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Dengue vaccine breakthrough infections reveal properties of neutralizing antibodies linked to protection

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The four serotypes of dengue virus (DENV1-4) are mosquito-borne flaviviruses that infect humans. Live attenuated tetravalent DENV vaccines are at different phases of clinical testing. DENV vaccine developers have relied on neutralizing antibodies (NAbs) as a correlate of protection. A leading tetravalent vaccine (Dengvaxia) stimulated NAbs to the 4 DENV serotypes, yet overall vaccine efficacy was low in children who were DENV seronegative at baseline before vaccination. We compared the properties of 1) NAbs induced by wild type DENV1 or 3 infections, which are strongly correlated with protection from repeat infections, and 2) NAbs induced by Dengvaxia in individuals who subsequently experienced DENV1 or DENV3 breakthrough infections. Wild type infections induced NAbs that recognized epitopes unique (type-specific) to each serotype, whereas the vaccine stimulated qualitatively different NAbs that recognized epitopes conserved (cross-reactive) between serotypes. Our results indicate that among children who were DENV seronegative at baseline, unbalanced replication of the DENV type 4 vaccine component in the tetravalent vaccine stimulates Abs capable of cross neutralizing DENV1 and 3 in vitro but not protect in vivo. In DENV seronegative individuals who are vaccinated, we propose that type specific NAbs are a better correlate of protection than total levels of NAbs.

Dengue Vaccine Breakthrough Infections Reveal Properties of Neutralizing Antibodies Linked to Protection

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Running title: DENV serotype-specific antibodies are correlated with durable protection

Conflict of interests: Matthew Bonaparte, Janice Moser, and Alina Munteanu are employees of the company (Sanofi Pasteur) that developed Dengvaxia. The other authors have declared that no conflict of interest exists.

ABSTRACT

The four serotypes of dengue virus (DENV1-4) are mosquito-borne flaviviruses that infect humans. Live attenuated tetravalent DENV vaccines are at different phases of clinical testing. DENV vaccine developers have relied on neutralizing antibodies (NAbs) as a correlate of protection. A leading tetravalent vaccine (Dengvaxia) stimulated NAbs to the 4 DENV serotypes, yet overall vaccine efficacy was low in children who were DENV seronegative at baseline before vaccination. We compared the properties of 1) NAbs induced by wild type DENV1 or 3 infections, which are strongly correlated with protection from repeat infections, and 2) NAbs induced by Dengvaxia in individuals who subsequently experienced DENV1 or DENV3 breakthrough infections. Wild type infections induced NAbs that recognized epitopes unique (type-specific) to each serotype, whereas the vaccine stimulated qualitatively different NAbs that recognized epitopes conserved (cross-reactive) between serotypes. Our results indicate that among children who were DENV seronegative at baseline, unbalanced replication of the DENV type 4 vaccine component in the tetravalent vaccine stimulates Abs capable of cross neutralizing DENV1 and 3 in vitro but not protect in vivo. In DENV seronegative individuals who are vaccinated, we propose that type specific NAbs are a better correlate of protection than total levels of NAbs.

INTRODUCTION

The four dengue virus serotypes (DENV1-4) are mosquito-transmitted flaviviruses estimated to infect over 100 million people every year.(1) DENV infections stimulate neutralizing antibodies (NAbs) that are correlated with protection. Several DENV vaccines are at different stages of clinical development (2-4). While the development of DENV vaccines has been guided by the presence of NAbs as a correlate of protection, recent studies indicate that NAbs to the four serotypes after vaccination is not a reliable correlate of protection (5-7). We compared the properties of NAbs induced by wild type DENV serotype 1 and 3 infections and a leading vaccine (Dengvaxia developed by Sanofi Pasteur) to improve our understanding of the properties of protective Abs.

A person infected with DENV for the first time (a primary infection) develops a durable serotype-specific (TS) NAb response that is correlated with resistance to re-infection by the same serotype (2-4, 8). After a primary infection, people are susceptible to second infections with new serotypes. DENV serotype cross-reactive (CR) Abs induced by primary infections have been linked to enhanced viral replication and more severe disease during secondary infections (9). Individuals who have recovered from secondary DENV infections develop new populations of serotype CR NAbs that are correlated with durable serotype cross protective immunity (4, 10, 11) To minimize the risk of DENV vaccines inducing Abs that enhance DENV infections, leading vaccines are based on tetravalent formulations to induce balanced protective immunity to all 4 serotypes.

Dengvaxia is a live attenuated chimeric tetravalent dengue vaccine (CYD-TDV) that was developed by modifying the yellow fever 17D live attenuated vaccine to contain the envelope (E) and pre-membrane proteins of each DENV serotype (12). The safety and efficacy of Dengvaxia was tested in a phase 2b trial (CYD23) in Thailand and two large phase 3 trials (CYD14 and CYD15) in Asia and Latin America, respectively (13-16). Efficacy was high in children with pre-existing immunity to DENVs who received the vaccine. In DENV-naïve children, the vaccine reliably stimulated NAbs to the 4 serotypes, yet overall efficacy was low (6, 16). More recently, preliminary efficacy data from another clinical trial (TAK-003 developed Takeda) also indicate that the presence of vaccine induced NAb alone is not a reliable indicator of protection in children who were DENV seronegative at baseline (5). In individual with no prior immunity to DENVs, we propose that TS NAbs directed to unique epitopes on each serotype are a better correlate of protection that total NAbs because the induction of TS Ab requires replication of the matched vaccine component (17, 18). To test this hypothesis, we characterized Dengvaxia induced Ab responses in baseline seronegative individuals who subsequently experienced symptomatic breakthrough infections with DENV1 or DENV3. As controls, we characterized the properties of Abs in baseline seronegative vaccine recipients who did not experience a breakthrough infection and in individuals exposed to primary wild type DENV1 or 3 infections, who are protected from repeat infections by the same serotype.

RESULTS AND DISCUSSION

Antibody response to wildtype DENV1 or DENV3 infections. We characterized the specificity of NAbs in individuals who experienced primary DENV1 (n=11) or DENV3 (n=8) infections (Supplementary **Table 1**) because wild type infections are known to induce durable protection from clinically symptomatic reinfection with the same serotype (2, 4). To measure levels of DENV1 or 3 TS Abs, we incubated the samples with beads coated with DENV serotypes not responsible for infection to deplete serotype CR Abs (Supplementary **Figure 1**). The depleted samples bound to DENV1 or DENV3, although at reduced levels compared to control depleted samples, demonstrating the presence of TS Abs to each serotype responsible for infection (Supplementary **Figure 1**). The CR Ab depleted samples neutralized the homologous serotype of infection (DENV1 or DENV3) at levels comparable to control depleted samples demonstrating that TS Abs were mainly responsible for functional neutralization (**Figure 1A and B**).

Dengue vaccine responses in children who experienced DENV1 or DENV3 breakthrough infections. Most children with no immunity to DENVs who received Dengvaxia developed NAbs to DENV1 and 3, yet vaccine efficacy against these two serotypes was low (6). We characterized the properties of vaccine induced Abs in baseline seronegative children who subsequently experienced DENV1 (n=15 subjects) or 3 (n=18 subjects) breakthrough infections (Supplementary **Table 2**). When vaccine immune sera from DENV1 breakthrough cases were depleted of CR Abs, we observed that nearly all DENV1 binding was due to CR Abs, whereas all subjects had DENV4 TS binding Abs (Supplementary **Figure 2A**). A similar pattern was observed when the Ab depleted samples were tested for neutralization of DENV1 and 4 (**Figure 2**). While all 15

subjects who experienced DENV1 breakthrough infections had developed DENV1 NAbs after vaccination (GMT=97), only 4/15 subjects had any DENV1 TS NAbs (GMT = 2.5) (**Figure 2A** and Supplementary **Figure 4A**). In contrast, 10/15 children had DENV4 TS NAbs (GMT = 15) (**Figure 2B** and Supplementary **Figure 4A**).

Among the 18 children who experienced DENV3 breakthrough infections, 14 had DENV3 NAbs (GMT =28) and all 18 had DENV4 NAbs (GMT = 122) (**Figure 3A**). After Ab depletion to remove CR Abs, only 4/18 subjects had DENV3 TS NAbs (GMT= 2) and 14/18 subjects had DENV4 TS NAbs (GMT = 19) (**Figure 3B** and Supplementary **Figure 4B**). Dengvaxia mainly stimulated DENV1 and 3 CR NAbs in children who subsequently experienced DENV1 or 3 breakthrough infections.

Dengue vaccine responses in the general population of children who were seronegative at baseline. Next, we characterized the properties of DENV1, 3 and 4 NAbs in baseline seronegative vaccine recipients (n = 11) who did not experience a breakthrough infection during the clinical trial. These subjects had variable NAb levels to the three serotypes tested, with the highest response to DENV4 followed by DENV3 then DENV1 (GMT= 103, 67 and 60 respectively) (Supplementary **Figure 3**). When the sera were subjected to Ab depletions to estimate levels of TS NAbs, we observed that 2/11 had DENV1 TS NAbs, 3/11 had DENV3 TS NAbs and 5/11 had DENV4 TS NAbs (Supplementary **Figure 3**). As the breakthrough and non-breakthrough cases had similar Ab profiles, we combined the results from all the children tested to define properties of DENV1, 3 and 4 Abs stimulated by the vaccine in children who were seronegative at

baseline (**Figure 4)**. Although nearly all children (42/44) had DENV1 NAbs, only 12 (27%) had DENV1 TS NAbs (**Figure 4A**). Similarly, 24/29 children tested had DENV3 NAbs but only 7 (24%) had DENV3 TS NAbs (**Figure 4B**). In contrast to DENV1 and 3, 28/44 (64%) children tested had TS NAbs to DENV4 (**Figure 4C**).

The clinical observation that Dengvaxia stimulated NAbs in baseline seronegative individuals that were not correlated with protection was unexpected because NAbs are correlated with the success of other flavivirus vaccines. The aim of our study was to identify improved Ab correlates of protection against DENV1 and 3 by comparing the properties of Abs in vaccine recipients who experienced breakthrough infections and individuals exposed to primary wildtype DENV 1 and 3 infections. Our results demonstrate that both wildtype infection and vaccinated individuals had NAbs to DENV1 and 3 but NAbs stimulated by wildtype virus infections targeted epitopes unique to DENV1 or DENV3 whereas the vaccine mainly induced NAbs to epitopes conserved between serotypes. In contrast to the DENV1 and 3 responses, most children developed DENV4 TS NAbs after vaccination. These results match our previous observation that adults immunized with Dengvaxia mainly develop TS NAbs to DENV4 and CR NAbs to other serotypes (19). The DENV4 component in Dengvaxia replicates to higher levels and/or infects more individuals than the other three components (12, 20, 21). We propose that in individuals with no pre-existing immunity to DENVs, the robust replication of the DENV4 vaccine component stimulates serotype CR Abs that neutralize DENV1 and 3 in cell culture, but these Abs are not protective or correlated with protection in vivo.

160 At first glance, our conclusion that total levels of NAbs are a poor correlate of vaccine
161 efficacy in baseline seronegative individuals appears to be inconsistent with recent
162 publications demonstrating that levels of NAbs induced by Dengvaxia are correlated with
163 vaccine efficacy (7, 22). However, these reports are based on a pooled analysis of
164 baseline seropositive and seronegative children that mainly consisted of seropositive
165 children. When the analysis was stratified by baseline serostatus, the correlation was
166 weak and imprecise in baseline seronegative children (7, 22). These two populations
167 require separate evaluation when evaluating immune correlates, vaccine efficacy and
168 safety given fundamental differences in how adaptive immunity is activated in individuals
169 who are seronegative and seropositive at the time of vaccination.
170
171 Recent studies provide explanations for why some Abs might neutralize DENVs in cell
172 culture but not protect in vivo (23). DENV stocks produced using laboratory cell lines
173 consist of virions at different stages of maturation with low specific infectivity (23). DENV1
174 virions circulating in humans were observed to be more mature and infectious than cell
175 culture derived virus used in cell culture neutralization assays (23). DENV1 TS
176 monoclonal Abs and sera from people exposed to primary DENV1 infections neutralized
177 both plasma and cell culture produced DENV1 efficiently (23). DENV serotype CR MAbs
178 and heterotypic immune sera from people exposed to primary DENV2 or DENV3
179 infections efficiently neutralized cell culture produced DENV1 and poorly neutralized or
180 failed to neutralize human plasma derived DENV1(23). Most human DENV serotype-
181 cross reactive antibodies target conserved epitopes at or near the fusion loop at the tip of
182 domain II of E protein. This region of E protein is partially exposed and accessible to

antibody binding in immature virions, whereas in mature virions the fusion loop is buried and not readily accessible to antibody binding(24, 25). DENV neutralizing Ab assays utilizing partially mature DENV stocks will measure NAbs to exposed and hidden epitopes on mature virions and overestimate levels of protective Abs.

For Dengvaxia, no significant efficacy against DENV1 or 3 was observed in dengue seronegative children who were vaccinated and monitored for 5-6 years (6). Therefore, our observation that baseline seronegative vaccinated individuals had similar Ab profiles in the breakthrough and non-breakthrough groups is not surprising. In the absence of subgroups within the seronegative vaccinated population for identifying immune correlates, we compared Ab responses in individuals exposed to primary DENV1 and 3 infections and individuals who were vaccinated to identify improved correlates. While not the perfect comparison, our finding that DENV1 and 3 TS NAbs are readily detected after natural infection and rarely in baseline seronegative vaccinated children who experienced breakthrough infections, indicates that TS NAbs are a more reliable correlate than total levels of NAbs to guide the development of DENV vaccines for use in this population.

We note that a few vaccinated individuals who developed low levels of TS NAbs to DENV1 or DENV3 experienced breakthrough infections with the matched serotype. Therefore, the presence of TS NAbs alone may not always be sufficient for protection. TS NAbs may have to be maintained above a certain threshold for protection. Individuals with TS NAb responses may be susceptible to infections caused by natural variant strains that differ at critical TS Ab epitopes from the vaccine strain.

In the current study, we focused on Ab responses to DENV1 and 3 in seronegative children who received Dengvaxia. In this population, the vaccine was also not efficacious against DENV2, while the clinical trials demonstrated significant efficacy against DENV4 (16). Studies are currently in progress to better understand vaccine responses to DENV2 and 4. Dengvaxia was efficacious in children with pre-existing immunity to DENVs who were vaccinated, and our study was not designed to identify vaccine correlates and mechanisms of protection in this population. In individuals with no baseline immunity to DENVs, we propose that TS NAbs are a better correlate of protection than the current standard of total NAbs.

Methods

Detailed Methods appear in supplemental material.

Statistics: Friedman's One-Way ANOVA test was used to establish statistical significance between the Control depleted samples and the Ab depleted ones. Comparisons of percentage serotype specific and absolute serotype specific neutralization titer were done by Wilcoxon signed ranks test (Figure 2 and 3) and Kruskal Wallis Test (Figure 4).

Study approval: The CYD14 (ClinicalTrials.gov ID NCT01373281) and CYD15 (ClinicalTrials.gov ID NCT01374516) trial protocols have been approved by all relevant ethics review boards (26, 27). The Institutional Review Board of the University of North

Carolina at Chapel Hill reviewed and approved the receipt and analysis of anonymized CYD14 and CYD15 clinical specimens at the University of North Carolina at Chapel Hill (protocol16-0793). The clinical specimens from individuals exposed to wild type DENV infections were obtained from the UNC Arbovirus traveler study to recruits individuals in the USA infected during foreign travel. The collection, storage and use of these samples for research has been approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (protocol 19-2187).

Author contributions: MB helped with the initial characterization and selection of clinical samples for this study. RB, AMS, SH, CA, MB, JM and AM designed the research studies and assisted with the analysis of results. CA and SH conducted experiments and wrote the first drafts of the manuscript. All the authors provided critical edits.

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Main Figures

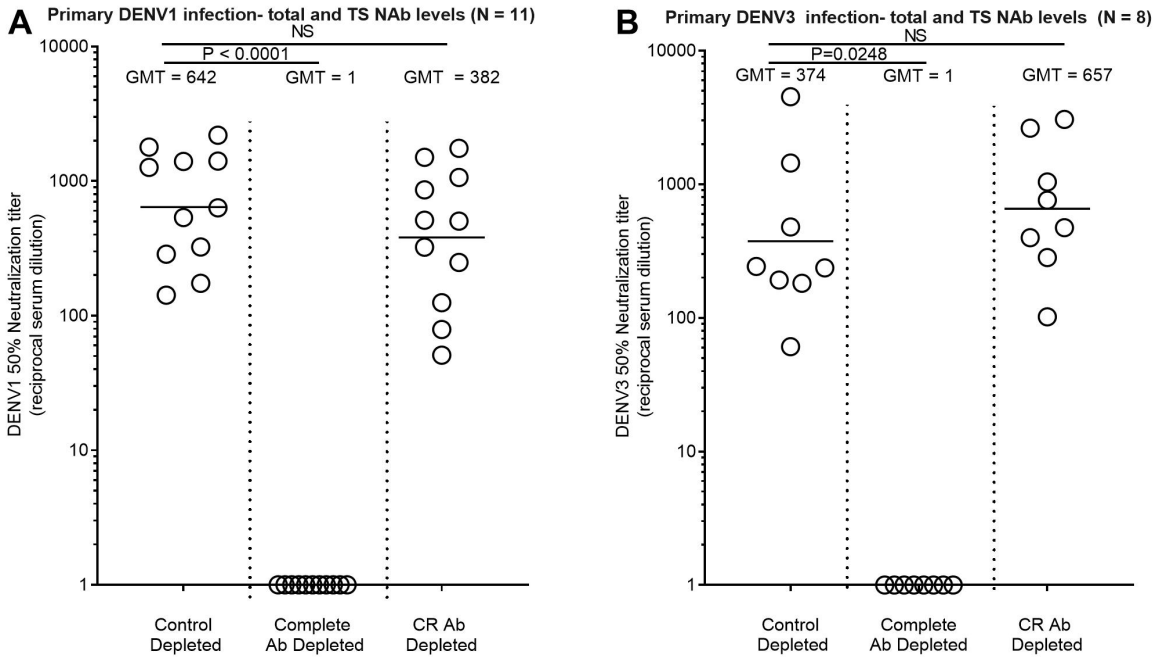

Figure 1. Antibody responses following primary DENV1 or DENV3 infection. Convalescent sera from people exposed to primary DENV1 **(A)** or DENV3 **(B)** infections were tested for NAb before (Control Depleted)) and after removal of total DENV Abs (Complete Ab Depletion) or DENV serotype CR Abs (CR Ab Depleted). Total Ab were depleted using DENV serotype responsible for infection as the depleting antigen (DENV1 or DENV3) and CR Abs were removed using a mix of antigen from heterologous DENV serotypes (DENV2 and 4 antigens for DENV1 immune sera and DENV1,2,4 antigens for DENV3 immune sera). Levels of DENV1 or 3 neutralizing Abs were not significantly reduced by the removal of CR Abs. Friedman's One-Way ANOVA test was used to establish statistical significance.

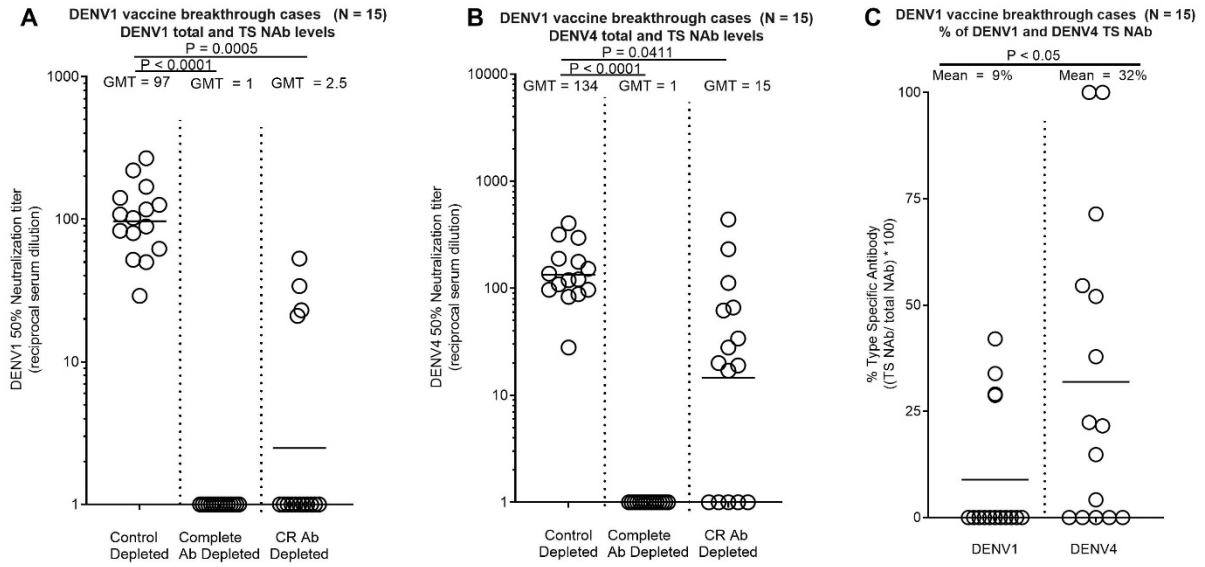

Figure 2. Vaccine induced Ab responses in children who experienced DENV1 breakthrough infections. Vaccine responses to DENV1 (A) and DENV4 (B) were analyzed in baseline seronegative children who received Dengvaxia and subsequently experienced DENV1 (n = 15) breakthrough infections. **(A)** DENV1 NAb responses after vaccination were measured without depleting any antibody (Control Depleted), after removal of all DENV1 binding Ab (Complete Ab Depletion with DENV1 antigen) and after removal of CR Ab (CR Ab depleted using a mix of DENV2 and 4 antigens). **(B)** DENV4 NAb responses after vaccination were measured without depleting any antibody (Control Depleted), after removal of all DENV4 binding Ab (Complete Ab Depletion with a mix of DENV2 and 4 antigens) and after removal of CR Ab (CR Ab depleted using DENV1 antigen). **(C)** The data were also analyzed to compare the percentage of DENV1 and DENV4 TS NAbs in the children after vaccination. Friedman's One-Way ANOVA test (panels A and B) or Wilcoxon signed ranks test (panel C) was used to establish statistical significance.

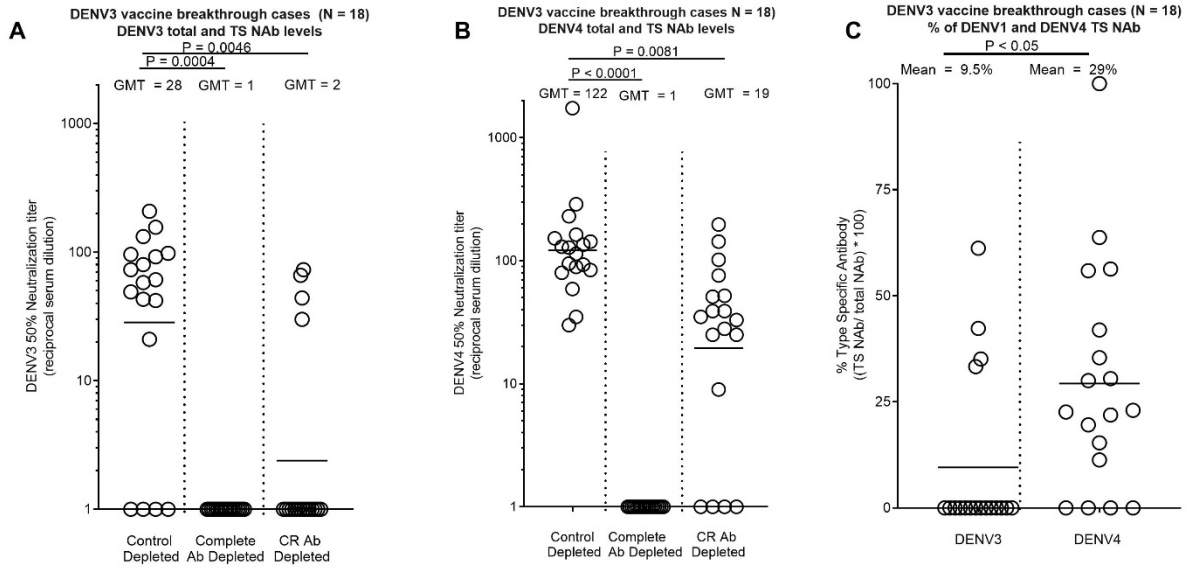

Figure 3. Vaccine induced Ab responses in children who experienced DENV3 breakthrough infections. Vaccine responses were analyzed in baseline seronegative children who received Dengvaxia and subsequently experienced DENV3 (n = 18) breakthrough infections. **(A)** DENV3 NAb responses after vaccination were measured without depleting any antibody (Control Depleted), after removal of all DENV3 binding Ab (Complete Ab Depletion with DENV3 antigen) and after removal of CR Ab (CR Ab Depleted using a mix of DENV1, 2 and 4 antigens). **(B)** DENV4 NAb responses after vaccination were measured without depleting any antibody (Control Depleted), after removal of all DENV4 binding Ab (Complete Ab Depletion with a mix of DENV1, 2 and 4 antigens) and after removal of CR Ab (CR Ab depleted using DENV3 antigen). **(C)** The data were also analyzed to compare the percentage of DENV3 and DENV4 TS NAbs in the children after vaccination. Friedman's One-Way ANOVA test (panels A and B) or Wilcoxon signed ranks test (panel C) was used to establish statistical significance.

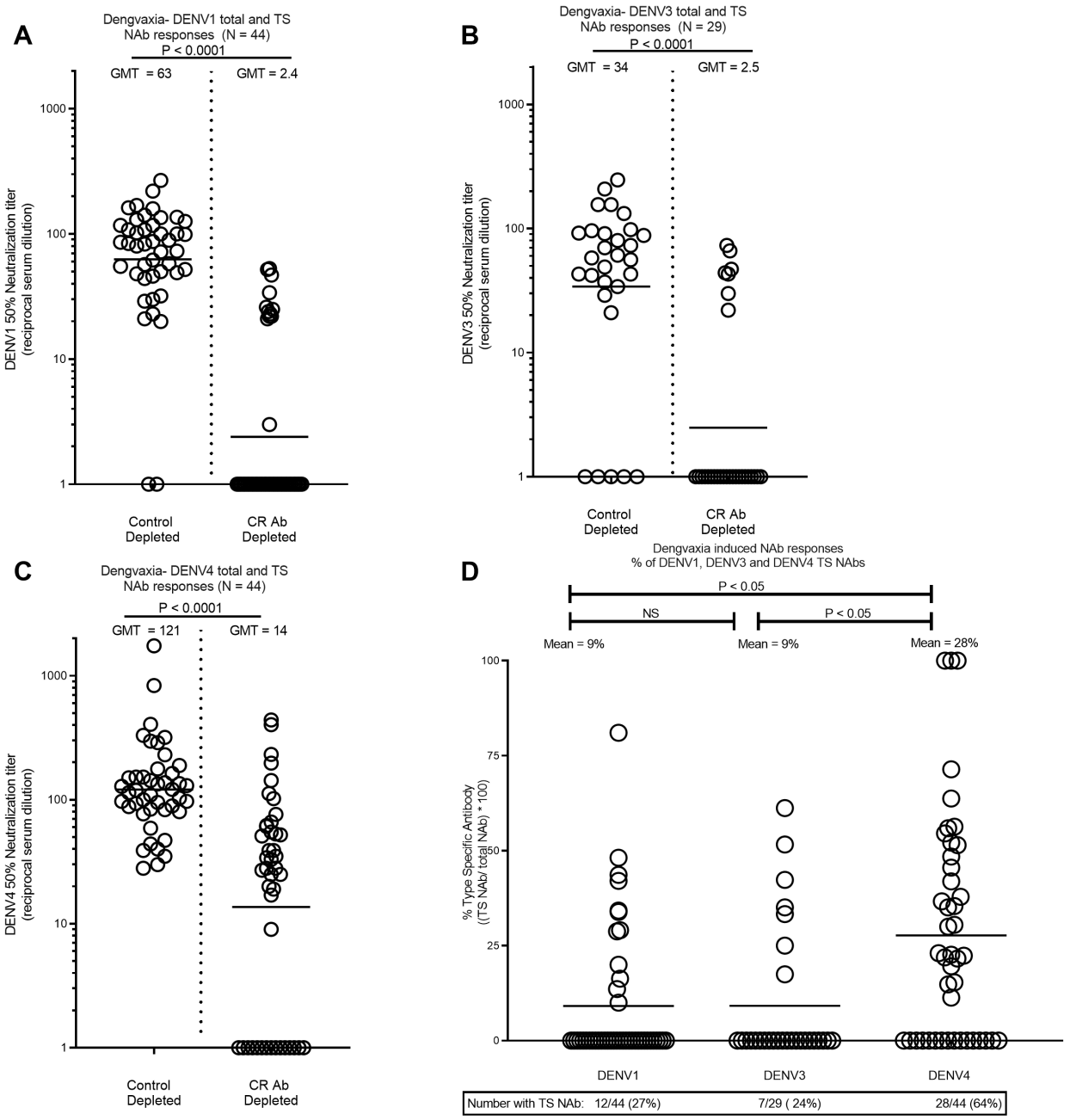

Figure 4. Dengvaxia induced Ab responses in children with no pre-exisiting immunity to DENVs. Dengvaxia recipients irrespective of subsequent outcome during CYD-TDV clinical trials were tested to determine levels of total and TS DENV1 (A), DENV3 (B) and DENV4 (C) NAbs. Wilcoxon signed ranks test was used to establish statistical significance. The data were also analyzed to compare the percentage (D) of vaccine induced DENV1, DENV3 and DENV4 TS NAbs. Kruskal Wallis test was used to compare Ab responses between serotypes. Supplementary Table 3 displays the DENV antigen combinations used for depletions to measure levels of TS NAbs to each serotype.

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