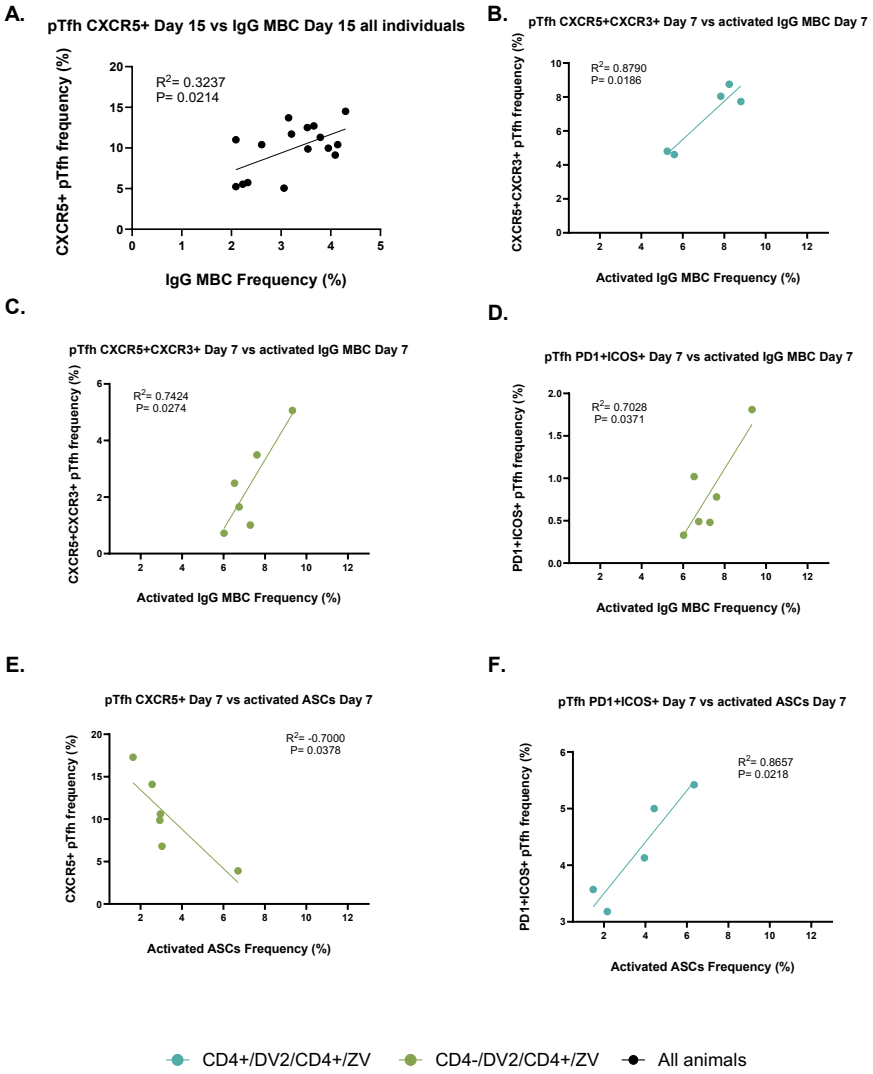


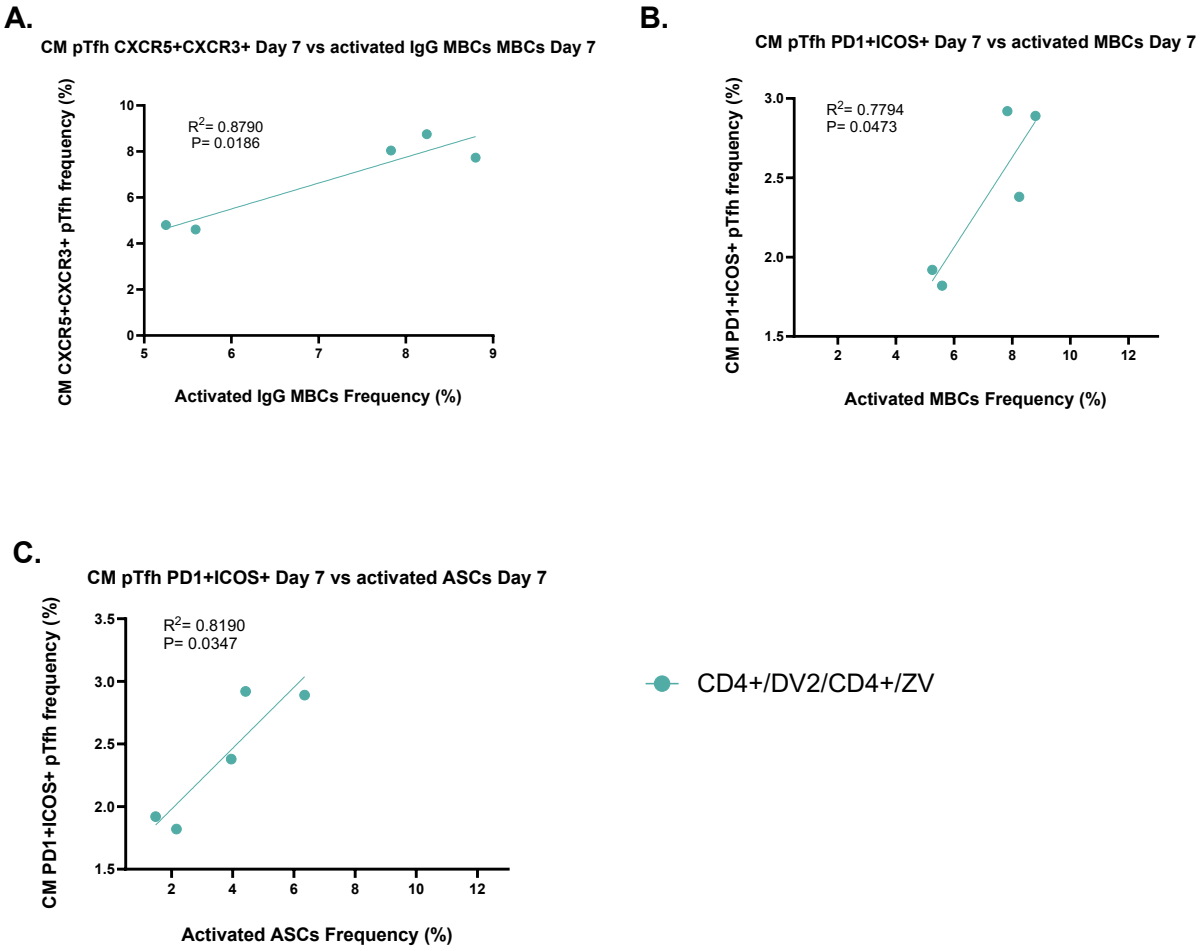
SFig 4. Depletion of CD4+ T cells before DENV infection increases DENV NS1 anti-IgG levels. DENV-NS1 anti-IgG levels were measured using commercial ELISA tests. **(A)** CD4-depleted animals are depicted in purple and undepleted animals are depicted in turquoise. DENV NS1 anti-IgG levels after DENV-2 infection. **(B)** Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), CD4+/DV2/CD4-/ZV (shown in yellow), CD4-/DV2/CD4-/ZV (shown in purple), and CD4+/DV2/CD8-/ZV (shown in red). DENV NS1 anti-IgG levels after ZIKV infection. No limit of detection or cut-off value is provided because this value will vary depending on flavivirus disease prevalence in the geographical location where the test is performed. Statistically significant differences among and within groups were calculated by two-way ANOVA using Tukey's multiple comparisons test and multiple t-tests.



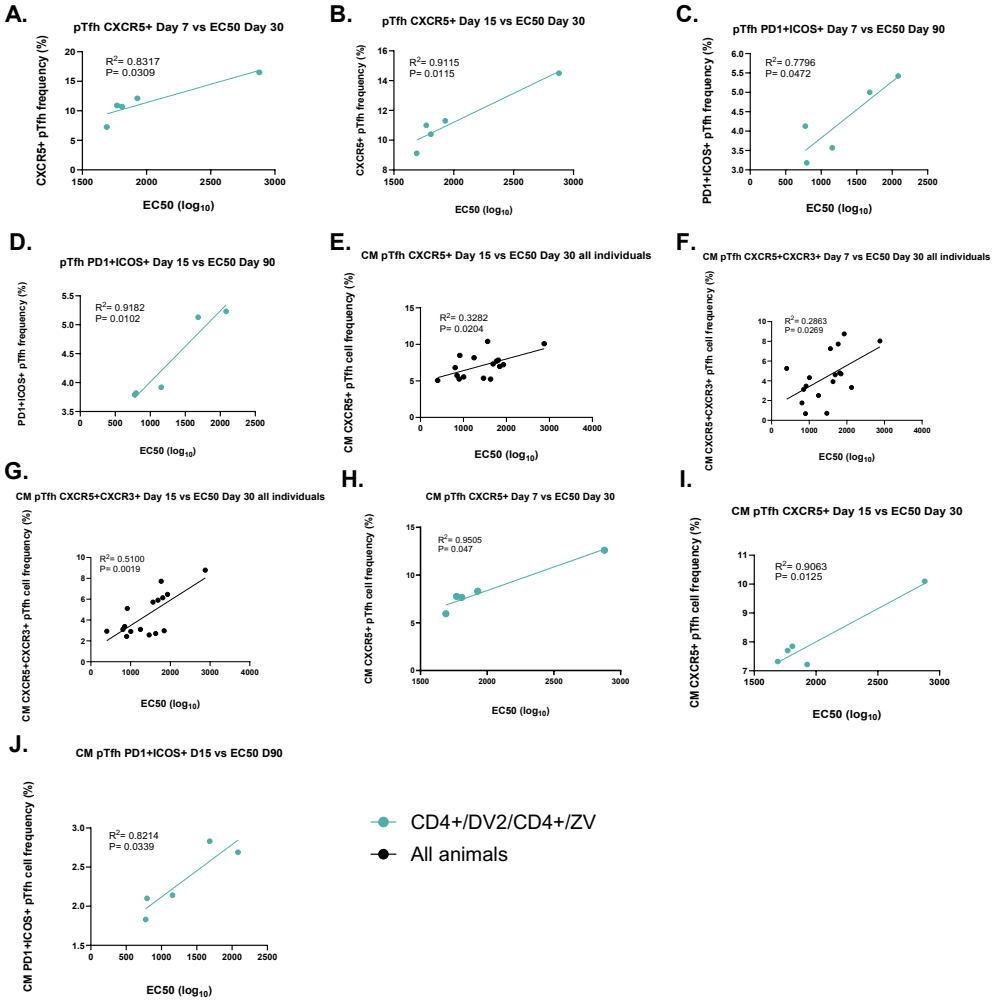
SFig 5. CD4+ T cell depletion status prior to DENV infection has no impact in production of ZIKV NS1-binding IgG after ZIKV infection. ZIKV NS1-IgG response after ZIKV infection in the presence of DENV immunity was assessed using a commercial ELISA test. Animal cohorts are depicted as follows based on treatment regime: Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise), CD4-/DV2/CD4+/ZV (shown in green), CD4+/DV2/CD4-/ZV (shown in yellow), CD4-/DV2/CD4-/ZV (shown in purple), and CD4+/DV2/CD8-/ZV (shown in red). Dotted lines indicate the limit of detection for each test. Binding capacity of ZIKV NS1-IgG antibodies from CD4-depleted and undepleted animals after ZIKV infection. Statistically significant differences among and within groups were calculated by two-way ANOVA using Tukey's multiple comparisons test and unpaired t-tests.



Sfig 6. **Frequencies of pTfh cells correlate with B cell response cell subsets such as total IgG$^+$ MBCs, activated IgG$^+$ MBCs, and activated ASCs during the secondary ZIKV infection.** Pearson correlations and simple linear regressions were performed on data from pTfh and B cell subsets. All experimental animals are shown in black. Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise) and CD4-/DV2/CD4+/ZV (shown in green). **(A)** The levels of total pTfh cells of the CXCR5$^+$ phenotype (shown as X5+ in graphs), measured via Flow Cytometry from PBMCs, isolated at day 15 p.c., correlated with frequencies of total IgG$^+$ MBCs, measured via Flow Cytometry from isolated whole blood at day 15 p.c.. **(B-C)** Correlations were performed comparing CXCR5$^+$CXCR3$^+$ pTfh cells (shown as X5+X3+) from day 7 p.c. with **(B)** activated IgG$^+$ MBCs from day 7 p.c., in the CD4+/DV2/CD4+/ZV group, and in the **(C)** CD4-/DV2/CD4+/ZV group. Correlations were performed comparing PD1+ICOS+ pTfh cells, from day 7 p.c., with **(D)** activated IgG$^+$ MBCs in the CD4-/DV2/CD4+/ZV group. A correlation was performed comparing CXCR5$^+$ (X5+) pTfh cells, from day 7 p.c., with **(E)** activated ASCs in the CD4-/DV2/CD4+/ZV group. A correlation was performed comparing PD1+ICOS+ pTfh cells, from day 7 p.c., with **(F)** activated ASCs in the CD4+/DV2/CD4+/ZV group (+/+). Pearson's rank correlation coefficients were used to calculate correlations, and R^2- and P-values are indicated. Linear regression is shown as lines and each dot represents one animal.

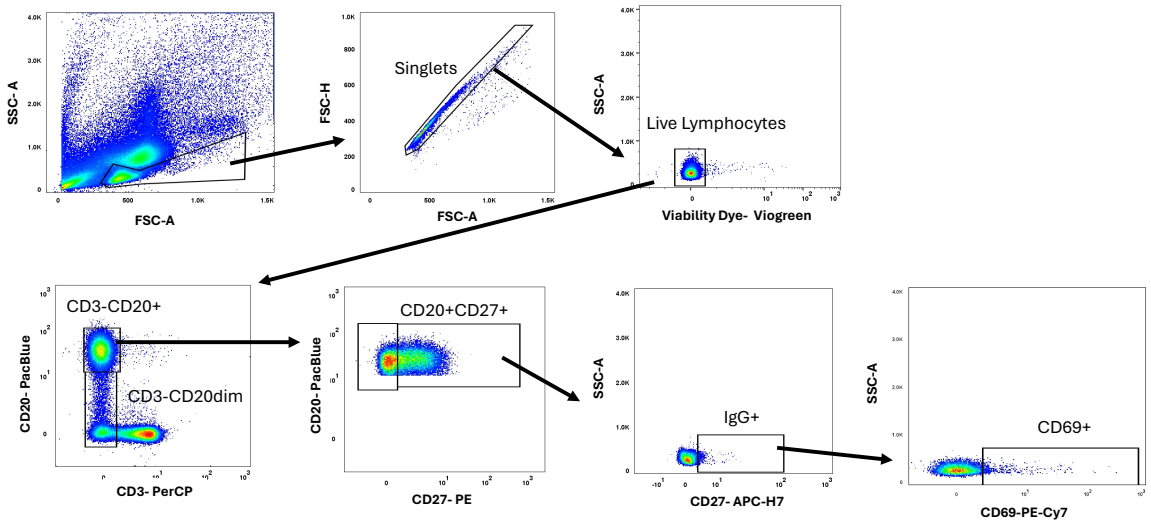


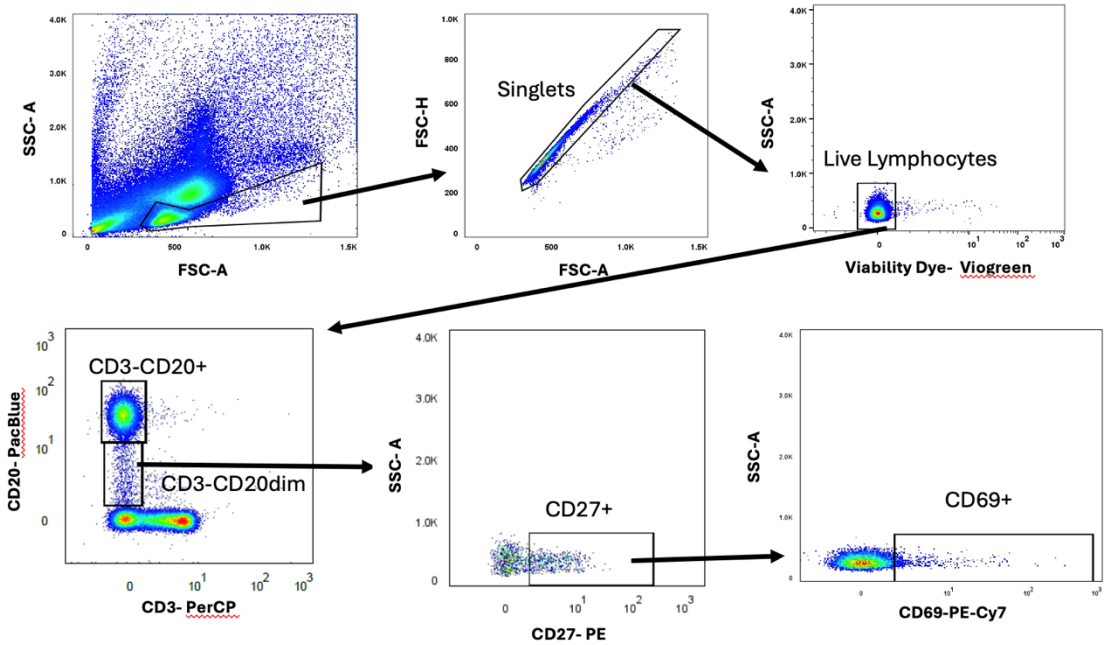
SFig 7. **Frequencies of CM pTfh cell subsets correlate with B cell response cell subsets.** Pearson correlations and simple linear regressions were performed using data from B cell subsets such as activated IgG^+ MBCs and ASCs during the secondary ZIKV infection**.** Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise). All significant correlations with CM pTfh cells were only observed in the CD4+/DV2/CD4+/ZV group. Correlations were performed and observed for the CM $CXCR5^+CXCR3^+$ pTfh cells (shown as X5+X3+), from day 7 p.c., with **(A)** activated IgG^+ MBCs, from day 7 p.c. in the CD4+/DV2/CD4+/ZV group. Correlations were performed for comparing CM PD1+ICOS+ pTfh cells, from day 7 p.c., with **(B)** activated IgG^+ MBCs in the CD4+/DV2/CD4+/ZV group. Correlations were performed for CM PD1+ICOS+ pTfh cells with **(C)** activated ASCs, from day 7 p.c., in the CD4+/DV2/CD4+/ZV group. Pearson's rank correlation coefficients were used to calculate correlations, and R^2- and P-values are indicated. Linear regression is shown as lines and each dot represents one animal.



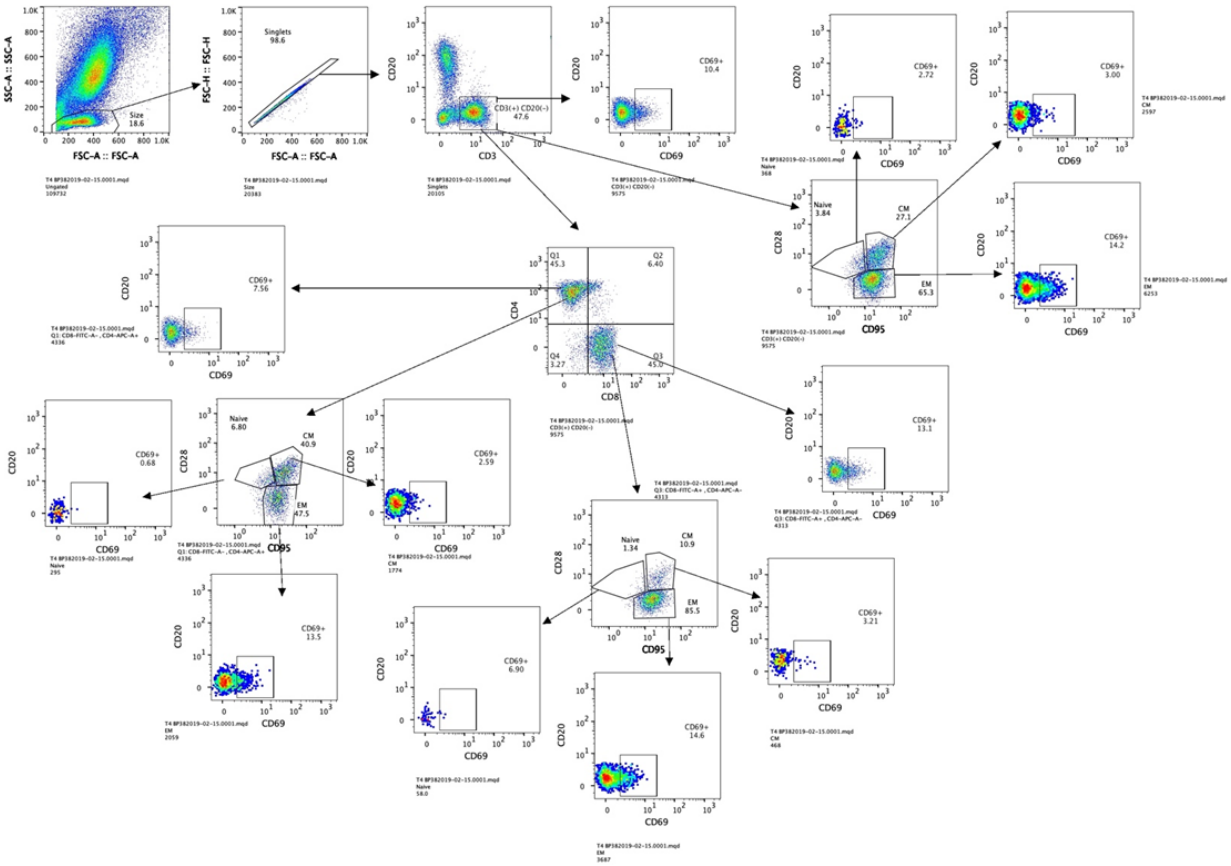
SFig 8. Frequencies of pTfh cell subsets correlate with EC50 (log_{10}) values. Data obtained from Focus/Plaque Reduction Neutralization Tests on collected sera from days 30 and 90 p.c. during secondary ZIKV infection was analyzed using Pearson and simple linear regression with pTfh cells. All experimental animals are shown in black. Animal cohorts are depicted as follows based on treatment regime: CD4+/DV2/CD4+/ZV (shown in turquoise). **(A-B)** Correlations were performed comparing $CXCR5^+$ pTfh cells (shown as X5+), from **(A)** days 7 and **(B)** days 15 p.c. with EC50 values from day 30 p.c., in the CD4+/DV2/CD4+/ZV group. **(C-D)** Correlations were performed comparing PD1+ICOS+ pTfh cells from **(C)** day 7 and **(D)** day 15 p.c. with EC50 values from day 90 p.c., in the CD4+/DV2/CD4+/ZV group. **(E-I)** Frequencies of CM pTfh cell subsets correlate with EC50 values from days 30 and 90 p.c.. **(E-G)** Correlations were performed using all experimental comparing **(E)** CM $CXCR5^+$ (X5+) pTfh cells, from day 15 p.c., with EC50 values from day 30 p.c., comparing **(F)** CM $CXCR5^+CXCR3^+$ (shown as X5+X3+) pTfh cells, from day 7 p.c., with EC50 values from day 30 p.c., and comparing **(G)** CM $CXCR5^+CXCR3^+$ (X5+X3+) pTfh cells, from day 15 p.c.. Correlations were performed comparing CM $CXCR5^+$ (X5+) pTfh cells, from **(H)** day 7 and **(I)** day 15 p.c., with EC50 values from day 30 p.c.. Correlations were performed comparing CM PD1+ICOS+ pTfh cells, from day 15 p.c., with **(J)** EC50 values from day 90 p.c.. Pearson's rank correlation coefficients were used to calculate correlations, and R^2- and P-values are indicated. Linear regression is shown as lines and each dot represents one animal.

IgG+ memory B cell Gating

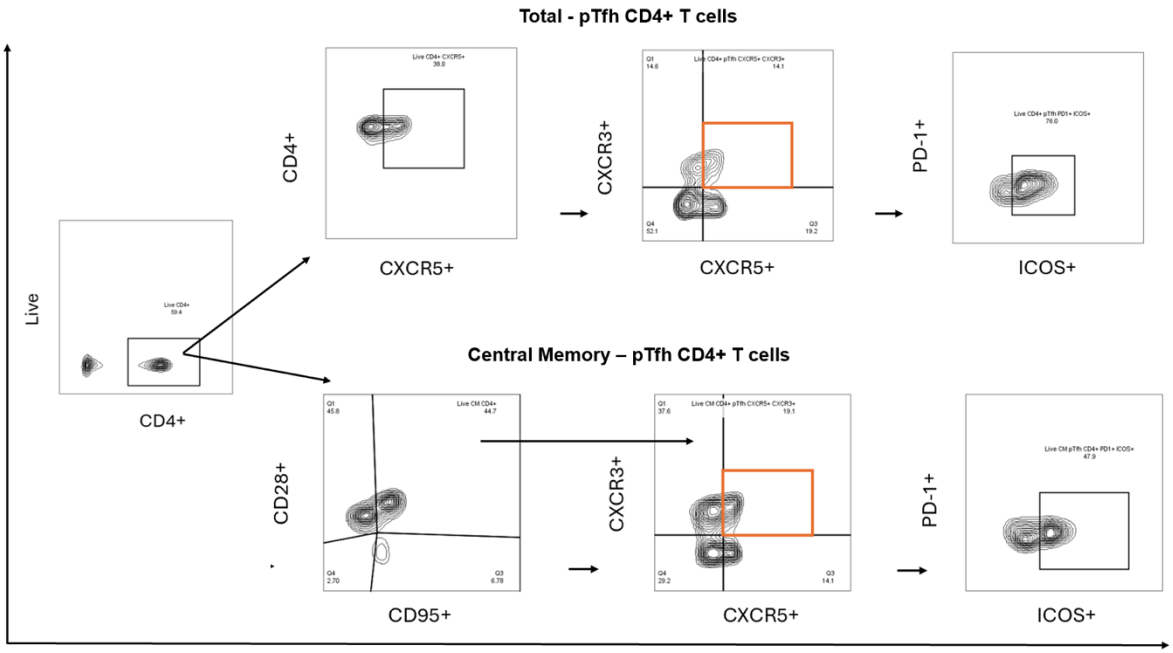




SFig 9. Gating strategy for B cells. Gating strategy used to define B cells and subsets. Lymphocytes were gated based on their characteristic forward and side scatter pattern (FSC, SSC). B cells were defined as CD20+CD3-.



SFig 10. Gating strategy for T cells. Gating strategy used to define T cells and subsets. Lymphocytes were gated based on their characteristic forward and side scatter pattern (FSC, SSC). T cells were defined as CD3+CD20+. CD4+ and CD8+ T cells were defined as CD3+CD4+ and CD3+CD8+, respectively.



SFig 11. Gating strategy for pTfh CD4+ T cells. Gating strategy used to define pTfh CD4+ T and total central memory (CM- pTfh) CD4+ T cells based on CD28+, CD95+, CXCR5+, CXCR3+, PD1+, and ICOS+ surface markers expression.

Parameter	Day of measurement for PCA
TFH_CD4_ ICOS_PD1	Day 7, Day 15
zikav_od_igm	Day 15, Day 30
zikav_od_igg	Day 15, Day 30, Day 90
denv_od_igm	Day 15
denv_od_igg	Day 15, Day, 30, Day 90
neutralization vs. ZikaV	Day 15, Day 30, Day 90
neutralization vs. DengueV	Day 15, Day 30, Day 90
CD4_T_cells	Day 15, Day 30, Day 90
CD8_T_cells	Day 15, Day 30, Day 90
T4_CM	Day 15, Day 30, Day 90
T4_CM_CD69	Day 15, Day 30, Day 90
T8_CM	Day 15, Day 30, Day 90
T8_CM_CD69	Day 15, Day 30, Day 90
T4_EM	Day 15, Day 30, Day 90
T4_EM_CD69	Day 15, Day 30, Day 90
T8_EM	Day 15, Day 30, Day 90
T8_EM_CD69	Day 15, Day 30, Day 90
CD4_Naive	Day 15, Day 30, Day 90
CD4_Naive_CD69	Day 15, Day 30, Day 90
CD8_Naive	Day 15, Day 30, Day 90
CD8_Naive_CD69	Day 15, Day 30, Day 90
B_Cells	Day 15, Day 30, Day 90
B_Cells_CD69	Day 15, Day 30, Day 90
CD27_CD69	Day 15, Day 30, Day 90
CD20_CD27	Day 15, Day 30, Day 90
EDIII_CRI_D1	DAY 90
EDIII_CRI_D2	DAY 90
EDIII_CRI_D3	DAY 90
EDIII_CRI_D4	DAY 90
EDIII_CRI_Z	DAY 90
NS1_CRI_D1	DAY 90
NS1_CRI_D2	DAY 90
NS1_CRI_D3	DAY 90
NS1_CRI_D4	DAY 90
NS1_CRI_Z	DAY 90
OD = Optical Density	
CM = Central Memory	
tfh = T Follicular Helper	
CRI = Cross Reactivity Index	

1445 Table S3**. Parameters used to perform PCA analysis.** PCA analysis was performed using parameters from
1446 days 7, 15, 30 and 90 post ZIKV infection.
1447

1448 **References**
1449

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