

Infection order outweighs the role of CD4⁺ T cells in tertiary flavivirus infection

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Abstract

Dengue (DENV) and Zika (ZIKV) are flaviviruses that co-circulate throughout the tropical and subtropical regions. The link between CD4⁺ T and B cells during immune responses to DENV and ZIKV and their roles in cross-protection during heterologous infection is an active area of research. Here we used CD4⁺ lymphocyte depletions to dissect the impact of cellular immunity on humoral responses during a tertiary flavivirus infection. We show that CD4⁺ depletion in DENV-primed animals followed by ZIKV-DENV resulted in delayed viremia followed by increased viral replication in tertiary infections and dysregulated adaptive immune responses compared to ZIKV-primed animals followed by DENV-DENV infections. We show a delay in DENV-specific IgM/IgG antibody titers and neutralization in the DENV-primed CD4-depleted animals but not in ZIKV-primed CD4-depleted animals. This study confirms the critical role of CD4⁺ cells in viremia control and priming of early and robust neutralizing antibody responses during sequential flavivirus infections. Our results also revealed differential cytokine profiles for both infection sequences regardless of CD4⁺ status. Our work here suggests that the order of flavivirus exposure affects the outcome of a tertiary infection. Our findings have implications for understanding complex immune responses induced by flaviviruses that co-circulate and develop effective flavivirus vaccines.

Introduction

Flaviviruses, including Dengue virus (DENV) and Zika virus (ZIKV), are principally arthropod-borne viruses that cause mild to severe diseases in humans worldwide. These members of the *Flaviviridae* family are transmitted primarily through the bite of *Aedes* spp. mosquitoes, imposing an enormous public health burden in tropical and subtropical areas ^{1,2}. Whereas ZIKV transmission has decreased in recent years, its initial emergence into the DENV-endemic regions of the western hemisphere ^{3,4} raised concerns, mainly due to immunological cross-reactivity limiting serological testing and the implications for the development of severe manifestations in populations exposed to sequential infections ^{5,6}. While ZIKV consists of a single serotype, there are four different serotypes of DENV based on antigenic differences of the envelope protein, all of which are pathogenic in humans ⁷⁻¹⁰. Exposure to one infecting serotype should confer lifelong protection against disease upon secondary homotypic infection. However, heterologous DENV infection can lead individuals to develop dengue or severe dengue, described as hemorrhagic fever or shock syndrome ^{11,12}. On the other hand, ZIKV cases are generally self-limiting febrile illnesses like dengue fever, but ZIKV has been associated with more severe outcomes such as Guillain-Barré syndrome (GBS) and birth defects ¹³.

During the peak of the ZIKV epidemic in 2016, there was little DENV transmission in the Americas. This has been linked to the extensive cross-reactivity between antibodies ^{6,14,15} and T cells ^{16,17} generated against DENV and ZIKV ¹⁸. The role of cellular immune responses in mediating clearance of subsequent DENV or ZIKV infections has been extensively studied in immunodeficient mice and human infections, highlighting the importance of DENV and ZIKV-specific CD8⁺ T cells against homotypic infections ^{8,19-23}. Similarly, CD4⁺ T cells have been shown to be important in flavivirus infections, displaying functional

plasticity exerting cytotoxic characteristics as a function of previous infections²⁴ and contributing to protection²⁵⁻²⁷. Recently, *Rouers et al.* provided a detailed dissection on the balance between protection or harm depending on T cells' phenotype in response to primary or secondary dengue infections²⁸. Previously, our group determined that cross-protection is associated with the interval of time between DENV and ZIKV infections and mediated by cellular immune responses, particularly CD4⁺ T cells²⁹. An area of active discussion is their role in the context of primary and secondary flavivirus infections where it ranges between being polarized to a T helper 1 cell³⁰ and aiding B cells in the germinal center (GC)³¹ to CD4-restricted HLAs associated with less severe infection outcome, expansion of T follicular cells promoting DENV-specific antibodies and cytotoxic subpopulations as a result of re-exposures^{24,32,33}. Detailed characterization of the maturation of the humoral immune response during secondary infections or vaccinations indicates that during secondary flavivirus exposure, the GC reaction, where the CD4⁺ T cells play a crucial role in naïve B cells activation and immunoglobulin switching, may not be critical for an optimal secondary immune response³⁴. Other groups also have confirmed the involvement of memory B cells (MBC) and MBC-derived plasmablasts in the humoral immune response more than the activation of naïve B cells during secondary flavivirus infections^{14,35-40} and emphasizing the role of cross-reactive CD4⁺ T cells³¹.

Despite these contributions, the changes in the functional quality of flavivirus-induced antibodies during immune recall responses remain less well characterized. More importantly, the role of CD4⁺ T cells in controlling flaviviral replication by generating polyfunctional responses and the quality of the antibodies produced by tertiary infections in flavivirus experienced humans or non-human primates (NHPs) is largely unknown. NHP models provide advantages such as human-like immune responses and control of external factors, such as injection method, amount of administered viral inoculum, and infection timing⁴¹. Moreover, their competent immune system resembles those of humans, which is essential for understanding the processes driving disease development and has been broadly used to study DENV and ZIKV responses⁴¹⁻⁴³. Using this model, our group provided the first evidence that prior DENV immunity is beneficial against ZIKV infection⁴⁴ which was confirmed later on by two other groups^{45,46}. These results from NHPs on the limited impact of pre-existing dengue immunity in ZIKV infection outcomes were first confirmed in humans by *Terzian et al.*⁴⁷ followed by other groups⁴⁸⁻⁵⁰.

To address the gaps in knowledge regarding the role of CD4⁺ T cells and the impact of flavivirus priming in sequential infections, we performed a longitudinal study focused on the adaptive immune responses. We assessed the contribution of CD4⁺ T cells in viral clearance and aid in producing a robust humoral response in rhesus macaques. To test this, DENV or ZIKV-primed CD4⁺ T cell-depleted, non-depleted, and flavivirus-naïve rhesus macaques were exposed to either DENV-2 ($n=8$) or DENV-4 ($n=20$). We found that the absence of CD4⁺ T cells in DENV-primed animals with a secondary ZIKV infection before a tertiary heterologous dengue challenge resulted in a significant drawback for the immune responses, including a delay in IgM and IgG responses, as well as a decrease in the overall magnitude of the antibody response, in addition to a reduction in the neutralization capacity of neutralizing antibodies. However, before a third

challenge, the lack of CD4⁺ T cells had no evident impact in ZIKV-primed animals, followed by a sequence of two consecutive heterologous dengue infections.

Furthermore, DENV-primed CD4-depleted cohorts showed plasmablast populations with delayed, impaired isotype switching and reductions in antibody binding during tertiary infections. Importantly, DENV-primed CD4⁺ T cells are necessary for a robust humoral response against a heterotypic infection with DENV, even in the context of a tertiary infection. However, in ZIKV-primed individuals, where DENV infections were consecutive, the role of CD4⁺ T cells in modulating the quantity and quality of the immune response was more limited, confirming implications of the order and timing of infections in the hierarchy of the non-neutralizing and neutralizing antibody maturation and function.

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

Methods

Viral stock

DENV-4 Dominique and DENV-2 New Guinea 44 (NGC) strains (kindly provided by Steve Whitehead, NIH/NIAID, Bethesda, MD) were used to obtain comparative results to our previously published data on DENV and ZIKV. Viruses were expanded and titered by plaque assay and qRT-PCR using protocols standardized in our laboratories. DENV-1 Western Pacific 74, DENV-3 Sleman 73 (provided by Steve Whitehead, NIH/NIAID, Bethesda, MD), and ZIKV PRVABC59 (ATCC VR-1843) were used for neutralization assays. DENV-4 Dominique and DENV-3 Sleman 73 strains were used to infect macaques in October 2013 and ZIKV PRVABC59 in November 2017. DENV-2 New Guinea 44 (NGC) strain was used to infect macaques in September 2016 and ZIKV PRVABC59 in September 2017.

Ethics statement

All procedures were reviewed and approved by the Institute's Animal Care and Use Committee at Medical Sciences Campus, University of Puerto Rico (IACUC-UPR-MS), and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (Animal Welfare Assurance number A3421; protocol number, 7890116). Procedures involving all study animals were

approved by the Medical Sciences Campus, UPR IACUC. They were conducted following the USDA Animal Welfare Regulations, the Guide for the Care and use of Laboratory Animals, and institutional policies. In addition, steps were taken to lighten sufferings, including the use of anesthesia and method of sacrifice if appropriate, following the Weatherall report, "The use of non-human primates in research: <http://www.acmedsci.ac.uk/more/news/the-use-of-non-human-primates-in-research/>.

Immunization, depletion, and viral challenge of macaques

Young adult rhesus macaques (6 – 9 years of age, male) seronegative for DENV and ZIKV were housed in the CPRC facilities, University of Puerto Rico, San Juan, Puerto Rico. Macaques in experimental cohorts were depleted of CD4+ lymphocytes by administering the anti-CD4 Ab CD4R1 (NHP Reagent Resource; <https://www.nhpreagents.org>). The initial subcutaneous administration (s.c.) of 50mg/kg (CD4⁺) at 15 days pre-challenge was followed by two intravenous administrations (i.v.) of 7.5mg/kg (CD4⁺) at 12- and 9-days pre-challenge. The DENV and ZIKV Flavi-POS controls were treated with PBS. For the DENV-2 challenge, macaques previously exposed to DENV3 or DENV4 in October 2013 and to ZIKV PRABCV59 in September 2017 (CD4 depleted $n=2$, DENV Flavi-POS control $n=2$) and DENV/ZIKV naïve macaques ($n=4$) were infected subcutaneously in the deltoid area with 500uL of virus diluted in PBS, using a dose of 5×10^5 pfu. For the DENV4 challenge, macaques previously exposed to DENV2 in September 2016 and to ZIKV PRABCV59 in September 2017 (CD4 depleted $n=3$, DENV Flavi-POS control $n=3$), to ZIKV PRABCV59 and DENV-2 in September 2017, respectively (CD4 depleted $n=3$, ZIKV Flavi-POS control $n=3$), and DENV/ZIKV naïve animals ($n=8$) were infected subcutaneously in the deltoid area with 500uL of virus diluted in PBS, using a dose of 5×10^5 pfu. Macaques were monitored after treatments and infections by trained veterinarians for evidence of disease and clinical status. Weights were taken on day 0 and every other day pre-treatment (days -15, -12, -9) and post-infection (days 1-15, 30 and 60). Rectal and external temperatures were taken daily during pre-treatment and post-infection periods. For consistency through the text, the groups are referred to as DENV/ZIKV or ZIKV/DENV CD4-depleted or immune-competent or naïve animals. In the figures, they are referred to as DV/ZV CD4(+) or CD4(-) and ZV/DV CD4(+) or CD4(-) and naïve.

qRT-PCR

DENV viral RNA for real-time PCR assay was extracted from 140 μ l virus isolate (previously tittered by plaque assay) and from acute serum samples using QIAmp Viral RNA mini kit (Qiagen, Valencia, CA) as per the manufacturer's instructions as described by our group before ^{29,44,51}.

ELISA for DENV and ZIKV

Seronegative status for DENV and ZIKV of naïve animals was assessed before DENV-2 or DENV-4 challenge using DENV IgG/IgM (Focus Diagnostics, CA) and ZIKV IgG (XpressBio, Frederick, MD) commercial kits. After DENV-2 or DENV-4 infection, seroreactivity to DENV was quantified using commercial IgM and IgG ELISA kits (Focus Diagnostics, CA) at baseline, 7-, 10-, 15-, 30- and 60-days post-infection. All assays were performed per the manufacturer's instructions and as described by our group before^{29,44,51}.

Endpoint dilution binding assay

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

DENV and ZIKV titration and neutralization assays

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

with the same conditions. After incubation, overlay was added and processed as previously described. Results were reported as the FRNT with a 60% or greater reduction in DENV foci (FRNT60). A positive neutralization titer was designated as 1:20 or greater, while <1:20 was considered a negative neutralization titer.

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

Immunophenotyping

Phenotypic characterization of macaques adaptive immune response was performed by 8-multicolor flow cytometry using fluorochrome-conjugated Abs at several time points (baseline, 1, 2, 3, 7, 10, 15, and 30 days post-infection). Aliquots of 150 μ L of whole heparinized blood were incubated with a mix of antibodies for 30 min. in the dark and at room temperature. After incubation, red blood cells are fixed and lysed with BD FACS fix and lyse solution, and cells are washed twice with BSA 0.05%. Samples were analyzed using a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec, CA). Antibodies used in this study were: CD3-PerCP (SP34), Ki67-Viogreen (B56), CD27-PE (M-T466), CD69-PeCy7 (FN50), CD4-APC (L200), CD95-PE (DX2) from BD-Biosciences; CD16-APC Vio 770 (REA423), CD28-APC Vio 770 (15E8) from Miltenyi; CD20-PacBlue (2H7) from Biolegend; CD8-FITC (DK25) from Sigma and CD14-FITC (26ic) from Caprico. For analysis, lymphocytes (LYM) were gated based on their characteristic forward

and side scatter pattern; T cells were selected with a second gate on the CD3 positive population. CD8⁺ T cells were defined as CD3⁺CD8⁺ and CD4⁺ T cells as CD3⁺CD4⁺. Naive (N=CD28⁺CD95⁻), effector memory (EM=CD28⁻CD95⁺) and central memory (CM=CD28⁺CD95⁺) T cell subpopulations were determined within CD4⁺ and CD8⁺ T cells. B cells were defined as CD20⁺CD3⁻, Memory (CD20⁺CD3⁻CD27⁺) and Plasma (CD20⁻CD3⁻CD27⁺Ki67⁺) B cell subpopulations were determined within CD20⁺ B cells. Activation marker CD69 and proliferation marker Ki67 were determined in each different lymphoid cell subpopulation. As we did not label cells with CD38⁺ or CD19⁺ markers, we could not differentiate from plasmablast (CD20⁻CD27⁺CD19⁺CD38⁺Ki67⁺) or plasma cells (CD20⁻CD27⁺CD19⁻CD38⁺⁺Ki67⁺). From here, throughout the text, the CD20⁻CD3⁻CD27⁺ are referred to as Antibodies Secreting Cells (ASC). Data analysis was performed using Flowjo (FlowJo LLC Ashland, OR).

Cellular immune response analysis

Intracellular cytokine staining of PBMCs from rhesus macaques was performed by multicolor flow cytometry

using methods similar to those described by Meyer et al. PBMC samples were thawed 1 day before stimulation. Approx. 1.5×10^6 PBMCs were infected overnight with DENV-4 (Dominique) at a MOI of 0.1, DENV-2 (NGC44) at a MOI of 0.1, or ZIKV at a MOI of 0.5 in RPMI medium with 5% FBS. The remaining PBMCs are rested overnight as described earlier in 5ml of RPMI with 10% FBS. These PBMCs were then stimulated for 6 h at 37°C/5%/CO₂ with DENV-4 E peptides (1.25 ug/ml-1), all in the presence of brefeldin A (10 ug/ml-1), a-CD107a-FITC (H4A3) (10 ul), and co-stimulated with a-CD28.2 (1 ug/ml-1) and a-CD49d (1 ug/ml-1). After stimulation, the cells were stained for the following markers: CD4- PerCP Cy5.5 (Leu-3A (SK3), CD8b-Texas Red (2ST8.5H7), CD3-PacBlue (SP34), CD20-BV605 (2H7), CD95-V510 (DX2), CD28.2-PE-Cy5, IFN-g-APC (B27) and TNF-a-PE-Cy7 (MAB11). The samples were run on an LSRII (BD) and analyzed using Flowjo (Treasar). Lymphocytes were gated based on their characteristic forward and side scatter pattern, T cells are selected with a second gate on the CD3⁺ population, and CD8⁺ T cells are defined as CD3⁺ CD20⁻ CD8⁺ Cytokine expression was determined by the number of CD8⁺ positive cells and then stained positive for IFN-g and TNF-a. CD107a production was also measured in these populations to determine functional cytotoxicity.

Multiplex cytokine analysis

Sera from rhesus macaques were analyzed for 11 cytokines and chemokines by Luminex using NHP XL Luminex Performance Assay (R&D Systems, MN). Evaluation of interleukin 12 (IL-12p70), granzyme

B, interferon-alpha (IFN α), interleukin 17A (IL-17A), and interferon gamma-induced protein 10 (IP-10, CXCL10) were included in this assay.

Statistical Methods

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

Results

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

The experimental design includes two cohorts of rhesus macaques (*Macaca mulatta*), subdivided by flaviviral immunological background and time of infections and immune depletion status (some were depleted of CD4⁺ T cells while others were not) and further challenged with DENV-2 NGC-44 (cohort A) or DENV-4 Dominique (cohort B) (Fig. 1). Experiment 1 served as a preliminary study cohort. Based on the results obtained in experiment 1, and the fact that after the introduction of ZIKV to DENV endemic areas, populations with primary ZIKV infections emerged, we decided to expand the study and the infection sequences. The depletion treatment was efficacious as a 90.1% CD4⁺ T cell depletion was reported for group A-1 (Supp Fig. 1A), 99.8% depletion for group B-1 (Supp Fig. 1B), and 99.9% depletion for group B-3 (Supp Fig. 1C). After depletion procedures and DENV challenge, neither symptomatic manifestations nor significant differences in weight or temperature were observed in any of the animals (Supp Fig. 2B-C & E-F). We noted differences in alanine transaminase (ALT) levels in the DENV-ZIKV-DENV groups in both experiments 1 and 2 with discrepant profiles. ALT values were significantly higher in the DENV/ZIKV immune-competent animals compared to the DENV/ZIKV CD4-depleted animals in experiment 1 (Supp Fig. 2H Day 7, $p=0.0407$) but vice versa in experiment 2 (Supp Fig. 2K, Day 2 and 7, $p=0.0246$ and 0.0020 respectively) despite having the same sequence of infections. On the other hand, no differences in aspartate transaminase (AST) and alkaline phosphatase were detected in either of the groups (Supp Fig. 2G, I, J, L M-O). However, in the animals with the sequence of infection ZIKV-DENV-DENV (groups B3 and B4), no differences in the enzyme profile between the CD4⁺ T cells-depleted and the control group were noted.

DENV-4 RNAemia profile is modified by CD4⁺ T cells hindrance in DENV-primed individuals

To determine if depletion of CD4⁺ T cells alters DENV replication kinetics, DENV RNAemia levels were measured in serum using qRT-PCR. For experiment 1, all flavivirus-positive animals experienced an early delay in viremia with a late peak compared to the naïve group (Fig. 2A). Contrary to our expectations, DENV3/4-ZIKV CD4-depleted animals showed the most noticeable delay during the acute phase of the DENV2 tertiary infection with a late peak on day 8 post-infection (p.i.) and detectable viremia by day 10 p.i. compared with the rest of the animals. For the DENV3/4-ZIKV immune-competent group, the RNA detection was consistent with typical heterotypic infection rates with lower replication than the naïve group and a late peak on day 6 p.i. However, when evaluating the area under the curve (AUC), we found that the immune-competent animals with prior flavivirus immunity could control the viremia to significantly better than the naïve group ($p=0.0212$). There were no differences in the total viremia between the DENV/ZIKV CD4-depleted and naïve animals. Additionally, the CD4-depleted animals had AUC values with a tendency, although not statistically significant, tendency to be higher than the DENV/ZIKV immune-competent animals (Fig. 2B). Next, we evaluated the average RNAemia days defined as the days with detectable viremia divided by total possible viremia days. We noted that animals depleted of CD4⁺ T cells had similar viremia days to the naïve group. In contrast, although not statistically significant, the DENV/ZIKV immune-competent group showed the lowest viremia days (Fig. 2C). For DENV2-ZIKV-DENV4 groups (Experiment 2, B-1, B-2 & B-5), we observed the same trend to an early delay in viremia with a late peak in all flavivirus-experienced animals (Fig. 2D). However, since DENV-4 replication is not as robust and consistent as DENV-2, we did not observe statistical differences in the AUC (Fig. 2E) or mean RNAemia days. Nevertheless, there was a tendency to higher values in the DENV/ZIKV CD4-depleted groups compared to the DENV/ZIKV immune-competent animals (Fig. 2F). Taken together, these results suggest that, although limited statistically significant differences among groups were observed, CD4⁺ T cell depletion negatively impacts the control of DENV viremia in DENV-primed individuals with a secondary ZIKV infection during a tertiary DENV infection regardless of the sequence of infecting DENV serotypes or timing of infections.

Depletion of CD4⁺ T cells modifies the serological profile during heterologous infections in DENV-primed individuals

To further explore the role of CD4⁺ T cells, we assessed their impact on the quantity of the humoral response against a tertiary DENV infection. All twenty-two DENV-primed animals were tested for seroreactivity against DENV. Fig. 3 shows a summary of the results for experiments 1 and 2. As expected, all flavivirus-experienced animals had a limited level of anti-DENV IgM in comparison to the induction of a primary IgM response in the naïve groups (Fig. 3A, Supp Fig. 3A & 3E). Total anti-DENV IgM levels were significantly lower in the DENV/ZIKV CD4-depleted groups compared to the DENV/ZIKV immune-competent groups (Fig. 3B, $p=0.0018$). This suggests a role of CD4⁺ T cells in supporting naïve B cells to become IgM-secreting plasmablast or an impact on IgM⁺ memory B cell (MBC) re-activation responses. DENV-ZIKV experienced animals all had detectable levels of anti-DENV IgG consistent with a heterotypic

DENV infection (Fig. 3C, Supp Fig. 3C & 3G). A statistically significant lower IgG production against the infecting serotype was detected in the DENV3/4-ZIKV CD4-depleted group of experiment 1 compared to their control group (Supp Fig. 3D, $p=0.0009$) but not in the DENV2/ZIKV CD4-depleted group of experiment 2 (Supp Fig. 3H). However, a limited fold-increase in the total IgG from day 0 to 15 and 0 to 30 p.i. and a significantly lower total IgG was observed in all DENV/ZIKV CD4-depleted groups compared to the DENV/ZIKV immune-competent animals (Fig. 3E, $p=0.0036$). Taken together, the IgG profile suggests that CD4⁺ T cell depletion impairs the amnestic response during tertiary flavivirus infection after sequential DENV-ZIKV infections.

Next, we measured the binding ability of the antibodies in the sera from depleted and non-depleted cohorts at 0, 15, and 30 days after DENV infection against the whole viral particle (DENV-2 and DENV-4 for experiments 1 and 2 and also DENV-3 for experiment 1) using an endpoint dilution approach. At baseline, all flavivirus-experienced animals showed cross-reactivity with titers up to a dilution of 1×10^4 with no differences in their AUC values (Fig. 4A & 4D). On day 15 p.i., the DENV3/4-ZIKV CD4-depleted group from experiment 1 showed a trend, although not statistically significant, to a lower AUC when compared to the DENV3/4-ZIKV immune-competent group (Fig. 4B). Moreover, the same tendency was noted for the DENV2/ZIKV CD4-depleted group from experiment 2, but with a statistically significant delay in the IgG-binding capabilities at day 15 (Fig. 4E, $p=0.0373$). By day 30 p.i. for both experiments, the differences in the binding abilities of the antibodies were still lower in depleted than non-depleted animals. (Fig. 4C & 4F). In summary, these results demonstrate the significant impact CD4⁺ T cells have in isotype switching and antibody binding response during tertiary flavivirus infection after sequential DENV-ZIKV infections. However, these cells have minimal or no role in modifying the recall humoral response irrespectively of the order of infections.

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

To address if depleting flavivirus-specific CD4⁺ T cells further hinders the antibody neutralizing capabilities in a tertiary infection, samples from experiments 1 and 2 were tested using focus reduction neutralization tests (FRNTs) and plaque reduction neutralization tests (PRNTs) against DENV serotypes and ZIKV, respectively. Neutralization assays against the infecting serotype were completed for acute and convalescent periods (Supp Fig. 4). The 50% effective concentration (EC_{50}) of neutralizing antibodies (nAbs) for experiments 1 and 2 are shown (Fig. 5A & 5C). The DENV3/4-ZIKV CD4-depleted animals from experiment 1 show a significant delay in the neutralization profile compared to their respective DENV3/4-ZIKV immune-competent groups. This tendency to lower titers is detectable on day 15 p.i. ($p=0.0424$), with a significant difference with the DENV/ZIKV immune-competent animals on day 30 p.i. (Fig. 5A, $p=0.0046$). However, this delay was partially recovered by day 60 p.i., having all flavivirus-experienced groups display a similar neutralization profile. For experiment 2, we observed a similar trend with the DENV2/ZIKV CD4-depleted group, which showed a trend towards lower neutralization magnitude by day 15 p.i. (Fig. 5C). Nevertheless, this group had a limited neutralization expansion against the infecting

serotype, compared to its respective DENV2/ZIKV immune-competent group (Fig. 5D, $p=0.0118$). Interestingly, the delay in the expansion of the neutralization in the depleted group is confirmed by the statistically significant increase in magnitude on day 60 p.i. ($p=0.0049$), which was apparent only in this group. That late increase resulted in a significant difference, with higher values in the depleted group compared to the DENV2/ZIKV immune-competent group (Fig. 5C, $p=0.0487$). Figures 5E and 5F summarize the neutralization titers for experiments 1 and 2. Per the EC50 values, the same trend was observed on the PRNT values. The DENV/ZIKV CD4-depleted groups showed a delay in neutralizing titers in acute and convalescent periods compared to all flavivirus-experienced animals (Fig. 5E). In addition, when analyzing the AUC, there was a slight non-significant trend to a lower AUC value in the DENV/ZIKV CD4-depleted groups compared to the immune-competent group (Fig. 5F). Collectively, these results confirm that regardless of the sequence or timing of the DENV infection, depletion of CD4⁺ T cells negatively impacts the magnitude of neutralization during a tertiary flavivirus infection in DENV-primed individuals followed by a secondary ZIKV infection.

Tertiary infection with DENV-4 is not enhanced in ZIKV-primed individuals

Since the introduction of ZIKV in DENV endemic areas, populations with primary ZIKV infections emerge. Therefore, to explore the role of flavivirus infection sequences in the functionality of CD4⁺ T cells and the outcome of a tertiary infection, we challenged six animals with DENV-4 two years after they had a primary infection with ZIKV and a secondary challenge with DENV-2 three months after (Fig. 1). For experiment 2 (groups B-3, B-4 & B-5), we noted the same tendency for DENV/ZIKV-groups, with an early delay in DENV-4 viremia more evident in the ZIKV/DENV2 CD4-depleted animals compared to the ZIKV/DENV2 immune-competent group (Fig. 6A). However, as in the previous experiment, the depleted group showed a higher and late peak viremia on day 5 p.i., suggesting a limited viremia control linked to the lack of CD4⁺ T cells. Nevertheless, different from the DENV/ZIKV cohorts, we did not observe differences in the AUC (Fig. 6B) or mean viremia days (Fig. 6C) among ZIKV/DENV groups. This result suggests that the role of CD4⁺ T cells controlling the viremia during a tertiary flavivirus infection may have different weights depending on the prior sequence and timing of infections.

CD4⁺ T cells depletion did not change the anti-dengue humoral profile in ZIKV-primed individuals

Next, we wanted to evaluate how the viremia of CD4⁺ depleted animals correlated to their serological profile, assessing the quantity and quality of the humoral response. Animals were tested for seroreactivity and binding ability of the sera-derived antibodies against the whole DENV-4 viral particle by an endpoint dilution approach. As expected, all flavivirus-experienced animals had a limited level of anti-DENV IgM in comparison to the induction of a primary IgM response in the naïve groups. Total anti-DENV IgM levels were slightly lower in the ZIKV/DENV CD4-depleted group than the ZIKV/DENV immune-competent group (Fig. 7A) but not as prominent as in the DENV/ZIKV-immune animals (Fig. 3B). Interestingly, there was no significant difference in the total IgG levels between ZIKV/DENV2 CD4-depleted and ZIKV/DENV2 immune-competent animals (Fig. 7B), as reported in the DENV/ZIKV-exposed groups (Fig. 3D). When we characterized the functional properties of the antibodies against DENV in ZIKV-primed individuals, at

baseline, all flavivirus-experience animals showed cross-reactivity with titers up to a dilution of 1×10^4 with no differences in their AUC values, respectively (data not shown). In addition, on day 15 p.i., no differences were observed between ZIKV/DENV2 groups (Fig. 7C & 7D), indicating that CD4⁺ T cell depletion did not affect the binding of the antibodies to DENV-4 when ZIKV was the initial flavivirus priming the immune system followed by a secondary DENV preceding a tertiary heterologous DENV infection. Lastly, we evaluated the antibody neutralizing capabilities during a tertiary infection against DENV4. Interestingly, ZIKV-DENV2-DENV4 CD4-depleted animals had a similar neutralizing profile to their control immune-competent animals against DENV4, with no differences observed in any of the time-points tested (Fig. 7E) as it was in the case for the DENV2-ZIKV-DENV4 sequence (Fig. 5C). These results suggest that the role of CD4⁺ T during a tertiary heterologous DENV infection can be modified by the sequence and timing of flavivirus infection.

Lack of CD4⁺ T cells does not affect the recall humoral immune response to previous infecting flaviviruses

Next, we reviewed if depleting CD4⁺ T cells changed the functional properties of the antibodies during the recall memory response to previous infecting serotypes. We measured the binding ability of the antibodies in the sera from depleted and non-depleted cohorts before and 15 days after DENV infection against the whole viral particle (DENV-3 & DENV-4 for experiment 1, DENV-2 for experiment 2, and ZIKV for both experiments) by an endpoint dilution approach (Supp Fig. 5). For experiment 1, we observed that for DENV-3 at day 0, animals with a primary infection against this serotype had a better binding than those with a primary infection against DENV-4 (Supp Fig. 5A). Interestingly, the DENV-2 challenge boosted the response on day 15 p.i. increasing the antibodies' affinity to DENV3 (Supp Fig. 5B). No differences were observed for DENV4 or ZIKV in any group (Supp Fig. 5C-F). In summary for experiment 1, the depletion had no impact on the recall memory response since there were no differences in the binding against the original infecting DENV serotype. For experiment 2, we observed the typical cross-reactive response against DENV2, the prior infecting serotype, at day 0 for both infection sequences (Supp Fig. 5G & K). Nevertheless, on day 15 p.i. the binding against DENV-2 in the DENV/ZIKV CD4 depleted animals showed a statistically significant decrease (Supp Fig. 5H, $p=0.0010$) in comparison to both the DENV2/ZIKV immune-competent group and the ZIKV-DENV2-DENV4 groups (Supp Fig. 5L). A meta-analysis was performed for the binding against ZIKV, in which results for DENV-ZIKV-DENV sequences for both experiments were combined. No differences were observed at day 0 (Supp Fig. 5M) and at day 15 p.i. (Supp Fig. 5N) between infecting sequences, but a slight although a not statistical trend to a lower AUC was present in the DENV/ZIKV CD4-depleted groups in comparison to the DENV/ZIKV-immune-competent groups. In summary, these results demonstrate the significant impact CD4⁺ T cells have in isotype switching and antibody binding response during tertiary flavivirus infection after sequential DENV-ZIKV infections. However, those cells have minimal or no role in modifying the recall humoral immune response irrespective of the order of infections suggesting that the antibodies are mainly plasmablast-derived.

To further explore how the role of CD4⁺ T cells can be modulated by order of infections, neutralizing capabilities against previous infecting flaviviruses (DENV-4, DENV-3, DENV-2, and ZIKV) were tested for experiments 1 and 2 (Supp Fig S6). The EC₅₀ of nAbs for experiments 1 and 2 are shown (Fig. 8). For experiment 1, DENV3/4-ZIKV-DENV2 immune animals had similar neutralizing responses against their previous infecting serotypes (DENV-3 or DENV-4) regardless of the status of CD4⁺ T cells (Fig. 8A & B). For experiment 2, the neutralization magnitude against DENV-2 remained unaffected regardless of the presence/absence of CD4⁺ T cells and order of infections. These results suggest that the lack of CD4⁺ T cells does not impact the recall humoral response regardless of the sequence of infection. Lastly, a meta-analysis was performed for neutralization against ZIKV in which were included results for experiments 1 and 2 of the DENV-ZIKV-DENV infection sequence (DENV3/4-ZIKV-DENV2 and DENV2-ZIKV-DENV4). The DENV/ZIKV CD4-depleted groups showed a delay in the neutralization response against ZIKV by day 15 p.i., which was statistically significant compared to the ZIKV/DENV2 CD4-depleted group (Supp Fig. 8E, p=0.0448), once again reaffirming the contribution of the order of infections to the recall humoral response to previous flaviviruses.

Next, we determined the neutralization hierarchy for experiments 1 and 2. For the sequence DENV3/4-ZIKV-DENV2 in experiment 1, the neutralization dominance was against the infecting DENV serotype (DENV-2) instead of the previous infecting viruses being DENV3, DENV4, or ZIKV. (Supp Fig. 7A & B). The naïve group had a typical type-specific neutralizing profile having dominance against DENV2 and a cross-reactive profile against the other tested serotypes (Supp Fig. 7C). As expected, the neutralization of the DENV2/ZIKV/DENV4 sequence (experiment 2) was significantly higher against the primary infecting DENV serotype (DENV-2) than to the heterologous current infecting (DENV-4) serotype (Supp Fig. 7D, p=0.0001). Even though the lack of CD4⁺ T cells did not impact the recall humoral response, there is a significant difference in individual responses between DENV/ZIKV and ZIKV/DENV-primed groups (Supp Fig. 7D-H). DENV/ZIKV CD4-depleted animals showed a noticeable tendency to lower neutralizing titers against all the serotypes compared to ZIKV/DENV CD4-depleted that had the highest titers of all the flavivirus-immune animals.

Bi-phasic adaptive immune response is modulated by the absence of CD4⁺ T cells and infection sequences in Flavi-immune animals

To further explore the role of CD4⁺ T cells and the hierarchy of infections in shaping the frequency, activation, and proliferation of adaptive immune cell subsets such as B (CD20⁺ CD3⁻) and T (CD3⁺ CD20⁻) cells, an analysis by flow cytometry was performed for experiment 1 and 2. (Supp Fig. 9 for gating strategy). No differences were detected in the total B cells between experiments 1 and 2 following DENV infection compared with baseline levels (Supp Fig. 8A-B) However, ZIKV/DENV groups had elevated levels of activated B cells (CD20⁺ CD3⁻ CD69⁺) in comparison to DENV/ZIKV, especially the ZIKV/DENV CD4-depleted animals (Supp Fig. 8C-D). We detected an expansion in antibody-secreting cells (ASC) (CD20⁻ CD3⁻ CD27⁺) in DENV/ZIKV groups at 7- and 10 p.i., while ZIKV/DENV groups maintained their levels (Supp Fig. 8E-F). No differences were detected in the memory B cells (CD20⁺ CD3⁻ CD27⁺) between

both infecting sequences. However, the ZIKV/DENV groups showed a higher frequency of activated cells than the DENV/ZIKV groups. Moreover, a higher frequency of (ASC) was detected in ZIKV/DENV-primed, but not in DENV/ZIKV-primed individuals, with a statistically significant difference at day 7 p.i. with their respective baselines (Fig. 9E-F $p=0.0003$, $p=0.0001$). Remarkably, there were no differences between the depleted and the non-depleted groups in that sequence of infection (Fig. 9F). To further explore the role of CD4⁺ T cells and the hierarchy of infections in shaping the frequency, activation, and proliferation of cellular immune cell subsets for experiment 2 we measured whether T-cell subpopulations, such as naïve (CD3⁺CD8⁺CD28⁺CD95⁻) effector memory (CD3⁺CD8⁺CD28⁻CD95⁺) and central memory (CD3⁺CD8⁺CD28⁺CD95⁺) T cells, within CD8⁺ T cell compartment were modulated by CD4⁺ T cell depletion and infection sequences following a tertiary DENV infection (Supp Fig. 10; Supp Fig. 11 for gating strategy). We observed different CD8⁺ T cell profiles depending on the sequence of infections (Supp Fig. 9. A-L). No major differences were observed when evaluating naïve (Supp Fig. 10. A-D) and effector memory CD8⁺ T cells among groups (Supp Fig. 10. E-H). Interestingly, the central memory CD8 T cells of the DENV2-ZIKV-DENV4 sequence of infection have a reduced number of these cells that cannot respond to DENV4 (Supp Fig. 9. I & K). In contrast, ZIKV-DENV2-DENV4 has central memory CD8 T cells at baseline and throughout the collection period capable of mounting an optimal response (Supp Fig. 10. J & L). Lastly, to assess the role of CD4⁺ T cells in the CD8⁺ T recall immune response, we measured their effector responses. CD8⁺ T cells expressing CD107a or producing IFN γ or TNF α in response to previous infecting serotypes were assessed for day 7 p.i. of experiment 2. No significant differences were observed between depleted groups or non-depleted groups and by infecting sequences in any of the parameters measured (Supp Fig. 11). Only a trend to lower frequency of IFN γ -producing cells was observed in the CD4⁺T cells-depleted group in the DENV/ZIKV cohort, suggesting that CD4⁺ T cells may have a limited role in priming CD8⁺ T recall memory responses during a tertiary infection.

The sequence of infection shapes the pro-inflammatory cytokine profile

To determine how the sequence of infection impacts the cytokine secretion during a tertiary DENV infection, we assessed the serum concentration (pg/mL) of several cytokines and chemokines at baseline, 1-, 3- and 7 p.i. in samples from experiment 2 (Supp Fig. 13). We found significant differences between the two sequences of infections (DENV2-ZIKV-DENV4 vs. ZIKV-DENV2-DENV4) regardless of the CD4⁺ T cell status. DENV2/ZIKV immune animals had significantly higher levels of pro-inflammatory cytokine IL-12p70 on day 1 p.i. (0.0012), responsible for enhancing Th1 and CD8⁺ T cytotoxic responses, compared to ZIKV/DENV2 immune animals (Supp Fig. 13A). This trend continued throughout the collection period, with detectable significant differences on days 3- and 7 p.i. ($p=0.0017$, $p=0.0001$). Also, this group showed significantly higher levels of granzyme B, a serine protease secreted by activated cytotoxic T cells and natural killer (NK) cells ($p=0.0178$, 1 p.i., $p=0.0152$, 3 p.i., and $p=0.0007$, 7 p.i.) on the same days in comparison with ZIKV/DENV immune animals (Supp Fig. 13B). Likewise, a significant increase in IFN α and IL-17A levels (Supp Fig. 13C-D), which are involved in innate immunity and neutrophil mobilization, was detected in DENV2/ZIKV experienced individuals on day 1 post-infection ($p=0.0018$, IL17-A) and day 7 p.i. ($p=0.0140$, IFN α and $p=0.003$, IL17-A) in comparison to ZIKV/DENV2

experienced animals. On the other hand, significantly higher CXCL10 (IP-10) levels (Supp Fig. 13E), a T-cell activating chemokine, and chemoattractant for many other immune cells were detected in ZIKV/DENV2 animals at day 7 p.i. ($p=0.0111$) than DENV2/ZIKV animals. No significant differences in the naïve group were observed. Collectively, these results demonstrate that the order of infecting flaviviruses prior to a tertiary infection can outweigh the role of the CD4⁺ T cells modulating the pro-inflammatory cytokine/chemokine profile.

Discussion

The role of CD4⁺ T cells in response to a flavivirus infection has been widely studied^{16,52,53}. However, there is still a debate in the field on their crucial contribution to protection²⁴⁻²⁷ vs. harm⁵⁴⁻⁵⁶, making it an area of active discussion in the context of primary and secondary flavivirus infections. However, most of those studies focus on the CD4⁺ T cell contribution to the recall cellular immune response more than on their role in coordinating the humoral immune response during sequential flavivirus infection. Our current study presents the results of depleting CD4⁺ T cells and the resulting impact on viral replication, as well as on the quantity and quality of the humoral immune response and the CD8⁺ T cells profile during tertiary flavivirus infection in NHPs. We provide results from two different flavivirus infection sequences: DENV-ZIKV-DENV vs. ZIKV-DENV-DENV. These scenarios resemble the reality of large geographic endemic areas with more than 390 million people at risk of acquiring multiple flavivirus infections⁵⁷. We are unaware of prior references studying tertiary immune responses combining DENV and ZIKV infections in different orders or addressing the CD4⁺T role and impact on the dynamic environment of humoral and cellular interactions in humans or NHPs.

Here we identified an early delay in the DENV/ZIKV/DENV infecting sequences (from experiments 1 and 2) in the DENV-2 or 4 viremias in all flavivirus-positive animals, including CD4-depleted animals. This result confirms that antibodies and CD8⁺ T cells may control early DENV viral replication^{19,22,58,59,60}. However, the DENV/ZIKV/DENV CD4-depleted animals showed a noticeable rebound on day 8 p.i., no resolution of the viremia by day 10, and a strong trend to higher viremia AUC values even in the presence of prior immunity, confirming that CD4⁺ T cells are needed for sustained viremia control when a secondary ZIKV infection precedes a tertiary DENV infection. This agrees with our previous work, including cohorts B1 and B2, where DENV specific CD4⁺ T cells induced during primary DENV-2 infection have a role in resolving viral replication during a secondary ZIKV infection²⁹. Moreover, *Weiskopf et al.* and others have shown that DENV CD4⁺ T cells are readily detectable early following DENV infection, and DENV-specific CD107a⁺ CD4⁺ T cells are associated with protection against DENV disease²⁴ and play a crucial role in controlling secondary flavivirus infections¹⁶. Our results suggest a critical role for the CD4⁺ T cells in controlling the viremia induced during a tertiary flavivirus infection when the two last consecutive infecting viruses (ZIKV/DENV4) do not belong to the same serocomplex but not in the infectious sequence ZIKV-DENV2-DENV4, where the two last infecting viruses are members of the same serocomplex,

Prior results indicate that sequential immunizations for flaviviruses sharing CD4⁺ epitopes should promote protection during a subsequent heterologous infection. However, as supported by our work, sequential infections with different Yellow Fever serocomplex viruses (DENV and JEV) result in different immune profiles³¹. Additionally, similar amounts of circulating ASC in primary and secondary viral infections helped postulate that pre-existing CD4⁺ T helper cells may not be required for optimal responses in some viral infections⁶¹⁻⁶⁴, and may depend on the nature of the antigen⁶⁵. Correspondingly, using a mouse model, *Yauch et al.* showed that during a primary DENV infection without prior flavivirus immunity, CD4⁺ T cell depletion did not impact the DENV-specific IgM/IgG Ab titers and their neutralizing activity⁶⁶. Our data add to the role of the CD4⁺ T cells in a more complex immune background, showing that CD4⁺ T cells depleted groups from the DENV-ZIKV-DENV infection sequence have a faster increase in total DENV-specific IgM and IgG antibodies, with lower affinity than CD4⁺ competent groups. Conversely, we were unable to identify the same effect in the ZIKV-DENV-DENV sequence. These differing results confirm a multifactorial setting controlling the contribution of CD4⁺ T cells in flavivirus infections.

It is known that the cross-reactive humoral response is broader in secondary DENV infections derived from MBC clonal expansion compared to predominant ZIKV-specific antibodies in primary ZIKV or DENV-ZIKV scenarios^{67,39}. We hypothesized that sequential secondary and tertiary DENV infections induce a CD4⁺ T cell-independent clonal expansion of DENV-specific MBC producing antibodies that, at a functional level, keep their high affinity contributing most strongly to DENV neutralization. On the other hand, during a secondary ZIKV infection, a virus outside the dengue serocomplex and group^{68,69} and with different immunodominant epitopes^{49,70,71}, more DENV-specific MBC generated during the primary DENV infection, may undergo hypersomatic mutation and lose cross-reactivity lowering the affinity, determined by the magnitude and specificity of the neutralization to the tertiary infecting DENV. This effect is confirmed by the lower affinity of the anti-DENV and ZIKV antibodies in the absence of CD4⁺ T cells. As shown before, the immune responses induced by ZIKV and DENV as secondary infecting viruses are different. ZIKV infection in DENV-naïve subjects or with prior DENV immunity induces both DENV-MBC and naïve B cells with the production of ZIKV type-specific antibodies in both cases. In contrast, cross-reactive MBC predominates after DENV infection^{14,67}. Essentially our results indicate that the dynamic of the CD4⁺ T and B cells interaction during a tertiary flaviviral infection is modulated by the prior, secondary, infecting virus. Results for the recall memory were also interesting. We found that the neutralization hierarchy in both sequences of experiment 2 during the tertiary DENV-4 challenge were similar (DENV2>ZIKV>DENV4) resembling the Original Antigen Sin (OAS) postulate. In both cases, it was broadly cross-reactive but of higher magnitude against DENV-2, which was the primary infecting DENV serotype. However, in the sequence DENV3/4-ZIKV-DENV2 (experiment 1), despite the limited number of animals, the neutralization was clearly dominated by DENV-2, the infecting serotype. The neutralization hierarchy was DENV2>ZIKV≥DENV3/4. Others have reported that during secondary DENV infections, the response is primarily directed to the current infecting serotype, most likely due to affinity maturation in the GCs, resulting in the selection of MBCs with antibodies directed to the current infecting serotype⁷².

Nevertheless, the hierarchy orders from our results, raise inquisitiveness on the potential immunodominance of DENV-2 over the other serotypes. Our results from experiment 2 agree with other works reporting cross-reactive plasmablasts more reactive towards the previous infecting DENV serotype^{40,73,74}. It has been documented that the plasmablast response during secondary DENV infection is mainly derived from MBC^{34,65,67}. However, there is no information on MBC or plasmablasts dynamic during tertiary flavivirus infection. We found a higher frequency of activated ASC in the ZIKV-DENV2-DENV4 cohorts, regardless of the CD4⁺ T cells status, compared to the DENV2-ZIKV-DENV4 groups (experiment 2) increasing by day 7 p.i. resembling the plasmablast dynamic after DENV infections^{39,40,73,74}. Also, the fact that in the ZIKV-DENV2-DENV4 sequence, with two consecutive dengue infections the neutralizing magnitude was higher against the priming DENV serotypes and not against the current infecting dengue serotype and that the neutralization against the infecting serotype was also independent of CD4⁺ T cells, strongly supports (*i*) that the antibodies originated from reactivation of MBC plasmablasts after the tertiary infection⁴⁰ and (*ii*) that the strong plasmablast response may neutralize the infecting agent without a secondary affinity maturation in GC^{14,34}, suggesting that the infection's order plays a crucial role in regulating the affinity maturation process in the GC.

The fact that the hierarchy of neutralization was unaffected regardless of the order of ZIKV infection related to the two DENV infections is also a noteworthy finding. However, it is in line with prior observations showing that DENV/ZIKV cross-reactive MBC response decreased over time post-ZIKV infection⁶⁷. In our case, the ZIKV infections occur at the same time, 13 months before the tertiary DENV infection in both sequential infections.

We evaluated the CD8⁺ T cell response on day 7 p.i. in both sequences of infections in experiment 2 by assessing the frequency of virus-specific CD8⁺ T producing IFN- γ ⁺ or TNF- α ⁺ or expressing CD107a⁺, a marker associated with cytotoxicity. We were unable to identify any statistically significant differences when the cells were stimulated with the whole DENV2, DENV4 or ZIKV. However, in animals with the sequence DENV2-ZIKV-DENV4, there was a trend towards lower IFN- γ ⁺ producing cells in the CD4⁺T cells, a trend that was not apparent in the ZIKV-DENV2-DENV4 sequence. Previous studies showed that expanded activated CD4⁺ T cells located near CD8⁺ T cells in the spleen after a primary DENV-2 infection did not affect the induction of DENV-2-specific CD8⁺ T cells⁶⁶. Others have highlighted the role of prior heterologous flavivirus immunity showing that T cell responses to DENV-1 infection are modulated by prior immunity to JEV and YFV to differing extents³¹. From those results and our work, it can be concluded that the specific contribution of the CD4⁺ T cells in the immune response to flavivirus is impacted by the order of flavivirus infection, more than the priming itself and that the order of infections plays a critical role in defining the recall cellular memory to a tertiary infection. Many others have focused on the characterization of CD4⁺ or CD8⁺ T cell-specific epitopes and their contribution in response to vaccination as well as their role in protecting or inducing damage during DENV^{75,76}, or more recently, ZIKV infections^{71,77,78}. However, there are still minimal studies addressing the interplay between those

two key players of the cellular immune response in the complex immunological scenario of flavivirus interactions.

Finally, we looked at the cytokine profile. Surprisingly, we found a pro-inflammatory pattern more associated with the sequence of infections than with the presence or absence of CD4⁺ T cells. This finding reinforces again the predominant role of infection sequences prior to a tertiary flavivirus infection over the contribution of CD4⁺ T cells shaping the immune response during the tertiary infection. Supported in the results presented here and other works from our group in NHP during secondary infection, it is possible to argue that the sequence of ZIKV-DENV infections induces a different immunological response in terms of neutralization magnitude, cytokine profile, and functionality of the cellular immune response compared to the DENV-ZIKV scenario ^{29,44,51}. Overall, our results reinforce the previous concept that ZIKV behaves like other flaviviruses outside the DENV serogroup ^{67 72} even in the context of tertiary flavivirus infection.

Previously we confirmed that the length of time between infections during secondary infection with ZIKV or DENV in DENV or ZIKV-primed animals, respectively, does impact the quality of the subsequent response ^{29,51}. Nevertheless, in the current work, it is unclear if the time between subsequent infections prior to a tertiary flavivirus infection plays a critical role in the results presented here. The DENV-2 challenge in experiment 2, in the sequence ZIKV-DENV2-DENV4 was performed only three months after a ZIKV priming ²⁹, limiting the full expansion of the humoral and cellular components of the ZIKV-specific immune response. Nonetheless, the immune profile from groups with the sequence of infections DENV-ZIKV-DENV (experiments 1 and 2) consisted of the same flavivirus infection sequence but at different time-points and share similar impairment in the humoral immune response associated with the lack of CD4⁺ T cells. This further suggests that order more than the timing of infections contributes to the significant differences in the contribution of CD4⁺ t cells in the DENV-ZIKV-DENV sequence. In contrast, in the ZIKV-DENV-DENV scenario, the lack of these cells had no detectable effect on the immune response, including affinity maturation, during a tertiary infection.

Our study has several limitations, including the number of animals per group, typical in works involving NHPs. Considering the quality of the animals and the multiple contributions in prior works using this model ⁷⁹⁻⁸², we believe in the accuracy and translational potential of the results presented here. Also, more in-depth studies characterizing the plasmablast and MBC profiles, in addition to B cells receptors repertoire and antibody populations at the single-cell level warrant a better understanding in the context of tertiary flavivirus infections. Also, an extensive characterization of virus-specific CD8⁺ T cells responses is needed in the context of tertiary flavivirus infections. Our group is currently addressing these gaps in the molecular mechanism behind our findings.

Beyond the drawbacks, our work adds new insight into the role of the CD4⁺ T cells in shaping the humoral immune response by the order and timing of infections during a tertiary infection. Also, it sheds new light on the dynamic of the neutralization hierarchy process, the potential mechanisms, and the multifactorial

nature of the immune response to flavivirus during sequential infections. It also anticipates what can be expected regarding the immune response and clinical outcome in extensive geographic endemic areas where DENV and ZIKV co-circulate. Flavivirus naïve people experimenting a primary ZIKV infection (most likely young people) followed by a secondary DENV infection will be in a better position to develop a protective response to a tertiary DENV exposure with very limited room for enhancing pathogenesis. On the other hand, DENV-immune people with a secondary ZIKV exposition would be more at risk to develop ADE during a tertiary flavivirus infection caused by DENV. Our contribution on the role of the order of primary and secondary flavivirus infection shaping the immune response during a tertiary exposition provides new insights for an effective flavivirus vaccine development.

Declarations

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Author contributions

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

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Figures

	Cohort	<i>n</i>	1° Infection	2° Infection	Depletion	3° Infection
Experiment 1 <i>n</i> = 8	A-1	2	DENV3/4 October 2013	ZIKVPR September 2017	CD4	DENV2 February 2019
	A-2	2	DENV3/4 October 2013	ZIKVPR September 2017	PBS	DENV2 February 2019
	A-3	4	DENV2 February 2019	N/A	N/A	N/A
Experiment 2 <i>n</i> = 20	B-1	3	DENV2 September 2016	ZIKVPR September 2017	CD4	DENV4 October 2019
	B-2	3	DENV2 September 2016	ZIKVPR September 2017	PBS	DENV4 October 2019
	B-3	3	ZIKVPR September 2017	DENV2 November 2017	CD4	DENV4 October 2019
	B-4	3	ZIKVPR September 2017	DENV2 November 2017	PBS	DENV4 October 2019
	B-5	8	DENV4 October 2019	N/A	N/A	N/A

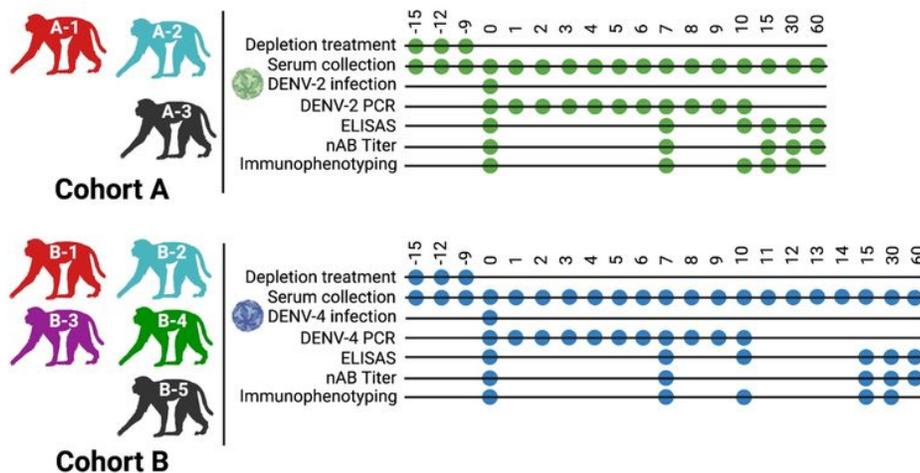


Figure 1

Experimental design for CD4⁺ T cell depletion and heterologous DENV challenge in flavivirus experienced and naïve macaques. Two cohorts of rhesus macaques (*Macaca mulatta*) were exposed to DENV and ZIKV virus at different time points. Cohort A: A-1 (*n*=2) and A-2 (*n*=2), were exposed to DENV-3 or DENV-4 (5×10^5 pfu s.c.) in October 2013 and to ZIKV PRABCV59 (1×10^6 pfu s.c.) in September 2017, along with a third subgroup (A-3) of flavivirus-naïve macaques (*n*=4); denoted experiment 1. Cohort B: B-1 (*n*=3) and B-2 (*n*=3) were exposed to DENV-2 (5×10^5 pfu s.c.) in September 2016 and to ZIKV PRABCV59 (1×10^6 pfu s.c.) in September 2017. B-3 (*n*=3) and B-4 (*n*=3) were exposed to ZIKV PRABCV59 (1×10^6 pfu s.c.) in September 2017 and to DENV-2 (5×10^5 pfu s.c.) in November 2017, along with a fifth subgroup (B-5) of flavivirus-naïve macaques (*n*=8); denoted experiment 2. Depletion of CD4⁺ T cells was performed on experimental groups (A-1 *n*=2, B-1 *n*=3 denoted DENV CD4 (-) and B-3 *n*=3 denoted ZIKV CD4 (-)) by initial subcutaneous administration of 50mg/kg (anti-CD4⁺) at 15 days pre-challenge followed by two intravenous administrations of 7.5mg/kg (anti-CD4⁺) at 12- and 9-days pre-challenge. Depletion control groups (A-2 (*n*=2), B-2 (*n*=3) denoted DENV Flavi-POS and B-4 (*n*=3) denoted ZIKV Flavi-POS) were treated

with PBS. All cohorts were challenged subcutaneously (deltoid area) with 5×10^5 pfu/500 μ l of either DENV-2 NGC44 (cohort A in February 2019) or DENV-4 Dominique (cohort B in October 2019). Sample collection was performed at various time points up to 60 days post-infection (dpi) for serum, whole heparinized blood, and PBMC isolation. Figure generated using BioRender.

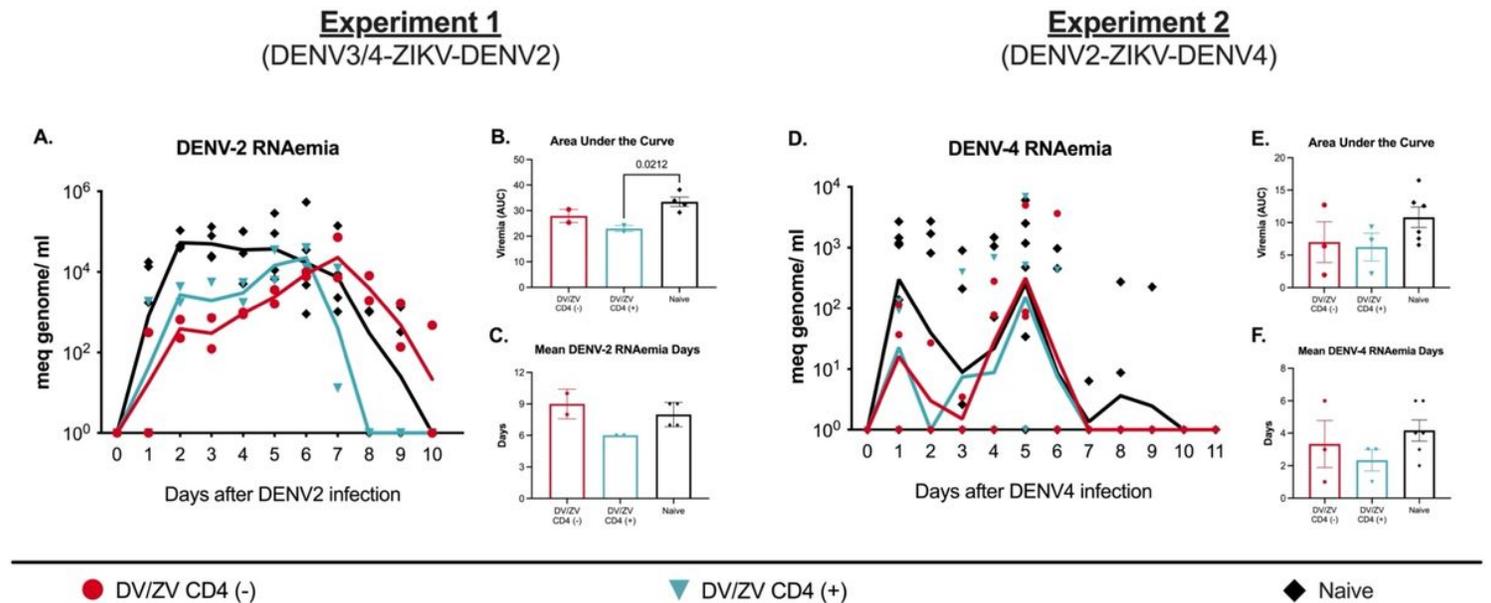


Figure 2

DENV/ZIKV infection sequences for experiments 1 & 2: DENV-2 and DENV-4 RNA kinetics of depleted and non-depleted macaques. RNAemia is negatively affected by the depletion of CD4⁺ T cells. In all panels, animals depleted of CD4⁺ T cells regardless of the DENV infection sequence are depicted in red. DENV/ZIKV immune-competent groups are depicted in blue and flavivirus-naïve animals in black. (A) For experiment 1 ($n=8$), DENV-2 genome copies/ml were measured in the serum to monitor viral replication during the first 10 days after infection. Statistically significant differences were observed using unpaired t-tests. Genome copies per mL are shown logarithmically. (B & E) The area under the curve (AUC) was calculated for individual values. (C & F) Average RNAemia days were calculated using the following formula: total viremia days divided by total possible viremia days. (D) For experiment 2 (DENV2-ZIKV-DENV4, $n=14$), DENV-4 genome copies/ml were measured in the serum to monitor viral replication during the first 11 days after infection. Statistically significant differences of AUC values were calculated using an unpaired t-test.

DENV-ZIKV-DENV (Experiments 1 & 2)

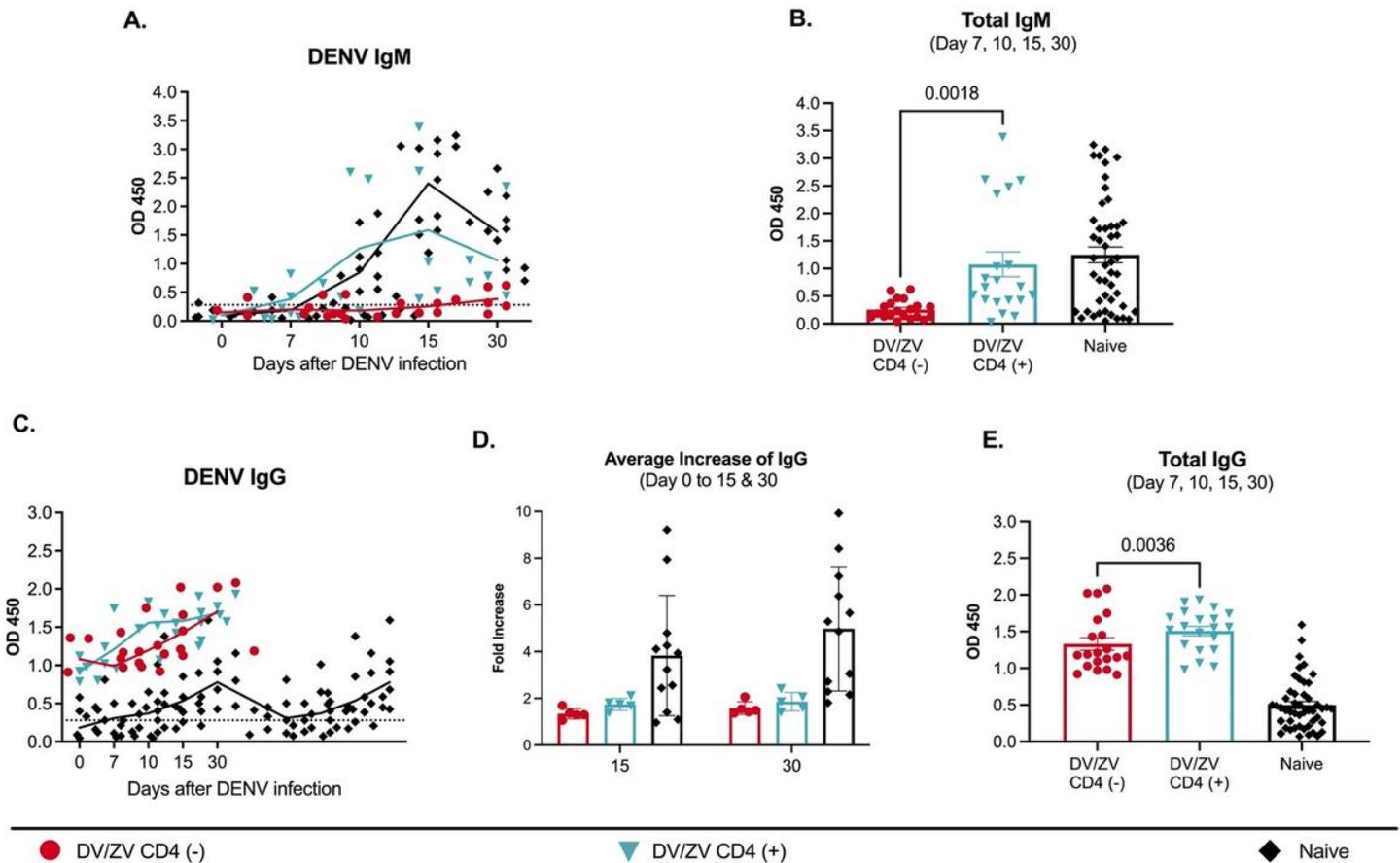


Figure 3

Meta-analysis DENV/ZIKV infection sequences for experiments 1 & 2: Depletion of CD4⁺ T cells modifies the serological profile during heterologous infections in DENV-primed individuals. The quantity of the humoral response was assessed using different commercial ELISA tests (A-E). Summary of the results for experiments 1 (*n*=8) and 2 (*n*=14). (B & E) Pool of OD readings for total antibody production against DENV from DENV-primed animals depleted and non-depleted with different infecting sequences is shown. Animals depleted of CD4⁺ T cells are depicted in red, DENV/ZIKV immune-competent animals are depicted in blue and flavivirus-naïve animals are depicted in black in all panels. Dotted lines indicate the limit of detecting for each test. Statistically significant differences among and within groups were calculated using Tukey's multiple comparisons tests and unpaired t-tests.

DENV3/4-ZIKV-DENV2

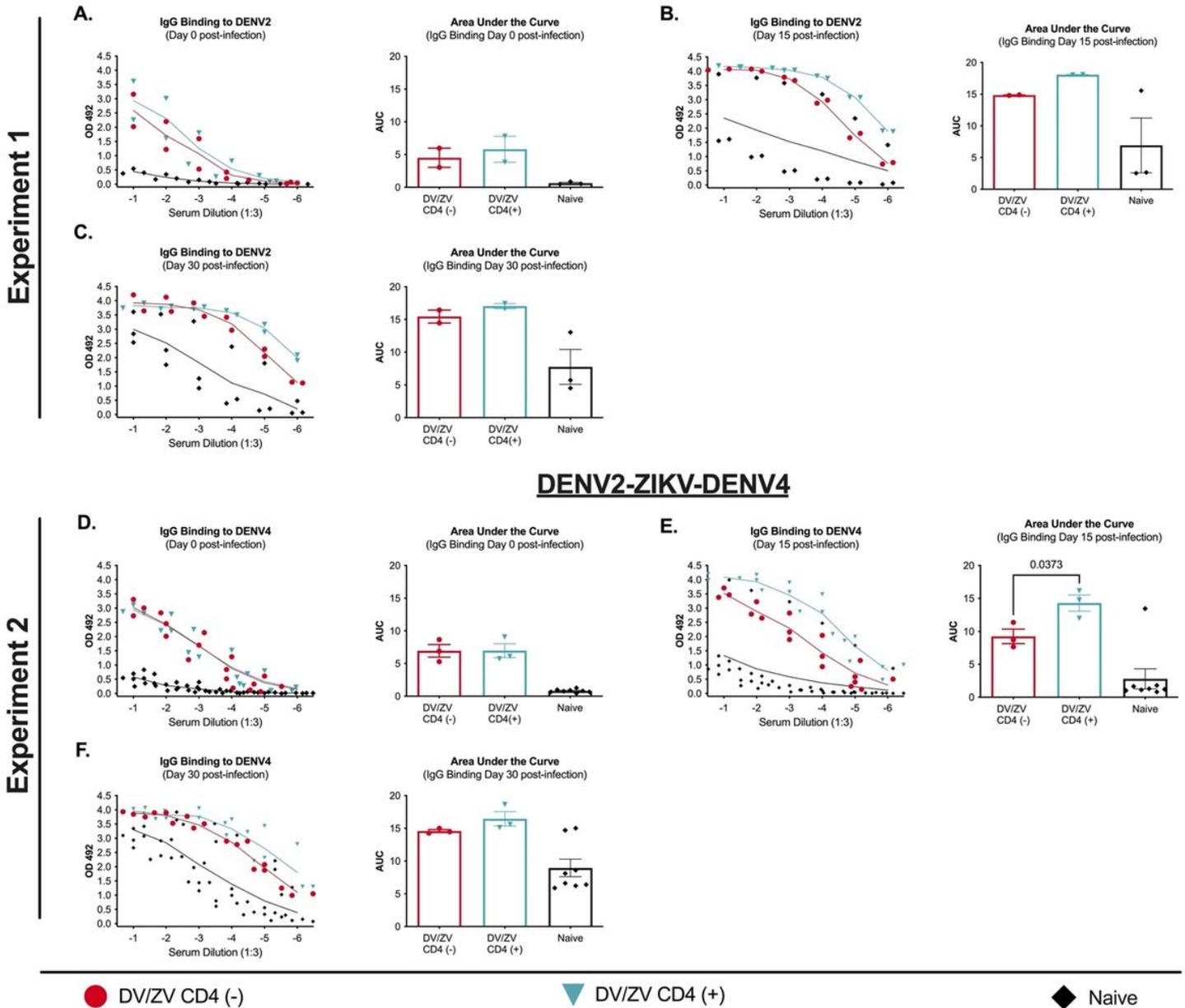


Figure 4

Depletion of CD4⁺ T cells modifies the IgG-binding capabilities of antibodies against DENV. The quality of the humoral response was assessed using an endpoint dilution binding ELISA (A-E). (A-C) Binding results for experiment 1 ($n=8$) against DENV2 for baseline, day 15, and day 30 p.i. are shown. (D-F) Binding results for experiment 2 ($n=14$) against DENV4 for baseline, day 15, and day 30 p.i. are shown. Animals depleted of CD4⁺ T cells are depicted in red, DENV/ZIKV immune-competent animals are depicted in blue and flavivirus-naïve animals are depicted in black in all panels. Statistically significant differences among groups were observed using multiple unpaired t-tests.

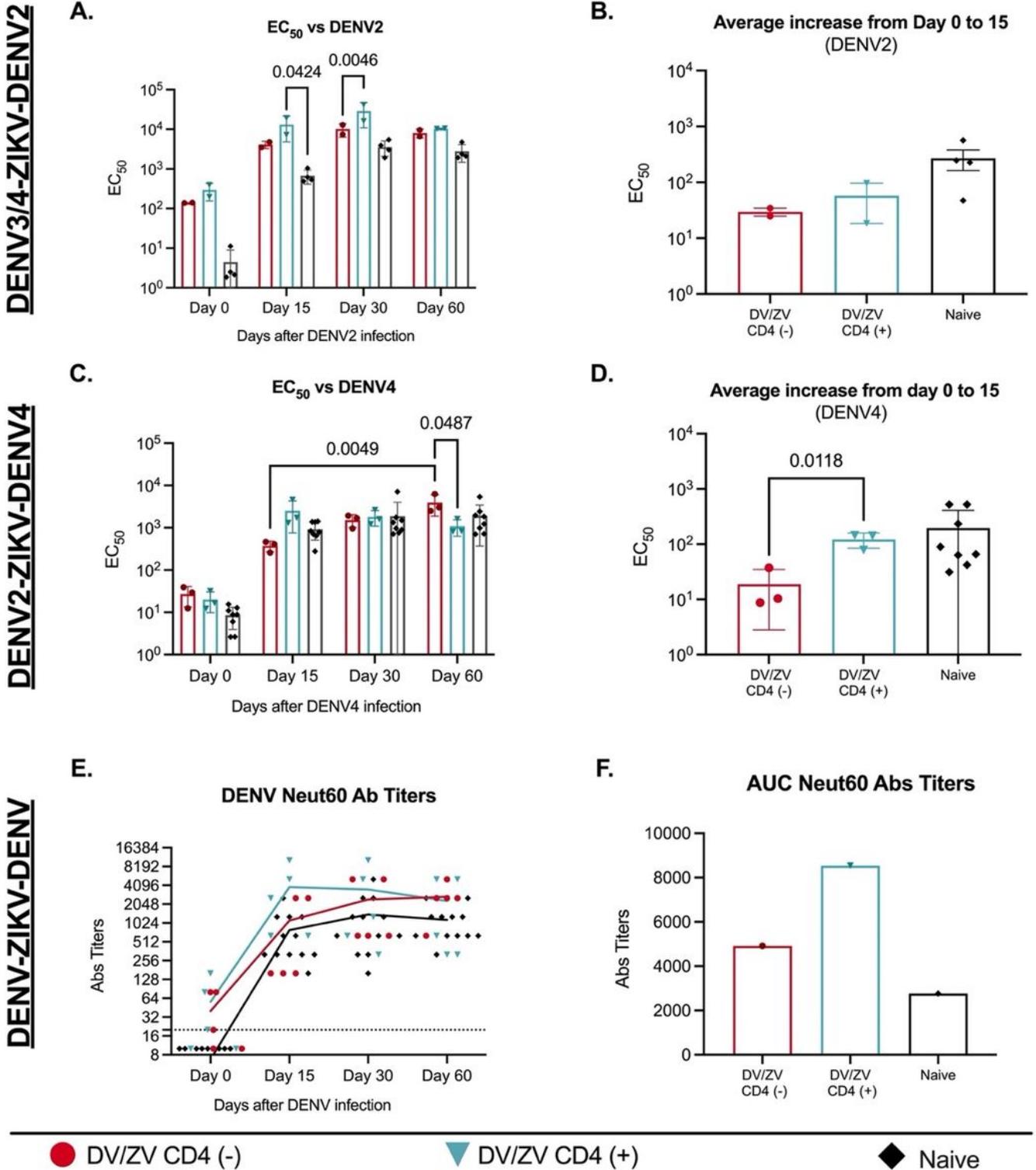


Figure 5

Meta-analysis DENV/ZIKV infection sequences for experiments 1 & 2: Geometric mean titers of DENV neutralizing antibodies. The 50% effective concentration of neutralizing antibodies was determined. Animals depleted of CD4⁺ T cells are depicted in red, DENV/ZIKV immune-competent animals are depicted in blue and flavivirus-naïve animals are depicted in black in all panels. Non-neutralizing sera were assigned a value of one-half of the detection limit for visualization and calculation of the geometric

means and confidence intervals. (A) EC50 values of neutralizing antibodies against DENV-2 after DENV-2 infection for experiment 1 ($n=8$) are shown. (B) The average increase for DENV-2 was calculated using the average time increase divided by the n . (C) EC50 values of neutralizing antibodies against DENV-4 after DENV-4 infection for experiment 2, DENV2-ZIKV-DENV4 sequence, ($n=14$) are shown. (D) The average increase for DENV-4 was calculated using the average time increase divided by the n . (E) Summary of FRNT titer values for experiments 1 & 2 ($n=22$). (F) Area under the curve (AUC) was calculated for FRNT individual titer values. Statistically significant differences among groups were calculated by two-way ANOVA using Tukey's multiple comparisons test and multiple unpaired t-tests.

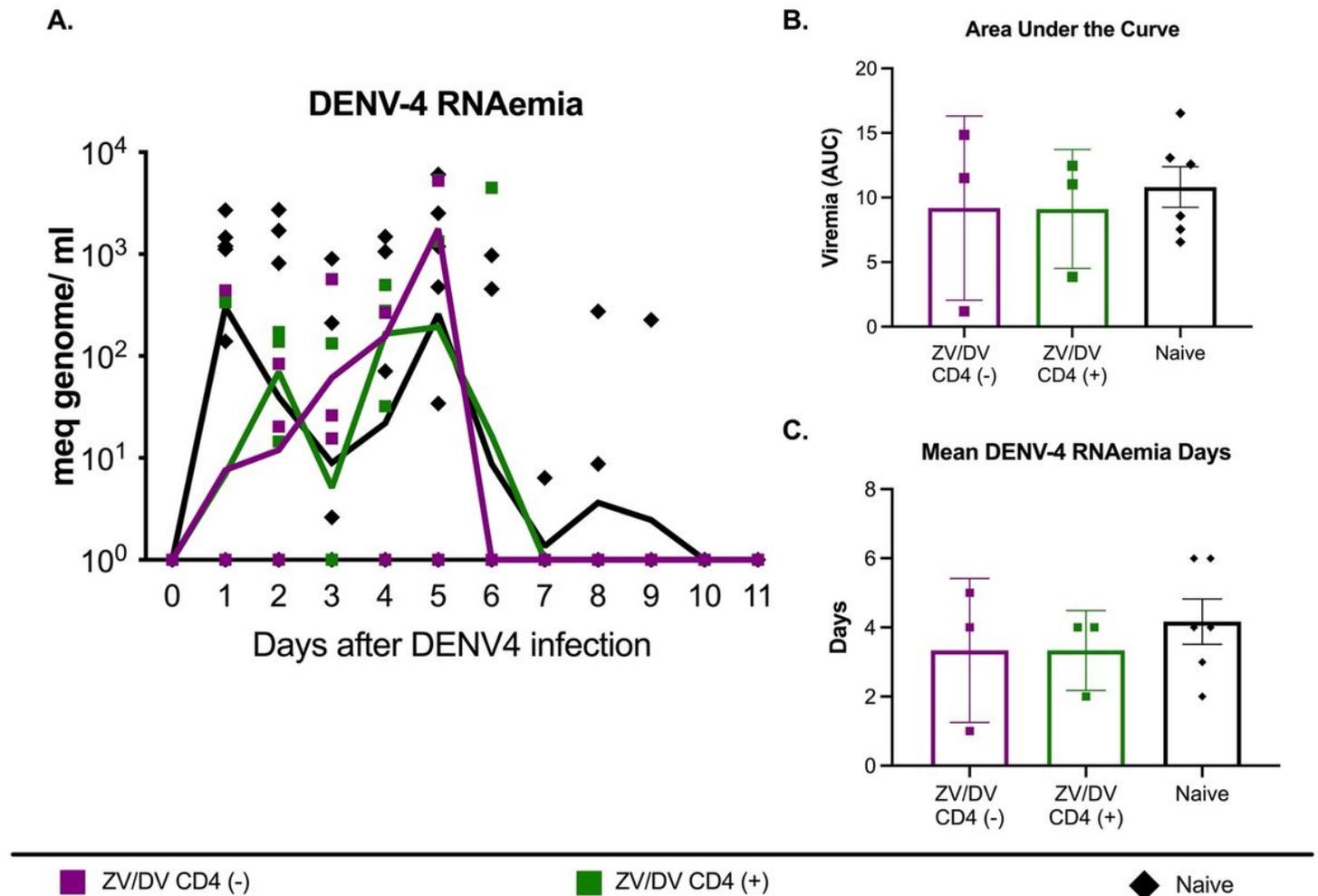


Figure 6

ZIKV/DENV infection sequence for experiments 2: DENV-4 RNA kinetics of macaques depleted or non-depleted of CD4⁺ T cells. RNAemia is limitedly affected by the depletion of CD4⁺ T cells. In all panels, animals depleted of CD4⁺ T cells are purple, ZIKV/DENV immune-competent animals are shown in green, and naïve animals in black. (A) For experiment 2 (ZIKV-DENV2-DENV4, $n=14$), DENV-4 genome copies/ml were measured in the serum to monitor viral replication during the first 11 days after infection. Genome copies per mL are shown logarithmically. (B) 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 whole possible viremia days. Statistically significant differences of AUC values were calculated using an unpaired t-test.

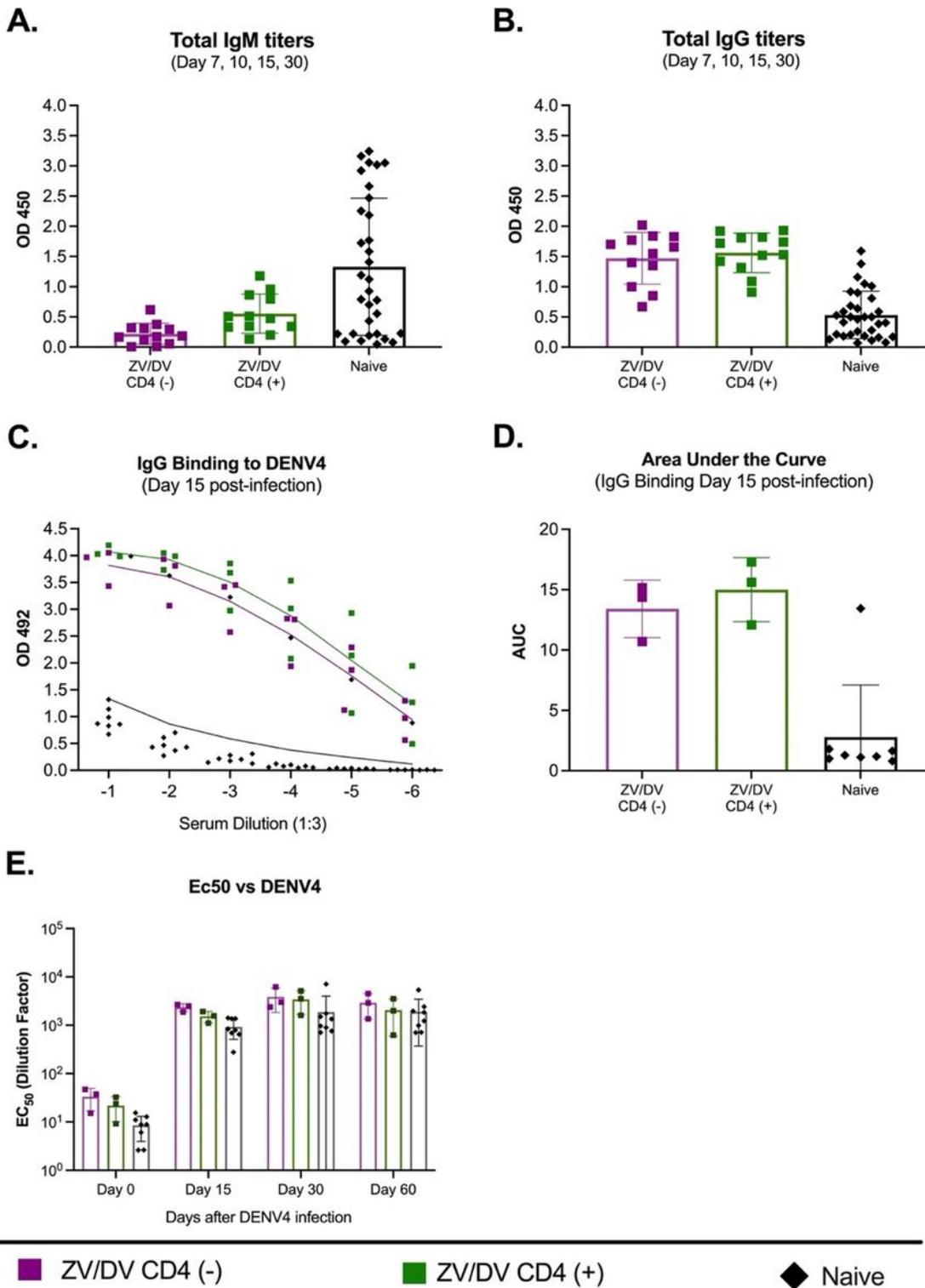


Figure 7

The quantity and quality of the humoral response are not affected by CD4⁺ T cell depletion in ZIKV-primed individuals. The quantity of the humoral response was assessed using different commercial

ELISA tests, and quality was assessed using an endpoint dilution binding ELISA. Animals depleted of CD4⁺ T cells are shown in purple, ZIKV/DENV immune-competent animals are shown in green and naïve animals are shown in black in all panels. (A & B) Pool of OD readings for total antibody production against DENV from animals depleted and non-depleted with different ZIKV and DENV infecting sequences is shown. (C & D) Endpoint dilutions for day 15 post-infection against DENV-4 from animals depleted and non-depleted for experiment 2 (ZIKV-DENV2-DENV4, *n*=14) are shown. The 50% effective concentration of neutralizing antibodies was determined. (E) EC₅₀ values of neutralizing antibodies against DENV-4 after DENV infection for experiment 2 (*n*=14) are shown. Statistically significant differences among groups were calculated by two-way ANOVA using Tukey's multiple comparisons test and multiple unpaired t-tests.

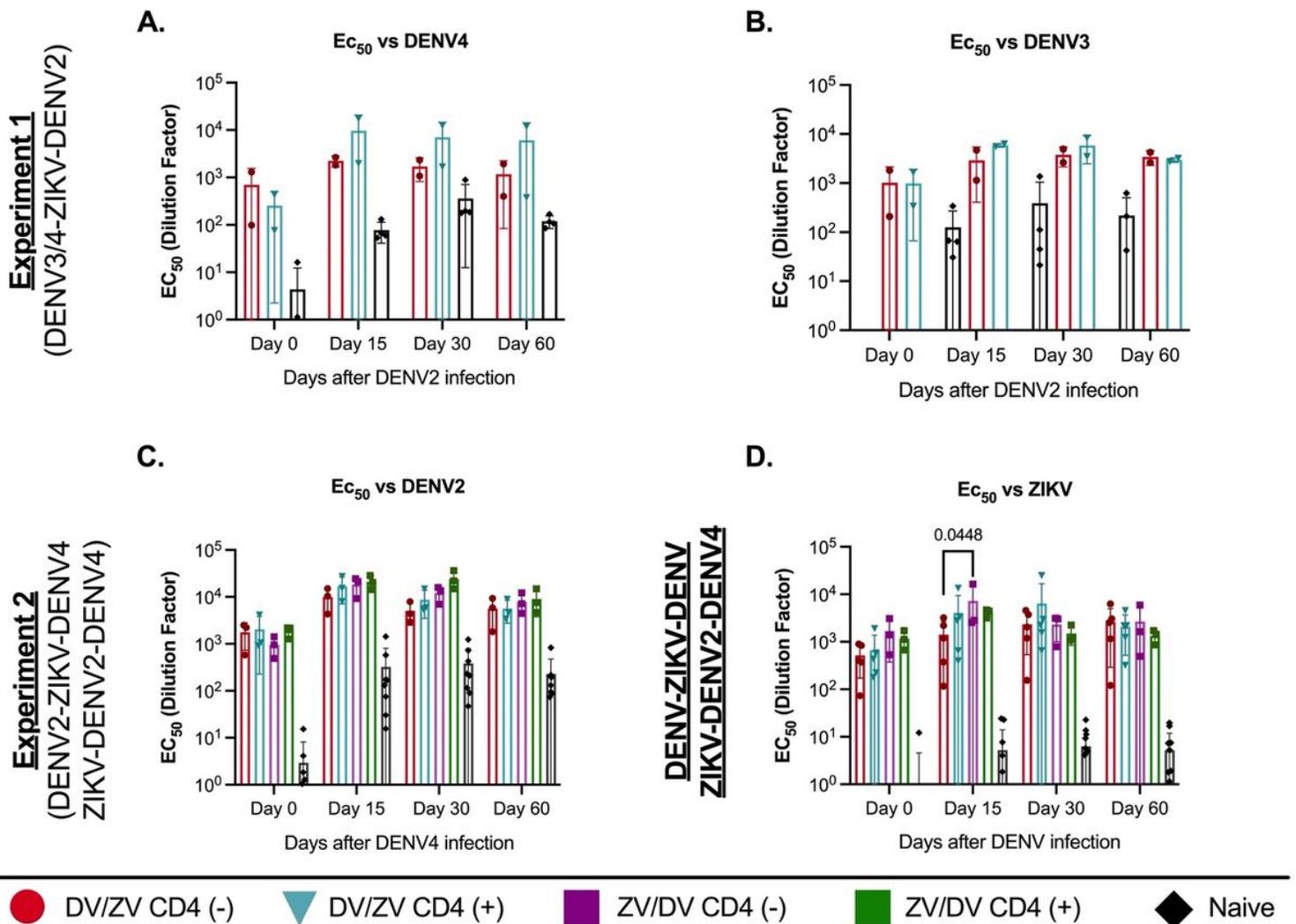


Figure 8

Recall humoral immune response: Geometric mean titers of DENV-4, DENV-2, and ZIKV neutralizing antibodies. The 50% effective concentration of neutralizing antibodies was determined. DENV/ZIKV CD4-depleted animals are depicted in red, DENV/ZIKV immune-competent animals are depicted in blue,

ZIKV/DENV2 CD4-depleted animals are depicted in purple, ZIKV/DENV2 immune-competent animals are depicted in green and flavivirus-naïve animals are depicted in black in all panels. Non-neutralizing sera were assigned a value of one-half of the detection limit for visualization and calculation of the geometric means and confidence intervals. (A & B) EC50 values of neutralizing antibodies against DENV-4 and DENV-3 after tertiary DENV-2 infection for animals of experiment 1 ($n=8$). (C) EC50 values of neutralizing antibodies against DENV-2 for animals of experiment 2 ($n=20$). (D) Meta-analysis of EC50 values of neutralizing antibodies against ZIKV after tertiary DENV infection for DENV-ZIKV-DENV, experiments 1 and 2, and ZIKV-DENV2-DENV4 infecting sequences ($n=28$). Statistically significant differences among groups are reported as multiplicity adjusted p values calculated by two-way ANOVA using Tukey's multiple comparisons test.

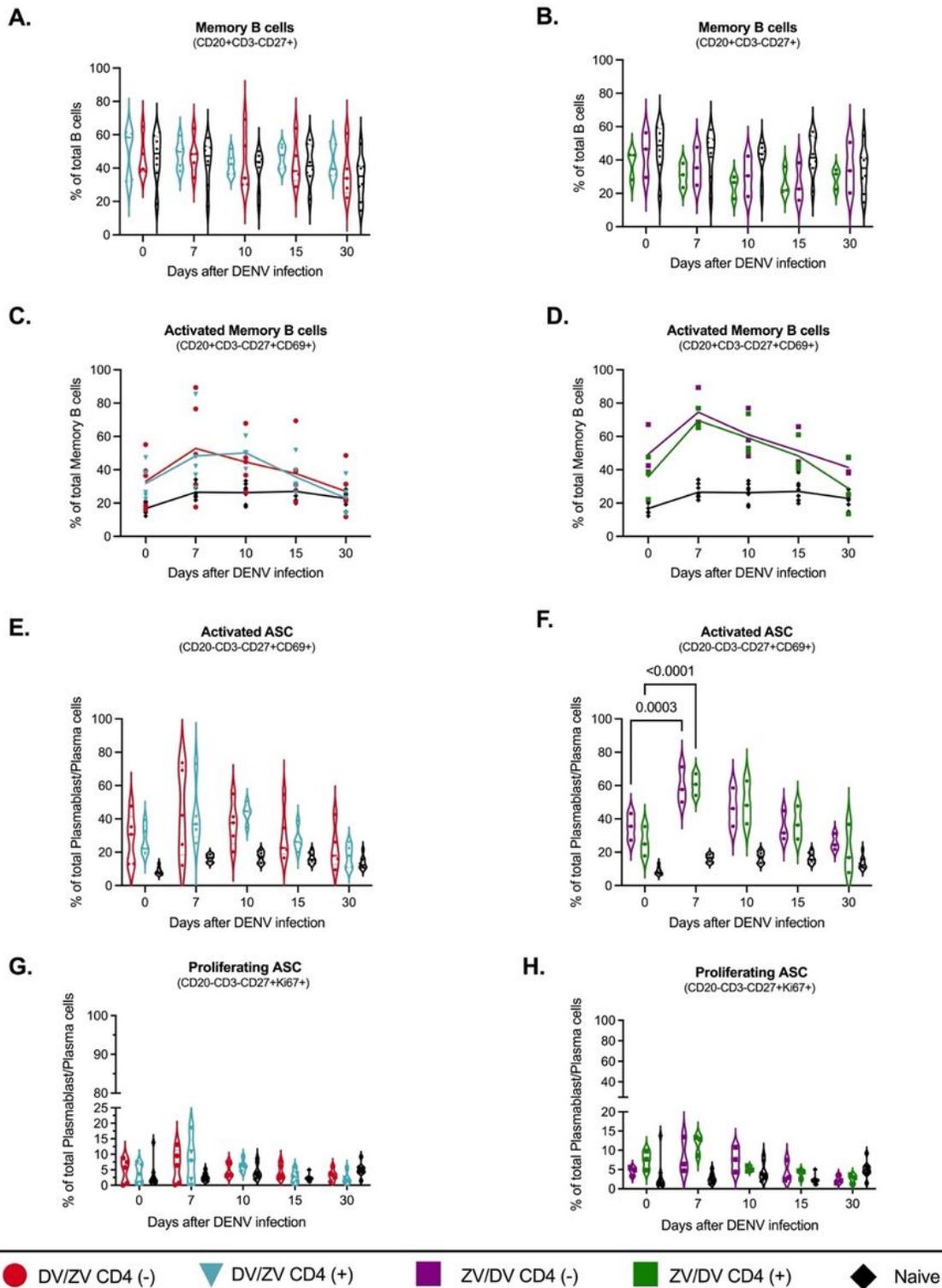


Figure 9

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

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

in all panels ($n=20$). (A-B) Percentage of total memory B cells ($CD20^+CD3^-CD27^+$). (C-D) Frequency of activated memory B cells ($CD20^+ CD3^-CD27^+CD69^+$). (E-F) Percentage of activated antibody-secreting cells (ACS) ($CD20^- CD3^-CD27^+CD69^+$). (G-H) Frequency of proliferating antibody-secreting cells (ACS) ($CD20^-CD3^-CD27^+Ki67^+$). Comparisons between groups are reported as multiplicity adjusted p values performed by two-way ANOVA using Tukey's multiple comparisons tests.

Supplementary Files

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