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Time elapsed between Zika and Dengue infections improves the immune response against Dengue without viremia enhancement in rhesus macaques

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Abstract

The role of a previous Zika virus (ZIKV) immunity on subsequent Dengue virus (DENV) infections is poorly understood. This is relevant to anticipate the dynamics of forthcoming DENV epidemics in areas with previous ZIKV exposure. It is still uncertain if the immunity conferred by the recent ZIKV epidemic may contribute to protection or worsening DENV cases severity. Accordingly, we have studied the effect of ZIKV infection with various strains on subsequent DENV immune response after 10 and 2 months of ZIKV infection. Our results in non-human primates showed that a subsequent DENV infection in animals with early- and middle-convalescent periods to ZIKV do not promote an increase in DENV viremia nor pro-inflammatory status. We found that previous ZIKV exposure improves the antibody and cell-mediated immune responses against DENV and that the time interval between infections impacted the magnitude and durability—more efficient after longer ZIKV pre-exposure—of the immune response. Furthermore, our data suggest that the elicited immune modulation between both ZIKV-immune groups after DENV infection are more influenced by the time elapsed between ZIKV and DENV infections and the maturation of the cross-reactive immune memory, rather than a possible effect due to ZIKV strain variation. Collectively, our findings provide evidence of a non-detrimental effect of ZIKV immunity in a subsequent DENV infection. This supports the implementation of ZIKV vaccines that could also boost immunity against future DENV epidemics.

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Introduction

Zika virus (ZIKV) is a re-emerging mosquito-borne *Flavivirus* that has captivated the attention of the scientific community by its explosive spread in The Americas[1], and severe neurological sequelae following infection[2-5]. ZIKV established itself in tropical and sub-tropical regions that are endemic to other closely-related flaviviruses such as Dengue virus (DENV). Both viruses belong to the Flaviviridae family and are transmitted by *Aedes spp.* mosquitoes. DENV is a global public health threat, having two-thirds of world's population at risk of infection, causing ~390 million infections annually, and possessing one of the highest mortality rate among arboviruses[6,7]. DENV exists as four genetically similar but antigenically different serotypes (DENV1-4)[8]. Exposure to one DENV serotype confers long-lived immunity against a homotypic secondary infection. However, secondary infection with a heterologous serotype of DENV is the major risk factor to induce severe DENV disease [9-11].

Due to the established history regarding the influence of cross-reactive immune interactions in dictating disease outcomes during heterologous infection and the genetic and consequently antigenic similarities between DENV and ZIKV, concerns have been raised regarding the impact of DENV-ZIKV cross-reactive immunity on the development of severe clinical manifestations[12,13]. In the last few years, multiple studies have aimed to understand the role of a prior DENV exposure in the outcome of ZIKV infection. It has been demonstrated that DENV-immune sera from humans can enhance ZIKV infection *in vitro*[14,15], and *in vivo* in immune-deficient mouse models[16]. However, recent results from our group and others have shown that previous flavivirus exposure—including DENV—may have no detrimental impact on ZIKV infection *in vivo* in non-human primates (NHP)[17,18] and humans[19]. Moreover, these studies and others suggest that previous DENV immunity may play a protective role during ZIKV infection involving humoral and cellular responses[20-24]. On the other hand, little is known about the opposite scenario, the role of a previous ZIKV exposure on a subsequent DENV infection, which is relevant to anticipate the dynamics of forthcoming DENV epidemics.

The recent ZIKV epidemic in the Americas resulted in the development of a herd immunity that may have an impact in subsequent infections with other actively circulating flaviviruses such as DENV. Thus, human sub-populations such as newborns, international travelers from non-flavivirus endemic areas or DENV-naïve subjects could be exposed to a ZIKV infection prior to DENV—since DENV declined in the Americas during ZIKV epidemic[25]. After the epidemic, herd immunity reduced ZIKV transmission and DENV will re-emerge and potentially infect these DENV-naïve ZIKV-immune sub-populations in The Americas or potentially in other geographic areas

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newly at risk [26,27]. An epidemiological study based on active Dengue surveillance in Salvador, Brazil, suggests that the reduction of DENV cases after the ZIKV epidemic is due to protection from cross-reactive immune responses between these viruses[28]. Prospective experimental studies are needed to confirm this hypothesis. For this purpose, we propose the use of NHPs as a suitable model. NHPs provide advantages such as an immune response comparable to humans, and the normalization of age, sex, injection route, viral inoculum and timing of infection[29]. Although clinical manifestations by flaviviral infections are limited in NHPs[30], they have been widely used as an advanced animal model for the study of DENV and ZIKV immune response, pathogenesis, and vaccine development[17,18,29,31-34].

ZIKV antibodies (Abs) are capable of enhancing DENV infection *in vitro*[35]. Characterization of the specificity of DENV and ZIKV cross-reactive response revealed that ZIKV monoclonal Abs—and more recently—maternally acquired ZIKV Abs can increase DENV severity and viral burden in immune-deficient mouse models[36,37]. However, little evidence is available concerning this phenomenon occurring *in vivo* in immuno-competent large animal models such as NHPs. *George et al.*, showed that an early convalescence to ZIKV induced a higher peak of DENV viremia and a pro-inflammatory status compared to ZIKV-naïve status in macaques[38]. Further characterization of ZIKV early convalescent sera from these macaques indicated that there is a delayed induction of the cross-reactive Ab response against DENV, supporting no cross-protection against the outcome of DENV infection[39]. Despite these findings, further studies are needed to dissect the complementary role of the innate, humoral and cellular immune response to mechanistically explain these findings. Particularly, there is no evidence of the modulation and functionality of the T cell immune response in the ZIKV-DENV scenario. Available studies rely upon pathogenesis and antibody studies, but there is no documented evidence as to whether cell-mediated immunity (CMI)—specifically the polyfunctional response of T cells—is modulated in a subsequent DENV infection by the presence of ZIKV immune memory.

The time interval between primary and secondary DENV infections have been shown to be an important predictor for the development of severe clinical outcomes in humans[10]. Shorter time interval between DENV infections result in a subclinical secondary infection, while symptomatic secondary infections and severe DENV cases have been related with longer periods between infections[40-43]. These findings suggest that high titers of cross-reactive Abs play a time-dependent protective role between heterotypic DENV infections. Despite this evidence from DENV sequential infections, it remains poorly understood if the same applies to the time interval between ZIKV-DENV sequential infections. Specifically, how do longer periods of convalescence after ZIKV infection impact the outcome of DENV infection. This scenario will more closely

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resemble the epidemiological setting and time intervals elapsed between the current circulation of related flaviviruses in the Americas. So currently, the role of multiple convalescent periods to ZIKV in the outcome of DENV and other flavivirus infections is in the forefront of discussions based on the limited studies available in experimental models and a lack of characterized human prospective cohorts of this scenario yet[28,44-46].

To address these knowledge gaps, the objective of our study is to investigate the immune modulatory role of an early- and mid-convalescence after ZIKV infection on the outcome of a subsequent DENV infection in a NHP model. To test this, NHP cohorts who were ZIKV immune for 10 months (mid-convalescence), 2 months (early-convalescence) or naïve for ZIKV were exposed to DENV. In each of these groups we assessed DENV pathogenesis, the elicited Ab response, and characterized the CMI. Based on our knowledge, this is the first characterization of CMI with this scenario—taking into account the synergistic effect between the Ab and cell-mediated responses. This study provides evidence that the presence of ZIKV immune memory contributes to improve the immune response—more efficient after longer ZIKV pre-exposure—against a DENV infection, without promoting enhancement of DENV viremia nor inducing higher levels of pro-inflammatory cytokines.

Results

DENV challenge and clinical status of rhesus macaques. The experimental design includes three cohorts of rhesus macaques (*Macaca mulatta*) that were challenged with DENV-2 (NGC-44 strain), monitored and bled during three months (Fig. 1). Two were previously exposed to ZIKV: cohort 1 (ZIKVPF-10mo) was comprised of 4 animals that had been exposed to ZIKV H/PF/2013 strain 10 months before DENV-2 challenge (mid-convalescence), and cohort 2 (ZIKVPR-2mo) comprised of 6 animals that had been exposed to ZIKV PRVABC59 strain two months before DENV-2 challenge (early-convalescence). Both ZIKV strains used for previous exposure of these groups are >99.99% comparable in amino acid identity (Supplementary Table 1). An additional cohort 3 (Naïve) included four animals naïve to ZIKV/DENV as a control group. After DENV challenge all macaques were extensively monitored and sample collection was performed at various timepoints up to 90 days post infection (dpi) for serum and PBMCs isolation.

The clinical status was monitored to determine if the presence of ZIKV immunity affected the clinical outcome of DENV infection. Vital signs such as weight (kg) and temperature (°C) were monitored. Also, complete blood cell counts (CBC) and comprehensive metabolic panel (CMP) were performed before (baseline: day 0) and after DENV infection at multiple timepoints (CBC: 0,

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7, 15 dpi; CMP: 0, 7, 15, 30 dpi). Neither symptomatic manifestations nor significant differences in weight or temperature were observed in any of the animals after DENV infection up to 90 dpi (Supplementary Fig. 1a-b). Likewise, no significant differences between groups were detected in CBC parameters: white blood cells (WBC), lymphocytes (LYM), neutrophils (NEU), monocytes (MON), and platelets (PLT) after DENV infection compared to basal levels of each group (Supplementary Fig. 1c-g). CMP levels showed no differences in alkaline phosphatase and aspartate transaminase (AST) (Supplementary Fig. 1h-i). Although within the normal range, levels of alanine transaminase (ALT) were significantly higher in the ZIKVPR-2mo group compared to its baseline at 7 dpi (p=0.0379, Two-way Anova Dunnett test), at 15 and 30 dpi values returned to baseline levels (Supplementary Fig. 1j). Overall, except for the isolated increase of ALT at 7 dpi in ZIKVPR-2mo, the clinical profile suggests that the presence of ZIKV-immunity did not significantly influence the clinical outcome of DENV infection.

DENV RNAemia is not enhanced by previous ZIKV immunity. RNAemia levels in NHPs serum were quantified by qRT-PCR at baseline, 1 to 10, and 15 dpi to determine if the presence of early- (ZIKVPR-2mo) or mid-convalescence (ZIKVPF-10mo) to ZIKV alters DENV kinetics. No significant differences between groups were observed in detected levels of DENV genome copies per ml of serum over time (Fig. 2a; Supplementary Table 2). We noted that in the ZIKVPF-10mo group 3 out of 4 animals were able to keep the RNAemia level below 10^3 genome copies the next day after DENV infection. This group started an early clearance of the RNAemia at 7 dpi, with only 1 out of 4 animals having detectable levels by days 8 and 9 pi. For ZIKVPR-2mo and naïve animals, the clearance of detectable RNAemia started at 8 dpi, in 4 out of 6 and 1 out of 4 of the animals, respectively. Naïve animals had the most delayed clearance of RNAemia with at least half of the animals with detectable levels of viral RNA until day 9 pi. RNAemia was completely resolved in all animals by 10 dpi. In summary, ZIKVPF-10mo had 7.25, ZIKVPR-2mo 7.5, and Naïve animals 8 mean days of detectable RNAemia after DENV infection (Fig. 2b; Supplementary Table 2). Together these results show that, although no statistically significant differences among groups were observed, previous immunity to ZIKV is not associated with an increase in DENV RNAemia set point or duration; even more, a mid-convalescence to ZIKV tended to develop a shorter viremic period.

Pro-inflammatory cytokines are not exacerbated by the presence of ZIKV immunity. To determine if the characterized cytokine profile of an acute DENV infection was modulated by ZIKV immunity we assessed the serum concentration (pg/ml) of 8 cytokines/chemokines by Luminex

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multiplex at baseline, 1, 2, 3, 5, 10, 15 and 30 dpi. The naïve group showed significant higher levels of pro-inflammatory cytokines (Fig. 3a-c). Type I interferon alpha (IFN-α) was highest at 5 dpi (Fig. 3a: $p<0.0001$ vs ZIKVPF-10mo and $p=0.0003$ vs ZIKVPR-2mo, Two-way Anova Tukey test). IFN-α has been demonstrated to be involved in the innate anti-viral immunity and elevated levels are associated with higher viral load and thus antigen availability. Interleukin-6 (IL-6), a multifunctional cytokine involved in immune response regulation and many inflammatory reactions showed the highest levels at 1 dpi in naïve animals (Fig. 3b: $p=0.0115$ vs ZIKVPF-10mo and $p=0.0185$ vs ZIKVPR-2mo, Two-way ANOVA Tukey test); Finally, a monokine induced by IFN-gamma (MIG/CXCL9), which is a potent chemoattractant involved in leucocyte trafficking demonstrated the highest levels at 10 dpi in naïve animals (Fig. 3c: $p=0.0004$ vs ZIKVPR-2mo, Two-way Anova Tukey test). On the other hand, the mid-convalescent ZIKVPF-10mo group showed higher levels of CXCL10 (IP-10) (Fig. 3g) at day 1 ($p=0.0198$ vs ZIKVPR-2mo, Two-way Anova Tukey test), 5 ($p=0.0487$ vs Naïve, Two-way Anova Tukey test) and 10 pi ($p=0.0009$ vs ZIKVPR-2mo, Two-way Anova Tukey test). CXCL10 is a T cell-activating chemokine and chemoattractant for many other immune cells. Also, this group showed higher levels of perforin (Fig. 3h) at day 10 ($p=0.0024$ vs Naïve and $p=0.0190$ vs ZIKVPR-2mo, Two-way Anova Tukey test) and 15 pi ($p=0.0178$ vs Naïve, Two-way Anova Tukey test). Perforin is an effector cytolytic protein released by activated cytotoxic CD8+ T cells and natural killer (NK) cells. No significant differences between groups were observed in monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1-beta (MIP-1β) and IL-1 receptor antagonist (IL-1RA) (Fig. 3d-f). Collectively, these results demonstrate that the presence of ZIKV immunity does not exacerbate pro-inflammatory status after DENV infection while mid-convalescence immunity to ZIKV stimulated levels of mediators mainly involved in the activation of cell-mediated immune response.

DENV and ZIKV cross-reactive antibody response is boosted by ZIKV immunity and is influenced by the time span of the previous ZIKV infection. An ELISAs-based serological profile was performed to determine the contribution of ZIKV immunity in the cross-reactive antibody response before and after DENV infection. We assessed the levels of DENV IgM and IgG, and cross-reactivity with ZIKV (IgM, IgG, NS1-IgG and EDIII-IgG) at multiple timepoints (Supplementary Fig. 2). Naïve cohort showed a significant higher peak of IgM (Supplementary Fig. 2a) characteristic of a primary DENV infection at 15 and 30 dpi ($p<0.0001$ vs ZIKVPF-10mo and $p=0.0004$ vs ZIKVPR-2mo, $p=0.0044$ vs ZIKVPF-10mo and $p=0.0179$ vs ZIKVPR-2mo, respectively, Two-way Anova Tukey test). This indicates the productive and acute DENV

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infection, while ZIKV immune groups showed lower levels of IgM resembling a heterotypic secondary infection. Total DENV IgG levels (Supplementary Fig. 2b) of both ZIKV immune groups were significantly higher compared to naïve since baseline (cross-reactive ZIKV-IgG Abs) and 7, 15, 30, 60 and 90 (the latter for ZIKVPF-10mo only) (ZIKVPF-10mo vs Naïve: p=0.0010, p<0.0001, p<0.0001, p<0.0001, p<0.0001, p=0.0016; ZIKVPF-2mo vs Naïve: p=0.0029, p=0.0002, p<0.0001, p<0.0001, p=0.0006; Two-way Anova Tukey test). The ZIKVPF-10mo group showed significant higher levels than ZIKVPR-2mo group at 30 and 90 dpi (p=0.0242 and p=0.0348, Two-way Anova Tukey test). Overall, ZIKVPF-10mo developed higher and long-lasting levels of DENV IgG.

In contrast to the elevated levels of DENV-specific IgM, ZIKV IgM levels were under or near the limit of detection in all groups over time after DENV infection despite several significant differences seen between groups (Supplementary Fig. 2c). ZIKV IgG levels (Supplementary Fig. 2d) were similarly high in both ZIKV-immune groups at baseline and 7 dpi compared to naive (p<0.0001 vs naïve, Two-way Anova Tukey test), suggesting that although different pre-infecting ZIKV strains, the previous elicited IgG response against heterologous ZIKV strains is comparable. After DENV infection, an increase of ZIKV IgG was shown and remain constantly high at 15, 30, 60 and 90 dpi in both ZIKV-immune groups (p<0.0001 vs naïve for all timepoints, Two-way Anova Tukey test), suggesting that DENV has the potential to stimulate ZIKV-binding Ab-producing plasmablasts. In addition, to elucidate the composition of similar ZIKV IgG levels in ZIKV-immune groups, we measured ZIKV-specific NS1 IgG (Supplementary Fig. 2e) and ZIKV-specific EDIII IgG (Supplementary Fig. 2f) levels. Although ZIKVPR-2mo showed significant differences compared to naïve at 30, 60 and 90 dpi (p<0.0001, p=0.0001, p=0.0159; Two-way Anova Tukey test), we observed a significantly higher expansion and long-lasting response of ZIKV NS1-specific Abs in the ZIKVPF-10mo group compared to the ZIKVPR-2mo group at baseline, 60 and 90 dpi (p=0.0036, p=0.0071, p=0.0294; Two-way Anova Tukey test) and also compared to naïve animals at all timepoints (p<0.0001, Two-way Anova Tukey test). Moreover, higher magnitude of ZIKV-specific EDIII IgG levels in the ZIKVPF-10mo group than in the ZIKVPR-2mo group was observed compared to naïve at baseline (ZIKVPF-10mo only), 15, 30 and 60 (ZIKVPF-10mo vs Naïve: p=0.0092, p<0.0001, p<0.0001, p=0.0034; ZIKVPR-2mo vs Naïve: p=0.0003, p=0.0014, p=0.0055; Two-way Anova Tukey test), suggesting that ZIKV mid-convalescence promotes an expansion of higher magnitude of ZIKV EDIII IgG Abs from ZIKV memory B cells. However, those higher cross-reacting levels decrease over time as expected. In summary, a boost of DENV and ZIKV Abs is triggered by the presence of ZIKV immunity and the expansion of specific- and cross-

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reactive Abs is of higher magnitude and long lasting when a mid-convalescence immunity to ZIKV is present.

Neutralizing antibody response against DENV-2 and heterologous serotypes is higher in magnitude and durability in presence of mid-convalescence to ZIKV. Neutralizing antibodies (NAbs) are essential to combat DENV and ZIKV infection. The maturation and potency of this response is known to define to a great extent the infection outcome[12,47]. Accordingly, we tested the neutralization capacity of NAbs in serum from ZIKV- immune and naïve animals before and after DENV infection, to determine whether an early- or mid-convalescence to ZIKV affected the neutralizing Ab response. Plaque Reduction Neutralization Test (PRNT) was performed to elucidate the NAb titers of all groups against all DENV serotypes and both ZIKV pre-infecting strains. Before infection with DENV the naïve groups had no detectable NAb levels (<1:20 PRNT60 titers) against all DENV serotypes, while ZIKV-immune groups showed low cross-NAb titers against DENV-2 and DENV-4 (Fig. 4a). These cross-reactive levels were higher in the ZIKVPF-10mo group than in the ZIKVPR-2mo group for both viruses. The peak of high NAb titers occurred at 30 days after DENV infection for all groups (ZIKVPF-10mo>ZIKVPR-2mo>Naïve) against all DENV serotypes (DENV-2>DENV-4>DENV-3>DENV-1) (Fig. 4b). The ZIKVPF-10mo group neutralized all DENV serotypes with significant higher potency than naïve animals (p<0.0001, p=0.0337, p<0.0001, p<0.0001 for DENV1-4; Two-way Anova Tukey test) and the ZIKVPR-2mo group, except for DENV-2, that both ZIKV-immune groups have comparable neutralization magnitude at 30 dpi (p=0.0002, p=0.7636, p=0.0016, p=0.0004; Two-way Anova Tukey test). However, the neutralization kinetics by sigmoidal response curves suggest higher percent of neutralization against DENV-2 overtime in the group with mid-convalescence to ZIKV (Supplementary Fig. 3). On the other hand, the ZIKVPR-2mo group showed significantly higher potency of the NAb response only against DENV-1 compared to naive animals (p=0.0146; Two-way Anova Tukey test) (Fig. 4b).

In addition, we tested whether the NAb titers that peak at 30 dpi for all groups remain constant over time (up to 90 dpi) against all DENV serotypes (Fig. 4c-f). In general, the neutralizing response of the ZIKVPF-10mo group was more long-lasting, maintaining higher NAb titers up to 90 dpi compared to ZIKVPR-2mo and naïve groups. Significant differences between ZIKVPF-10mo and ZIKVPR-2mo groups were observed against DENV-1,-3 and -4 at day 30 pi (p=0.0002, p=0.0016, p=0.0004; Two-way Anova Tukey test) and at day 60 pi against DENV-2 and DENV-3 (p=0.0179, p=0.0047; Two-way Anova Tukey test). The neutralizing Ab response of the ZIKVPF-10mo group was even more significantly higher compared to the naïve group at day

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15 (only performed for the infecting serotype to monitor early neutralizing activity), day 30, 60 and 90 pi against DENV-2 (p=0.0022, p=0.0337, p=0.0146, p=0.0337; Two-way Anova Tukey test); at day 30 pi against DENV-1 (p<0.0001, Two-way Anova Tukey test); at day 30 and 60 pi against DENV-3 (p<0.0001, Two-way Anova Tukey test); and at day 30 pi against DENV-4 (p<0.0001, Two-way Anova Tukey test). In contrast, the ZIKVPR-2mo group showed a neutralizing Ab response with a magnitude and long-lasting levels comparable to the naïve group, except at day 15 and 30 pi against DENV-2 and DENV-1, respectively (p=0.0067, p=0.0146; Two-way Anova Tukey test). The neutralizing response was long-lasting in the ZIKVPF-10mo group compared to other groups as supported by the data from days 30 and 60 p.i. At day 90 pi, although no significant differences were observed between all groups, the ZIKVPF-10mo group showed a consistent trend to maintain higher NAb titers against all DENV serotypes indicating a higher and long-lasting breadth of cross-neutralization within DENV serocomplex.

Interestingly, comparing DENV-2 NAb titers and DENV genome copies at early and late phase of RNAemia timeframe (baseline NAb titer vs 1 dpi genome copies: early phase; and 7 dpi NAb titers vs 8 dpi genome copies: late phase), an inverse proportion was observed among elevated NAb levels and a subsequent lower (early phase) or no detection (late phase) of DENV genome copies (Supplementary Fig. 4a). This proportion (>NAb titers: <genome copies) was stronger for the ZIKVPF-10mo group. Therefore, this observation is consistent with the trend of ZIKVPF-10mo group to develop a shorter viremic period (Fig. 2). Although no statistically significant differences between groups in NAbs at baseline and 7 dpi, we observed that only the ZIKVPF-10mo group have PRNT60 cross-reactive NAb titers capable to neutralized 60% or more (~75%) of DENV-2 infection at baseline, but only at the more concentrated serum dilution (1:20) (Supplementary Fig. 4b). All groups had the property to neutralize 60% or more of DENV-2 one week after infection. However, the ZIKVPF-10mo and ZIKVPR-2mo groups retained this property even at higher serum dilutions, 1:20-1:160 and 1:20-1:80, respectively, compared to naïve animals (Supplementary Fig. 4c). Collectively, these results demonstrate that a mid-convalescence to ZIKV provokes a boost of the magnitude and durability of the neutralizing response against all DENV serotypes more effectively than in animals with an early-convalescence to ZIKV, but also higher compared to a *de novo* DENV-specific NAb response of the naïve animals. Also, our results suggest that the low-to-intermediary levels of cross-neutralizing Abs to DENV-2 detected prior DENV infection (induced by previous ZIKV infection) may play an early role controlling DENV set point RNAemia. This qualitative effect seems to be associated to a longer period of convalescence (10 months) but not to a recent ZIKV infection (2 months earlier).

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Potency of ZIKV cross-neutralizing antibody response is strain-independent and higher in magnitude and durability in the presence of mid-convalescence to ZIKV. Previous exposure to heterologous ZIKV strains in ZIKV-immune groups developed long-lasting high levels of cross-reactive, non-neutralizing, and neutralizing Abs before DENV infection (baseline). To determine if this memory Ab response is strain-specific and if the difference in convalescence period to ZIKV alters the efficacy and modulation after DENV infection, we assessed the NAb levels in ZIKV-immune (ZIKVPF-10mo and ZIKVPR-2mo) and ZIKV-naïve (Naïve) serum with both pre-infecting contemporary Asian-lineage H/PF/2013 and PRVABC59 ZIKV strains at multiple timepoints after DENV infection. At baseline, both ZIKV-immune groups showed high NAb titers against H/PF/2013 strain, which suggest that irrespective of pre-exposure to heterologous ZIKV strains and different convalescent periods the Ab response remains similarly effective (Fig. 5a). As early as day 15 after DENV infection, a potent boost of NAb titers in both ZIKV-immune groups was developed. However, elevated NAb titers were significantly higher in the ZIKVPF-10mo group compared to the ZIKVPR-2mo and naïve groups at day 15 pi (p=0.0005, p<0.0001; Two-way Anova Tukey test) and day 30 pi (p=0.0067, p=0.0012; Two-way Anova Tukey test). As expected, this elevated ZIKV cross-reactive NAb levels decreased gradually over time after 15 dpi in both ZIKV-immune groups. Nevertheless, the ZIKVPF-10mo group retained higher NAb titers until 90 dpi while the titers of the ZIKVPR-2mo group returned to baseline levels. In addition to this trend in NAb titers, the difference in durability is clearly demonstrated by the calculation of half maximal (50%) effective concentration (EC_{50}). The serum of the ZIKVPF-10mo group maintained the neutralization capacity at significantly more diluted concentrations compared to the ZIKVPR-2mo group at later timepoints (p<0.0001 for 60 dpi and p=0.0028 for 90 dpi; Two-way Anova Tukey test) (Fig. 5b). Of note, the NAb titers of the naïve group were considered as negative in all timepoints and failed to neutralize ZIKV throughout DENV infection even at concentrated levels of the antibodies (Fig. 5a-b). These results are confirmed by the behavior of neutralization kinetics by sigmoidal response curves where the ZIKVPF-10mo group retained elevated magnitude of ZIKV neutralization overtime (Supplementary Fig. 5).

To determine if the strain of ZIKV used in the previous exposures played a role in the modulation of the cross-NAb response triggered by a subsequent DENV infection, NAb titers were measured against both ZIKV strains before and 30 days after DENV infection. The ZIKVPF-10mo group showed significant higher NAb titers to the pre-infecting and heterologous ZIKV strains compared to the ZIKVPR-2mo group before DENV infection (p=0.0093, p=0.0141; Two-way Anova Tukey test) (Fig. 5c). Subsequently, DENV infection promote an equally 8-fold increase of

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NAb titers against both strains in the ZIKVPF-10mo group, significantly higher than the 4-fold increase in the ZIKVPR-2mo group (p=0.0025, p=0.0011; Two-way Anova Tukey test) (Fig. 5d). Altogether, these results demonstrate that DENV infection results in a significant increase in the magnitude and durability of the cross-neutralizing Ab response against ZIKV in animals with a mid-convalescent period from ZIKV infection. The elicited changes in neutralization capacity were likely driven more by the longevity of the immune memory maturation and the associated memory recall of the ZIKV immunity than by a strict dependency of the specific pre-exposed ZIKV strain.

Frequency, early activation and proliferation of immune cell subsets are modulated by the presence or absence of ZIKV immunity. We performed immunophenotyping by flow cytometry to assess the frequency, early activation and proliferation of multiple immune cell subsets and how these parameters are affected by the presence of pre-existing immunity to ZIKV on a subsequent DENV infection (Supplementary Fig. 6 for gating strategy; Supplementary Table 3 for Ab panel). As part of the innate immune response, the frequency of dendritic cells (DCs) and natural killer (NK) cells subpopulations were measured. Plasmacytoid DCs (pDCs: lin$^-$HLA-DR$^+$CD123$^+$) are known to respond to viral infection by production of IFN-α, while myeloid DCs (mDCs: lin$^-$HLA-DR$^+$CD11c$^+$) interacts with T cells. The frequency of pDCs was not significantly altered by DENV infection in any group compared to baseline levels (Supplementary Fig. 7a). At day 2 pi we detected a significant increase of mDCs in the ZIKVPF-10mo group (p=0.0082; Two-way Anova Dunnett test) (Supplementary Fig. 7b). Furthermore, we determined the frequency of NK subpopulations including: NK8, NK56, NKp30 and NKp46 (Supplementary Fig. 8). In general, no differences were detected between baseline and after DENV infection in all groups for all NK subpopulations and receptors with the exception of the ZIKVPR-2mo group that showed a significant increases in the following subpopulations: NKG2A$^+$NKp30 and NKp30$^+$NKp46$^+$ at 7 dpi (p=0.0495, p=0.0006; Two-way Anova Dunnett test) and NKp46$^+$NKp30$^+$ at 7 and 10 dpi (p=0.0005, p=0.0001; Two-way Anova Dunnett test) (Supplementary Fig. 8j, o, s).

We next investigated cell subsets that are part of the bi-phasic (humoral/cellular) adaptive immune response such as B (CD20$^+$CD3$^-$) and T (CD3$^+$CD20$^-$) cells, to determine if convalescent immunity to ZIKV alters responses to subsequent DENV infection (Supplementary Fig. 6 for gating strategy). No differences were detected in total B cells between groups following DENV infection compared to baseline levels (Supplementary Fig. 9a), but ZIKV-immune groups had elevated levels of activated B cells (CD20$^+$CD3$^-$CD69$^+$) since baseline and a trend to increase these levels more than the naïve group over time (Supplementary Fig. 9b). We detected a significant decrease of proliferating B cells (CD20$^+$CD3$^-$Ki67$^+$) in naïve animals at 7 and 10 dpi (p=0.0031, p=0.0345;

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Two-way Anova Dunnett test), while ZIKV-immune groups retained their proliferating levels (Supplementary Fig. 9c). Interestingly, the ZIKVPF-10mo group showed a significant increase of B cells that were proliferating and activated simultaneously ($CD20^+CD3^-CD69^+Ki67^+$) as early as in day 1 pi (p=0.0240; Two-way Anova Dunnett test) and maintained higher levels up to 10 dpi (Supplementary Fig. 9d) in comparison to other groups. Together, these phenotyping results of B cells are consistent with the early and boosted production of binding and neutralizing Abs in the ZIKV-immune groups compared to naïve animals. The frequency of total T cells ($CD3^+CD20^-$) and $CD4^+/CD8^+$ T cells subsets, was comparable at all time points before and after infection with DENV in all groups of animals (Supplementary Fig. 10a-c). Likewise, the frequency of total T cells ($CD3^+$) and $CD4^+/CD8^+$ T cells subsets, was similar at all time points before and after infection with DENV in all groups of animals (Supplementary Fig. 10a-c). Moreover, the T cell response was activated by DENV infection in all groups of animals (Supplementary Fig. 10d-f).

Previous studies have demonstrated that DENV and ZIKV specific $CD4^+$ and $CD8^+$ T cells are enriched in certain memory subsets[24,48]. Thus, we measured whether the early activation of T cell subpopulations, such as effector memory ($CD3^+CD4^+CD28^-CD95^+$) and central memory ($CD3^+CD4^+CD28^+CD95^+$) T cells (T-EM and T-CM), within each T cell compartment was modulated following DENV infection in presence or absence of convalescence to ZIKV (Fig. 6). The ZIKVPF-10mo group showed significant higher frequency of activated $CD4^+$ and $CD8^+$ T-EM ($CD3^+CD4^+CD28^-CD95^+CD69^+$ and $CD3^+CD8^+CD28^-CD95^+CD69^+$) following DENV infection compared to basal levels ($CD4^+$ T-EM at 7 and 10 dpi: p=0.0001, p=0.0072; $CD8^+$ T-EM at 2 and 7 dpi: p=0.0291, p=0.0001; Two-way Anova Dunnett test) (Fig. 6a, d). Interestingly, the ZIKVPR-2mo group showed a very limited frequency and activation of the $CD4^+$ and $CD8^+$ T-EM compared to the ZIKVPF-10mo and naïve groups. However, this group with an early convalescent period to ZIKV, contrary to the other two groups, showed a very limited but significant activation of $CD8^+$ T-CM ($CD3^+CD8^+CD28^+CD95^+CD69^+$) at day 7 and 10 pi ($p$=0.0007, p=0.0147; Two-way Anova Dunnett test). (Fig. 6e).In contrast, naïve animals did not show any significant activation of these cell subsets after DENV infection. Collectively, the B and T cell results suggest that following DENV infection: (i) animals with a mid-convalescence ZIKV immunity have a more dynamic B cell response and are able to rapidly produce more activated effector memory T cells from both T cell compartments; (ii) animals with an early-convalescence to ZIKV induced activation of central memory T cells in the $CD8^+$ compartment with a very limited T-EM frequency and activation profile compatible with a contraction phase of the T cells compartments; (iii) and animals without previous exposure to ZIKV exhibited a limited B cell response and minimal modulation of T cell subpopulations at early timepoints as the ZIKV-immune groups.

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T cell polyfunctional effector response against DENV and ZIKV is shaped by the longevity of ZIKV-immunity. To further characterize the cross-reactive T cell response we investigated if different convalescent periods of ZIKV immunity impacted the outcome of the effector role of $CD4^+$ and $CD8^+$ T cells following DENV infection. PBMCs were isolated and stimulated with peptide pools from DENV and ZIKV envelope (E) proteins and from ZIKV non-structural proteins (ZIKV-NS) (Supplementary Table 5 for peptide sequences). Then, intracellular cytokine staining using flow cytometry analysis (Supplementary Fig. 11 for gating strategy; Supplementary Table 4 for Ab panel) was performed to quantify the production of effector immune markers such as the cytotoxic marker CD107a, IFN-γ, and TNF-α by $CD4^+$ and $CD8^+$ T cells at baseline, 30, 60, and 90 days after DENV infection (Fig. 7).

To assess the ZIKV-primed specific- or cross-reactive effector T cell response we studied the response against ZIKV or DENV stimuli before DENV infection. In general, before DENV infection, we found that the ZIKV-primed effector T cell response was higher in $CD8^+$ (Fig. 7m, q, u) than in $CD4^+$ (Fig 7a, e, i) T cells. Of note, significant higher levels of CD107a, INF-γ and TNF-α producing $CD8^+$ T cells were found only in the ZIKVPF-10mo group before DENV infection (ZIKVPF-10mo vs ZIKVPR-2mo for CD107a: p=0.0002; ZIKVPF-10mo vs Naïve for CD107a: p=0.0401; ZIKVPF-10mo vs ZIKVPR-2mo for INF-γ: p=0.0020; ZIKVPF-10mo vs ZIKVPR-2mo for TNF-α: p=0.0033; ZIKVPF-10mo vs Naïve for TNF-α: p=0.0354; Two-way Anova Tukey test) (Fig. 7m, q, u). This basal effector response of $CD8^+$ T cells in the ZIKVPF-10mo group is predominated by cross-reactive $CD8^+$ T cells against DENV E protein. Very low effector T cell response against ZIKV NS proteins was detected for all groups (ZIKVPF-10mo>ZIKVPR-2mo>Naïve). In summary, results of T cell functional response before DENV infection suggest that a mid-convalescence to ZIKV provoke a higher $CD8^+$ T cell effector response capable to cross-react efficiently with DENV E protein.

After DENV infection, we were able to determine the modulation of the ZIKV-primed effector $CD4^+$ and $CD8^+$ T cell responses of ZIKV-immune groups and the *de novo* response of ZIKV-naïve animals. The ZIKVPF-10mo and naïve groups significantly boosted their CD107a expression in both T cell compartments stimulated mainly by DENV E protein at 30 and up to 90 days after DENV infection ($CD4^+$ T cells: ZIKVPF-10mo vs ZIKVPR-2mo: p<0.0001 at 30 dpi, p<0.0001 at 60 dpi; Naïve vs ZIKVPR-2mo: p<0.0001 at 30 dpi, p=0.0018 at 60 dpi; ZIKVPF-10mo vs Naïve: p=0.0204 at 30 dpi. $CD8^+$ T cells: ZIKVPF-10mo vs ZIKVPR-2mo: p<0.0001 at 30 dpi, p<0.0001 at 60 dpi, p=0.0008 at 90 dpi; Naïve vs ZIKVPR-2mo: p=0.0039 at 30 dpi, p<0.0001 at 60 dpi; p=0.0081 at 90 dpi; ZIKVPF-10mo vs Naïve: p=0.0194 at 30 dpi; Two-way

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Anova Tukey test) (Fig. 7b, c, n, o, p). Also, these groups boosted the CD107a cytotoxic signature reacting against ZIKV E and NS proteins by cross-reactive $CD4^+$ T cells 30 days after DENV infection (ZIKVPF-10mo vs ZIKVPR-2mo: p=0.0025 for ZIKV E, p<0.0001 for ZIKV NS; Naïve vs ZIKVPR-2mo: p=0.0025 for ZIKV E, p=0.0002 for ZIKV NS; Two-way Anova Tukey test) (Fig. 7b). The ZIKVPF-10mo group showed a remarkable significant increase of the IFN-γ producing $CD4^+$ T cells against DENV E protein since 60 dpi and is maintained up to 90 dpi compared to other groups (ZIKVPF-10mo vs ZIKVPR-2mo at 60 and 90 dpi: p<0.0001, p=0.0024; ZIKVPF-10mo vs Naïve at 60 and 90 dpi: p<0.0001, p=0.0037; Two-way Anova Tukey test) (Fig. 7g, h), and was the only group with significant increase in the IFN-γ producing $CD8^+$ T cell compartment at 60 dpi (ZIKVPF-10mo vs ZIKVPR-2mo: p=0.0253; Two-way Anova Tukey test) (Fig. 7s). On the other hand, the ZIKVPR-2mo group exhibited a significant increase of IFN-γ producing $CD4^+$ T cells earlier than other groups at 30 dpi (ZIKVPR-2mo vs ZIKVPF-10mo: p<0.0001; ZIKVPR-2mo vs Naïve: Two-way Anova Tukey test) (Fig. 7f). Interestingly, the naïve group showed an increase of cross-reactive TNF-α producing $CD4^+$ T cells against ZIKV NS proteins 30 days after DENV infection (Naïve vs ZIKVPR-2mo: p=0.0359; Two-way Anova Tukey test) (Fig. 7j). The ZIKVPF-10mo group developed a significant effector T cell response by TNF-α producing $CD4^+$ T cells against DENV and ZIKV E proteins at 60 days after DENV infection (ZIKVPF-10mo vs ZIKVPR-2mo against DENV/ZIKV E protein: p=0.0163, p=0.0172; Two-way Anova Tukey test) (Fig. 7k). Although all groups showed a boosted TNF-α effector response in the $CD8^+$ T cell compartment up to 90 days after DENV infection, no significant differences between groups were observed.

Collectively, these results after DENV infection suggest that a mid-convalescence to ZIKV translate in a more complete polyfunctional T cell response characterized by: (i) a cytotoxic $CD107a^+$ phenotype directed to DENV E protein for both T cell compartments comparable to the DENV-specific *de novo* response of the naïve group, (ii) developed CD107a, IFN-γ and TNF-α producing $CD8^+$ T cell effector response that cross-react efficiently with DENV E protein since baseline and is boosted after DENV infection, (iii) and promoted the higher T cell effector response against ZIKV NS proteins. An early-convalescence to ZIKV results in (iv) a very limited cytotoxic activity (limited expression of CD107a marker) which is in line with a very limited activation of the T-EM observed and with failed capability to react efficiently against E or NS proteins. The ZIKV-naïve group response was characterized by: (v) production of a DENV-specific *de novo* functional T cell response with similar magnitude between both T cell compartments, (vi) capable to cross-react against ZIKV E and NS proteins, (vii) and able to mount a DENV-specific cytotoxic $CD107a^+$ phenotype.

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Discussion

We found that previous infection to ZIKV improved the immune response against subsequent DENV infection without an enhancement of DENV viremia nor pro-inflammatory status, and that this improvement relies in the longevity of ZIKV convalescence—more efficient after longer ZIKV pre-exposure. This scenario seems to be independently of the ZIKV pre-exposure strain when those strains belong to the same phylogenetic group. The results presented herein provide insights on the anticipated immune modulation by previous ZIKV immunity in the expected re-emergence of DENV in the same geographical regions. The aftermath of the recent ZIKV epidemic has been related to a remarkable decrease in DENV cases in Brazil [28], and also in most of Latin American and Caribbean countries (http://www.paho.org/data/index.php/es/temas/_indicadores-dengue/dengue-nacional/9-dengue-pais-ano.html?start=2)[25]. Yet, little is known about the role of previous ZIKV immunity in the outcome of a subsequent DENV infection in human populations, and if ZIKV immunity is supporting this epidemiological phenomenon observed post-ZIKV epidemic[28]. To evaluate the hypothesis of a potential ZIKV-DENV cross-protection in humans characterizing the immunological history of prospective cohorts[46] will be necessary, but human samples for this purpose are scarce yet. Because of this, non-human primates are key to provide knowledge and anticipate different immunological scenarios when DENV epidemics re-emerge in human populations with previous immunity to ZIKV.

Animals with pre-existing ZIKV immunity do not show an enhancement of DENV-induced RNAemia, regardless of the period of convalescence from previous ZIKV infection (10 or 2 months) and the heterologous pre-infecting ZIKV strains. A convalescence period of 10 months is associated with a trend of less RNAemia days during subsequent DENV infection. Previous work reported that a period of early-convalescence (56 days) to ZIKV (PRVABC59 strain) in rhesus macaques was associated with a significant increase of DENV-2 RNAemia at day 5 after DENV infection and a pro-inflammatory cytokine profile. However, very similar to our results, it was noteworthy a delay at early timepoints and an early clearance in late timepoints of the DENV-2 RNAemia in ZIKV-immune macaques in comparison to the naïve ones[38]. The lack of significant DENV RNAemia enhancement found in the group with the early-convalescence period in our work, compared to previous results[38], may be attributable to the different DENV-2 strains used for the challenge [New Guinea/1944 strain vs Thailand/16681/1964 strain, from Asian II and Asian I Genotype, respectively]. This fact is of relevance because it suggests that the effect of previous ZIKV immunity on a subsequent DENV infection may differ between DENV serotypes or even

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within genotypes. Another possible explanation is the genetic heterogeneity of rhesus macaques used in these two studies as they are derived from different breeders. The importance of selecting genetic well-characterized macaques have been discussed previously[49]. Furthermore, we are showing that presence of low-to-intermediary levels of cross-NAbs against DENV induced by a previous ZIKV infection may play a role in controlling the early DENV RNAemia set point. This effect is associated with a middle- but not to an early-convalescent period after ZIKV infection. This finding contrasts with previous results from our group showing also low-to-intermediary levels of NAbs against ZIKV after DENV infection but with very limited or no impact in the early ZIKV RNAemia set point independently of the time elapsed between DENV and ZIKV infections (Serrano-Collazo et al, 2019; under review). Together, these results strongly suggest that the dynamic of the neutralization is qualitatively different in the two different sequences of DENV-ZIKV or ZIKV-DENV infections. Further studies are urgently needed to dissect these differences.

Due to limited availability of ZIKV-immune cohorts we used animals infected with two different ZIKV strains for our subsequent challenge with DENV. However, extensive revision of the literature up to date reveals a broad consensus that these two contemporary ZIKV strains behave very similar from an antigenic point of view[12,50-52]. Our results are confirmatory of those results showing that both ZIKV strains were neutralized with same efficacy by serum within each ZIKV-convalescent group, explained by the broadly neutralization activity against multiple ZIKV strains irrespective of the infecting strain[51]. However, the magnitude of the neutralization of both strains was statistically higher in animals exposed to DENV 10 months (mid-convalescence) after ZIKV infection compared to the animals with a shorter ZIKV convalescence (2 months). The mid-convalescent group showed a higher fold-increase in NAb titers against both ZIKV strains induced after DENV infection. These results suggest that the differences in the neutralization profile between the two ZIKV-immune groups are associated to the interval of time between ZIKV and DENV sequential infections. This may be more attributable to the maturation of the cross-reactive immune memory during memory recall elicited by the heterologous infection, and not restricted to elicited changes in the immune response by antigenic differences in the pre-infecting ZIKV strains. This statement is reinforced by the fact that the basal neutralization magnitude and titers fold-increase after DENV infection against both ZIKV strains were identical within each ZIKV-immune group. Altogether, these results suggest that the longevity of ZIKV convalescence has a significant impact in the magnitude of the neutralization against ZIKV in animals with a middle-convalescent period to ZIKV, displaying an early and potent deployment of NAbs compared to the early-convalescent group.

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The period of convalescence further had an impact in the maintenance of the neutralization magnitude against ZIKV and DENV overtime. The maturation of the memory B cells (MBCs) and the affinity of the produced Abs during memory recall in the ZIKV middle-convalescent group may be involved in such differences. Based upon studies of human monoclonal Abs, plasmablasts response during secondary DENV infection is mainly of MBC origin, resulting in a mature response characterized by cross-neutralizing Abs *in vitro*[53]. However, there are very limited studies on how the affinity maturation develops during the initial viral encounter and whether the affinity of MBCs is modified during a secondary heterologous infection or as in this work, during a secondary DENV infection following a primary ZIKV infection. From our results we can further suggest that the maturation of the immune response to ZIKV has significant consequences in the breadth of neutralization within the DENV serocomplex. While the neutralization against the infecting serotype 2 is very similar in magnitude in both ZIKV-immune groups, the mid-convalescent group retain significant higher magnitude compared to the early-convalescent group after one month post-infection until the end of the study. Most importantly, both ZIKV-immune groups developed a significant more rapid and robust neutralizing response against DENV compared to ZIKV-naïve, which exhibited a limited proliferation of B cells after DENV infection. This is in contrast with previous findings were ZIKV-convalescent macaques show a delayed induction of DENV NAbs compared to ZIKV-naïve overtime after DENV infection[39]. Of relevance, the magnitude and durability of the neutralization against heterologous DENV serotypes significantly increased in the presence of ZIKV convalescence compared to the *de novo* response of ZIKV-naïve animals. This improvement is more efficient in animals with mid-convalescence to ZIKV, the group with elevated proportion of early activated and proliferating B cells. These are seminal contributions to forecast and understand the cross-neutralization capacity of further heterologous DENV epidemics in the context of previous ZIKV-DENV immunity. Interestingly, ZIKV-convalescent animals showed some degree of cross-neutralization against DENV-2 and DENV-4 before DENV infection. This is consistent with our previous results showing that DENV-naïve ZIKV-infected animals also preferentially neutralized DENV-4 followed by DENV-2 after ZIKV infection[17]. Longitudinal data of cross-neutralization of DENV serotypes in DENV-naïve ZIKV-infected human subjects showed low cross-neutralization against all DENV serotypes, but DENV-4 followed by DENV-2 were neutralized more efficiently up to 6 months after ZIKV infection with comparable basal titers reported here[54]. After ZIKV infection in human DENV-naïve subjects, the ZIKV/DENV cross-reactive MBC response increased in magnitude (39% of total MBC proportion) after longer periods of ZIKV convalescence (~8 months post-ZIKV infection)[55], similar to the 10 months in the ZIKV mid-convalescent group that exhibited higher

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DENV cross-neutralization prior DENV infection. The highly conserved identity of E protein between ZIKV and DENV suggests the development of cross-binding/cross-neutralizing functional Abs against both viruses[56]. However, there is no data yet that delineates shared cross-neutralizing epitopes between ZIKV and DENV-2/-4, but it is known that DENV-4 genotypic diversity impact the capacity of its neutralization[57].

One factor that plays a critical role in the induction of enhancement and disease severity is the time elapsed between sequential heterologous DENV infections[10]. At this time, it is not known whether this factor play a role when DENV accounts for a secondary infection following ZIKV. Based on our previous works (*Serrano-Collazo et al.*, 2019; under review)[17], it is possible to argue that the sequence of DENV-ZIKV infections induce a different immunological response—in terms of the neutralization magnitude, cytokines profile and functionality of the cellular immune response—compared to the ZIKV-DENV scenario shown here. However, in both scenarios, the role of the time interval between infections seems to play a critical role in the quality and quantity of the immune response. Recently, *Fowler et al.* showed that maternally-acquired ZIKV Abs enhance DENV disease severity in mice lacking type I interferon receptors in myeloid cells[37]. However, they found similar increased DENV severity in pups from ZIKV early- and late-convalescent mothers. This is in contrast with our results and human findings in DENV sequential infections where the window of cross-protection and immune memory maturation was influenced by the longevity of convalescence[40,41,54,58]. Of relevance, in contrast to DENV sequential infections—suggesting cross-neutralizing protection by short time intervals—our results by ZIKV-DENV sequential infections reveal that longer convalescence to ZIKV induce higher boost of cross-neutralizing response with potentially less risk of DENV pathogenesis than short period of convalescence. However studies using NHP cohorts with longer period between ZIKV and DENV infections and prospective human cohorts, when available, are necessary to provide more conclusive answers.

Early studies of T cells associate their contribution towards immunopathogenesis in DENV secondary infections explained by the original antigenic sin[59], but increasing evidence suggest their protective role during primary and secondary DENV infections[60]. Recently, with the introduction of ZIKV into The Americas, T cells from DENV immunity are being implicated in mediating cross-protection against ZIKV[22-24]. However, the role and kinetics of T cells from ZIKV immunity in a subsequent DENV infection remains unknown. We found that animals with a mid-convalescence to ZIKV developed an early activation of $CD4^+$ and $CD8^+$ effector memory T cells after DENV infection. This early activation has been observed for the opposite scenario in DENV-immune ZIKV-infected patients[24]. Interestingly, the ZIKV early-convalescent group display a

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modest activation (T-CM>T-EM) early after DENV infection. Since this group was infected with ZIKV only two months before DENV it is possible that after viral clearance and development of ZIKV-specific T cell response, the T cell compartments were still under the contraction phase at the time of the DENV challenge. Yellow fever virus (YFV) and vaccinia virus vaccinations in humans demonstrate that T cell contraction start as early as approximately one-month post-vaccination and at least for almost three months is still ongoing[61]. Also, a study shows that re-stimulation using alphavirus replicons during T cell response contraction does not have significant impact modulating the pre-existing T cell response[62].

The profile of ZIKV-specific $CD8^+$ T cells in humans with convalescence to ZIKV is characterized by the production of IFN-γ, and expression of activation and cytotoxic markers[63]. Presence of sustained levels of IFN-γ prior and early after DENV challenge in vaccinees has been associated with protection against viremia and/or severe disease[64,65]. We observed a similar phenotype of the polyfunctional response of $CD8^+$ T cells prior DENV infection in animals with longer convalescence to ZIKV. Strikingly, this response recognize more efficiently peptides from DENV E protein than from ZIKV E protein. However, ZIKV-specific $CD8^+$ T cells direct 57% of their response against structural proteins, which may suggest these cells can recognize conserved epitopes between ZIKV and DENV structural proteins. Cross-reactivity of T cells between heterologous flavivirus infections is explained by selective immune recall of memory T cells that recognize conserved epitopes between DENV and ZIKV[24], which also has previously been demonstrated during secondary heterotypic DENV infections[66,67]. In addition an increased cytotoxic profile as demonstrated by the higher frequency of CD107a-expressing $CD4^+$ and $CD8^+$ T cells in the ZIKV mid-convalescent group correlates with the synchronously early activation of $CD4^+$ and $CD8^+$ effector memory T cells and elevated levels of perforin release. Since $CD8^+$ T cells are known for their role in early clearance of DENV in heterologous secondary infections[68], it is possible that the T cell cross-reactivity with DENV E detected in the ZIKV mid-convalescent group at baseline together with the basal levels of cross-neutralizing Abs against DENV-2 before DENV infection, provide a synergistic and partial cross-protective effect.

Higher proportion of IFN-γ and TNF-α producing T cells before a secondary heterologous DENV infection has been associated to a subsequent subclinical outcome[69]. Herein, we observed that the ZIKV mid-convalescent group had elevated levels of IFN-γ and TNF-α producing T cells since baseline. In this group, DENV infection stimulated a higher frequency of these cells, but remarkably, also increased highly cross-reactive IFN-γ-producing $CD4^+$ T cells directed to DENV E, and ZIKV E/NS proteins. A study showed that cross-reactive ZIKV-primed $CD4^+$ T cells recognized conserved homologous sequences of other related flaviviruses such as West Nile

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virus (WNV), YFV, and of relevance for our study, cross-react with E protein epitopes of all DENV serotypes[70]. Moreover, IFN-γ-producing CD4$^+$ T cells have a role in providing help to B cells while B cells present DENV antigens to CD4$^+$ T cells to produce IFN-γ and other immune mediators that induced B cell activation and subsequent efficient Ab production[71]. Memory CD4$^+$ T cells are also required to generate an effective humoral response against ZIKV[72]. Based on this, the higher proportion of DENV-E-reactive IFN-γ-producing CD4$^+$ T cells may play a role in the induction of the robust Ab response in the ZIKV mid-convalescent group against ZIKV and all DENV serotypes. The polyfunctional response of T cells was detected up to 90 days after DENV infection with higher frequency in ZIKV-immune animals, suggesting a more long-lasting T cell polyfunctional response that may play an effector role in a subsequent heterologous DENV infection. However, this hypothesis needs to be confirmed by longitudinal studies with subsequent DENV heterotypic challenges. Despite the lack of evidence on T cell response in this scenario (ZIKV-DENV), the majority of the modulation exhibited after DENV infection can be explained by previous findings of sequential DENV heterologous infections. This reinforce the similarities of these two flaviviruses and their effect in shaping memory recall and T cells cross-reactivity, as reported for sequential infections between other related flaviviruses such as Japanese Encephalitis virus (JEV), YFV and DENV[73]. On the other hand, we showed that naïve animals with DENV *de novo* response did not cross-neutralized ZIKV at all, which state that although similar, antigenic differences are sufficient to mount predominantly type-specific rather than cross-reactive responses during a primary infection[50,55].

A lack of ZIKV immunity promoted a more pro-inflammatory profile characterized by significant elevated levels of IFN-α, IL-6 and MIG/CXCL9. These are part of the cytokine storm produced by DENV infection and correlate with DENV severe disease. IFN-α is known to be actively produced by human pDCs—increasing frequency of pDCs in naïve animals—during acute DENV infection *in vitro* and *in vivo*[74]. Elevated levels have been correlated with severity in DHF patients, and to act as a marker for elevated DENV replication[75,76]. IL-6 has been detected in high levels during secondary DENV infections in children[77], and the day patients suffer from shock (DSS)[78] or died from DHF[79]. MIG/CXCL9 is known to be a risk factor for DENV severity involved in vascular permeability[80]. Its detection vary between primary and secondary (higher levels) DENV infections[81], which may explain non-significant peaks within ZIKV-immune groups during a secondary DENV infection. However, it remains unclear if this can be extrapolated to the scenario of ZIKV-DENV sequential infections. On the other hand, the presence of a longer ZIKV convalescence induced increased levels of CXCL10/IP-10 and perforin. CXCL10 is an immune mediator for T cells proliferation, recruitment of CD4$^+$ and CD8$^+$ activated T cells and IFN-γ-

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producing CD8$^+$ T cells, required to control DENV infection *in vivo*[82,83]. Levels of CXCL10 are amplified in presence of DENV-specific T cells[84]. This correlated with higher proportion and activation of both T cell compartments and subsequent polyfunctional T cell response against DENV-E-specific peptides in the group with longer convalescence to ZIKV. Perforin is involved in the cytotoxic degranulation process against virus-infected cells. In DENV infection, perforin is part of the anti-DENV cytotoxic phenotype of CD8$^+$ and CD4$^+$ T cells[48,85]. Perforin levels were significantly elevated only in the ZIKV mid-convalescent group after DENV infection. Accordingly, this coincide with a significant activation of CD8$^+$ and CD4$^+$ effector memory T cells, and degranulation functional response of both T cell compartments, suggesting an enhanced perforin-producing cytotoxic role of T cells in presence of longer convalescence to ZIKV.

In summary, dissecting our main findings per previous ZIKV-immune status we found that a ZIKV middle-convalescence: (i) results in shorter DENV viremic period, (ii) lowest pro-inflammatory status with upregulation of cellular immune response mediators, (iii) robust neutralizing antibody response higher in magnitude and durability against ZIKV strains and DENV serotypes, (iv) elevated activated and proliferating B cells, (v) early activation of cross-reactive CD4$^+$ and CD8$^+$ effector memory T cells, (v) and a major breadth of polyfunctional T cell response. For ZIKV early-convalescence we demonstrated: (i) average DENV viremic period and no exacerbation of pro-inflammatory status, (ii) neutralizing antibody response with high magnitude but less durability against ZIKV strains and DENV serotypes compared to the ZIKV middle-convalescent group, (iii) early activation of central memory CD8$^+$ T cells, (iv) and very limited activation of effector memory T cells. For the ZIKV-naïve group we demonstrated: (i) longer DENV viremic period and pro-inflammatory status, (ii) a more delayed *de novo* neutralizing antibody response against DENV serotypes and inability to neutralize ZIKV strains, (iii) a limited B cell response, (iv) and an overall *de novo* T cell response lower in magnitude and cross-reactivity compared to ZIKV-immune groups.

This work reinforce the usefulness of NHPs as a suitable model to characterize the immune response elicited by heterologous and consecutive flavivirus infections and to identify differential modulation of the immune response influenced by the time interval between infections. This proof-of-concept and other prospective studies of ZIKV/DENV pathogenesis and cross-immune relationships are urgently needed even as the peak of the ZIKV epidemic has passed as there is a high probability for ZIKV to establish a sylvatic transmission cycle using neotropical primates and mosquitoes in the Americas that will sustain ZIKV circulation and potential re-emergence[86,87]. Our data show a positive scenario that supports the implementation of ZIKV vaccine programs, since it suggests that a vaccine-acquired ZIKV-immunity will not worsen DENV

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pathogenesis and may ameliorate immune response against a subsequent infection with DENV. Similarly, the implementation of DENV vaccines is also supported in the context of previous ZIKV immunity, since ZIKV convalescence may boost the vaccine-acquired anamnestic immune response to DENV without predisposing to an enhanced pathogenesis. However, the selection of the vaccine schedule may be critical to induce the optimal immune response when more than one dosis is planned.

Figures

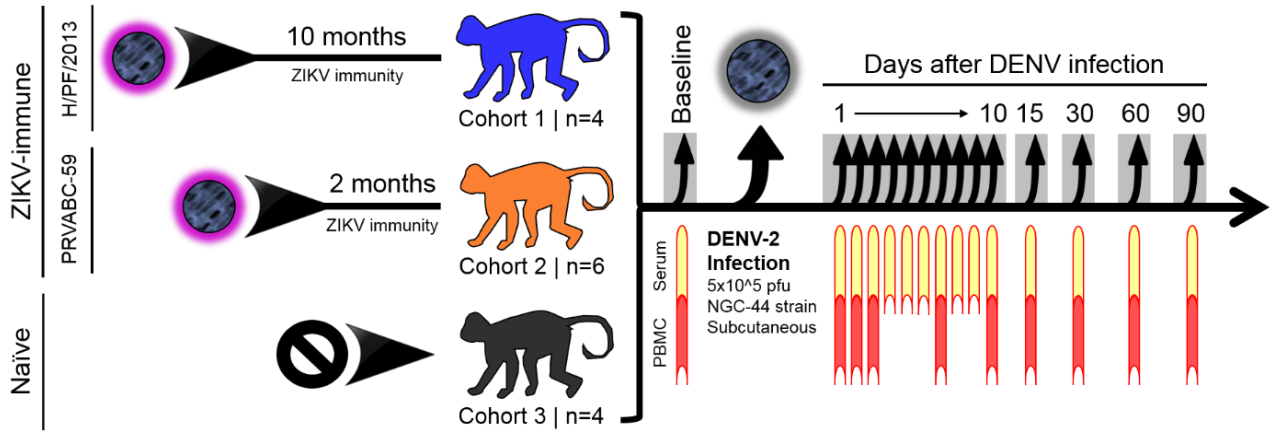

Figure 1 | Experimental design for DENV-2 challenge of ZIKV-immune and naïve rhesus macaques. 14 young adult male rhesus macaques (*Macaca mulatta*), matched in age and weight, were divided in three cohorts. Cohort 1 (ZIKVPF-10mo, n=4, blue): composed of four animals (5K6, CB52, 2K2, and 6N1) that were inoculated with $1x10^6$ pfu/500 ul of the ZIKV H/PF/2013 strain subcutaneously 10 months before (mid-convalescence) DENV-2 challenge. Cohort 2 (ZIKVPR-2mo, n=6, orange): composed of six animals (MA067, MA068, BZ34, MA141, MA143, and MA085) that were inoculated with $1x10^6$ pfu/500 ul of the contemporary ZIKV PRVABC59 strain two months before (early-convalescence) DENV-2 challenge. Both ZIKV strains used for previous exposure of these groups are >99.99% comparable in amino acid identity (Supplementary Table 1). Cohort 3 (Naïve, n=4, black): composed of four ZIKV/DENV naïve animals (MA123, MA023, MA029, and MA062) as a control group. Prior to DENV-2 challenge all animals were subjected to quarantine period. All cohorts challenged subcutaneously (deltoid area) with $5x10^5$ pfu/500 ul of DENV-2 New Guinea 44 strain (NGC44). After DENV-2 challenge all animals were extensively monitored for evidence of disease and clinical status by vital signs such as external temperature (°C), weight (Kg), CBC and CMP panels at the Caribbean Primate Research Center (CPRC). Blood samples were collected longitudinally at baseline, 1 to 10, 15, 30, 60 and 90 days after DENV infection (gray arrows). In all timepoints the blood samples were used for serum separation (yellow), and for PBMCs isolation (red) at baseline, 1, 2, 3, 7, 10, 15, 30, 60, and 90 days after DENV infection.

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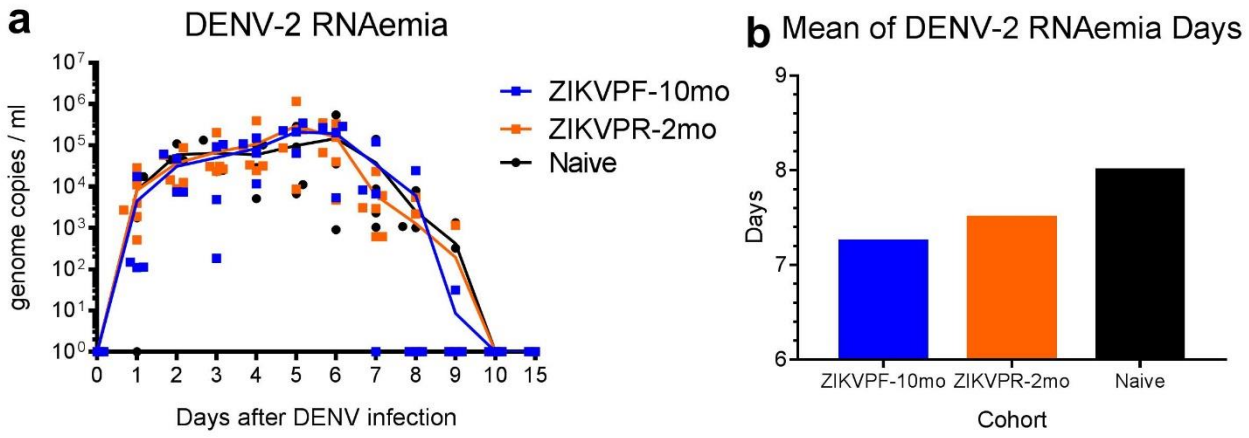

Figure 2 | Previous ZIKV immunity does not contribute to an increase of DENV RNaemia. (**a**) DENV-2 RNA kinetics in ZIKV-immune and naïve animals at baseline, sequentially from day 1 to day 10, and day 15 after DENV infection. Genome copies (Log10) per ml of serum were measured by qRT-PCR. Symbols represent individual animals per cohort: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Lines indicate the mean number of genome copies detected for each cohort over time. Statistically significant differences between groups were determined using Two-Way Anova (Tukey's multiple comparisons test). (**b**) Mean of days that DENV-2 RNAemia was detected per cohort. Bars represent mean values for ZIKVPF-10mo (blue), ZIKVPR-2mo (orange) and Naïve (black).

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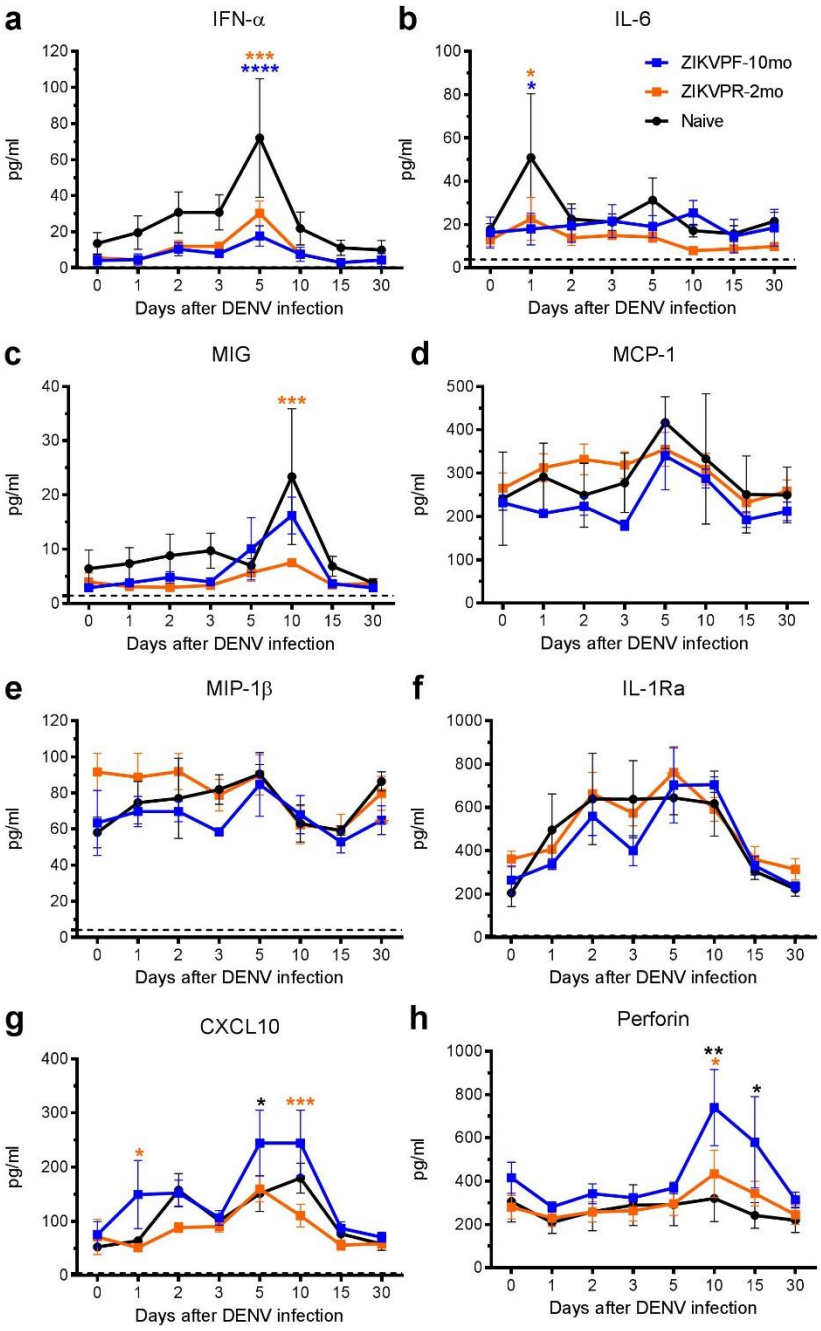

Figure 3 | ZIKV immunity does not exacerbate levels of pro-inflammatory cytokines. Cytokines and chemokines expression levels were determined in serum (pg/ml) by multiplex bead assay (Luminex) at baseline, 1, 2, 3, 5, 10, 15 and 30 days after DENV infection. The panel includes: (**a**) interferon alpha (IFN-α), (**b**) interleukin-6 (IL-6), (**c**) monokine induced by IFN-gamma (MIG/CXCL9), (**d**) monocyte chemoattractant protein 1 (MCP-1/CCL2), (**e**) macrophage inflammatory protein 1-beta (MIP-1β/CCL4), (**f**) IL-1 receptor antagonist (IL-1RA), (**g**) C-X-C motif chemokine 10 (CXCL10/IP-10) and (**h**) perforin. Symbols connected with lines represent mean expression levels detected of each cytokine/chemokine per cohort over time: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Error bars indicate the standard error of the mean (SEM) for each cohort per timepoint. Statistically significant differences

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between groups were calculated using Two-Way Anova Tukey's multiple comparisons test. Significant multiplicity adjusted *p* values (* <0.05, ** <0.01, *** <0.001, **** <0.0001) are shown colored representing the cohort against that particular point where is a statistically significant difference between groups.

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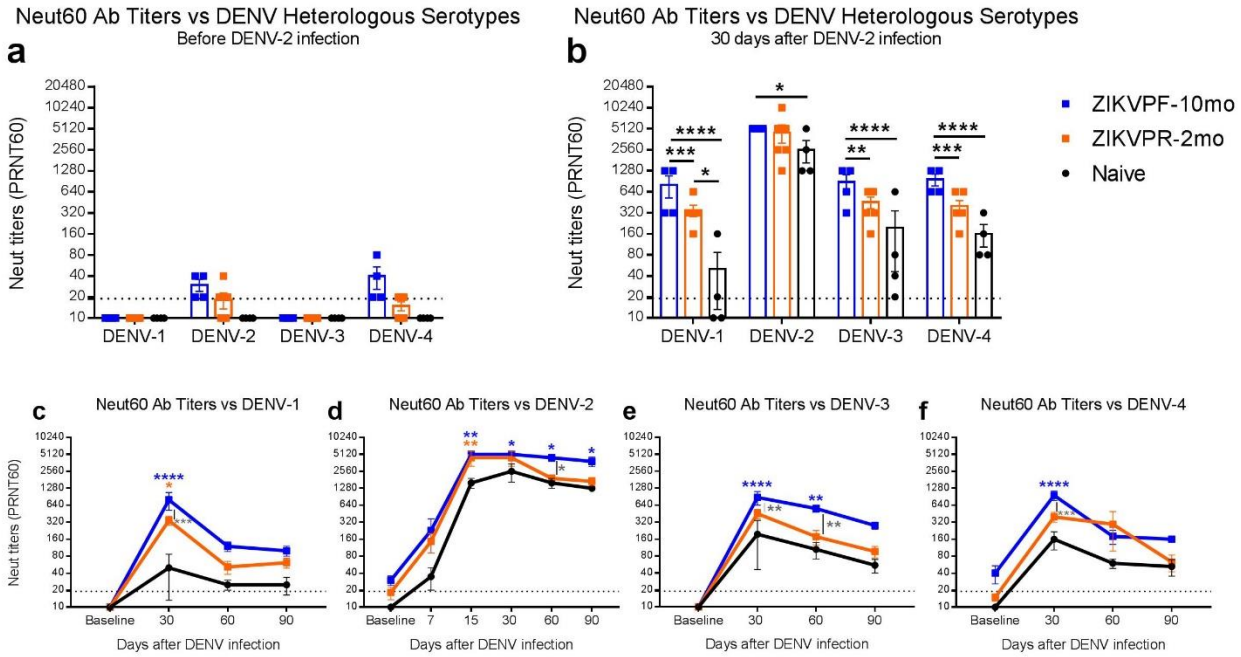

Figure 4 | Neutralization of DENV-2 and heterologous serotypes is higher in magnitude and durability by animals with mid-convalescence to ZIKV. The magnitude of the neutralizing antibody (NAb) response was determined (**a**) before and (**b**) 30 days after DENV infection by Plaque Reduction Neutralization Test (PRNT) against all DENV serotypes. (**c-f**) The durability of the neutralizing response was assessed measuring NAb titers up to 90 dpi against all DENV serotypes. Symbols connected with full lines indicate mean levels of NAb titers detected per cohort over time: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Error bars represent the standard error of the mean (SEM). PRNT60: NAb titer capable of reduce 60% or more of DENV serotypes plaque-forming units (pfu) compared with the mock (control of virus without serum). A PRNT60 1:20 titer was considered positive, and <1:20 as a negative Neut titer. Dotted line mark <1:20 for negative results. Non-neutralizing titers (<1:20) were assigned with one-half of the limit of detection for graphs visualization (1:10). Statistically significant differences between groups were calculated using Two-Way Anova Tukey's multiple comparisons test with 95% CI. Significant multiplicity adjusted *p* values (* <0.05, ** <0.01, *** <0.001, **** <0.0001) are shown. Blue and orange asterisks represent significant difference between the corresponded ZIKV immune groups and naive group, and gray asterisks indicate a significant difference between ZIKV immune groups.

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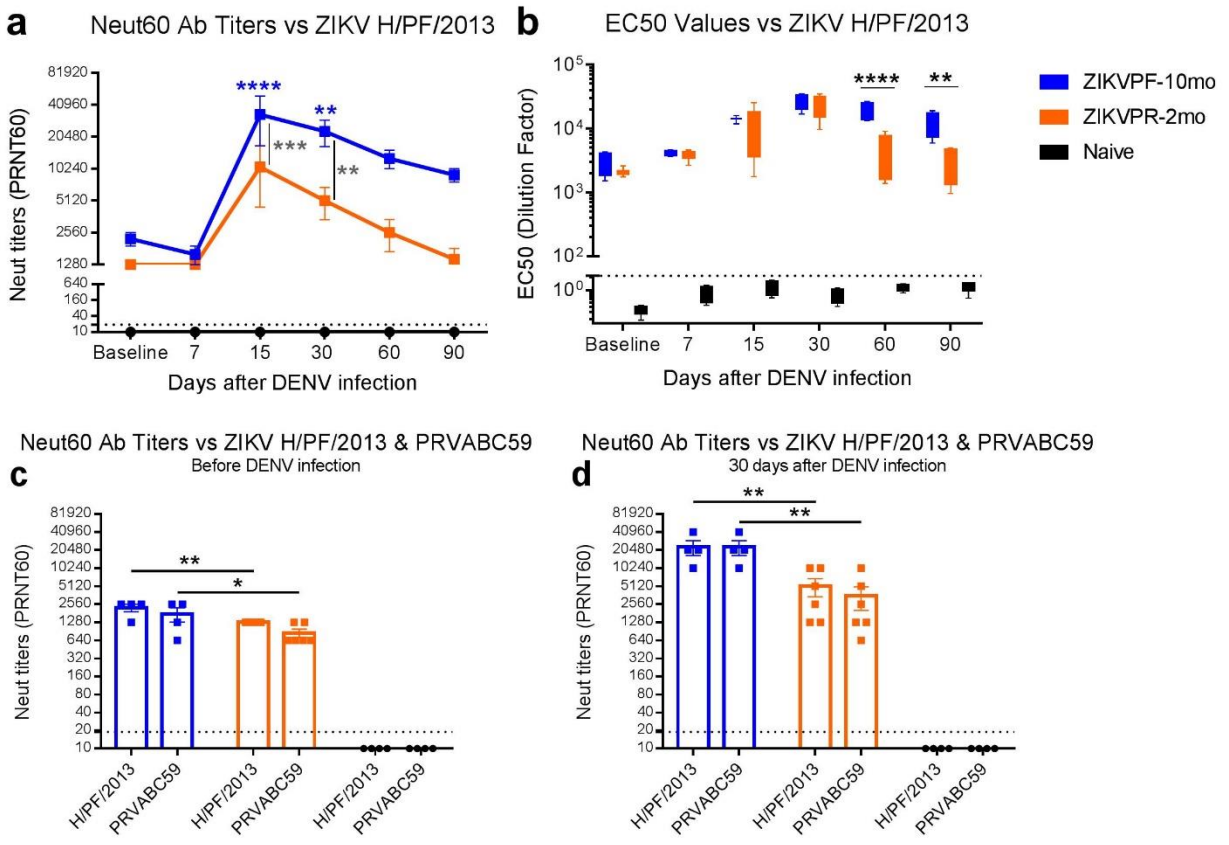

Figure 5 | ZIKV neutralization is more potent and durable in animals with mid-convalescence to ZIKV and is independent of the pre-infecting ZIKV strain. (**a**) NAb titers and (**b**) EC_{50} against ZIKV H/PF/2013 were determined by PRNT60 at baseline, 7, 15, 30, 60 and 90 days after DENV infection. Comparison of NAb titers between pre-infecting and heterologous ZIKV strains was performed (**c**) before and (**d**) after DENV infection. Symbols connected with full lines indicate mean levels of NAb titers detected per cohort over time: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Error bars represent the standard error of the mean (SEM). PRNT60: NAb titer capable of reduce 60% or more of ZIKV strains plaque-forming units (pfu) compared with the mock (control of virus without serum). A PRNT60 1:20 titer was considered positive, and <1:20 as a negative Neut titer. Dotted line mark <1:20 for negative results. Non-neutralizing titers (<1:20) were assigned with one-half of the limit of detection for graphs visualization (1:10). Statistically significant differences between groups were calculated using Two-Way Anova Tukey's multiple comparisons test with 95% CI. Significant multiplicity adjusted *p* values (* <0.05, ** <0.01, *** <0.001, **** <0.0001) are shown. Blue and orange asterisks represent significant difference between the corresponded ZIKV-immune groups and naive group, and gray asterisks indicate a significant difference between ZIKV-immune groups. Half maximal (50%) effective concentrations (EC_{50}) were calculated based on the % of neutralization of ZIKV for all timepoints. The EC_{50} was defined as the Ab dilution factor that is successfully capable of block 50% of the viral infection in the PRNT assay.

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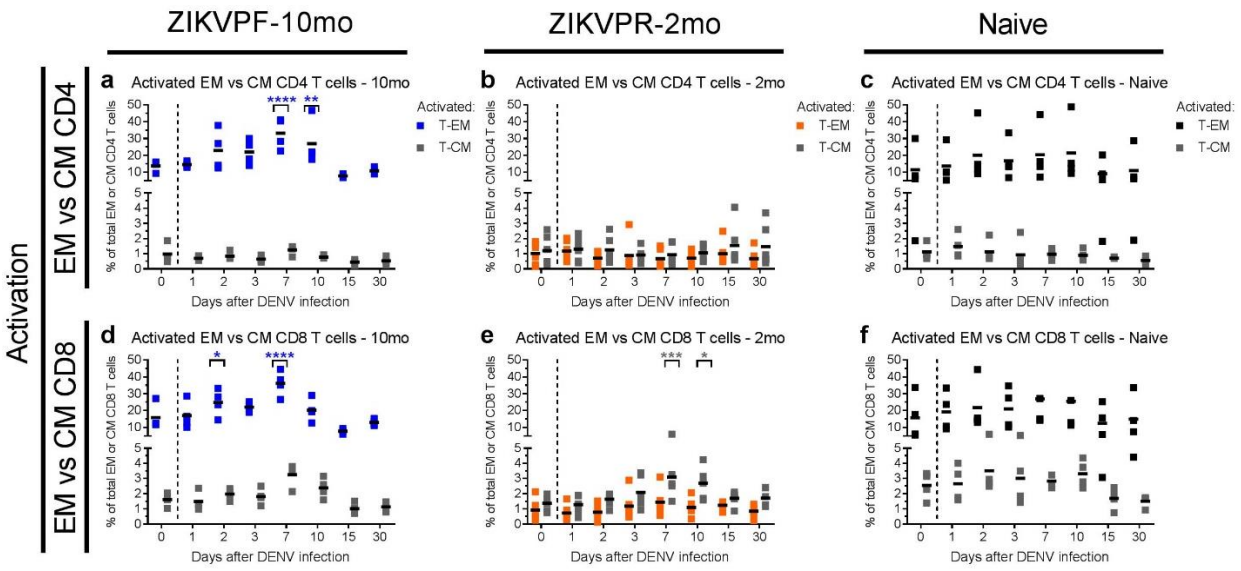

Figure 6 | Effector and central memory T cells within CD4$^+$ and CD8$^+$ T cell compartments are activated after DENV infection. Activation (CD69$^+$) of effector memory (T-EM: CD3$^+$CD4$^+$CD28$^-$CD95$^+$) and central memory (T-CM: CD3$^+$CD4$^+$CD28$^+$CD95$^+$) T cells within (**a-c**) CD4$^+$ and (**d-f**) CD8$^+$ T cell compartments before and after DENV infection. Percent of cells were determined by immunophenotyping using flow cytometry (Supplementary Fig. 6 for gating strategy). Blue, orange and black squares represent T-EM for ZIKVPF-10mo, ZIKVPR-2mo and Naïve, respectively. Gray squares represent T-CM for each group. Short black lines mark mean value for each group per timepoint. Cutted line divide % of T-EM and T-CM cells quantified before and after DENV infection. Statistically significant differences between groups were determined using Two-Way Anova Dunnett's multiple comparisons test (comparison of each cohort response at each timepoint versus baseline) and reported as multiplicity adjusted *p* values (* <0.05, ** <0.01, *** <0.001, **** <0.0001) with 95% CI. Asterisks represent significant difference between the corresponded timepoint and baseline within the same group.

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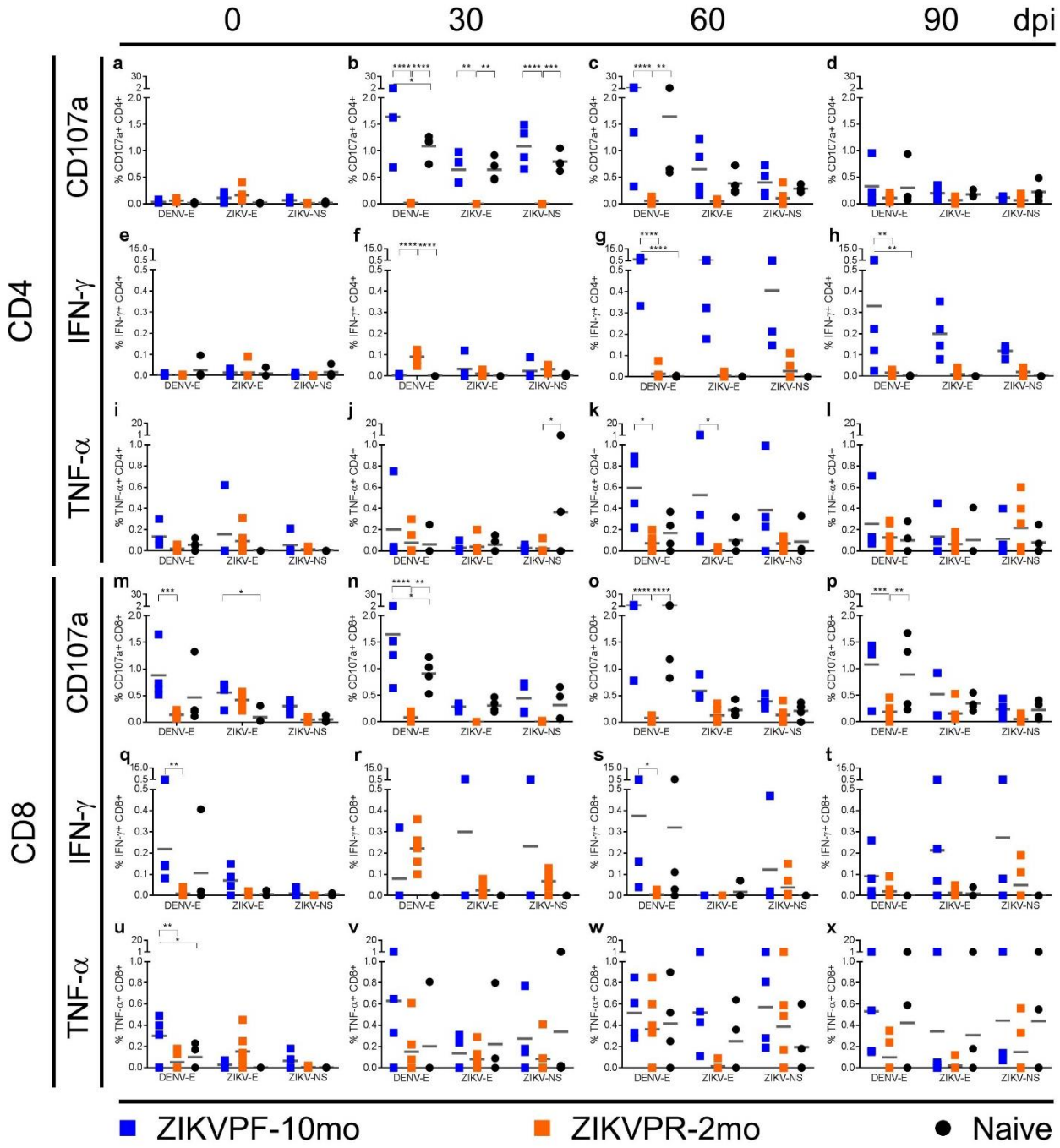

Figure 7 | Longevity of ZIKV immunity shapes the polyfunctional response of CD4⁺ and CD8⁺ T cells. T cell polyfunctional effector response was determined by the quantification (%) of (**a-d; m-p**) CD107a-expressing and (**e-h; q-t**) IFN-γ or (**i-l; u-x**) TNF-α producing CD4⁺ and CD8⁺ T cells before (0) and 30, 60 and 90 days after DENV infection. Responses to several peptide pools that encode for DENV and ZIKV envelope (E) proteins or ZIKV non-structural (NS) proteins were quantified. After antigenic stimulation intracellular cytokine staining was performed using flow cytometry analysis (Supplementary Fig. 11 for gating strategy). Individual symbols represent each animal per antigenic stimulation over time: blue squares (ZIKVPF-10mo), orange squares

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(ZIKVPR-2mo) and black circles (Naïve). Short gray lines mark mean value for each group. Statistically significant differences between groups were calculated using Two-Way Anova Tukey's multiple comparisons test with 95% CI. Significant multiplicity adjusted *p* values (* <0.05, ** <0.01, *** <0.001, **** <0.0001) are shown. Asterisks represent significant difference between indicated groups.

Methods

Cell lines. Aedes *albopictus* cells, clone C6/36 (ATCC CRL-1660), whole mosquito larva cells, were maintained in Dulbecco Minimum Essential Medium (DMEM) (GIBCO, Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (P/S) (Gibco). C6/36 were used to produce previous ZIKV and DENV viral stocks with high titers in 150-175 cm^2 cell culture flasks (Eppendorf), and incubated at 33°C and 5% CO_2. Vero cells, clone 81 (ATCC CCL-81), African green monkey kidney epithelial cells, were maintained with DMEM supplemented with 10% FBS and 1% of P/S, HEPES, L-glutamine and non-essential amino acids (NEAA) in 75 cm^2 cell culture flasks, and incubated at 37°C and 5% CO_2. Vero-81 cells were used for the cells monolayer in viral titrations by plaque assays and plaque reduction neutralization test (PRNT) in flat-bottom 24-well plates (Eppendorf).

Viral stocks. The DENV-2 New Guinea 44 (NGC) strain (kindly provided by Steve Whitehead, NIH/NIAID, Bethesda, Maryland, USA), known to replicate well in rhesus macaques, was used for the challenge in order to obtain comparative results with previous published studies from our group on DENV and ZIKV challenge studies[17,34]. We have standardized the assays to quantify this virus by Plaque assay, as described in our previous work[17]. The titer of DENV-2 for the challenge was $5x10^7$ pfu/ml. In addition, ZIKV H/PF/2013 strain (kindly provided by CDC-Dengue Branch, San Juan, Puerto Rico), ZIKV PRVABC59 (ATCC VR-1843), DENV-1 Western Pacific 74, DENV-3 Sleman 73, and DENV-4 Dominique strains (kindly provided by Steve Whitehead from NIH/NIAID, Bethesda, Maryland, USA) were propagated in C6/36 cells, titrated and used for Plaque Reduction Neutralization Test (PRNT) assays.

Viral titration plaque assay. DENV titrations by plaque assay were performed seeding Vero-81 (~$8.5x10^4$ cells /well) in flat bottom 24-well cell culture well plates (Eppendorf) in supplemented DMEM the day before. Viral dilutions (10-fold) were made in diluent media [Opti-MEM (Invitrogen) with 2% FBS (Gibco) and 1% P/S (Gibco)]. Prior to inoculation, growth medium was removed and cells were inoculated with 100 ul/well of each dilution in triplicates. Plates were incubated for 1 hr, 37°C, 5% CO_2 and rocking. After incubation, virus dilutions were overlaid with 1 ml of Opti-

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MEM [1% Carboxymethylcellulose (Sigma), 2% FBS, 1% of NEAA (Gibco) and P/S (Gibco)]. After 3 to 5 days of incubation (days vary between DENV serotypes), overlay was removed and cells were washed twice with phosphate buffered saline (PBS), fixed in 80% methanol (Sigma) in PBS, and incubated at room temperature (RT) for 15 minutes. Plates were blocked with 5% Non-fat dry milk (Denia) in PBS for 10 minutes. Blocking buffer was discarded and 200 ul/well of primary antibodies mix [anti-E protein monoclonal antibody (mAb) 4G2 and anti-prM protein mAb 2H2 (kindly provided by Aravinda de Silva and Ralph Baric, University of North Carolina Chapel Hill, North Carolina, USA), both diluted 1:250 in blocking buffer] were added and incubated for 1 hr, 37°C, 5% CO_2 and rocking. Plates were washed twice with PBS and incubated in same conditions with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Sigma), diluted 1:1000 in blocking buffer. Plates were washed twice with PBS and 150 ul/well of TrueBlue HRP substrate (KPL) were added and plates were incubated from 1-10 minutes at RT until plaque-forming units (pfu) were produced and visible. Then 200 ul/well of distilled water were added to stop the substrate reaction, plates get dry and pfu were counted to calculate viral titers.

Macaques and viral challenge. From 2008 to 2015, the Caribbean Primate Research Center (CPRC) funded a large DENV research program. Multiple studies made available several cohorts of rhesus macaques (*Macaca mulatta*) infected with different DENV serotypes in distinct timelines and also naïve cohorts were available as well. After our laboratories prioritized ZIKV research since 2016, DENV pre-exposed and naïve cohorts were infected with ZIKV and pre-exposed animals became available for this study. All animals were housed within the Animal Resources Center facilities at the University of Puerto Rico-Medical Sciences Campus (UPR-MSC), San Juan, Puerto Rico. All the procedures were performed under the approval of the Institutional Animal Care and Use Committee (IACUC) of UPR-MSC and in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC file # 000593; Animal Welfare Assurance number A3421; protocol number, 7890116). Procedures involving animals were conducted in accordance with USDA Animal Welfare Regulations, *the Guide for the Care and use of Laboratory Animals* and institutional policies to ensure minimal suffering of animals during procedures. All invasive procedures were conducted using anesthesia by intramuscular injection of ketamine at 10-20 mg kg^{-1} of body weight. Rhesus macaques from the CPRC are very well genetically characterized from a common stock introduced in 1938 at Cayo Santiago, an islet located in the southeast of Puerto Rico. These macaques with Indian genetic background are part of the purest colony used in the United States for comparative medicine and biomedical research[49]

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The experimental design was based on 14 young adult male rhesus macaques, matched in age and weight, in three different cohorts. Cohort 1 (ZIKVPF-10mo): composed of four animals (5K6, CB52, 2K2, and 6N1) that were inoculated with $1x10^6$ pfu/500 ul of the ZIKV H/PF/2013 strain subcutaneously[17] 10 months before DENV-2 challenge. Cohort 2 (ZIKVPR-2mo): composed of 6 animals (MA067, MA068, BZ34, MA141, MA143, and MA085) that were inoculated with $1x10^6$ pfu/500 ul of the ZIKV PRVABC59 strain two months before DENV-2 challenge. Both ZIKV strains used for previous exposure of these groups are >99.99% comparable in amino acid identity (Supplementary Table 1). Cohort 3 (Naïve): composed of four ZIKV/DENV naïve animals (MA123, MA023, MA029, and MA062) as a control group. Prior to DENV-2 challenge all animals were subjected to quarantine period. All cohorts were bled for baseline and challenged subcutaneously (deltoid area) with $5x10^5$ pfu/500 ul of DENV-2 New Guinea 44 strain. After DENV-2 challenge all animals were extensively monitored by trained and certified veterinary staff for evidence of disease and clinical status: external temperature (°C) with an infrared device (EXTECH Instruments, Waltham, MA), weight (Kg), CBC and CMP. All animals were bled continuously from day 1 to day 10, 15, 30, 60 and 90 days post-infection (dpi). In all timepoints the blood samples were used for serum separation, and also for PBMCs isolation (Baseline, 7, 30, 60, 90 dpi only). Additional heparin samples were obtained for immunophenotyping by flow cytometry using fresh whole blood. Fig. 1 shows the experimental design and samples collection timeline.

DENV RNAemia. DENV viral RNA extraction was performed from acute serum samples (Baseline, 1-10, and 15 dpi) using QIAamp Viral RNA mini kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's instructions. RNAemia levels were measured by a One-Step qRT-PCR detection kit (Oasig, Primerdesign Ltd., UK) and using DENV RT primer/probe Mix kit (Genesig, Primerdesign Ltd., UK) according to the manufacturer's protocol. Assays were performed in an iCycler IQ5 Real-Time Detection System with Optical System Software version 2.1 (Bio-Rad, Hercules, CA, USA). Furthermore, in order to correlate RNAemia levels with DENV pathogenesis we will monitor the clinical status for injury and/or clinical manifestations. Complete Blood Counts (CBC) were performed for all animals in several timepoints (Baseline, 7, and 15 dpi) to determine the absolute number (10^6 cells/ml) and percent (%) of lymphocytes (LYM), monocytes (MON), white blood cells (WBC), neutrophils (NEU) and platelets (PLT). Also, Comprehensive Metabolic Panel (CMP) were evaluated in several timepoints (Baseline, 7, 15 and 30 dpi) to measure concentration (U/L) of alkaline phosphatase and liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

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ELISA. Seroreactivity to DENV and cross-reactivity to ZIKV was measured at different timepoints before and after DENV-2 challenge. DENV-IgM (Focus Diagnostics, Cypress, CA, USA) was quantified at baseline, 5, 10, 15 and 30 dpi. DENV-IgG was quantified at baseline, 7, 15, 30, 60 and 90 dpi (Focus Diagnostics, Cypress, CA, USA). To determine the modulation of serological profile against ZIKV we assessed: levels of anti-ZIKV IgM (InBios, Seattle, WA, USA) at baseline, 5, 10, 15 and 30 dpi; anti-ZIKV IgG (XPressBio, Frederick, MD, USA) at baseline, 7, 15, 30, 60 and 90 dpi; anti-ZIKV NS1-IgG (Alpha Diagnostics, San Antonio, TX, USA) at baseline, 30, 60 and 90 dpi (including additional timepoints prior baseline for both ZIKV-immune groups); and anti-ZIKV EDIII-IgG (Alpha Diagnostics International, San Antonio, TX, USA). All ELISA-based assays were performed following the manufacturers' instructions. This serological characterization allow us to assess the dynamics of DENV and ZIKV cross-reactivity but without discerning between cross-reactive binding Abs and cross- or type-specific neutralizing Abs.

Plaque Reduction Neutralization Test (PRNT). Selected serum samples (baseline, 30, 60 and 90 dpi) were challenged to neutralized ZIKV (H/PF/2013, PRVABC59), DENV-1 Western Pacific 74, DENV-2 NGC 44, DENV-3 Sleman 73, and DENV-4 Dominique strains. For the infecting serotype (DENV-2) and ZIKV the NAbs were measured in early timepoints as well (7 and 15 dpi). For the PRNT, serum samples were inactivated, diluted (2-fold), mixed with a constant inoculum of virus (volume necessary to produce ~35 pfu/well) and then incubated for 1 hr at 37°C and 5% CO_2. After incubation, virus-serum mix dilutions were added to Vero-81 cells monolayer in flat bottom 24-well plates seeded the day before for 1 hr at 37°C and 5% CO_2, finally overlay medium was added and incubated by several days (serotype dependent). Results were reported as PRNT60 titers, NAb titer capable of reduce 60% or more of DENV serotypes or ZIKV strains pfu compared with the mock (control of virus without serum). A PRNT60 1:20 titer was considered a positive Neut titer, and <1:20 as a negative Neut titer. Non-neutralizing titers (<1:20) were assigned with one-half of the limit of detection for graphs visualization. In addition, the half maximal (50%) effective concentrations (EC_{50}) were calculated based on the % of neutralization of DENV-2 and ZIKV infection for all timepoints. The EC_{50} was defined as the Ab dilution factor that is successfully capable of block 50% of the viral infection in the PRNT assay. For this, the data was transformed into Log_{10}, non-linear regression (curve fit) analysis was performed and calculated by the sigmoidal dose response (variable slope) equation in Prism 7. Reported values were validated by a R squared < 0.75 and Hill Slope absolute value < 0.5, and the geometric mean with 95% confidence interval was also calculated.

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Multiplex cytokine profile analysis. A total of 8 cytokines/chemokines were measured (pg /ml^{-1}) by Luminex at baseline, 1, 2, 3, 5, 10, 15 and 30 dpi, including: interferon alpha (IFN-α), interleukin-6 (IL-6), monokine induced by IFN-gamma (MIG/CXCL9), monocyte chemoattractant protein 1 (MCP-1/CCL2), macrophage inflammatory protein 1-beta (MIP-1β/CCL4), IL-1 receptor antagonist (IL-1RA), C-X-C motif chemokine 10 (CXCL10/IP-10) and perforin. The multiplex assay was conducted as previously described[17,88].

Immunophenotyping. Flow cytometry (MACSQuant Analyzer 10, Miltenyi Biotec) analysis was performed to determine the frequency, activation and proliferation of cell populations of the innate and adaptive immune response based on the phenotyping strategy of a previous study[17] (Supplementary Fig. 6 for gating strategy; Supplementary Table 3 for Ab panel). Phenotypic characterization of macaque PBMCs from fresh whole blood samples was performed by 8-multicolor flow cytometry using fluorochrome conjugated Abs at several timepoints (Baseline, 1, 2, 3, 7, 10 dpi; and 15 and 30 dpi for B/T cell panel only). Single cells (singlets) were selected by their FSC area (FSC-A) and height (FSC-H) patterns. Lymphocytes (LYM) were gated based on their characteristic forward and side scatter pattern (FSC, SSC). T cells were selected gating on the CD3$^+$ population. CD4$^+$ and CD8$^+$ T cells were defined as CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$, respectively. 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$^-$. The activation of B and T cell memory subpopulations (EM and CM) was assessed by the presence of the early activation marker CD69. Proliferation of total and activated B cells was quantified by the expression of the intracellular marker Ki67. Natural killer (NK) cells were defined as CD3$^-$CD20$^-$CD14$^-$ and analyzed by the expression of the following NK cell markers: CD8, CD56, NKG2A, NKp30, and NKp46. Dendritic cells (DC) were separated in two populations within the Lineage-DR+ (HLA-DR$^+$ CD3$^-$ CD14$^-$ CD16$^-$ CD20$^-$ CD8$^-$ NKG2A$^-$) by the expression of CD123 (plasmacytoid, pDC) or CD11c (myeloid, mDCs) and their percentages were calculated from total PBMCs. The phenotyping assays were optimized and performed as previously published[17,34,89].

T cell polyfunctional response analysis. Intracellular cytokine staining of macaques PBMCs was performed by multicolor flow cytometry using methods previously described (Supplementary Fig. 11 for gating strategy; Supplementary Table 4 for Ab panel)[17,89]. Polyfunctional effector response of CD4$^+$ and CD8$^+$ T cells was measured before and after DENV infection. Antigen-

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specific $CD4^+$ and $CD8^+$ T cell effector responses were measured at baseline to determine basal levels in presence (ZIKVPF-10mo, ZIKVPR-2mo) or absence (Naïve) of previous immunity to ZIKV. Also, 30, 60 and 90 dpi were assessed to determine how this pre-existing functional response is modulated after DENV infection and if is maintained over time. PBMCs were thawed one day before stimulation. For peptide pools stimulation, PBMCs were stimulated for 6 hr at 37°C and 5% CO_2. The peptides used for DENV-E, ZIKV-E and ZIKV-NS were 15-mers overlapped by 10 amino acids at 1.25 ug/ml^{-1}, 2.5 ug/ml^{-1}, 475 ng/ml^{-1} per peptide, respectively. PBMCs were *ex vivo* stimulated with DENV and ZIKV supernatants of infection, envelope (E) proteins, and ZIKV-NS peptide pools (Supplementary Table 5 for peptide sequences). The stimulation with peptides was performed in presence of brefeldin A at 10 ug/ml^{-1}. After stimulation, the cells were stained for the following markers: CD3, CD4, CD8, CD20 (excluded), CD107a (functional cytotoxicity). Levels of IFN-γ and TNF-α also were measured in gated lymphocytes cell populations. Samples were measured and data was collected on a LSRII (BD).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). The statistical significance between the means of all groups were determined using Two-way ANOVA Multiple Comparison Tukey Test, and to compare each mean against the baseline mean within same group Two-way ANOVA Multiple Comparison Dunnett Test was performed. Significant multiplicity adjusted *p* values (* <0.05, ** <0.01, *** <0.001, **** <0.0001) show statistically significant difference between groups (Tukey Test) or timepoints within a group (Dunnett Test).

Legends for Supplementary Figures

Supplementary Figure 1 | Clinical status and vital signs kinetics in ZIKV-immune and naïve macaques. (**a**) Weight (kg) was measured at baseline, 1, 3, 5, 7, 9, 15, 30, 60 and 90 dpi. (**b**) Temperature (°C) was monitored with an infrared device at baseline, 1-10, 15, 30, 60 and 90 dpi. Complete blood cell counts (CBC) parameters (thou/ul and/or % of total WBC) such as (**c**) white blood cells (WBC), (**d**) lymphocytes (LYM), (**e**) neutrophils (NEU), (**f**) monocytes (MON), and (**g**) platelets (PLT) were screened at baseline, 7, and 15 dpi. Comprehensive metabolic panel (CMP) was performed to assess levels (U/L) of (**h**) alkaline phosphatase (ALK PHOSPHATASE) and liver enzymes (**i**) aspartate transaminase (AST), and (**j**) alanine transaminase (ALT) at baseline, 7, 15 and 30 dpi. Normal range of AST and ALT are depicted for reference. Symbols represent mean level detected for each parameter per cohort per timepoint: blue squares (ZIKVPF-10mo),

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orange squares (ZIKVPR-2mo) and black circles (Naïve). Lines connect mean values detected over time. Error bars indicate the standard error of the mean (SEM) for each cohort per timepoint. Statistically significant differences between groups were determined using Two-Way Anova Tukey's multiple comparisons test. For differences in ALT levels Two-Way Anova Dunnett's multiple comparisons test (comparison of each cohort response at each timepoint versus baseline) was performed due to levels divergence between cohorts since baseline. Statistically differences are reported as multiplicity adjusted *p* values (* <0.05) and 95% CI.

Supplementary Figure 2 | Serological cross-reactivity is boosted by ZIKV immunity. Levels of DENV (**a**) IgM and (**b**) IgG, and ZIKV (**c**) IgM, (**d**) IgG, (**e**) NS1-IgG and (**f**) EDIII-IgG were measured by ELISA at multiple timepoints before and after DENV infection. Symbols connected with full lines represent mean levels of Abs detected per cohort over time: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Error bars indicate the standard error of the mean (SEM) and dotted line mark the limit of detection for each individual ELISA. Results were read at OD 450, 405 or using ISR (Immune Status Ratio) following manufacturer's instructions. Statistically significant differences between groups were calculated using Two-Way Anova Tukey's multiple comparisons test with 95% CI. Significant multiplicity adjusted *p* values (* <0.05, ** <0.01, *** <0.001, **** <0.0001) are shown. Blue and orange asterisks represent significant difference between the corresponded ZIKV immune groups and naive group, and gray asterisks indicate a significant difference between ZIKV immune groups.

Supplementary Figure 3 | Neutralization kinetics against DENV-2 of mid- and early-convalescent and naïve animals. Percentage of DENV-2 neutralization of each animal per group calculated by the transformation of PRNT60 Neut 2-fold titers into Log10 (1/serum dilution), and sigmoidal-dose response curves were generated. Each column of panels represent the % of DENV-2 neutralization for each group (ZIKVPF-10mo: blue squares/curves; ZIKVPR-2mo: orange squares/curves; Naïve: black circles/curves) and each row of panels represent a timepoint before and after DENV infection (baseline, 7, 15, 30, 60, 90 dpi).

Supplementary Figure 4 | Elevated titers of neutralizing antibodies correlate with decreased or no detectable levels of genome copies at early and late phase of DENV RNAemia kinetics. (**a**) Comparison of NAb titers by PRNT60 and DENV genome copies (log10) per ml of serum detected by qRT-PCR. Empty and full symbols represent NAbs and genome copies, respectively. Symbols and the shape represent individual animals per cohort: blue

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squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Gray short lines indicate the mean and error bars show the SEM. PRNT60: NAb titer capable of reduce 60% or more of DENV serotypes plaque-forming units (pfu) compared with the mock (control of virus without serum). A PRNT60 1:20 titer was considered positive, and <1:20 as a negative Neut titer. Dotted line mark <1:20 for negative results. Non-neutralizing titers (<1:20) were assigned with one-half of the limit of detection for graphs visualization (1:10). (**b-c**) Percentage of DENV-2 neutralization before and 7 days after DENV infection. This was calculated by the transformation of PRNT60 Neut 2-fold titers (NAb titer capable of reduce 60% or more of DENV-2 pfu compared with the mock) into Log10 (1/serum dilution). Symbols connected with full lines indicate mean levels of neutralization percentage detected per cohort for each serum dilution: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Dotted line indicates 60% of DENV-2 neutralization by PRNT60 titers.

Supplementary Figure 5 | Neutralization kinetics of ZIKV mid- and early- convalescent animals and naïve animals against ZIKV. Percentage of ZIKV (H/PF/2013) neutralization of each animal per group calculated by the transformation of PRNT60 Neut 2-fold titers into Log10 (1/serum dilution), and sigmoidal-dose response curves were generated. Each column of panels represent the % of ZIKV neutralization for each group (ZIKVPF-10mo: blue squares/curves; ZIKVPR-2mo: orange squares/curves; Naïve: black circles/curves) and each row of panels represent a timepoint before and after DENV infection (baseline, 7, 15, 30, 60, 90 dpi).

Supplementary Figure 6 | Gating strategy for immunophenotyping and activation of B cells, and memory T cell subpopulations.

Supplementary Figure 7 | Dendritic cells subsets modulation depends of the presence or absence of ZIKV immunity. The frequency (% of total PBMCs) of dendritic cells (DCs) subsets including (**a**) plasmacytoid (pDCs: HLA-DR^{+}CD123^{+}) and (**b**) myeloid (mDCs: HLA-DR^{+}CD11c^{+}) was assessed before and up to 10 days after DENV infection by immunophenotyping using flow cytometry analysis. Symbols represent individual animals per group for each timepoint: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Short gray lines depict mean value for each group detected over time. Cutted line divide % of DCs quantified before and after DENV infection. Statistically significant differences between groups were determined using Two-Way Anova Dunnett's multiple comparisons test (comparison of each cohort response at each timepoint versus baseline) and reported as multiplicity adjusted *p* values

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(* <0.05, ** <0.01, *** <0.001, **** <0.0001) with 95% CI. Asterisks represent significant difference between the corresponded timepoint and baseline within the same group.

Supplementary Figure 8 | Natural killer cell subpopulations and their differential receptors expression in presence or absence of ZIKV immunity. Natural killer (NK) cell subpopulations and the relative expression of multiple NK receptors within each subpopulation: (**a-d**) NK8, (**e-h**) NK56, (**i-l**) NKG2A, (**m-p**) NKp30 and (**q-t**) NKp46 were quantified by immunophenotyping using flow cytometry analysis before and up to 10 days after DENV infection. Individual symbols represent each animal per group over time: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Short gray lines mark mean value for each group. Cutted line divide % of NK cells quantified before and after DENV infection. Statistically significant differences between groups were determined using Two-Way Anova Dunnett's multiple comparisons test (comparison of each cohort response at each timepoint versus baseline) and reported as multiplicity adjusted *p* values (* <0.05, ** <0.01, *** <0.001, **** <0.0001) with 95% CI. Asterisks represent significant difference between the corresponded timepoint and baseline within the same group. ND (Not Done) in panels *8o* and *8s* refers that for ZIKVPF-10mo and Naïve the $NKp30^{+}NKp46^{+}$ and $NKp46^{+}NKp30^{+}$ subpopulations were not measured.

Supplementary Figure 9 | B cells proliferation and activation levels are higher in ZIKV-immune groups. The (**a**) total (% of total Lymphocytes), (**b**) activated, (**c**) proliferating, and (**d**) proliferating/activated B cells (% of total B cells) were determined at baseline and following DENV infection by immunophenotyping using flow cytometry analysis. B cells proliferation and activation were monitored since baseline up to 10 and 30 dpi, respectively. Symbols represent individual animals per group for each timepoint: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Short gray lines depict mean value of B cells percent in each group of animals per timepoint. Cutted line divide % of B cells quantified before and after DENV infection. Statistically significant differences between groups were determined using Two-Way Anova Dunnett's multiple comparisons test (comparison of each cohort response at each timepoint versus baseline) and reported as multiplicity adjusted *p* values (* <0.05, ** <0.01) with 95% CI. Asterisks represent significant difference between the corresponded timepoint and baseline within the same group.

Supplementary Figure 10 | T cells frequency and activation before and after DENV infection. The (**a**) total T cells (% of total Lymphocytes), (**b**) $CD4^{+}$ and (**c**) $CD8^{+}$ T cell

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compartments (% of total T cells) frequencies and their activation (**d-f**) were quantified at baseline and following DENV infection up to 30 dpi by immunophenotyping using flow cytometry. Symbols represent individual animals per group for each timepoint: blue squares (ZIKVPF-10mo), orange squares (ZIKVPR-2mo) and black circles (Naïve). Short gray lines mark mean value of T cells percent in each cohort per timepoint. Cutted line divide % of T cells quantified before and after DENV infection. Statistically significant differences between groups were determined using Two-Way Anova Dunnett's multiple comparisons test (comparison of each cohort response at each timepoint versus baseline).

Supplementary Figure 11 | Gating strategy for CD4+ and CD8+ T cell polyfunctional response.

Legends for Supplementary Tables

Supplementary Table 1 | Sequence alignment and amino acid identity of ZIKV strains PRVABC59 and H/PF/2013.

Supplementary Table 2 | DENV RNAemia kinetics in ZIKV-immune and naïve animals.

Supplementary Table 3 | Antibody panel for Immunophenotyping.

Supplementary Table 4 | Antibody panel for T cell polyfunctional response assessment.

Supplementary Table 5 | Peptide sequences for stimulation of T cell polyfunctional response.

Data availability. All relevant data is in main figures and supplementary information, any additional details are available from authors upon request.

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Author contributions. C.A.S. and E.X.P.-G. developed the experimental design. I.V.R. supervised and performed sample collection and animals monitoring. E.X.P.-G., P.P., C.S.-C., M.A.H., A.O.-R., V.H., L.P., L.C., and T.A. performed the experiments. E.X.P.-G., C.A.S., V.H., M.A.H., L.J.W., A.d.S., and D.W. analyzed the data. E.X.P.-G. and C.A.S. drafted the manuscript. C.A.S., E.X.P.-G., D.W., A.K.P., J.D.B., M.A.H., L.G., L.J.W., and A.d.S. revised the manuscript.

Additional Information

Supplementary Information available at Supplementary Information File linked to the online version of this manuscript.

Competing interests: The authors declare no competing financial interests.

References

1 Metsky, H. C. *et al.* Zika virus evolution and spread in the Americas. *Nature* **546**, 411-415, doi:10.1038/nature22402 (2017).

2 Garcez, P. P. *et al.* Zika virus impairs growth in human neurospheres and brain organoids. *Science (New York, N.Y.)* **352**, 816-818, doi:10.1126/science.aaf6116 (2016).

3 Li, C. *et al.* Zika Virus Disrupts Neural Progenitor Development and Leads to Microcephaly in Mice. *Cell stem cell* **19**, 672, doi:10.1016/j.stem.2016.10.017 (2016).

4 Mlakar, J. *et al.* Zika Virus Associated with Microcephaly. *The New England journal of medicine* **374**, 951-958, doi:10.1056/NEJMoa1600651 (2016).

5 de Oliveira, W. K. *et al.* Zika Virus Infection and Associated Neurologic Disorders in Brazil. *The New England journal of medicine* **376**, 1591-1593, doi:10.1056/NEJMc1608612 (2017).

6 Guo, C. *et al.* Global Epidemiology of Dengue Outbreaks in 1990-2015: A Systematic Review and Meta-Analysis. *Frontiers in cellular and infection microbiology* **7**, 317, doi:10.3389/fcimb.2017.00317 (2017).

7 Bhatt, S. *et al.* The global distribution and burden of dengue. *Nature* **496**, 504-507, doi:10.1038/nature12060 (2013).

8 Katzelnick, L. C. *et al.* Dengue viruses cluster antigenically but not as discrete serotypes. *Science (New York, N.Y.)* **349**, 1338-1343, doi:10.1126/science.aac5017 (2015).

9 Khurram, M. *et al.* Dengue hemorrhagic fever: comparison of patients with primary and secondary infections. *Journal of infection and public health* **7**, 489-495, doi:10.1016/j.jiph.2014.05.005 (2014).

10 Guzman, M. G. *et al.* Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. *Rev Panam Salud Publica* **11**, 223-227, doi:S1020-49892002000400003 [pii] (2002).

11 Guzman, M. G., Alvarez, M. & Halstead, S. B. Secondary infection as a risk factor for dengue hemorrhagic fever/dengue shock syndrome: an historical perspective and role of

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antibody-dependent enhancement of infection. *Archives of virology* **158**, 1445-1459, doi:10.1007/s00705-013-1645-3 (2013).

12 Barba-Spaeth, G. *et al.* Structural basis of potent Zika-dengue virus antibody cross-neutralization. *Nature* **536**, 48-53, doi:10.1038/nature18938 (2016).

13 Butler, D. Brazil asks whether Zika acts alone to cause birth defects. *Nature* **535**, 475-476, doi:10.1038/nature.2016.20309 (2016).

14 Dejnirattisai, W. *et al.* Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. *Nature immunology*, doi:10.1038/ni.3515 (2016).

15 Priyamvada, L. *et al.* Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 7852-7857, doi:10.1073/pnas.1607931113 (2016).

16 Bardina, S. V. *et al.* Enhancement of Zika virus pathogenesis by preexisting antiflavivirus immunity. *Science (New York, N.Y.)* **DOI 10.1126/science.aal4365**, doi:10.1126/science.aal4365 (2017).

17 Pantoja, P. *et al.* Zika virus pathogenesis in rhesus macaques is unaffected by pre-existing immunity to dengue virus. *Nature communications* **8**, 15674, doi:10.1038/ncomms15674 (2017).

18 McCracken, M. K. *et al.* Impact of prior flavivirus immunity on Zika virus infection in rhesus macaques. *PLoS pathogens* **13**, e1006487, doi:10.1371/journal.ppat.1006487 (2017).

19 Terzian, A. C. B. *et al.* Viral Load and Cytokine Response Profile Does Not Support Antibody-Dependent Enhancement in Dengue-Primed Zika Virus-Infected Patients. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **65**, 1260-1265, doi:10.1093/cid/cix558 (2017).

20 Gordon, A. *et al.* Prior dengue virus infection and risk of Zika: A pediatric cohort in Nicaragua. *PLoS medicine* **16**, e1002726, doi:10.1371/journal.pmed.1002726 (2019).

21 Kam, Y. W. *et al.* Cross-reactive dengue human monoclonal antibody prevents severe pathologies and death from Zika virus infections. *JCI insight* **2**, doi:10.1172/jci.insight.92428 (2017).

22 Wen, J. *et al.* Dengue virus-reactive CD8(+) T cells mediate cross-protection against subsequent Zika virus challenge. *Nature communications* **8**, 1459, doi:10.1038/s41467-017-01669-z (2017).

23 Wen, J. *et al.* Identification of Zika virus epitopes reveals immunodominant and protective roles for dengue virus cross-reactive CD8(+) T cells. *Nature microbiology* **2**, 17036, doi:10.1038/nmicrobiol.2017.36 (2017).

24 Grifoni, A. *et al.* Prior Dengue virus exposure shapes T cell immunity to Zika virus in humans. *Journal of virology* **DOI 10.1128/jvi.01469-17**, doi:10.1128/jvi.01469-17 (2017).

25 Perez, F. *et al.* The decline of dengue in the Americas in 2017: discussion of multiple hypotheses. *Tropical medicine & international health : TM & IH*, doi:10.1111/tmi.13200 (2019).

26 Solimini, A. G., Manica, M., Rosa, R., Della Torre, A. & Caputo, B. Estimating the risk of Dengue, Chikungunya and Zika outbreaks in a large European city. *Scientific reports* **8**, 16435, doi:10.1038/s41598-018-34664-5 (2018).

27 Gutierrez-Lopez, R. *et al.* Vector Competence of Aedes caspius and Ae. albopictus Mosquitoes for Zika Virus, Spain. *Emerging infectious diseases* **25**, 346-348, doi:10.3201/eid2502.171123 (2019).

28 Ribeiro, G. S. *et al.* Does immunity after Zika virus infection cross-protect against dengue? *The Lancet. Global health* **6**, e140-e141, doi:10.1016/s2214-109x(17)30496-5 (2018).

29 Sariol, C. A. & White, L. J. Utility, limitations, and future of non-human primates for dengue research and vaccine development. *Frontiers in immunology* **5**, 452, doi:10.3389/fimmu.2014.00452 (2014).

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30 Clark, K. B., Onlamoon, N., Hsiao, H. M., Perng, G. C. & Villinger, F. Can non-human primates serve as models for investigating dengue disease pathogenesis? *Frontiers in microbiology* **4**, 305, doi:10.3389/fmicb.2013.00305 (2013).

31 Koraka, P., Benton, S., van Amerongen, G., Stittelaar, K. J. & Osterhaus, A. D. Characterization of humoral and cellular immune responses in cynomolgus macaques upon primary and subsequent heterologous infections with dengue viruses. *Microbes and infection* **9**, 940-946, doi:10.1016/j.micinf.2007.03.012 (2007).

32 Borges, M. B. *et al.* Characterization of recent and minimally passaged Brazilian dengue viruses inducing robust infection in rhesus macaques. *PLoS One* **13**, e0196311, doi:10.1371/journal.pone.0196311 (2018).

33 White, L. J. *et al.* An alphavirus vector-based tetravalent dengue vaccine induces a rapid and protective immune response in macaques that differs qualitatively from immunity induced by live virus infection. *Journal of virology* **87**, 3409-3424, doi:10.1128/JVI.02298-12 (2013).

34 Sariol, C. A. *et al.* Decreased Dengue Replication and an Increased Anti-viral Humoral Response with the use of Combined Toll-Like Receptor 3 and 7/8 Agonists in Macaques. *PLoS One* **6**, 9 (2011).

35 Kawiecki, A. B. & Christofferson, R. C. Zika Virus-Induced Antibody Response Enhances Dengue Virus Serotype 2 Replication In Vitro. *The Journal of infectious diseases* **214**, 1357-1360, doi:10.1093/infdis/jiw377 (2016).

36 Stettler, K. *et al.* Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. *Science (New York, N.Y.)* **353**, 823-826, doi:10.1126/science.aaf8505 (2016).

37 Fowler, A. M. *et al.* Maternally Acquired Zika Antibodies Enhance Dengue Disease Severity in Mice. *Cell host & microbe* **24**, 743-750.e745, doi:10.1016/j.chom.2018.09.015 (2018).

38 George, J. *et al.* Prior Exposure to Zika Virus Significantly Enhances Peak Dengue-2 Viremia in Rhesus Macaques. *Scientific reports* **7**, 10498, doi:10.1038/s41598-017-10901-1 (2017).

39 Valiant, W. G. *et al.* Zika convalescent macaques display delayed induction of anamnestic cross-neutralizing antibody responses after dengue infection. *Emerging microbes & infections* **7**, 130, doi:10.1038/s41426-018-0132-z (2018).

40 Anderson, K. B. *et al.* A shorter time interval between first and second dengue infections is associated with protection from clinical illness in a school-based cohort in Thailand. *The Journal of infectious diseases* **209**, 360-368, doi:10.1093/infdis/jit436 (2014).

41 Montoya, M. *et al.* Symptomatic versus inapparent outcome in repeat dengue virus infections is influenced by the time interval between infections and study year. *PLoS neglected tropical diseases* **7**, e2357, doi:10.1371/journal.pntd.0002357 (2013).

42 Kliks, S. C., Nimmanitya, S., Nisalak, A. & Burke, D. S. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. *Am J Trop Med Hyg* **38**, 411-419 (1988).

43 Bhoomiboonchoo, P. *et al.* Sequential dengue virus infections detected in active and passive surveillance programs in Thailand, 1994-2010. *BMC public health* **15**, 250, doi:10.1186/s12889-015-1590-z (2015).

44 Arien, K. K., Michiels, J., Foque, N., Heyndrickx, L. & Van Esbroeck, M. Can Zika virus antibodies cross-protect against dengue virus? *The Lancet. Global health* **6**, e494, doi:10.1016/s2214-109x(18)30122-0 (2018).

45 Ribeiro, G. S. *et al.* Can Zika virus antibodies cross-protect against dengue virus? - Authors' reply. *The Lancet. Global health* **6**, e495, doi:10.1016/s2214-109x(18)30123-2 (2018).

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46 Katzelnick, L. C. & Harris, E. The use of longitudinal cohorts for studies of dengue viral pathogenesis and protection. *Current opinion in virology* **29**, 51-61, doi:10.1016/j.coviro.2018.03.004 (2018).

47 Katzelnick, L. C., Montoya, M., Gresh, L., Balmaseda, A. & Harris, E. Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 728-733, doi:10.1073/pnas.1522136113 (2016).

48 Weiskopf, D. *et al.* Dengue virus infection elicits highly polarized CX3CR1+ cytotoxic CD4+ T cells associated with protective immunity. *Proceedings of the National Academy of Sciences of the United States of America* **112**, E4256-4263, doi:10.1073/pnas.1505956112 (2015).

49 Kanthaswamy, S. *et al.* The Population Genetic Composition of Conventional and SPF Colonies of Rhesus Macaques (Macaca mulatta) at the Caribbean Primate Research Center. *Journal of the American Association for Laboratory Animal Science : JAALAS* **55**, 147-151 (2016).

50 Collins, M. H. *et al.* Lack of Durable Cross-Neutralizing Antibodies Against Zika Virus from Dengue Virus Infection. *Emerging infectious diseases* **23**, 773-781, doi:10.3201/eid2305.161630 (2017).

51 Dowd, K. A. *et al.* Broadly Neutralizing Activity of Zika Virus-Immune Sera Identifies a Single Viral Serotype. *Cell reports* **16**, 1485-1491, doi:10.1016/j.celrep.2016.07.049 (2016).

52 Swanstrom, J. A. *et al.* Dengue Virus Envelope Dimer Epitope Monoclonal Antibodies Isolated from Dengue Patients Are Protective against Zika Virus. *mBio* **7**, DOI 10.1128/mBio.01123-01116, doi:10.1128/mBio.01123-16 (2016).

53 Priyamvada, L. *et al.* B Cell Responses during Secondary Dengue Virus Infection Are Dominated by Highly Cross-Reactive, Memory-Derived Plasmablasts. *Journal of virology* **90**, 5574-5585, doi:10.1128/jvi.03203-15 (2016).

54 Montoya, M. *et al.* Longitudinal Analysis of Antibody Cross-neutralization Following Zika Virus and Dengue Virus Infection in Asia and the Americas. *The Journal of infectious diseases* **218**, 536-545, doi:10.1093/infdis/jiy164 (2018).

55 Andrade, P. *et al.* Impact of pre-existing dengue immunity on human antibody and memory B cell responses to Zika. *Nature communications* **10**, 938, doi:10.1038/s41467-019-08845-3 (2019).

56 Priyamvada, L., Hudson, W., Ahmed, R. & Wrammert, J. Humoral cross-reactivity between Zika and dengue viruses: implications for protection and pathology. *Emerging microbes & infections* **6**, e33, doi:10.1038/emi.2017.42 (2017).

57 Gallichotte, E. N. *et al.* Genetic Variation between Dengue Virus Type 4 Strains Impacts Human Antibody Binding and Neutralization. *Cell reports* **25**, 1214-1224, doi:10.1016/j.celrep.2018.10.006 (2018).

58 Yu, L. *et al.* Delineating antibody recognition against Zika virus during natural infection. *JCI insight* **2**, doi:10.1172/jci.insight.93042 (2017).

59 Mathew, A., Townsley, E. & Ennis, F. A. Elucidating the role of T cells in protection against and pathogenesis of dengue virus infections. *Future microbiology* **9**, 411-425, doi:10.2217/fmb.13.171 (2014).

60 St John, A. L. & Rathore, A. P. S. Adaptive immune responses to primary and secondary dengue virus infections. *Nature reviews. Immunology*, doi:10.1038/s41577-019-0123-x (2019).

61 Miller, J. D. *et al.* Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. *Immunity* **28**, 710-722, doi:10.1016/j.immuni.2008.02.020 (2008).

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62 Knudsen, M. L. *et al.* Kinetic and phenotypic analysis of CD8+ T cell responses after priming with alphavirus replicons and homologous or heterologous booster immunizations. *Journal of virology* **88**, 12438-12451, doi:10.1128/jvi.02223-14 (2014).

63 Grifoni, A. *et al.* Cutting Edge: Transcriptional Profiling Reveals Multifunctional and Cytotoxic Antiviral Responses of Zika Virus-Specific CD8(+) T Cells. *J Immunol* **201**, 3487-3491, doi:10.4049/jimmunol.1801090 (2018).

64 Gunther, V. J. *et al.* A human challenge model for dengue infection reveals a possible protective role for sustained interferon gamma levels during the acute phase of illness. *Vaccine* **29**, 3895-3904, doi:10.1016/j.vaccine.2011.03.038 (2011).

65 Wijeratne, D. T. *et al.* Quantification of dengue virus specific T cell responses and correlation with viral load and clinical disease severity in acute dengue infection. *PLoS neglected tropical diseases* **12**, e0006540, doi:10.1371/journal.pntd.0006540 (2018).

66 Weiskopf, D. *et al.* Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. *Proceedings of the National Academy of Sciences of the United States of America* **110**, E2046-2053, doi:10.1073/pnas.1305227110 (2013).

67 Mathew, A. *et al.* Predominance of HLA-restricted cytotoxic T-lymphocyte responses to serotype-cross-reactive epitopes on nonstructural proteins following natural secondary dengue virus infection. *Journal of virology* **72**, 3999-4004 (1998).

68 Zellweger, R. M. *et al.* CD8+ T Cells Can Mediate Short-Term Protection against Heterotypic Dengue Virus Reinfection in Mice. *Journal of virology* **89**, 6494-6505, doi:10.1128/jvi.00036-15 (2015).

69 Hatch, S. *et al.* Intracellular cytokine production by dengue virus-specific T cells correlates with subclinical secondary infection. *The Journal of infectious diseases* **203**, 1282-1291, doi:10.1093/infdis/jir012 (2011).

70 Reynolds, C. J. *et al.* T cell immunity to Zika virus targets immunodominant epitopes that show cross-reactivity with other Flaviviruses. *Scientific reports* **8**, 672, doi:10.1038/s41598-017-18781-1 (2018).

71 Rivino, L. *et al.* Differential targeting of viral components by CD4+ versus CD8+ T lymphocytes in dengue virus infection. *Journal of virology* **87**, 2693-2706, doi:10.1128/jvi.02675-12 (2013).

72 Elong Ngono, A. *et al.* CD4+ T cells promote humoral immunity and viral control during Zika virus infection. *PLoS pathogens* **15**, e1007474, doi:10.1371/journal.ppat.1007474 (2019).

73 Saron, W. A. A. *et al.* Flavivirus serocomplex cross-reactive immunity is protective by activating heterologous memory CD4 T cells. *Science advances* **4**, eaar4297, doi:10.1126/sciadv.aar4297 (2018).

74 Gandini, M. *et al.* Dengue virus activates membrane TRAIL relocalization and IFN-alpha production by human plasmacytoid dendritic cells in vitro and in vivo. *PLoS neglected tropical diseases* **7**, e2257, doi:10.1371/journal.pntd.0002257 (2013).

75 Kurane, I. *et al.* High levels of interferon alpha in the sera of children with dengue virus infection. *Am J Trop Med Hyg* **48**, 222-229 (1993).

76 Singla, M. *et al.* Immune Response to Dengue Virus Infection in Pediatric Patients in New Delhi, India--Association of Viremia, Inflammatory Mediators and Monocytes with Disease Severity. *PLoS neglected tropical diseases* **10**, e0004497, doi:10.1371/journal.pntd.0004497 (2016).

77 Restrepo, B. N. *et al.* Serum levels of interleukin-6, tumor necrosis factor-alpha and interferon-gamma in infants with and without dengue. *Revista da Sociedade Brasileira de Medicina Tropical* **41**, 6-10 (2008).

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78 Hober, D. *et al.* Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 beta) in dengue-infected patients. *Am J Trop Med Hyg* **48**, 324-331 (1993).

79 Chen, L. C. *et al.* Correlation of serum levels of macrophage migration inhibitory factor with disease severity and clinical outcome in dengue patients. *Am J Trop Med Hyg* **74**, 142-147 (2006).

80 Dalrymple, N. A. & Mackow, E. R. Endothelial cells elicit immune-enhancing responses to dengue virus infection. *Journal of virology* **86**, 6408-6415, doi:10.1128/jvi.00213-12 (2012).

81 Huang, J. *et al.* Serum Cytokine Profiles in Patients with Dengue Fever at the Acute Infection Phase. *Disease markers* **2018**, 8403937, doi:10.1155/2018/8403937 (2018).

82 Dufour, J. H. *et al.* IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J Immunol* **168**, 3195-3204 (2002).

83 Hsieh, M. F. *et al.* Both CXCR3 and CXCL10/IFN-inducible protein 10 are required for resistance to primary infection by dengue virus. *J Immunol* **177**, 1855-1863 (2006).

84 Dejnirattisai, W. *et al.* A complex interplay among virus, dendritic cells, T cells, and cytokines in dengue virus infections. *J Immunol* **181**, 5865-5874 (2008).

85 Gagnon, S. J., Ennis, F. A. & Rothman, A. L. Bystander target cell lysis and cytokine production by dengue virus-specific human CD4(+) cytotoxic T-lymphocyte clones. *Journal of virology* **73**, 3623-3629 (1999).

86 Althouse, B. M. *et al.* Potential for Zika Virus to Establish a Sylvatic Transmission Cycle in the Americas. *PLoS neglected tropical diseases* **10**, e0005055, doi:10.1371/journal.pntd.0005055 (2016).

87 Favoretto, S. R. *et al.* Zika Virus in Peridomestic Neotropical Primates, Northeast Brazil. *EcoHealth*, doi:10.1007/s10393-019-01394-7 (2019).

88 Giavedoni, L. D. Simultaneous detection of multiple cytokines and chemokines from nonhuman primates using luminex technology. *Journal of immunological methods* **301**, 89-101, doi:10.1016/j.jim.2005.03.015 (2005).

89 Meyer, C., Haberthur, K., Asquith, M. & Messaoudi, I. Flow Cytometry-Based Methods to Characterize Immune Senescence in Nonhuman Primates. *Methods in molecular biology* **1343**, 65-80, doi:10.1007/978-1-4939-2963-4_6 (2015).